CHARACTERISATION OF NON-SPECIFIC ESTERASE ISOENZYME FORMS IN NORMAL AND LEUKAEMIC MYELOID CELLS

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The candidate confirms that the work submitted is his own and that appropriate credit has been given where reference has been made to the work of others.

SUMMARY

The generic term 'esterases' broadly represents enzymes that hydrolyse aliphatic or aromatic esters, and within this definition is a group of esterases that show a preference for short acyl chain esters such as α-naphthyl acetate, propionate or butyrate. These are often collectively referred to as 'non-specific esterases' (NSE), but a more accurate and descriptive designation is according to the substrate used. The demonstration of esterases by azo-dye techniques has found particular applications in haematological cytochemistry, and, with specific reference to normal myeloid cells, α-naphthyl acetate esterase (ANAE) cytochemical reactions of moderate-strong diffuse type are typically associated with cells of monocyte/macrophage lineage. In contrast, granulocytes at all levels of morphological differentiation are cytochemically ANAE negative. Isoelectric focusing (IEF) studies of ANAE isoenzymes have further revealed the existence of two species with apparent differences in lineage affiliation. The first (ComEst) is expressed by both granulocytes and monocytes, and comprises a series of isoenzymes with isoelectric points (pI) ranging from 6.3 to 7.9, whereas a second (MonEst) species that is specifically expressed by haemopoietic cells of monocytic/macrophage origin is seen on IEF zymograms as a series of isoforms within a relatively narrow pI range of 5.5-6.2.

These present studies were undertaken in order to extend what is currently known of the cellular, kinetic and molecular features of the two main myeloid esterase species. It was considered that these investigations were necessary to clarify the nature of atypical cytochemical reactions in leukaemic and dyshaemopoietic myeloid cells, to establish whether or not the two species were related or distinct enzymes, to gain further insights into their possible functional role(s), and to provide molecular details of relevance for the longer term aim of cloning MonEst protein in particular.

Studies reported here of normal myeloid cells confirmed the lineage affiliation of the two main esterase species, and analysis of a large number of acute myeloid leukaemias also resolved the nature of atypical ANAE cytochemistries. Abnormally increased focal and granular reactions of myeloid blasts was shown to be due to over-expression of ComEst, and the lack of ANAE cytochemical staining in a significant proportion of monocytic leukaemias was shown to result from a failure to synthesise MonEst. As a prelude to the biochemical purification of myeloid esterases, the ComEst and MonEst species were also investigated to determine their chromatographic characteristics. This involved an evaluation of a wide range of column gels including ion-exchange, hydrophobic interaction, affinity, and gel filtration. The purification protocol resulting from these evaluations successfully permitted the purification of ComEst to a highly enriched state and MonEst to homogeneity. Subsequent molecular and kinetic analyses revealed that enzymatically active MonEst exists in its native state as an apparent trimer which, under non-reducing conditions, dissociates to inactive 63 kDa monomers. In contrast, native ComEst was shown to be a 68 kDa monomer which retained enzymatic activity following SDS treatment, and was not dissociated under reducing conditions. Lectin affinity studies

confirmed that both esterase species were glycoproteins but differed in that MonEst contained oligomannosidic-type glycan(s) whilst ComEst contained a mixture of fucosylated and non-fucosylated biantennary N-acetyllactosamine-type glycan(s). Neuraminidase, α-mannosidase, α-L-fucosidase, and endoglycosidase H were shown to have no effect on the pI distribution of individual ComEst or MonEst isoforms, but endoglycosidase treatment did reduce the Mr of MonEst from 63 to 60 kDa. Enzyme kinetic studies also revealed that purified ComEst preferentially hydrolysed esters of short acyl chain length (C2 and C3) whilst MonEst hydrolysed esters of higher acyl chain length (butyrate > propionate > acetate). However, MonEst failed to hydrolyse a wide range of natural and synthetic peptidase substrates thus tending to exclude its functional role in peptide processing. Possible differences in reaction mechanisms of the two esterase species were also evaluated by examining the inhibitory effects of representative enzyme inhibitors which demonstrated that serine and histidine residues were required for MonEst but not ComEst activity. N-terminus amino acid sequencing of purified MonEst indicated almost complete identity with human alveolar macrophage esterase, differing only in a Val-Thr substitution at position 12, and close similarities with rabbit liver carboxylesterase.

In summary, substrate and inhibitor studies strongly suggested that the MonEst and ComEst species should be classified as carboxylesterases (EC 3.1.1.1) and acetylesterases (EC 3.1.1.6) respectively and that, together with distinct differences in their molecular and biochemical characteristics, it is concluded that these are unrelated myeloid enzymes which share only the ability to hydrolyse α-naphthyl acetate. Although yet to be established, the kinetic and molecular differences reported here may have fundamental relevance with respect to the biofunctional role(s) of these enzymes.

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CHAPTER ONE

GENERAL INTRODUCTION

1.1 INTRODUCTION

Cytochemical analyses of leucocyte enzyme systems represent an important supplement to traditional morphological classifications of leukaemic cells. Since the early part of this century, many cytochemical techniques have evolved for the demonstration of leucocyte lineage-associated enzymes. One of the enzyme groups of normal and neoplastic blood cells that has been extensively studied by cytochemical techniques, and more recently with biochemical and electrophoretic methodologies is the so-called "non-specific esterase" system.

Nonspecific esterases (NSE) are widely distributed in numerous cell types and in leucocytes they are demonstrated by the hydrolysis of short acyl chain esters of alpha-naphthol. Although their physiological role remains poorly understood, the cytochemical demonstration of esterase activity represents an integral part of the classification of acute leukaemia. In particular, recent biochemical studies provide substantial evidence that the specific monocytic maturational commitment from pluripotential myeloid precursors is associated with the synthesis and expression of distinct esterase isoenzyme forms. This Chapter will discuss the general concepts of myeloid cell maturation, the known cytochemical and biochemical characteristics of leucocyte esterases and the theoretical aspects of isoenzymes. Subsequent Chapters analyse the cytochemical and molecular (electrophoretic and chromatographic) characteristics of myeloid esterases, including their purification, and discuss the findings in the context of current concepts of myeloid cell differentiation programmes.

1.2 NORMAL AND LEUKAEMIC MYELOID CELL MATURATION

1.2.1 Myeloid maturation: General Concepts Conventional morphological features have proved useful for establishing cell function (particularly at the ultrastructural level) as well as for delineating patterns of maturation for normal and neoplastic cells. Traditional morphological boundaries are defined by criteria such as cell size, ratio of size of nucleus to cytoplasm, fineness of chromatin, shape of nucleus, the presence or

absence of nucleoli, the presence and type of cytoplasmic granules, and the cytoplasmic colouration of Romanowsky-stained cells. Since changes in nuclear chromatin and cell size occur during each replication stage, and as the formation of granules and other cytoplasmic constituents occur gradually during the stages of cell development, morphological definitions are necessarily arbitrary and do not always parallel biochemical or physiological changes. In some cases, it is difficult to classify a cell into one category or another, since cell development does not occur as step-like transformations but by gradual transition. Consequently, the individual myeloid differentiation stages described below should be regarded simply as 'snap-shots' of a maturational continuum. One other basic consideration is that since a cell is composed of several component parts, each must undergo transformation. In normal haemopoiesis, these transformations are simultaneous and closely associated (i.e. developmental 'synchronism'). However, abnormal and neoplastic process may be characterised by differential maturation between, particularly, the nucleus and cytoplasm. This aberrant process is referred to as 'asynchronism' or 'maturation asynchrony'.

1.2.2 Granulocytic differentiation: The earliest recognisable granulocyte precursor is the myeloblast. With progressive maturation this sequentially develops to the promyelocyte, myelocyte, metamyelocyte, 'band' form and finally the mature, functional granulocyte (polymorphonuclear neutrophil). The transformation of a myeloblast to a mature granulocyte takes 10-15 days, the greater part of this process being in the bone marrow, resulting in an eight fold increase in their number. Cellular division occurs up to and including the myelocyte stage, and this is followed by unidirectional maturation to the neutrophil. It is generally agreed that neutrophil, eosinophil and basophil precursors are indistinguishable by light microscopy until the myelocyte stage, when specific granules (i.e. neutrophilic, eosinophilic and basophilic) are formed.

1.2.2.1 Myeloblast: These cells are often indistinguishable from lymphoid precursors and comprise approximately 1-5 percent of normal marrow cells. Morphologically, the

cytoplasm is moderate in quantity and deeply basophilic due to the presence of RNA. Cytoplasmic granulation is usually insignificant but some granules may occasionally be seen in more mature myeloblast forms. As myeloblasts are mitotic and represent one of the earliest stages of myeloid development, their morphological and cytochemical appearances show considerable variation. This is particularly true in acute leukaemia where the proliferating myeloblasts may show different levels of 'maturation arrest'. Another morphological feature associated with leukaemic myeloblasts, is the presence of rod-shaped cytoplasmic inclusions derived from primary granules [White, 1967; Wetzel, 1970]. These distinctive structures, which are widely known as Auer bodies (rods), share the cytochemical characteristics of primary granules [Harada, 1951; Goldberg, 1964].

1.2.2.2 Promyelocyte: Developmental stages following the myeloblast have been classically differentiated on the basis of the number and type of granules. The 'non-specific azurophilic' or primary granules are predominantly synthesised at the promyelocyte stage and have been identified by fine structural studies as being a common characteristic of the neutrophil, eosinophil and basophil series [Ackerman, 1968; Scott & Horn 1970; Bainton et al., 1971]. Morphologically, the promyelocyte is the largest of the granulocyte precursors. The nucleus has a similar chromatin pattern to the myeloblast but the nucleoli tend to be less conspicuous. Cytoplasmic granulation is however distinctive and although the synthesis of these primary granules is restricted to promyelocytes, primary granules persist through all subsequent developmental levels including mature polymorphonuclear forms [Bainton & Farquhar, 1966; Scott & Horn 1970; Bainton et al., 1971]. Primary granules characteristically contain lysosomal enzymes such as acid phosphatase, myeloperoxidase, esterases, lysozyme, and beta-glucuronidase [Bainton & Farquhar, 1966, 1968a, b; Wetzel et al., 1967; Zeya & Spitznagel, 1969; Bainton et al., 1971]. Compared to their normal counterparts, the promyelocytes of most acute promyelocytic leukaemias (APL-M3) are typically hypergranular; the cytoplasm of these cells being densely populated with coarse, intensely staining azurophilic granules, which

tend to obscure the nuclear border. In addition, the nuclear contour is frequently irregular and in some cells the nucleus may be folded, bilobed or reniform in appearance, resembling an immature monocyte nucleus [Hillestad, 1957; Bernard et al., 1963; Sultan et al., 1973; Bennett et al., 1976]. The cytoplasm often exhibits randomly distributed bundles of Auer rods ('faggots') characteristic of a leukaemic process [Breton-Gorius & Houssay, 1973; Sultan et al., 1973; Bennett et al., 1976; McKenna et al., 1982]. A variant form of acute promyelocytic leukaemia (M3-Hypo), in which the promyelocytes show relatively little cytoplasmic granulation has also been described [Golomb et al., 1980; Bennett et al., 1980]. In common with the hypergranular form of APL-M3 however, these hypogranular promyelocytes exhibit distinctive nuclear lobulation or convolutions and patients with M3-Hypo also show a chromosomal 15:17 translocation, [Rowley et al., 1977; Van Den Berghe et al., 1979; Golomb et al., 1980].

1.2.2.3 Myelocyte: The myelocyte may be defined as the stage of granulocytic differentiation at which specific (secondary) granules appear in the cytoplasm. Consequently, these cells can be identified as being neutrophilic, eosinophilic or basophilic depending on lineage commitment. Concomitant with the production of secondary granules, the active synthesis of primary granules ceases at this maturational stage and their numbers decrease with successive mitotic divisions (mitodilution) [Ackerman & Bellios, 1955; Bainton & Farquhar, 1966]. Neutrophilic secondary (specific) granules are smaller than primary granules and are formed in increasing numbers on the convex surface and lateral borders of the somewhat less prominent Golgi cisternae [Bainton & Farquhar, 1966; Ackerman, 1968]. These granules show little peroxidase or acid phosphatase activity but do contain aminopeptidases, lysozyme, collagenase and other basic proteins [Wetzel et al., 1967; Zeya & Spitznagel, 1969; Bainton et al., 1971].

1.2.2.4 Metamyelocyte and 'Band' Forms: As the myelocyte matures to the non-mitotic metamyelocyte, the cytoplasm assumes the characteristics of mature granulocytes. The

nuclear chromatin becomes coarser, the nucleus indented and cytoplasmic granulation becomes particularly evident. The intermediate stage between the metamyelocyte and the mature polymorphonuclear granulocyte is variously described as the 'band', 'stab' or 'juvenile' form. In these granulocytes, the nuclear chromatin is further condensed but the nuclear shape, which may be curved (horseshoe) or coiled, remains non-segmented. The cytoplasm becomes progressively less basophilic and the number of organelles, other than secondary granules, decrease. This cell type is the most immature cell of the granulocyte series that may be seen in normal peripheral blood.

1.2.2.5 Polymorphonuclear Granulocytes: The mature neutrophilic granulocyte is essentially differentiated from its immediate (non-segmented) precursor by distinct nuclear lobulation. The cytoplasm lacks basophilia and contains fine secondary granules. The primary function of a mature neutrophil is phagocytosis of small foreign particles such as bacteria. The neutrophil, being a migratory cell, performs most if not all of its functions after leaving the blood stream; which it achieves via the endothelial cells of venules [De Vargas-Linares & Burgos, 1964]. Neutrophil migration is stimulated by chemotactic factors, produced in response to formation of antigen-antibody complexes, leucocyte debris and many other physiological factors. This migration occurs continuously into sites such as the oral cavity, lungs, and gastro-intestinal tract [Ambrus & Ambrus, 1959]. Neutrophil phagocytosis can occur in the absence of antibody or complement protein although, the process is greatly enhanced if the foreign particle is opsonised (i.e. coated by antibody or complement).

1.2.3 Monocytic differentiation: Monocytes develop from immature myeloid precursors in the bone marrow. Mature monocytes migrate from the blood to tissue sites such as the liver, spleen, lung, and lymph nodes where they function in their terminally differentiated state as free or fixed macrophages. Promonocytes are the earliest recognisable precursors of monocytes found in normal bone marrow. The immediate precursor of the promonocyte is not known although possible candidates include the

hypothetical monoblast, myeloblast or promyelocyte. Morphologically, the promonocyte cytoplasm is relatively basophilic, containing peroxidase positive azurophilic granules and the nucleus is folded with fine chromatin and prominent nucleoli. Granule numbers decrease with subsequent division and they are not replaced by specific granules [Hirsch & Fedorko, 1970].

Mature monocytes may be readily discriminated from other cells in Romanowsky-stained preparations. The nuclear chromatin is moderately condensed, the nucleus is indented or folded and no nucleoli are observed. Cytoplasm is abundant, grey-blue in colour and contains fine dust-like peroxidase positive granules, which give the monocyte cytoplasm its characteristic ground-glass appearance. Monocyte granules contain acid phosphatase, beta-glucuronidase and cathepsin [Cohn & Benson, 1965]. Other hydrolases such as esterase, acid ribonuclease and lysozyme are also found in monocytes though their sub-cellular location is unclear. Lysosomes (dense granules) arise by a process in which enzyme-containing vesicles (primary lysosomes budding from the Golgi apparatus) fuse with pinocytic vacuoles. They differ from the specific granules of granulocytes, which are formed at the promyelocyte stage and represent primary lysosomes. Synthesis of lysosomal enzymes by monocytes is stimulated by activation of the cell, for example during phagocytosis of biological material [Cohn & Benson, 1965; Cohn, 1970].

1.3 LEUCOCYTE ESTERASES

1.3.1 Cytochemical characteristics

1.3.1.1 Evolution of cytochemical methods: Esterases are a group of enzymes capable of hydrolysing a variety of aliphatic and aromatic esters. 'Tweens' were introduced as substrates in 1945 [Gomori, 1945] for the demonstration of esterases in fixed tissue sections, but these gave crude results and received only cursory descriptive studies. Several years later, the first azo dye coupling method for esterases, adapted from earlier methodology by Menten et al., [1944] for alkaline phosphatase, was introduced by Nachlas & Seligman [1949]. This involved the hydrolysis of beta-naphthyl acetate by

esterases present in acetone-fixed tissue sections with the simultaneous capture of the liberated naphthol by a diazonium salt. Although this resulted in a brightly coloured and insoluble azo-dye-product complex, the staining was not topographically precise as lateral diffusion occurred due to slow coupling. Gomori [1952] subsequently recommended that beta-naphthyl acetate be replaced by the alpha-isomers or by naphthol AS acetate (2-acetoxy-3 naphthoic anilide) in order to minimise diffusion artefacts. Naphthol AS acetate in particular was hydrolysed more slowly than the simpler naphthyl acetate, but yielded a highly insoluble and substantive azo-dye product, with a corresponding improvement in localisation. Other investigators [Pearse, 1954; Burstone, 1957; Gossner, 1958] achieved somewhat more precise localisation of azo-dyes, in relation to sites of enzymatic activity, by substituting the more insoluble naphthol AS derivatives for the parent compound. Wachstein & Wolf [1958] later used naphthol AS acetate as substrate and demonstrated esterase activity in all types of human blood and marrow cells. However, when using alpha-naphthyl acetate as substrate, Braunstein [1959] was able to demonstrate esterase activity only in monocytes. Subsequently, Fischer and Schmalzl [1964] showed that the esterase activity of monocytes was sensitive to sodium fluoride (NaF) inhibition whilst that of granulocytes was relatively resistant to NaF. Additionally, Gomori [1953] reported the use of chloroacyl esters as histochemical substrates for the demonstration of chloroacetate esterases. Consequently, the differing reaction patterns with various substrates, together with the observation that the predominant esterase species in monocytes but not granulocytes was NaF sensitive, strongly suggested the existence of multiple esterase forms in human blood cells.

1.3.1.2 Demonstration of cellular esterases - Reaction Principles: The cytochemical visualisation of esterases is achieved by the formation of insoluble coloured precipitates at, or near, the site of substrate hydrolysis. The most commonly employed substrates currently used for the demonstration of leucocyte esterases are acetate, propionate and butyrate esters of alpha-naphthol (ANA, ANP and ANB respectively) and naphthol AS-D chloroacetate (NASDCA). Alpha-naphthyl acetate, propionate and butyrate substrates

show progressive increases in acyl chain lengths of C2, ($CO.CH_3$), C3, ($CO.C_2H_5$) and C4, ($CO.C_3H_7$) respectively and are produced by reactions between alpha-naphthol and acetic, propionic or butyric acid. As a result of enzymic hydrolysis, free alpha-naphthol is liberated and this subsequently forms an insoluble coloured (azo-dye) product through its spontaneous coupling with a suitable and soluble diazonium salt (e.g. hexazotised pararosaniline, New Fuchsin or Fast Blue BB; Table 1.1). As diazonium salts are unstable compounds, which readily form more stable but inactive isodiazotates [Pearse, 1968], active salts need to be prepared immediately prior to use by diazotising base dyes with nitrous acid. Figure 1.1 schematically illustrates the hydrolysis of alpha-naphthyl acetate and subsequent capture of liberated alpha-naphthol by the diazonium salt to produce an insoluble azo-dye. Similar reaction principles are applicable to the hydrolysis of propionate and butyrate esters.

1.3.1.3 Esterase cytochemistry: Leucocyte esterases are divided into two main groups; the chloroacetate esterases and the so-called 'non-specific' esterases (NSE). NSE enzymes hydrolyse simple esters of alpha-naphthol although they may also act on amide derivatives [Myers, 1960] and fatty acid esters [Pearse, 1972]. In haematological cytochemistry, esterases are commonly referred to by the substrates used for their demonstration, hence the terms alpha-naphthyl acetate esterase (ANAE) and chloroacetate esterase (CAE). The NSE reaction is routinely performed using either alpha-naphthyl acetate or butyrate as substrate [Yam et al., 1971; Li et al., 1973, 1986], both of which are hydrolysed by esterases associated with cells of monocytic-lineage, megakaryocytes, platelets, lymphocytes and plasma cells (Table 1.2). The butyrate substrate has been reported to show a more specific reaction for monocytic esterases [Ainsley et al., 1971; Li et al., 1973], although macrophages, megakaryocytes and mature T-lymphocytes may also show positive reactions. Some hydrolysis of alpha-naphthyl esters by chloroacetate esterase may occur although this is only observed following prolonged incubation at an alkaline pH [Li et al., 1973; 1986].

Compared to the normally unreactive mature granulocytic elements, some myeloblasts tend to show weak cytoplasmic ANAE staining, of granular or diffuse type, which may be very weak or absent with the butyrate substrate. Monocytes and macrophages usually exhibit intense diffuse cytoplasmic staining with both acetate and butyrate substrates, the reaction being relatively sensitive to sodium fluoride (NaF) inhibition [Li et al., 1973]. Electron microscopic (EM) studies have revealed that the distinctive esterases of monocytes are localised on the external surface of the plasma membrane and are thus ectoenzymes [Monahan et al., 1981]. In contrast to monocyte esterases, lymphocyte esterases appear to be associated with clusters of smooth membrane vesicles in the cytoplasm [Marec et al., 1981; Monahan et al., 1981; Zicca et al., 1981], and cytochemical reactivity of T-lymphocytes is usually seen as one to three discrete 'dots' in the cytoplasm. This dot or focal type reaction observed in lymphocytes has been correlated with mature helper-T-lymphocytes, exhibiting CD2, CD3 and helper-T-cell antigen (CD4) [Zicca et al., 1981; Bernard & Dufer, 1983]. B-lymphocytes usually show negligible NSE cytochemical staining as do leukaemic B- and T-lymphoblasts. Megakaryocytes may also show an intense diffuse cytoplasmic reaction with the acetate ester but a much weaker reaction with the butyrate substrate [Li et al., 1973]. Platelets show similar ANAE staining patterns to monocytes; again the reaction products have been shown by EM to be localised to the external surface of the plasma membrane [Bozdech & Bainton, 1981; Boesen, 1984].

1.3.1.4 Diagnostic value of NSE cytochemistry in leukaemia: The cytochemical demonstration of leucocyte alpha-naphthyl acetate (ANAE) or butyrate (ANBE) esterase reactivity is widely used for the delineation of haemopoietic cells showing monocytic differentiation. ANAE/ANBE reactivity of normal and leukaemic monocytes is characteristically of a moderate to strong diffuse type and is invariably weak or absent in normal granulocytic components. Consequently, this cytochemical procedure is widely used for the identification of monocytic components in acute monocytic leukaemia (AMoL-M5), acute myelomonocytic leukaemia (AMML-M4) and chronic

myelomonocytic leukaemia (CMML). Although these enzymes are reliable markers for the demonstration of monocytic involvement in myeloid leukaemias, recent studies have shown that monocytic components in approximately 15% of morphologically and immunologically defined AMoL-M5 cases show weak or negative ANAE and ANBE reactions [Hayhoe & Cawley, 1972; Rodgers et al., 1982; Scott et al., 1983, 1985, 1987; Milligan et al., 1984]. Furthermore, whilst normal myeloblasts and cells of granulocytic lineage typically lack demonstrable ANAE or ANBE activity, weak to moderate ANAE reactivity is often seen in leukaemic myeloblasts and the promyelocytes of acute promyelocytic leukaemia (APL-M3 hypergranular variant) [Bennett et al., 1976, 1980; Scott et al., 1989]. As atypical esterase cytochemistry in acute leukaemias may be difficult to interpret, in terms of diagnostic classification and assessment of lineage, a number of modifications to standard cytochemical procedures have been introduced. These include both the use of alternative short acyl chain (C2-C4) substrates and the incorporation of enzyme inhibitors. For example, it has been suggested that the use of alpha-naphthyl butyrate may be more definitive than acetate for the demonstration of monocytic esterase activity [Ainsley et al., 1971; Li et al., 1973], and the incorporation of sodium fluoride in the staining procedure has been promoted as a specific inhibitor of monocytic esterase activity [Li et al., 1973; Hayhoe & Quaglino, 1980].

In addition to standard esterase (NSE) cytochemical procedures, it is also possible to simultaneously demonstrate ANAE and chloroacetate esterase (CAE) on the same blood or bone marrow film. This is most effectively achieved by sequentially staining for the two esterase enzyme types with different colours in two separate incubation mixtures [Yam et al., 1971]. The technique is of particular value in observing the atypical esterase staining of granulocytes in the hypergranular variant of acute promyelocytic leukaemia and some myelodysplastic syndromes (MDS). In hypergranular AML-M3, the promyelocytes often stain for both ANAE and CAE [Grusovin & Castoldi, 1976; Rydell, 1979; Bennett et al., 1980; Stavem et al., 1981; Scott et al., 1983; Tomonaga et al., 1985], a phenomenon rarely observed in other leukaemias.

1.3.2 Biochemical characteristics

1.3.2.1 Introduction: Early attempts at classifying esterases were largely based on their ability to hydrolyse aliphatic (ali-esterases) or aromatic (arom-esterases) esters. Aldridge [1953] subsequently extended this by proposing the categorisation of esterases based on their relative resistance or sensitivity to organophosphorous compounds. In this way, esterases which were not inhibited by organophosphates were designated 'A-esterases' and those that were sensitive were classified as 'B-esterases'. Additionally, a third type known as 'C-esterases' were described [Kirsch, 1971] and these were defined by both their resistance to organophosphates and their preferential hydrolysis of acetic acid esters. The biochemically-based Enzyme Commission [IUPAC-IUB, 1978] classification for enzymes acting on ester bonds has recommended their subdivision into various parent groups (EC 3.1.1 through 3.1.31) depending on the chemical nature of the ester bond being hydrolysed. The histochemically important esterases are grouped under the generic title, 'carboxylic ester hydrolases' (EC 3.1.1) and are further categorised into three main groups; carboxylesterases (EC 3.1.1.1), arylesterases (EC 3.1.1.2) and acetylesterases (EC 3.1.1.6) based on the type of substrate being hydrolysed and sensitivity to various inhibitors (Tables 1.3 and 1.4). In general, ali- or B-esterases are broadly represented by the carboxylesterases, the arom- or A-esterases by arylesterases and the C-esterases by acetylesterases. In addition, carboxylesterases characteristically hydrolyse straight-chain (aliphatic) esters, as well as naphthol esters, arylesterases hydrolyse cyclic (aromatic) esters and (strictly speaking) esters of phenol, whereas acetylesterases preferentially hydrolyse esters of acetic acid and are resistant to the action of organophosphates and sulphydryl blocking agents.

Classifications based on substrate characteristics are clearly valid for most enzyme systems. However, due to their wide substrate specificity, the sub-categorisation of esterases may be of limited value [Pen & Beintema, 1986; Wienker & Deimling, 1987]. According to the recommendations of the IUB [1978], most leucocyte esterases would be

categorised as carboxylesterases (EC 3.1.1.1) as they hydrolyse carboxylic esters and do not share the inhibitor/activator characteristics of the other groups of histological esterases described by EC 3.1.1 (Table 1.3 and 1.4) [Holmes & Masters, 1967; Oertel & Kastner, 1984]. In haematological terms, the rather vague term 'nonspecific esterase' (NSE) is applied to all enzymes which hydrolyse simple esters (e.g. alpha-naphthyl acetate), even though their cellular origins and cytochemical reactivities may vary considerably. For the purposes of this current study, and in order to avoid unnecessary confusion, the so-called 'nonspecific' and 'specific' leucocyte esterases described in the text will subsequently be referred to as alpha-naphthyl acetate (or butyrate) esterases (ANAE/ANBE) and 'chloroacetate esterases' (CAE) respectively. The other groups of carboxylic ester hydrolases such as acetylcholinesterases (EC 3.1.1.7) and cholinesterases (EC 3.1.1.8) may be of some histochemical interest, but their concentration in leucocytes is generally low [Zajicek et al., 1954].

1.3.2.2 Substrate reactivities of esterases: Using the combined techniques of zone electrophoresis and histochemical staining [Hunter & Markert, 1957], multiple isoenzyme forms of esterases have been shown to occur in the human liver [Paul & Fottrell, 1961], brain [Barron et al., 1963; Lagnado & Hardy, 1967], muscle [Ecobichon & Kalow, 1965], kidney [Ecobichon & Kalow, 1964], red cells [Tashian, 1961, 1969], leucocytes [Shows, 1972a; Li et al., 1973; Radzun et al., 1980; Yourno & Mastropaolo, 1981] and plasma [Harris et al., 1962]. In certain cases, these isoenzymes are relatively specific for particular substrates and are identified as tissue-specific enzymes (e.g. cholinesterases and acetylcholinesterases). However, the majority of esterases exhibit broad and frequently overlapping inhibitor and substrate reactivities (Tables 1.3 and 1.4). Indeed, not only do esterases act on ester bonds but they may also catalyse the hydrolysis of amide bonds and be involved in processes of transacylation [Myers et al., 1957; Mentlein et al., 1980]. Consequently, there may occasionally be difficulties in assigning an esterase to a particular enzyme group since it may display properties characteristic of more than one group. For example, some proteolytic enzymes such as trypsin (EC 3.4.21.4)

and chymotrypsin (EC 3.4.21.1) are capable of hydrolysing carboxylic esters and can be inhibited by esterase inhibitors such as diisopropyl fluorophosphate and diethyl-p-nitrophenyl phosphate [Dixon et al., 1958].

Leucocyte-associated esterases in their strictest sense catalyse the hydrolysis of uncharged carboxylic esters [Kirsch, 1971] but may also act on esters of fatty acids or glycerol [Nachlas & Seligman, 1949], a property usually associated with lipases. Despite these overlaps in substrate specificity, carboxylesterases may be regarded, in general terms, as enzymes which act on esters of short-chain glyceryl and simple monoesters (acyl chain-lengths of C2-C4) whereas lipases act on longer chain (fatty acid) esters (C8 or more) [Khoo & Steinberg, 1975].

1.3.2.3 Inhibitor studies of esterases: Due to the heterogeneous nature of esterases and lack of knowledge of physiological substrates, their characterisation has been additionally dependent on inhibitor studies. The classification of arylesterases, carboxylesterases and acetylesterases is traditionally based on their activities in presence of organophosphates such as diisopropyl fluorophosphate (DFP) or diethyl p-nitrophenyl phosphate (E600) [Dixon & Webb, 1964; Chayen et al., 1969]. Organophosphate inhibition appear to result from the ability of these compounds to compete with the ester substrate for binding to the active site of the enzyme. Thereafter, instead of an enzyme-acyl complex formation, as is the case with the normal substrate, the enzyme becomes phosphorylated. The rate at which the phosphorylated enzyme can be hydrolysed is much slower than the acylated form consequently, resulting in inhibition. With weaker inhibitors, this hydrolysis does occur at a measurable rate and the enzyme may eventually become fully active again. On the basis of organophosphate inhibition, carboxylesterases are generally sensitive whereas acetylesterases and arylesterases are resistant. For comparison, trypsin, chymotrypsin, plasmin and thrombin are also sensitive to these organophosphate compounds.

Sodium fluoride (NaF) is the most widely investigated inhibitor of esterase cytochemical activity. Early observations indicated that the inhibitory action of this compound could be used to specifically differentiate cells of monocytic lineage. However, the atypical esterase cytochemical activity of some myeloblastic leukaemias may also be inhibited at higher NaF concentrations (1.0mg/ml) [Li et al., 1973], even though there are clear differences in monocytic and non-monocytic reactions when examined according to relative inhibitor sensitivity. Indeed, detailed inhibition studies of monocytic esterase isoenzymes, separated by electrophoretic techniques, indicate a thirteen-fold increased sensitivity of these isoenzymes to NaF as compared to isoenzyme forms of higher pI (Section 1.3.2.4) common to both monocytes and granulocytes [Scott et al., 1984a, b, c]. Consequently, the specificity of NaF inhibition for monocytic esterases should be regarded as a 'relative' (rather than specific) phenomenon because both myeloid esterase activities (monocyte-associated and common) have been demonstrated to be inhibited by this compound [Scott et al., 1984a, b, c; Drexler et al., 1985]. NaF inhibition of myeloid esterase activity has been found to be reversible, but it is not known whether NaF directly inactivates the 'native' esterase or acts on the acylated enzyme [Scott et al., 1984c]. For comparison, chloroacetate esterases are resistant to NaF inhibition [Moloney et al., 1959; 1960].

Other compounds, such as sodium taurocholate, have been used [Burstone, 1962] to differentiate (by activation rather than inhibition) lipases and esterases, and cholinesterases may be further characterised by their relative sensitivity to eserine [Pearse, 1972]. Sulphydryl blocking reagents such as p-Hydroxymercuribenzoic acid (HMBA) and related compounds have also been reported to inhibit arylesterases and activate acetylesterases [Burstone, 1962; Webb, 1966; Holmes & Masters, 1967; Bulmer & Fisher, 1970].

1.3.2.4 Haemopoietic cell esterase isoenzymes: Distinct differences in the esterase cytochemical staining patterns of normal and neoplastic blood cells stimulated a number

of biochemical and electrophoretic investigations which were designed to assess whether or not these variable cytochemistries could be attributed to differences in esterase isoenzyme distributions.

Li et al., [1973] first detailed the substrate specificity and cellular distribution of leucocyte esterases separated by polyacrylamide gel electrophoresis (PAGE) at pH 4.0. A total of nine different enzyme bands, displaying esterolytic activity, were resolved using this technique. Bands 1, 2, 7, 8 and 9 were best demonstrated with naphthol AS-D chloroacetate as substrate (CAE activity), and bands 3, 4, 5, and 6 preferentially hydrolysed alpha-naphthyl esters. With the recognition of multiple isoenzyme forms, alternative methods were applied in an attempt to clarify the esterase isoenzyme distribution of normal and neoplastic cell lineages. Other investigators, using the techniques of vertical or horizontal PAGE confirmed and extended the findings of Li and colleagues [Kass & Peters, 1978; Yourno & Mastropaolo, 1981]. These standard electrophoretic techniques, while demonstrating for the first time major differences between esterases of different normal and leukaemic cell types, also revealed areas of diffuse staining suggesting the possible occurrence of additional unresolved components [Kass & Munster, 1979a; 1979b].

The introduction of isoelectric focusing (IEF), using ampholyte carriers to generate pH gradients within a polyacrylamide gel, considerably improved the resolution of individual isoenzyme components which were otherwise not resolved by conventional electrophoretic methods. Using the high-resolution IEF technique, multiple esterase isoenzymes with differing isoelectric points (pI) have been demonstrated and shown to exist in a cell-associated manner [Radzun et al., 1980; Radzun & Parwaresch, 1980; Scott et al., 1984a; Oertel & Kastner, 1984; Cohn et al., 1987]. Composite IEF zymograms for most haemopoietic elements have been documented by a number of independent investigators [Radzun et al., 1980; Parwaresch et al., 1981; Scott et al., 1984a, b; Cohn et al., 1987]. Whilst minor discrepancies have been reported in esterase isoenzyme

distribution profiles, these differences could largely be attributed to differences in experimental procedures. Of particular importance, was the finding that the cytochemical esterase activity of normal and leukaemic monocytes (and macrophages) was specifically associated with a group of acidic esterase isoenzymes within a narrow pI range of 5.5 to 6.2. In contrast, esterase zymograms of purified granulocytes were reported to show up to 11 isoenzyme forms displaying a broader pI range of 6.3 to 8.3. These latter isoenzymes were additionally found to be expressed by other myeloid cell types [Radzun et al., 1980].

Fractionation of esterases from leukaemic monocytes, by a combination of anion- (DEAE, pH 7.5) cation-exchange (CM, pH 5.0) chromatography, has been reported to result in four peaks of enzyme activity, each displaying differing substrate specificities [Lam et al., 1978]. Attempts have also been made to determine the molecular weights of different esterase enzyme species, using various electrophoretic and gel filtration techniques. However, many of the initial studies examined animal liver carboxylesterases and molecular weights of 160-170 kDa are frequently quoted [reviewed by Krisch, 1971]. Considerably lower molecular weights, in the region of 50-60 kDa, have also been reported for animal carboxylesterases [Barker & Jencks, 1969; Ecobichon, 1969; Heymann et al., 1971; Cohn et al., 1987] but inter-relationships between the two molecular weight species remains poorly understood.

1.4 ISOENZYMES: THEORETICAL CONSIDERATIONS

1.4.1 Introduction: Enzymes often occur as various molecular forms, each of which may display differing physicochemical properties. The original terminology used to describe these different forms of the same enzyme was isozymes [Markert & Moller, 1959], this was later changed to isoenzymes [Wroblewski & Gregory, 1961]. Isoenzymes essentially describe enzyme molecular and/or biochemical heterogeneity. This protein heterogeneity can occur as a result of various factors and these may be categorised into two main types; (a) genetic or primary factors, where multiple genes

encoding different enzyme subunits are present, or (b) post-translational or secondary factors, where homogeneous enzymes or subunits comprising the active enzyme, are modified differentially leading to the synthesis of multiple isoenzyme forms from a single gene product. The current definition recommended by the Commission of Biological Nomenclature [1977] states that the term isoenzyme should only be used to describe multiple forms of enzymes arising within a single species from genetically determined differences in the primary structure. Strictly speaking, this therefore excludes multiple enzyme forms derived from post-transcriptional or post-translational modifications, which are often referred to as secondary isoenzymes.

1.4.2 Primary isoenzymes: Primary isoenzymes are genetically derived, either as a result of multiple allelism at a single gene locus, determining structurally distinct versions of a particular polypeptide chain, or as a result of multiple gene loci, coding for structurally distinct polypeptide chains of the enzyme.

1.4.2.1 Multiple allelism: In a diploid genome, each gene locus possesses two alleles for which an individual may be homozygous (both alleles identical) or heterozygous (both alleles different). Homozygous individuals will produce a single species of protein, this enzyme varying from individual to individual, depending on the alleles present at the locus in question [Harris & Hopkinson, 1976]. In the heterozygous individual the two different allelic variants encode two different polypeptide chains thus in heterozygous individuals monomeric enzymes can be present as two different isoenzyme forms, dimeric enzymes as three isoenzyme forms, trimeric as four forms and so on assuming unrestricted random association of subunits. Within any individual, the degree of enzyme heterogeneity resulting from multiple allelism is limited. However, intra-species variations may be considerable depending on the variety of allelic forms, for the locus, present in the species gene pool. Isoenzymes resulting from multiple allelism are also referred to as "allozymes".

1.4.2.2 Multiple gene loci: Although multiple gene loci coding for structurally distinct isoenzymes are well established for many enzyme systems, their occurrence defies to some extent the normal biological regulatory mechanisms which occur during gene duplication [Markert, 1987]. For example, it is known that mutations can arise during gene duplication, resulting in the formation of variant genes. In general however, only one copy of each structural gene is allowed to be present in the genome and consequently, the replicate variant is destroyed through specific degradation if it is not a perfect copy of the original gene. In the case of multilocus isoenzyme systems these replicate variant genes are preserved and furthermore, are thought to acquire the status of independent regulation. Survival of the replicated genes appears to be enhanced by translocation to a different part of the genome, either within the same chromosome or to a different chromosome. It is possible that a new genomic position facilitates independent regulation. Isoenzymes derived by way of multiple gene loci therefore may not only display structural and physicochemical differences but, more significantly, may be synthesised in a cell or tissue specific manner [Markert & Moller, 1959; Markert, 1963; 1964]. These characteristics are attributed to the belief that replicate genes acquire the status of independent regulation.

In a multilocus isoenzyme system, each gene locus encodes a related but distinct polypeptide chain. For monomeric enzymes, the isoenzyme forms generated are usually distributed in different cellular compartments; examples of this include cytoplasmic and mitochondrial isoenzyme forms of malate dehydrogenase, isocitrate dehydrogenase, superoxide dimutase, glutamate oxaloacetate transaminase and fumarate hydratase [Harris & Hopkinson, 1976]. In all these cases the cytoplasmic and mitochondrial forms are encoded by separate genes and the gene loci for these enzymes may be located on different chromosomes, as in the case of human isocitrate dehydrogenase. For this particular enzyme, the soluble cytoplasmic form is encoded by a gene on chromosome 2 and the mitochondrial form by a gene on chromosome 15 [Shows, 1972b; Turner et al., 1974].

For oligomeric enzymes, each different gene locus encodes a distinct subunit of the functionally active enzyme. The subunit associations in these enzymes can then lead to variant forms of true isoenzymes displaying different biochemical and physicochemical properties [Moss, 1982]. The number of isoenzyme forms generated effectively depends on the number of gene loci in the particular multilocus system, and also the numbers of subunits required to produce an active enzyme molecule. For example, in the multilocus system encoding the isoenzymes of lactate dehydrogenase (LDH), which is a tetrameric enzyme, there are three separate gene loci which give rise to 15 different isoenzymes (i.e. three homogeneous and 12 heterogeneous). Thus, if the subunits of LDH are designated A, B, and C, the subunit composition of the possible isoenzymes (assuming random subunit association) would be A4, A3B, A2B2, AB3, B4, A3C, A2C2, AC3, C4, B3C, B2C2, BC3, ABC2, A2BC, and AB2C.

The complexity of isoenzyme forms may be further compounded if allelic variation occurs in addition to multiple loci. Again using vertebrate LDH as an example, there are allelic variants of subunits A and B. An organism doubly heterozygous for these variants could synthesise five different subunits and consequently a much larger range of tetrameric isoenzymes.

The main difference between multiple allelism and gene loci as causes of isoenzyme formation is that multiple allelism results in differences in the pattern of isoenzymes of individual members of a species, while multiple gene loci are in general, common to all members of a species.

1.4.3 Secondary isoenzymes: Isoenzymes generated by modification at post-translational level, rather than at genetic level, are not true isoenzymes and are generally referred to as secondary isoenzymes [Shaw, 1969]. These post-synthetic modifications, either by addition of carbohydrate residues, limited proteolysis, or covalent modification of amino acid side chains may all lead to the generation of multiple molecular enzyme

forms with apparent differences in molecular charge, molecular weight, substrate specificity, and inhibitor characteristics. Post-synthetic alterations, leading to heterogeneity in homogeneous proteins, is a well documented phenomenon [Multiple Molecular Forms of Enzymes, 1968; Williamson et al., 1973] and the Sections below briefly outlines some of the most commonly encountered post-translational modifications which can lead to secondary isoenzyme formation.

1.4.3.1 Deamidation: Secondary isoenzymes may be derived from deamidation ($-CONH_2 \rightarrow -COO^-$) of asparagine or glutamine residues. This alteration causes a change in the net negative charge and consequently, isoenzymes resulting from deamidation may be detected by electrophoretic techniques (particularly isoelectric focusing). Microheterogeneity observed for muscle aldolase fully illustrates secondary isoenzyme generation as a result of deamidation. In vertebrates, aldolase is encoded by three genetic loci and although only one of these, the alpha locus, is expressed in skeletal muscle, two closely related but different subunits alpha and beta can be isolated from the tissue on the basis of charge. Tryptic maps of separated peptides show extensive regions of homology for the two subunits [Koida et al., 1969] and the alpha subunit is thought to be the original gene product which is converted, with time, to the beta subunit by deamidation of an asparagine residue near the C-terminus [Koida et al., 1969; Lai et al., 1970; Midelfort & Mehler, 1972]. Deamidation of glutamine residues may occur in other enzyme systems including L-amylase [Lehrner & Malacinski, 1975; Karn et al., 1975; Doane et al., 1975], lysozyme [Robinson & Tedro, 1973], arginine kinase [Blethen, 1972], and carbonic anhydrase C [Lin & Deutsch, 1972]. Human L-amylase is encoded by two different gene loci; AmyA is demonstrated as being active in the salivary glands and AmyB in pancreatic cells. The salivary form is both deamidated and deglycosylated and shows different electrophoretic and gel filtration profiles from the pancreatic enzyme, which is deamidated only.

1.4.3.2 Proteolysis: Multiple molecular enzyme forms, resulting from limited proteolysis of the parent enzyme protein, may also be considered as secondary isoenzymes. In most instances, trypsin is the enzyme involved in proteolytic cleavage. Isoenzymes derived by way of limited proteolysis, which occur in higher and lower molecular weight forms, include those of the enzyme galactosyl transferase [Magee et al., 1974], neuraminidase [Francus et al., 1975], pyruvate kinase [Marie et al., 1977] and beta-glucuronidase [Dean, 1974; Keller & Touster, 1975; Stahl & Touster, 1971]. The enzyme system of beta-glucuronidase has been extensively studied with respect to its molecular structure and genetics. The active mammalian enzyme exists as a tetrameric glycoprotein of molecular weight 300 kDa. However, six different isoenzyme forms have been demonstrated exhibiting molecular weights ranging from 260 to 470 kDa [Swank & Paigen, 1973]. These differences in molecular weights have been shown to be due to varying amounts of a glycoprotein, egasyn which is associated with the enzyme [Swank & Paigen, 1973]. This glycoprotein is thought to be complexed with glucuronidase when the enzyme is membrane-bound and is removed by tryptic proteolysis when the enzyme is lysosomal [Lusis & Paigen, 1977]. Cleavage of single amino acid residues may also lead to the generation of secondary isoenzymes, as illustrated by the alkaline phosphatase isoenzymes in E. coli. Here, three isoenzymes of alkaline phosphatase have been demonstrated, each derived by step-wise proteolysis of the amino-terminal arginine residue from each of the identical subunits of the active dimer.

1.4.3.3 Glycosylation: Secondary isoenzymes resulting from variations in the carbohydrate content may be subdivided into (a) isoenzymes displaying different electrophoretic or charge characteristics and (b) isoenzymes displaying different affinity chromatography binding characteristics. Enzyme heterogeneity in the first group of isoenzymes results from the differential presence of acidic or basic carbohydrate moieties, especially neuraminic (sialic) acid, whereas in the second group enzyme heterogeneity is caused by differences in the incorporation of neutral sugar residues (glucose, fucose and

mannose). Numerous isoenzyme systems exist which can be associated with differences in sialic acid content. In general, an increase in the number of sialic acid residues per molecule leads to an increase in net negative charge. Conversely, treatment with neuraminidase leads to a decrease in the number of sialic acid residues producing an increase in the net positive charge and, consequently, a higher isoelectric point (pI). This effect has been demonstrated for the isoenzyme systems of acid phosphatase [Smith & Whitby, 1968; Ostrowski et al., 1970] and catalase [Jones & Masters, 1972]. However, even though variations in sialic acid content can result in marked charge heterogeneity, the molecular weight differences are usually negligible.

Numerous multiple molecular forms of enzymes differ in their content of neutral sugars, such as glucose and mannose [Swallow, 1977]. Isoenzymes resulting from differences in the content of neutral sugar residues can be demonstrated using affinity chromatography media (e.g. *Concanavalin-A, Lentil lectin* and *Wheatgerm lectin*). Some of these isoenzyme forms have been shown to be tissue restricted; for example, adenosine deaminase and esterase extracted from human liver and several other organs bind to Con-A, whereas both enzymes from erythrocytes do not [Swallow, 1977].

1.4.3.4 Oxidation/reduction of thiol groups: The oxidation or reduction of thiol groups ($SH \leftrightarrow S^-$), situated either within or outside the active site of an enzyme, may lead to the generation of secondary isoenzymes. Modification of thiol groups which are part of the active site will usually cause changes to enzyme activities (substrate specificities/inhibitor susceptibilities). Thiol groups located away from the active site may undergo several different reactions with no apparent change in enzymic activity. Such reactions include the formation of intramolecular S-S bonds and the reaction of SH groups with low-molecular weight disulphides. The formation of mixed disulphides between a low-molecular weight thiol compound such as glutathione and free SH groups of an enzyme may lead to alteration of molecular charge and weight. The formation of intramolecular S-S bonds can lead to polymerisation and hence a considerable

heterogeneity of both molecular charge and weight. The enzyme activity may also be altered in these isoenzymes depending on the accessibility of substrate to the active sites of the polymerised molecule.

1.4.3.5 Other post-translational changes: When the post-translational modification process is very active in some tissues, compared to others, the result will be an apparent tissue specific distribution of secondary isoenzymes (mimicking the effect of multilocus isoenzymes). An example of this type of post-translational modification is seen with pyruvate kinase (PK). The subunits of PK are encoded at three independent genetic loci in mammals and the active enzyme is a tetramer. One of the subunits, the L-type (also referred to as type I), is localised in liver and erythrocytes. As the liver L-type PK subunit, but not the erythrocyte form, undergoes rapid proteolytic cleavage, the L-type isoenzymes associated with these two tissue sites differ from each other in molecular size (gel filtration chromatography) and electrophoretic charge (isoelectric focusing) [Ibsen, 1977]. As PK plays an important role in the regulation of glycolysis, these two L-type subunits which show different regulatory properties are likely to be of physiological relevance.

Conformational isomerism has also been suggested as a possible cause of isoenzyme formation. In this particular case, multiple molecular forms have identical primary structures but differ in their tertiary and quaternary structures because their polypeptide chains can exist in more than one stable configuration. If several multiple molecular forms represent a series of conformers, it may be assumed that any single member of the series could generate the complete set of isoenzymes. Mitochondrial malate dehydrogenase and erythrocyte acid phosphatase both provide possible examples of this phenomenon [Epstein & Schechter, 1968; Schechter & Epstein, 1968].

When interpreting electrophoretic (isoelectric focusing and gradient gel electrophoresis) and chromatographic (ion-exchange, gel filtration and affinity) characteristics of secondary isoenzymes it is of fundamental importance to establish that the apparent

observed heterogeneity is genuine (present *in-vivo*) and not artefactual (resulting from *in-vitro* extraction procedures). For example, extraction techniques which involve subcellular organelle disruption may expose the enzyme of study to proteases or glycosidases, thus leading to apparent heterogeneity which would not be observed *in-vivo* in their compartmentalised state. In addition, extraction procedures that utilise agents such as detergents may lead to artefactual heterogeneity as a result of differences in solubilisation.

1.5 PRESENT STUDY

Previous studies of myeloid cell esterases suggest that granulocytic and monocytic myeloid differentiation is associated with the expression of distinct isoenzyme zymograms. Traditional histochemical assessments, together with analyses of their biochemical characteristics following partial purification, provide further indications of differences in both the reactivity of these isoenzymes with substrate esters of short acyl chain lengths and their sensitivity to inhibitors. Whilst these observations provide circumstantial evidence that granulocyte- and monocyte-associated esterases are of different origin and possibly encoded by separate structural genes, more definitive proof requires studying the molecular and biochemical characteristics of extensively purified enzyme forms. Indeed, it has been suggested [Yourno et al., 1984] that these various esterase isoenzyme components could result from post-translational modifications of a common gene product which 'inter-converts' as a consequence of changes in myeloid differentiation programmes. In order to further investigate these possibilities, this study proposes to undertake an extensive analysis of normal and leukaemic myeloid cell esterases in order to (a) confirm the relationships between myeloid cell differentiation and esterase isoenzyme patterns, and (b) to specifically analyse by chromatographic, molecular and kinetic techniques whether or not the esterase isoenzyme species that are expressed by granulocytic and monocytic cells represent different gene products or are variant post-translational forms derived from the same gene product.

To achieve this, the study is divided into three main Sections. In the first part, a representative number of normal and leukaemic granulocytic and monocytic cell fractions will be investigated to assess the distribution of esterase isoenzymes with respect to cell-lineage and level of maturation. Comparisons will be then be undertaken to establish potential correlations between esterase isoenzyme distributions and cytochemical staining patterns; an area or particular importance both to the understanding of esterase regulation and for diagnostic interpretation.

The second part of the study will extensively evaluate the chromatographic characteristics of esterase isoenzyme species. This will include gel filtration, ion-exchange (anion and cation), lectin and non-lectin affinity chromatography, and hydrophobic interaction. The recognition of molecular differences between the various myeloid esterase isoenzyme forms by such techniques is particularly relevant to the final Section which applies these procedures to the full-scale purification of the main esterase species. Purified esterase components will then be characterised with respect to their molecular, biochemical, and kinetic (including inhibition) properties. Where possible, these studies will include the determination of a limited N-terminal amino acid sequence of the purified protein, which could subsequently enable the design of specific DNA probes for a more discriminating approach to the classification myeloid leukaemias. Additionally, as myeloid esterases are glycoproteins [Cohn et al., 1987], the study will also include analysis of the glycan structure of the two esterase species through specific lectin affinity binding. Collectively, the molecular and biochemical studies will enable a better understanding of the inter-relationships between myeloid esterase isoenzyme forms.

CHAPTER 1

Figure 1.1 and Tables 1.1 to 1.4

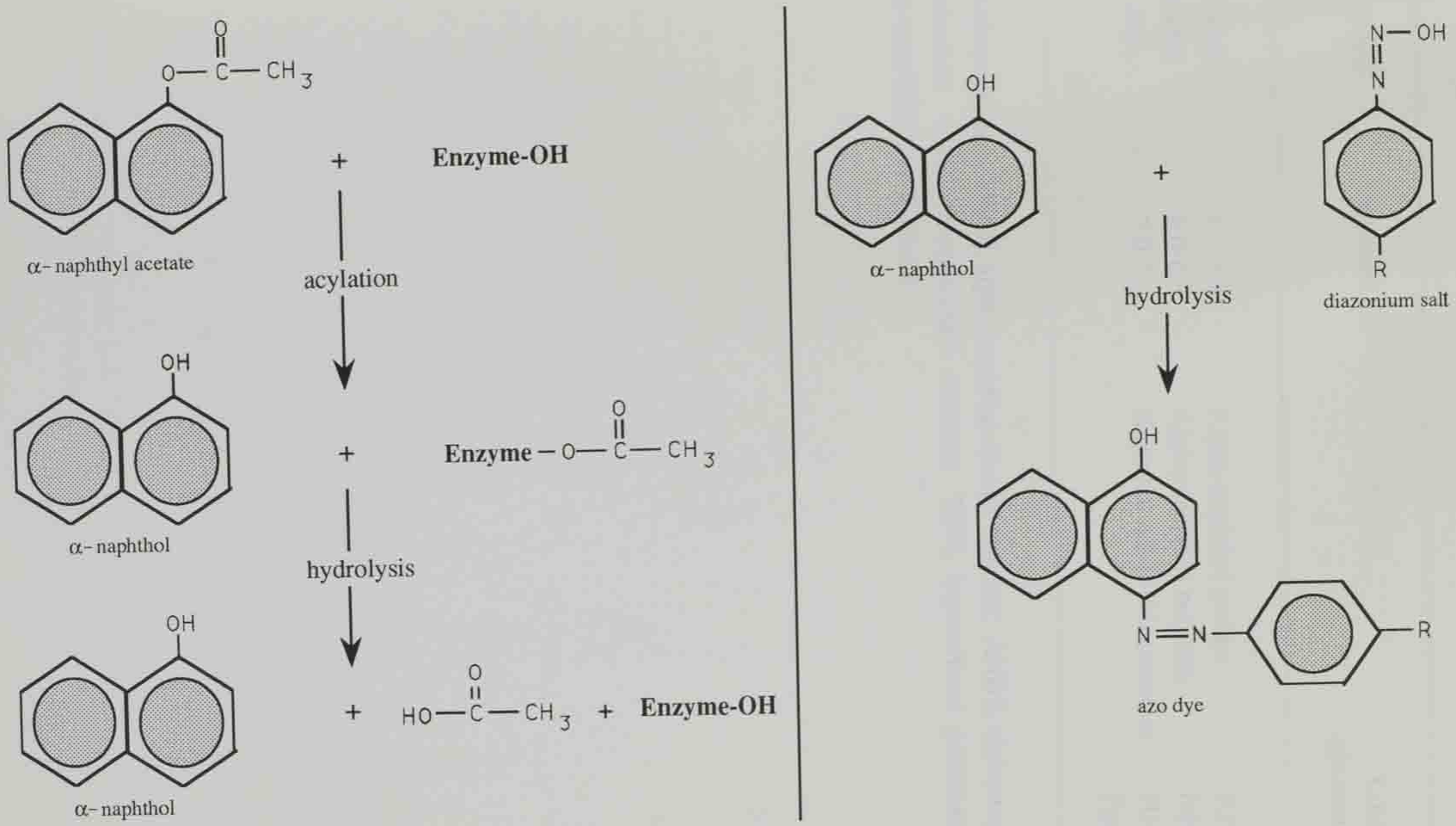

Figure 1.1: *Hydrolysis of alpha-naphthyl acetate by the enzymic action of leucocyte alpha-naphthyl acetate esterase (ANAE), resulting in the liberation of alpha-naphthol. In the absence of a diazonium salt, alpha-naphthol will either diffuse from the site of hydrolysis or react with organic acid to reform the naphthol ester. In the cytochemical reaction mixture, free naphthol combines spontaneously with a diazonium salt to form an insoluble azo-dye complex at, or near to, the site of hydrolysis.*

Table 1.1 Esterase substrates and diazonium salt couplers commonly used for the cytochemical demonstration of esterases in haemopoietic cells

Esterase	Optimum pH	Substrate	Coupler (diazonium salt)
ANAE	6.0-6.3	Alpha-naphthyl acetate	HPR
ANBE	6.0-6.3	Alpha-naphthyl butyrate	HPR
CAE	7.0-7.6	Naphthol AS-D chloroacetate	HNF or Fast Blue BB

Abbreviations: ANAE, alpha-naphthyl acetate esterase; ANBE, alpha-naphthyl butyrate esterase; CAE, chloroacetate esterase; HPR, hexazotised pararosaniline; HNF, hexazotised New Fuchsin.

Table 1.2 Esterase cytochemistry of normal and leukaemic blood elements

Leucocyte-type	Leucocyte Esterase [a]		
	ANAE	ANBE	CAE
Normal myeloid elements			
Neutrophils	0	0	+++
Eosinophils	0	0	0
Basophils	0	0	0/+
Monocytes	++/+++	++/+++	0/+
Megakaryocytes	++/+++	0/+	0
Normal lymphoid elements			
T-helper	+/++ (F)	+/++ (F)	0
T-suppressor	0	0	0
B-cells	0	0	0
Plasma cells	++/+++	+	0
Leukaemic myeloid elements [b]			
AML-M0	0	0	0
AML-M1	0	0	0
AML-M2	0	0	0/+/++
APL-M3 Hyper	0/+/++/+++ (G)	0	+++
APL-M3 Hypo	0	0	0/+
AMML-M4	+/++ [c]	+/++ [c]	+/++
AMoL-M5	++/+++	++/+++	0
AML-M6	+++	0	0
AML-M7	+++	0/+	0
Leukaemic lymphoid elements			
T-lymphoblasts	0/+(F)	0/+(F)	0
B-lymphoblasts	0	0	0

[a] Leucocyte esterase ANAE (alpha-naphthyl acetate esterase), ANBE (alpha-naphthyl butyrate esterase) and CAE (chloroacetate esterase) reactivities graded from 0 (negative) to +++ (strong); (F) indicates a focal reaction and G indicates granular reactivity.

[b] Leukaemic myeloid cells categorised in accordance with the FAB classification [Bennett et al., 1976, 1980].

[c] ANAE and ANBE reactivities in AMML are predominantly restricted to the monocytic component of the leukaemic population.

Table 1.3 Inhibition characteristics of histochemically important carboxylic ester hydrolases

	INHIBITORS [a]			
	DFP (10^{-4}M)	HMBA (10^{-3}M)	Eserine (10^{-5}M)	Ach I (10^{-3}M)
Carboxylesterases (EC 3.1.1.1)	+	-	-	-
Arylesterases (EC 3.1.1.2)	-	+	-	-
Acetylesterases (EC 3.1.1.6)	-	A	-	-
Acetylcholinesterases (EC 3.1.1.7)	+	-	+	+
Cholinesterases (EC 3.1.1.8)	+	-	+	-

[a] Inhibitors: DFP, diisopropyl fluorophosphate; HMBA, p-hydroxymercuribenzoic acid; and Ach I, acetylcholine iodide. + indicates enzyme inhibited; - indicates enzyme resistant; and A indicates enzyme activated.

Table 1.4 Hydrolysis of alpha-naphthyl esters of increasing chain length (C2-C4) by histochemically important carboxylic ester hydrolases

	ALPHA-NAPHTHYL ESTER		
	Acetate	Propionate	Butyrate
Carboxylesterases (EC 3.1.1.1)	+++	++	++
Arylesterases (EC 3.1.1.2)	+++	+	+
Acetylesterases (EC 3.1.1.6)	+++	-	-
Acetylcholinesterases (EC 3.1.1.7)	+++	+++	+++
Cholinesterases (EC 3.1.1.8)	+++	+++	+++

Reaction intensities shown as negative (-), weak (+), moderate (++) and strong (+++).

CHAPTER TWO

MYELOID CELL ESTERASE ISOENZYME EXPRESSION: QUALITATIVE AND QUANTITATIVE STUDIES

2.1 INTRODUCTION

This Chapter primarily deals with the characterisation of myeloid cell esterase isoenzymes by isoelectric focusing (IEF), and the relationships between IEF esterase zymograms and cytochemical staining patterns. Evaluation of these features is important to the understanding of myeloid cell differentiation, as maturational commitment from pluripotential myeloid precursors appears to be associated with the synthesis and expression of distinct esterase isoenzyme forms. These investigations are also important in helping to define the criteria used for the subsequent purification of myeloid esterase species (Chapters 3 and 4). The various components involved in this Chapter, sequentially detail the theoretical aspects of cellular enzyme extraction/solubilisation and IEF, the techniques used for preparation and characterisation of normal and leukaemic cell fractions, cytochemical and immunophenotypic methodologies, esterase zymogram studies of myeloid cells, and the quantitative expression of myeloid esterases.

2.1.1 Extraction of cellular enzymes:

2.1.1.1 General extraction principles: In practice, extraction procedures for the solubilisation of any given protein need to be experimentally optimised and are designed to result in a high recovery with minimal denaturation. Extraction of soluble cytoplasmic enzymes is achieved with relative ease, by disruption of the plasma membrane in an isotonic buffer at physiological pH. Disruption of the plasma membrane may be achieved by homogenisation (organelles remain intact), alternate freezing and thawing, or by ultrasonication (plasma and subcellular membranes disrupted).

Procedures employed for the extraction of membrane bound enzymes, are governed by the type of protein-membrane association involved for the particular enzyme of interest. There are two major groups of membrane proteins; the integral (intrinsic) proteins and peripheral (extrinsic) proteins [Singer & Nicolson, 1972; Singer, 1974]. Integral proteins are strongly associated with the membrane through hydrophobic interactions between the proteins and the 'core' of the membrane. They possess hydrophilic

domains, formed by ionic and polar groups (including carbohydrates) and hydrophobic domains, rich in apolar amino acid residues. The polar groups are ultimately exposed to an aqueous environment at the surface of the membrane while the apolar residues form a strong hydrophobic association with the core of the membrane lipid bilayer [Lenard & Singer, 1966; Wallach & Zahler, 1966]. Consequently, the distribution of hydrophobic and hydrophilic regions of a protein determines its orientation within the membrane structure. Integral proteins assume three positions within the lipid bilayer; spanning or transmembrane proteins have hydrophilic regions which interact with the aqueous phase on both sides of the membrane, whereas a second type of integral protein does not span the membrane and has hydrophilic regions exposed to an aqueous phase on only one side of the membrane. The third type of integral protein is totally immersed within the hydrophobic phase of the bilayer and does not interact with the aqueous phase [Singer & Nicolson, 1972].

2.1.1.2 Extraction of peripheral proteins: Peripheral proteins located on membrane surfaces are fundamentally different from integral proteins in that their attachments are strictly polar in nature (electrostatic or hydrogen-bonding). Peripheral proteins are associated either with the polar heads of the lipid bilayer or with the hydrophilic regions of integral proteins [Singer & Nicolson, 1972]. Some of these proteins show restricted binding to the cytoplasmic side of the bilayer [Kant & Steck, 1973], which may infer the involvement of specific binding sites. Whatever the nature of the association, solubilisation of peripheral proteins in general can be achieved by a number of techniques involving chelating agents, or manipulation of ionic strength and pH. These procedures effectively decrease electrostatic interactions between the peripheral protein and charged lipids or integral proteins. Once in solution, peripheral proteins generally resemble soluble proteins although some degree of aggregation may occur [Steck, 1974].

2.1.1.3 Extraction of integral proteins: Solubilisation, by definition, involves the disintegration of the lipid bilayer, which may be achieved using various membrane-

solubilising agents. Amongst the most widely used reagents are detergents, which solubilise the membrane and also act as a solvent medium for stabilising membrane proteins [Helenius & Simons, 1975].

Detergents used for membrane solubilisation may be subdivided into three types; (a) nonionic, such as octylglucoside and the polyoxyethylenes (which include Triton X-100, Lubrol PX and Nonidet P-40); (b) zwitterionic, such as 3-[(3-cholamidopropyl) dimethyl-ammonio]-1-propanesulphonate (CHAPS) and sulphobetaine; and (c) ionic, such as cetylammonium bromide and sodium dodecyl sulphate (SDS), which possess strongly acidic or basic polar head groups. Naturally occurring bile salts, such as cholate, tauracholate and deoxycholate also act as ionic detergents. Detergents are amphiphilic molecules and in aqueous solutions exist as monomers and micelles, providing the monomer concentration exceeds the 'critical micellar concentration' (CMC) [Mukerjee & Mysels, 1971; Tanford, 1973; Helenius & Simons, 1975]. Above the CMC, both monomers and micelles exist in equilibrium; below the CMC, detergent molecules exist as monomers only. Micelles are fairly monodisperse compact aggregates of detergent monomers, where the apolar groups of the molecules are sequestered into the centre and the polar groups are exposed to the aqueous phase. The micellar dimensions and the CMC of a given detergent are dependent on the apolar moiety of the detergent molecule [Helenius et al., 1979].

In devising an approach for the isolation of a specific membrane protein, one must consider the nature of subsequent requirements for the purification and characterisation of the isolated molecule. For example, it is of paramount importance when isolating membrane enzymes that the extraction procedure does not result in a loss of biological activity because enzymatic activity is usually required for both, monitoring the fractionation procedure and for the subsequent characterisation of the purified product. Consequently, nonionic and zwitterionic detergents are generally used for solubilisation of functional proteins.

The mechanisms by which detergents interact with membrane lipids and proteins vary considerably. The nature of the detergent-membrane association is fairly complex and is reviewed in greater depth by Helenius and Simons [1975]. Briefly, detergent monomers substitute for membrane lipids at low detergent concentrations (below CMC), the degree of substitution increasing with increasing detergent concentration. At the CMC, sufficient lipid displacement by detergent occurs to allow disruption of the lamellar membrane structure [Bont et al., 1969; Tanford, 1972; Helenius & Soderlund, 1973]. This disruption (solubilisation) results in the generation of mixed detergent-lipid and detergent-protein-lipid micelles which co-exist with the pure detergent micelles already present in solution. When higher concentrations of detergent are added to the mixed micelle populations, than are required to induce the lamellar-micellar phase transition, lipids are separated from proteins (delipidation) resulting in two separate populations, detergent-protein and detergent-lipid, of mixed micelles. The quantities of detergents required depend both on the composition of the membrane and on the type of detergent used. The mixed micellar size, buoyant density, charge and binding affinity will vary, depending on the constituents of the micelles, and these properties may therefore be used to separate different mixed micelles in a heterogeneous population. Gel filtration is the technique most frequently chosen for the separation of protein-free and protein-containing micelles, as it results in high protein recovery and allows easy monitoring of the separation. The technique exploits the differential micellar dimensions (molecular weight and shape) exhibited by the mixed and homogeneous micelles. It is important to recognise that a micelle, mixed or homogeneous, is a fluid, noncovalently linked aggregate and as such its size and shape are susceptible to environmental changes. Therefore, in order to achieve reproducible results with detergent-solubilised proteins, it is imperative to standardise parameters such as ionic strength, pH, temperature, detergent and protein concentration.

Lipids and proteins of native membranes interact with each other in complex fashions that differ depending on the nature of the membrane. Furthermore, protein structures are often affected by their environment and, consequently, the ability to retain biological

activity during and following extraction also varies from protein to protein. In general, nonionic detergents are most widely used for the solubilisation of leucocyte membrane enzymes, as these have several advantages over ionic detergents when used for extracting integral membrane enzymes. These rarely cause enzyme inactivation and, as they are electrically neutral, they do not interfere with subsequent separation procedures based on protein charged groups (e.g. ion-exchange chromatography, chromatofocusing or isoelectric focusing techniques). An additional advantage of using nonionic detergents is that although they are efficient in breaking membrane lipid-lipid and lipid-protein interactions, they do not disrupt protein-protein interactions. Consequently, the subunit structure of a native enzyme usually remains unaltered [Helenius & Simons, 1972]. One disadvantage in the use of such detergents however is that they may be difficult to remove from proteins, due to the formation of stable high molecular weight mixed detergent-protein micelles [Furth et al., 1984; Renswoude & Kempf, 1984]. However, if removal of the detergent becomes necessary for further protein characterisation and providing the extracted enzyme proves to be stable in an aqueous environment, then low molecular weight nonionic detergents may be used (i.e. octylglucoside). These low molecular weight detergents often have a higher CMC and are readily dialysed out of a protein-detergent mixture [Keesey, 1987]. One other factor that is important to the design of an enzyme purification procedure is that some detergents may absorb strongly in the 260-280nm range and may limit the ability to monitor chromatographic fractionation using a conventional UV flow cell. Triton X-100 is such a detergent but in instances where absorption profiles are required, alternative nonionic detergents including Thesit or isotridecylpoly (ethylene glycol ether) may be used.

2.1.2 Isoelectric focusing:

Isoelectric focusing (IEF) involves the electrophoretic migration of ampholytes (i.e. polypeptides and proteins) in a pH gradient. Biomolecules possessing a net positive or negative charge migrate in an electric field towards the pH where they are isoelectric (i.e. possess no net charge). This pH is referred to as the isoelectric point (pI) and at this

position in the pH gradient, they have zero electrophoretic mobility and concentrate in narrow zones. The principle of this so-called focusing effect is essentially a steady-state equilibrium between electrophoretic migration towards the isoelectric point and diffusion in the opposite direction. IEF plates for flat bed electrophoresis are commercially available as two main types; (a) Ampholine plates (Pharmacia), which consist of an inert support matrix (polyacrylamide or agarose) containing a mixture of low molecular weight ampholytes (synthetic and natural amino acids and peptides); and (b) Immobiline plates (Pharmacia), consisting of an inert matrix which has a preformed, immobilised pH gradient generated by covalently bonded buffering groups. In an electric field, the ampholytes incorporated into ampholine plates migrate rapidly to their isoelectric points and form a stable pH gradient [Svenson, 1962]. The larger sample protein molecules undergo a slower migration, governed by the initial pH environment, and this migration continues until the pI has been reached. The technique has an extremely high and reproducible resolving power and can differentiate between proteins with pI differences of as little as 0.01 pH units [O'Farrell 1975; Radola, 1984]. As a result it has become increasingly employed in the characterisation of isoenzyme forms. Furthermore, if individual isoenzyme forms are stable at their pI, they can be visualised by simply immersing the plate in an appropriate substrate reaction mixture.

2.2 METHODS

2.2.1 Preparation of normal and leukaemic cell fractions:

2.2.1.1 Preparation of normal blood granulocyte (neutrophil) fractions: Peripheral blood granulocytes were prepared from whole blood of 11 different normal individuals by sequential removal of erythrocytes, followed by density sedimentation on Lymphoprep (Nyegaard). The first part of this procedure involved the preparation of leucocyte-rich plasma by selective removal of erythrocytes. To achieve this, 40ml of whole blood was mixed with a 1:5 volume of Plasmagel (Laboratoire Roger Bellon). Following rapid sedimentation at 1g in a narrow bore tube, the leucocyte-rich supernate was removed and the cells washed three times in Hanks balanced-salt solution (HBSS;

Flow Laboratories). These cells were then resuspended in 10ml of autologous cell-free plasma and layered onto an equal volume of Lymphoprep (density 1.077g/ml) in siliconised glass tubes of 22mm internal diameter. The tubes were subsequently centrifuged at 600g for 30 mins at room temperature after which the leucocytes separated into an interface, containing mononuclear cells (lymphocytes, monocytes) and platelets, and a pellet comprising granulocytes and a small number of residual erythrocytes. The interface was carefully removed, ensuring no mixing with the pellet, and this was subsequently used for the monocyte enrichment procedure described below (Section 2.2.1.2). The cell pellet was then removed, and contaminating erythrocytes lysed with 10ml of 0.86% aqueous ammonium chloride at 37°C. Following erythrolysis, the red cell free, granulocyte-rich fraction was washed in HBSS and resuspended in the same prior to further processing.

2.2.1.2 Preparation of normal blood monocyte fractions: In this study, two different types of blood monocyte fractions were prepared. The first comprised a cell suspension containing both lymphocytes and monocytes with approximate relative frequencies of 80% and 20% respectively, which was used to examine correlations between the expression of membrane CD14 and cytoplasmic alpha-naphthyl acetate esterase (ANAE) activity by normal blood monocytes. The second cell suspension was specifically prepared for chromatographic and electrophoretic analyses of cellular esterases and comprised a predominance (mean 77%) of monocytes with the remaining cells being of lymphoid origin.

Monocyte enrichment for cytochemical and phenotypic studies: Peripheral blood mononuclear cells (lymphocytes and monocytes) were prepared from 19 individual normal EDTA-anticoagulated blood samples. Preliminary studies indicated that platelet removal prior to leucocyte fractionation was desirable because of their tendency to adhere to monocytes and cause clumping. Their removal was facilitated by centrifugation of EDTA blood at 60g for 5 min; the platelet-rich plasma, containing few leucocytes, was

removed and the platelets pelleted by high speed centrifugation. The resulting platelet-free plasma was re-mixed with the packed cells and the procedure repeated twice more. The platelet-depleted blood was then layered onto an equal volume of Lymphoprep and centrifuged at 600g for 30 min. The resulting interface comprised 10-25% morphologically-defined monocytic cells, with the remainder being of lymphoid origin.

Monocyte enrichment for esterase isoenzyme studies: Nine different mononuclear cell fractions, obtained from the Lymphoprep stage of the granulocyte fractionation procedure (Section 2.2.1.1), were washed twice and resuspended to a volume of 10ml in HBSS. This was then layered onto 10ml of Percoll solution (Pharmacia), diluted to give a density of 1.054g/ml, and centrifuged at 600g for 15 mins at room temperature. After centrifugation, the Percoll interface, primarily consisting of platelets, was discarded and the cell pellet containing monocytes and lymphocytes was washed twice and resuspended to 10ml in HBSS. This mononuclear cell fraction was subsequently layered onto an equal volume of Percoll solution of density 1.062g/ml and centrifuged at 600g for 30 mins at room temperature. The resulting monocyte-rich interface was carefully removed, washed with HBSS and resuspended in the same prior to further processing.

2.2.1.3 Fractionation of myeloid leukaemia blasts: Peripheral blood and/or bone marrow samples from a total of 204 patients with acute myeloid leukaemia were layered onto equal volumes of Lymphoprep and centrifuged at 600g for 30 min at room temperature. In each case the interface, which comprised high proportions of leukaemic blasts, was removed and washed in phosphate-buffered saline (PBS) pH 6.8 containing 0.01% sodium azide prior to further studies.

2.2.2 Characterisation of normal and leukaemic cell fractions:

2.2.2.1 Preliminary studies of normal blood myeloid cell fractions: Enriched normal blood granulocyte (neutrophil) and monocyte fractions were examined morphologically (Romanowsky stain) and cytochemically (alpha-naphthyl acetate esterase, ANAE stain)

following cytocentrifugation at 100g for 5 min onto clean glass slides. Morphological examination, by means of a 200 cell differential, was undertaken to assess the proportions of individual leucocyte components present in each fraction. ANAE cytochemical staining permitted an assessment of the proportions of monocytic cells in both the monocyte-enriched fractions and, as a potential contaminate, in the granulocyte-enriched fractions. The monocyte fractions were additionally tested by immunological procedures (Section 2.2.4) for the proportions of cells expressing membrane monocyte-associated CD14 (63D3 and My4 monoclonal antibodies) determinants.

2.2.2.2 Characterisation of leukaemic blast cell fractions: Fractionated leukaemic blasts (Section 2.2.1.3) were examined morphologically, following cytocentrifugation, to determine the proportions of blasts. Only those cases with >80% blasts, together with high viability, were subsequently processed for electrophoretic and chromatographic isoenzyme analysis. All myeloid leukaemias studied were diagnostically classified by the following criteria:

Morphology: Leukaemic peripheral blood/bone marrow smears were fixed in methanol and stained by a standard Romanowsky technique (May-Grunwald-Giemsa). Morphological assessments of the acute leukaemias were based on previously published standardised features, as defined by the French-American-British (FAB) Group [Bennett et al., 1976; Bennett et al., 1985], and leukaemias were thus categorised as acute myeloblastic (AML- M1 or M2), acute promyelocytic (APL- M3), acute myelomonocytic (AMML- M4) or acute monocytic (AMoL- M5). Further sub-categorisation of the M3 cases into hypergranular (M3-hyper) and hypogranular (M3-hypo) variants was based on the degree of cytoplasmic granulation [Bennett et al., 1976; Bennett et al., 1980; Golomb et al., 1980], and further division of the M5 cases into M5a and M5b was based on the level of blast cell maturation [Bennett et al., 1976].

Cytochemistry: All acute leukaemias studied were additionally examined by conventional cytochemical techniques for the presence of various myeloid and/or monocyte-associated

staining patterns (Table 2.1). These included Sudan Black B (SBB), myeloperoxidase (MPO), chloroacetate esterase (CAE) and alpha-naphthyl acetate esterase (ANAE), the reaction procedures for which are outlined in Section 2.2.3.

Immunophenotyping: All cases of myeloid leukaemia included in this investigation were examined for the presence of myeloid lineage-associated membrane CD13 and/or CD33 determinants, defined by My7 (Coulter Electronics) and My9 (Coulter Electronics) monoclonal antibodies respectively. In addition, expression of membrane monocyte-associated CD14 determinants was assessed with at least two different monoclonal antibodies from a panel comprising FMC17 (Sera-Lab), FMC32 (Sera-Lab), Mo2 (Coulter Electronics), My4 (Coulter Electronics), LeuM3 (Becton-Dickinson), UCHM1 (University College Hospital, London), and 61D3 (Bethesda Research Laboratories). The full monoclonal antibody panel used for the routine immunophenotypic characterisation of myeloid leukaemias analysed in this study is summarised in Table 2.2.

2.2.3 Cytochemical techniques:

2.2.3.1 Sudan Black B (SBB): Peripheral blood/bone marrow smears were fixed in formaldehyde vapour and stained according to the method of Sheehan and Storey [1947]. Following incubation in the staining solution, the slides were destained twice with 70% ethanol and counterstained by Romanowsky procedure.

2.2.3.2 Myeloperoxidase (MPO): Peripheral blood/bone marrow smears were fixed with cold buffered formol-acetone and stained for myeloperoxidase using a modification of the ICSH method [Elias, 1980; ICSH, 1985]. Slides were immersed in an incubation mixture comprising 1.7mM diaminobenzidine tetrahydrochloride (Sigma) and 0.12% hydrogen peroxide in 50mM tris/HCl pH 7.5. After incubation at room temperature for 15 minutes, the slides were counterstained with 0.6% neutral red.

2.2.3.3. Chloroacetate esterase (CAE): Peripheral blood/bone marrow smears were fixed with cold buffered formol-acetone and stained according to the method of Yam et

al., [1971]. Slides were immersed in a staining solution containing 0.35mM naphthol AS-D chloroacetate substrate (Sigma) and hexazotised New Fuchsin (Sigma) in phosphate buffer at pH 7.2 for 20 minutes. After incubation at room temperature, the slides were counterstained with Haematoxylin.

2.2.3.4 Alpha-naphthyl acetate and butyrate esterase (ANAE/ANBE): Peripheral blood/bone marrow smears were fixed with buffered formol-acetone and stained for 60 minutes in a reaction mixture comprising 5.0mM alpha-naphthyl acetate, or alpha-naphthyl butyrate (Sigma), and hexazotised pararosaniline (Sigma) [Yam et al., 1971] in 50mM phosphate buffer at pH 6.2 (ANAE) or 6.4 (ANBE). After completion of staining, the slides were counterstained with aqueous methyl green.

2.2.4 Immunophenotyping techniques:

Various different immunophenotyping procedures were used to characterise membrane antigen expression by normal myeloid cells and leukaemic blasts in this study. Included among these are techniques for the determination of antigen expression, either as single or multiple analyses, as well as a combined immunophenotyping/cytochemical procedure specifically designed to correlate membrane CD14 and cytoplasmic ANAE expression at the single cell level (Section 2.2.4.4).

2.2.4.1 Indirect (manual) immunofluorescence: Fractionated cell suspensions were adjusted to a concentration of approximately 5.0 x 10^6/ml in PBS containing 0.1% azide. 50µl of each cell suspension was incubated at 4°C with a pre-determined amount of appropriate primary monoclonal antibody for 30 min. Following incubation, the cell suspension was washed twice with PBS-azide and 25µl of fluorescein isothiocyanate (FITC) or rhodamine (TRITC) conjugated goat anti-mouse immunoglobulin (TAGO) was added. Primary and secondary antibody working concentrations were pre-determined by titration against antigen expressing and non-expressing cells (data not shown). The suspension was incubated for a further 30 min, the cells washed twice and the labelled

cells examined under a fluorescent microscope for membrane fluorescence. In each case, the proportion of cells expressing any given determinant was recorded. Control studies using irrelevant monoclonal antibodies of the same isotypes and second antibodies alone were routinely carried out.

2.2.4.2 Indirect immunorosetting: Fractionated leukaemic myeloid blasts were adjusted to a concentration of approximately 5.0 x 10^6/ml in PBS-azide. 10µl of cell suspension was then incubated for 30 min at 4°C with a predetermined amount of appropriate primary antibody. Following incubation, the cells were washed twice with PBS-azide and resuspended to a volume of 100µl. An equal volume of 1% ox erythrocytes (oxE), coated with goat anti-mouse immunoglobulin (GAM-Ig, TAGO) by a chromic chloride coupling technique [MacKarill et al., 1987], was added to the cell suspension and the mixture centrifuged at 350g for 1 min. Following centrifugation, the leucocyte/oxE pellet was gently resuspended in a dilute solution of acridine orange/ethidium bromide and examined under a fluorescent microscope. Viable leukaemic blasts were considered antigen-positive when more than three oxE were seen to be clearly bound to the cell membrane. Control studies, comprising blasts incubated with irrelevant monoclonal antibodies, were routinely undertaken.

2.2.4.3 Automated flow cytometry: Acute myeloid leukaemias were examined for the expression of lineage-associated determinants by flow cytometry using two-colour combinations of FITC and phycoerythrin (PE) conjugated monoclonal reagents (Table 2.2). For this, fractionated myeloid blasts were examined by direct immunofluorescent techniques using flow cytometry as described by Richards & Scott [1990]. Briefly, 10-20µl of each cell suspension (containing 10^6 cells) was incubated at 4°C with a predetermined amount of appropriate FITC- or PE-conjugated primary antibody in microtitre plates [Campana & Janossy, 1986] for five minutes. Following incubation, the cell-antibody mixture was pelleted and the cells washed twice with 150µl of PBS-azide to

remove excess antibody. The cells were resuspended in 500µl of Isoton (Coulter Electronics) prior to analysis by flow cytometry.

Analyses were carried out with a FACSCAN flow cytometer (Becton-Dickinson) using Consort 30 software as previously described [Richards & Scott, 1990; Scott et al., 1990]. Forward scatter (FSC), side scatter (SSC), FITC (FL1) and PE (FL2) data were acquired and stored in list mode files. Single and dual antigen-positive components were determined using contour (quadrant) graphs of FL1 versus FL2. By utilising appropriate gate settings, the antigenic characteristics of the blast cell populations could be assessed.

2.2.4.4 Combined ANAE cytochemistry and CD14 immunophenotyping: The CD14 monoclonal antibodies used for these particular experiments were LeuM3 (Becton Dickinson), My4 (Coulter Electronics) and 61D3 (Bethesda Research Laboratories), and a total of 19 normal blood mononuclear fractions (Section 2.2.1.2) were investigated. To simultaneously analyse membrane CD14 expression and cytoplasmic ANAE staining, a pre-determined amount of CD14 monoclonal antibody (LeuM3, My4 or 61D3) was added to 2.0 x 10^6 of mononuclear cells and incubated at 4°C for 30 min. Following incubation, the cells were washed twice with complement fixation diluent (CFD) buffer (Oxoid) at pH 7.2 and resuspended to a concentration of 1.0 x 10^6/ml. 100µl of this cell suspension was added to an equal volume of 1% ox erythrocytes (oxE) coated with goat anti-mouse immunoglobulin (GAM-Ig) and the mixture centrifuged at 350g for 1 min [MacKarill et al., 1987]. Following centrifugation, the leucocyte/oxE pellet was gently resuspended and cytocentrifuged (50g for 5 min) onto clean glass slides. The resulting cell (leucocyte/oxE) monolayers were stained by either a conventional Romanowsky technique or for ANAE (Section 2.2.3.4) activity. ANAE-stained cytocentrifuge preparations were examined for monocytes expressing cytoplasmic ANAE reactivity and/or membrane CD14 determinants. Typical monocytes showed moderate/strong M-type ANAE reactions (defined as ANAE$^+$); mononuclear cells with granular or 'dot' type reactions were excluded, as were cells displaying non-monocytic cytology.

Morphologically characterised monocytes binding a minimum of three oxE were defined as being $CD14^+$ and, for each sample analysed, a minimum of 10^3 cells were examined. In this way, it was possible to subdivide normal monocytic components into three sub-populations; (a) monocytes expressing both membrane CD14 and cytoplasmic ANAE ($CD14^+ANAE^+$), (b) monocytes lacking membrane CD14 but showing typical cytoplasmic M-type ANAE activity ($^+$), and (c) monocytes expressing membrane CD14 but showing insignificant cytoplasmic ANAE staining ($CD14^+ANAE^-$). Cells with monocytoid morphology but lacking both membrane CD14 and cytoplasmic ANAE were occasionally seen although, in all mononuclear fractions studied, these represented only a minor proportion of those cells assessed.

2.2.5 Extraction of cellular esterases:

2.2.5.1 Optimisation of cellular esterase extraction: In order to determine the efficiency of cellular esterase extraction, an artificially prepared mixture of normal monocytes and granulocytes (50:50) was tested with a variety of extraction procedures and detergents. In this way, the quantitative and qualitative features of liberated esterases could be determined. The five solubilising agents investigated were: (a) 0.25M potassium chloride (KCl); (b) a zwitterionic detergent, 10mM 3-[(3-cholamidopropyl)dimethyl-ammonio]-1-propanesulphonate (CHAPS); (c) the nonionic detergent isotridecylpoly(ethylene glycol ether) (IEGE) at a concentration of 10mg/ml; (d) the nonionic detergent n-octylglucoside (30mM); and (e) the nonionic detergent Triton X-100 (10mg/ml). These agents were all made up in 20mM morpholino-ethane sulphonic acid (MES) pH 6.4 and their solubilising efficiency, compared with this buffer alone. Zwitterionic and nonionic detergents (all obtained from a single commercial source, Boehringer) were chosen for the extraction procedures as they are generally non-denaturing and do not interfere with many of the chromatographic and electrophoretic fractionation procedures subsequently evaluated in this study. They are also known to help preserve enzyme activity, particularly in cases where native enzymes are membrane bound. The degree of solubilisation of esterases was assessed by carrying out (a) quantitative (fluorimetric) enzyme assays using 4-

methylumbelliferyl acetate (4MB-Ac; Koch-Light) as substrate; and (b) qualitative analytical isoelectric focusing (IEF), with histochemical staining of the gel for ANAE.

To carry out this experiment, the monocyte/granulocyte cell mixture was pre-cooled in an ice bath and disrupted by ultrasonication (MSE Soniprep 150) for 30 secs. The sonicated sample was then aliquoted into six fractions and each diluted with an equal volume of one of the above extraction reagents. These cell fractions were then incubated in the presence of extraction reagents at 37°C for 15 min, following which the cell debris was pelleted by centrifugation at 10^4g for 30 min. The supernates, containing extracted solubilised proteins, were removed and the cell pellets resuspended to the original sonicate volume in 20mM MES pH 6.4.

The volumes of sonicate supernate and the resuspended pellet from each of the extracted fractions were then measured and 20µl of each analysed for acetate esterase activity by fluorimetric assay (Section 2.3.6.4) using 4MB-Ac as substrate. Total acetate esterase activity was calculated and the percentage enzyme extracted by each procedure determined. The supernates from each extracted fraction were also electrophoresed on pI range 3.5 - 9.5 IEF plates (Section 2.2.6.1) and stained for ANAE activity. The resulting ANAE isoenzyme profiles were compared and the relative staining intensities of each isoenzyme form (defined by differences in pI) assessed.

2.2.5.2 Effect of different Triton X-100 concentrations on the relative molecular weight (Mr) species of raw sonicate esterases: Based on the results of the above procedures, Triton X-100 was selected to supplement 20mM MES (pH 6.4) in the extraction reagent. As detergents often promote micelle formation, with consequent effects on the apparent distribution of molecular weight species, it was necessary to establish whether the relative concentration of Triton X-100 in the extraction system could affect or contribute to the presence of different esterase molecular weight forms.

Fractionated leukaemic blasts from a case of AMML-M4, known to express the full complement of myeloid esterase isoenzyme forms as defined by IEF, were sonicated by standard procedures in 20mM MES pH 6.4 and the sonicate subsequently divided into six equal fractions. Triton X-100 was then added to each fraction to give final concentrations of: (a) 0%; (b) 0.25%; (c) 0.5%; (d) 1.0%; (e) 5.0%; or (f) 10.0%. These sonicate-detergent mixtures were then incubated at 37°C for 15 min, following which, the cell debris was removed by centrifugation at 10^4g for 30 min. The supernates, containing solubilised proteins, were then individually examined for the presence of different esterase molecular weight species by Fast Protein Liquid Chromatography (FPLC) gel filtration using a Superose-12 column (Pharmacia) as described in Section 3.2. Briefly, 100µl of each sample was passaged through the Superose-12 column, at a flow rate of 0.35ml/min, in 50mM phosphate buffer (pH 7.2) containing the appropriate concentration (0 to 10%) of Triton X-100. One minute (0.35ml) fractions were collected and 100µl of each assayed by fluorimetry for esterase activity with a substrate mixture of 0.25mM 4MB-acetate and 0.25mM 4-methylumbelliferyl-butyrate (Section 2.3.6.4).

2.2.6 Isoelectric focusing (IEF) of myeloid cell esterases:

2.2.6.1 Cases studied and standard electrophoretic procedure: A total of 11 normal blood granulocyte-enriched and nine monocyte-enriched fractions were examined in this study, in addition to blast cell fractions from 204 cases of acute myeloid leukaemia. These leukaemias comprised 85 cases of AML-M1/M2, 11 cases of APL-M3, 63 cases of AMML-M4, and 45 cases of AMoL-M5. Normal and leukaemic myeloid cells were initially isolated by the appropriate fractionation procedures outlined earlier (Section 2.2.1) and esterases extracted by ultrasonication and detergent solubilisation (Section 2.2.5).

Esterase isoenzymes were fractionated by analytical isoelectric focusing (IEF) and the isoenzyme patterns of cell extracts examined following alpha-naphthyl acetate esterase (ANAE) histochemical staining. To achieve this, 30-50µl aliquots of normal or

leukaemic cell extracts were applied, using micracloth sample application pads, to 5% polyacrylamide gels (pH 3.5-9.5 PAG plates; Pharmacia) containing 2.5% w/v ampholine. The samples were focused for 1400 volt hours (Vhrs) at a constant current of 20mA and the gel temperature kept at approximately 5-10°C by carrying out the electrophoresis on a water cooled glass plate. Commercial, pre-stained protein standards (Sigma) of known isoelectric points (pI) were simultaneously focused with the extract samples. On completion of electrophoresis, the relative migratory positions of the protein standards were measured, and additionally, the pH gradient across the gel determined by use of a surface pH electrode (Philips). Focusing was considered complete when a linear pH gradient, ranging from 3.5-9.5, was generated across the gel. Gels were subsequently immersed in a staining solution containing alpha-naphthyl acetate and hexazotised pararosaniline at a pH of 6.2 (Section 2.2.3.4) and the pI of individual ANAE isoenzyme forms calculated with respect to both standard proteins and the gel surface pH measurements.

2.2.6.2 Substrate specificity of myeloid esterase isoenzymes: In order to determine the relative substrate affinities of the various myeloid isoenzyme components, five AMML-M4 and two AMoL-M5 leukaemic cell extracts expressing the full of complement myeloid isoenzyme forms were prepared. Three replicate IEF strips were run for each cell extract and on completion of electrophoresis, the individual lanes were cut and immersed in esterase staining solutions containing either (a) 5.0mM alpha-naphthyl acetate (ANA), (b) 5.0mM alpha-naphthyl propionate (ANP), or (c) 5.0mM alpha-naphthyl butyrate (ANB). Hexazotised pararosaniline was employed as coupler in each reaction mixture and a standard pH of 6.3 was used. The relative staining intensities of esterase isoenzymes, with respect to the three substrates examined, were subjectively assessed after a 30 minute staining period.

2.2.6.3 Inhibitor studies of myeloid esterase isoenzymes: To determine the relative sensitivity of the major myeloid esterase isoenzymes to various inhibitors, an AMML-M4

leukaemic cell extract containing all the major myeloid isoenzyme forms was electrophoresed (IEF) in multiple lanes. On completion, the individual lanes were segmented and pre-incubated for 10 min in a 50mM pH 6.4 phosphate buffer containing one of the inhibitors being investigated. This was followed by immersion in an ANAE reaction mixture also containing the same inhibitor. The inhibitors examined in this study were 5.0mM phenylmethylsulphonyl fluoride (PMSF; Boehringer), 2.4mM sodium fluoride (NaF; Sigma), 10mM neostigmine (Sigma), 0.1mM eserine (Sigma) and 1.0mM p-hydroxymercuribenzoic acid (HMBA; Sigma). In addition, the inhibitory effects of NaF and PMSF were examined over a series of concentrations (ranging from 0.0 to 1.5mg/ml for NaF and 0.0 to 10.0mM for PMSF).

2.2.7 Protein estimations using the BCA assay:

The BCA assay is a colourimetric procedure for the estimation of protein concentrations which is related to the Lowry technique, in that it relies on the conversion of Cu^{2+} to Cu^{+}. Under alkaline conditions, Cu^{2+} forms a complex with protein peptide bonds and is reduced to Cu^{+} which, in turn, is captured by bicinchoninic acid and forms an intense purple reaction product with an absorption maximum at 562nm. BCA assay reagents are commercially available (Pierce Chemical Company) and, using this procedure, protein concentrations can be estimated in a simple one-step process. In this current study, protein concentrations of normal and leukaemic cell extracts were estimated using the BCA procedure; cell extracts were diluted as required and assayed in parallel with a range of protein standards (Sigma Diagnostics). The absorbance at 562 (A562) was measured and the protein concentration of cell extracts estimated from a standard curve.

2.2.8 Optimisation of UV and fluorimetric esterase assays:

2.2.8.1 UV spectrophotometric assay of alpha-naphthyl acetate esterase (ANAE): A previously described assay for leucocyte alpha-naphthyl acetate esterase (ANAE) used an ultraviolet (UV) spectrophotometric procedure [Mastropaolo & Yourno, 1981] where the hydrolysis of the substrate (alpha-naphthyl acetate) to alpha-naphthol was monitored at 235nm. Under standard conditions, the rate of hydrolysis is linear and directly proportional to the esterase concentration; where the enzymatic hydrolysis of 1nmol of alpha-naphthyl acetate results in an increase in A235 of 0.023 absorbance units. In order to validate this procedure and, if necessary, modify it for use in this study, a number of preliminary experiments were carried out to establish the effects of substrate concentration, assay time and enzyme concentration.

Effect of substrate concentration: A leukaemic blast cell (AMML-M4) extract, containing all the major myeloid esterase isoenzyme forms, was tested for ANAE activity by UV spectrophotometric assay with a range (0.05mM to 0.75mM) of substrate concentrations, prepared by diluting a stock 50mM ANA solution (dissolved in 2-methoxyethanol; Sigma) in 10mM MES pH 6.3. 20µl of 1:2 and 1:4 dilutions of the cell extract were added to 1.0 ml of each working substrate solution (pre-warmed to 30°C) in a semimicro quartz cuvette. The cuvette contents were then mixed and the rate of increase in absorption at 235nm (A235) measured over a period of 5 min (PU 8800 UV/VIS spectrophotometer; Pye Unicam). The observed rates of change for each substrate concentration were corrected for spontaneous substrate hydrolysis (control incubation of substrate in the absence of enzyme), thus giving the rate of increase in A235 due to specific enzymic hydrolysis. The effect of substrate concentration on reaction rate, additionally allowed a broad estimate of the Michaelis-Menten constant (K_m) to be made for the hydrolysis of alpha-naphthyl acetate by unpurified esterases from this particular blast cell extract.

Reaction rate linearity: Esterases extracted from an AMML-M4 blast cell fraction, containing all the major myeloid esterase isoenzyme forms, were tested for ANAE activity by UV spectrophotometric assay over a period of 10.0 min. Preliminary studies were carried out at a substrate concentration of 0.2mM, as originally described [Mastropoalo & Yourno, 1981], but this was modified to 0.5mM as a result of the above substrate investigations. For these experiments, 20µl of neat, 1:2, 1:4 and 1:8 dilutions of the blast cell extract were added to 1.0ml of working substrate solution in a semiquartz cuvette. The cuvette contents were mixed and the increase in absorbance at 235nm (A235) was measured at 0.5 min intervals over a period of 10.0 min. The rate of change in A235, over the 10 min period, was then analysed with respect to the leukaemic extract dilutions examined.

Effect of enzyme (cellular extract) concentration: Serial dilutions of cell sonicate, ranging from neat (undiluted) to 1:8, were prepared for one case each of AMML-M4 and AMoL-M5 (both of which contained all the major myeloid isoenzymes), and two cases of AML-M1/M2 (which contained all myeloid isoenzyme forms with the exception of those specifically associated with monocytic differentiation). Cell extracts, at all the dilutions examined, were assayed by UV spectrophotometry for ANAE activity in order to determine the maximum rate of change which remained linear throughout a 5 min assay period. To achieve this, 20µl of each dilution of cell extract was added to 1ml of working (0.5mM) alpha-naphthyl acetate substrate solution at 30°C in a semimicro quartz cuvette. The cuvette contents were mixed and the rate of increase in A235 was measured. After correcting the observed rates for spontaneous substrate hydrolysis, the maximum sustainable hydrolysis rate which remained linear through a 5 min assay period was determined.

2.2.8.2 Fluorimetric assays for acetate and butyrate esterases: As an alternative to UV spectrophotometry, it is possible to measure solubilised acetate and butyrate esterase activities by an end-point fluorimetric assay with the synthetic substrates 4-

methylumbelliferyl acetate (4MB-Ac) and 4-methylumbelliferyl butyrate (4MB-But; Koch-Light). Fluorimetric assays, which are particularly suitable for the analysis of large sample numbers (e.g. chromatography fractions) are based on the principle that fluorogenic compounds, when excited at a particular wavelength, subsequently emit light energy in the form of fluorescence at a higher wavelength. This is due to the emission of energy when these compounds return to 'ground state' following excitation; excitation being a process causing the outermost electrons of such compounds to reach a higher energy state. 4MB-Ac and 4MB-But substrates show negligible fluorescence, compared to the enzyme hydrolysis product 4-methylumbelliferone (4MB), and by standardising the excitation and emission wavelengths for these particular compounds it is possible to quantitate the rate of formation of 4MB resulting from 4MB-Ac or 4MB-But hydrolysis.

Effect of substrate concentration: An AMML-M4 leukaemic blast cell extract, containing all the major myeloid isoenzyme forms, was tested for acetate and butyrate esterase activities by fluorimetric assay with 4MB-Ac and 4MB-But substrate concentrations ranging from 0.05mM to 1.0mM. For this, 20µl of a 1:2 dilution of the blast cell extract was incubated at 37°C with 250µl of 4MB-Ac or 4MB-But substrate solutions in 50mM MES pH 6.3 for 60 min, following which an additional 500µl of MES was added. As determined by preliminary studies of fluorescent spectra for 4-methylumbelliferone (data not shown), the fluorescence emission was measured at 460nm with an excitation wavelength of 400nm using a LS-2 Micro Filter Fluorimeter (Perkin-Elmer). The observed values were then corrected for non-specific hydrolysis, resulting from protein-substrate interactions, by incubating a 100°C heat-inactivated cellular extract under identical assay conditions.

Reaction rate linearity: The time course of esterase hydrolysis for both acetate and butyrate substrates, was established using esterases from an AMML-M4 blast cell extract containing all the major myeloid isoenzyme forms. Reaction rates were determined by adding 20µl of neat, 1:2 and 1:4 dilutions of the blast cell extract to replicate 250ul

volumes of 0.5mM substrate (4MB-Ac or 4MB-But) solution at 37°C. At 10 min intervals, ranging from 0 to 60 mins, the reaction mixtures were made to 750µl with 50mM MES pH 6.4, and the fluorescence emission measured at 460nm with an excitation wavelength of 400nm.

Effect of enzyme (cellular extract) concentration: Serial dilutions of blast cell extracts, ranging from neat to 1:8, were prepared for two cases of myelomonocytic leukaemia (one each of AMML-M4 and AMoL-M5) which contained all the major myeloid isoenzyme forms, and two cases of AML-M1/M2 which contained all myeloid esterase isoenzyme forms with the exception of those specifically associated with monocytic differentiation. These leukaemic cell extracts were assayed for acetate or butyrate esterase activity using substrate concentrations of 0.5mM; 20µl of each extract dilution was added to 250µl of pre-warmed (37°C) substrate solution and the reaction mixture incubated at 37°C for 60 min. At this time, the reaction mixtures were made to 750µl with 50mM MES pH 6.3 and the fluorescence emission at 460nm measured. Observed hydrolysis rates were corrected for non-specific hydrolysis, determined by assay of heat-inactivated samples, and the rate of reaction which remained constant throughout the assay period was determined.

2.2.9 Correlations between UV and fluorimetric esterase assays:

2.2.9.1 Effect of substrate concentration: A leukaemic AMML-M4 blast cell extract, containing all the major myeloid isoenzyme forms as defined by isoelectric focusing (IEF), was diluted 1:2 and 20µl of this assayed for acetate esterase activity by both the UV (Section 2.2.8.1) and fluorimetric (Section 2.2.8.2) assay procedures at substrate (alpha-naphthyl acetate or 4-methylumbelliferyl acetate) concentrations ranging from 0.05 to 0.5mM. The rate of substrate hydrolysis for both assay procedures at each substrate concentration was determined and compared.

2.2.9.2 Effect of enzyme concentration: Esterases extracted from AMML-M4 blasts, containing all the major myeloid isoenzyme forms, and a case of AML-M1/M2 containing all myeloid isoenzymes with the exception of those specifically associated with monocytic differentiation, were assayed for acetate esterase activity by both the optimised UV spectrophotometric and fluorimetric assays in order to determine the degree of correlation between the two procedures. To achieve this, the blast cell extracts were serially diluted from neat to 1:8 and 20µl of each assayed for esterase activity using either alpha-naphthyl acetate (5 min UV assay) or 4MB-Ac (60 min fluorimetric assay) at 0.5mM substrate concentrations. Rates of substrate hydrolysis for both assays were determined and compared for each extract dilution studied.

2.2.10 Quantitation of cell extract esterase activities:

Acetate and butyrate esterase activities of leukaemic myeloid cell extracts were determined using the standardised UV spectrophotometric assay (Section 2.3.5.5). The extracts, which represented various ANAE-IEF isoenzyme pattern types, comprised 56 acute myeloid leukaemias (AML-M1/M2, n=22; APL-M3, n=3; AMML-M4, n=19 and AMoL-M5, n=12). In contrast, a series of normal granulocyte (n=11) and monocyte (n=9) cell extracts were analysed for their esterase activities using the standardised fluorimetric assay (Section 2.3.6.4). This particular assay was used for the normal myeloid cell extracts because their esterase concentrations were found to be too low for accurate quantitation by UV spectrophotometry. All normal and leukaemic samples showed, prior to sonication and detergent extraction (Section 2.2.5), >70% population homogeneity with high viabilities, and protein concentrations of these extracts were determined with the BCA assay procedure (Section 2.2.7).

2.3 RESULTS

2.3.1 Preparation and characterisation of myeloid cell fractions:

2.3.1.1 Normal peripheral blood granulocytes: Peripheral blood granulocytes (neutrophils) were fractionated from 11 different normal adult blood samples as detailed in Methods (Section 2.2.1.1). With this standardised procedure, the resulting granulocyte fractions in all cases exceeded 98% purity (Figure 2.1a), contained insignificant (<1.0%) monocytic components as adjudged by ANAE cytochemistry, and had viabilities in excess of 95%. Recovery of granulocytes from each blood examined was greater than 90% (range 91-98%) and, consequently, the loss of granulocytes at the interface of the Lymphoprep fractionation stage was minimal. Cytochemically, the normal granulocytes were unreactive for alpha-naphthyl acetate (ANAE) and butyrate (ANBE) esterase but were strongly positive for myeloperoxidase (MPO) and chloroacetate esterase (CAE).

2.3.1.2 Normal peripheral blood monocytes: Normal peripheral blood monocytes were fractionated from nine individual normal blood samples using the three-step density sedimentation procedure described in Section 2.2.1.2. Analysis of the resulting cell suspensions by morphology (Figure 2.1b), ANAE cytochemistry (Figure 2.2b) and CD14 immunophenotyping revealed that this fractionation procedure provided monocyte purities ranging from 68-90% (mean 77%; Table 2.3) with viabilities in excess of 90%. The contaminating non-monocytic cells in these suspensions were predominantly of lymphoid lineage and were easily distinguishable from the monocytic components which showed ANAE cytochemical reactions of moderate-strong diffuse (M-type) strength. Furthermore, almost all (mean, 95%; range, 89-98%) of the platelets present in the original blood samples were removed as a result of including the 40% Percoll solution in the fractionation procedure, with no significant (<5%) loss of monocytes. In addition to determining the efficiency of monocyte purification by this fractionation method, these studies also analysed the yields (i.e. actual recovery/theoretical maximum). Although the incorporation of EDTA in the washing procedures and the use throughout of siliconised

glass vessels minimised monocyte loss, it was evident that the relatively low monocyte recoveries for the nine different blood samples studied (mean, 41%; Table 2.3) were primarily due to losses occurring within the gradients. Nevertheless, as the cytochemical staining of monocyte-enriched cell suspensions revealed that virtually all ANAE activity was associated with the monocytic components, and not with contaminating lymphoid elements, it was considered that these fractions were sufficiently pure for subsequent qualitative IEF analysis.

2.3.1.3 Leukaemic blast cell fractions: Leukaemic blasts from 85 cases of acute myeloid leukaemia (AML-M1/M2), 11 cases of acute promyelocytic leukaemia (APL-M3), 63 cases of acute myelomonocytic leukaemia (AMML-M4) and 45 cases of acute monocytic leukaemia (AMoL-M5) were fractionated by the density sedimentation technique described in Section 2.2.1.3. In all cases, the proportion of leukaemic blasts in the resulting cell suspensions exceeded 80% (defined morphologically) and, with the exception of the monocytic proliferations, contaminating mature monocytic components (defined by ANAE cytochemistry and CD14 immunophenotyping) were minimal (<2%). Viabilities of all cell fractions were in excess of 90%.

2.3.1.4 Simultaneous demonstration of membrane CD14 and cytoplasmic ANAE: Enriched peripheral blood monocyte suspensions prepared by density sedimentation (Section 2.2.1.2) were examined by a combined analytical procedure designed to simultaneously assess membrane CD14 and cytoplasmic ANAE expression (Section 2.2.4.4). These particular monocyte fractions, comprising 10-25% monocytes with the remainder being of lymphoid lineage (Figure 2.2a), of >90% viability were stained with a CD14 monoclonal antibody (LeuM3, 61D3 or My4), followed by incubation with goat anti-mouse immunoglobulin (GAM-Ig) coated ox erythrocytes (oxE). Cytocentrifuge preparations of the oxE-labelled mononuclear cells were examined by morphology and ANAE cytochemistry. Cells exhibiting moderate/strong diffuse (M-type) ANAE reactions were considered positive whilst those showing granular or 'dot' type were

excluded, as were cells with non-monocytic morphology. Cell differentials (10^3 leucocytes) of oxE rosettes and/or ANAE-positive cells were carried out to determine correlations between CD14 expression and ANAE staining. Three subgroups of normal monocytes were identified; (i) cells showing both CD14 expression and M-type ANAE ($CD14^+ANAE^+$; mean 92%); (ii) cells lacking CD14 expression but showing M-type ANAE staining ($^+$; mean 2.7%); and (iii) cells expressing CD14 but showing insignificant M-type ANAE staining ($CD14^+ANAE^-$; mean 4.9%). Monocytoid cells expressing neither CD14 nor ANAE were not seen. The relative distributions of these three normal monocyte sub-populations were remarkably similar for all three CD14 monoclonal antibodies studied (Table 2.4).

2.3.2 Leukaemic (AML) case classification:

2.3.2.1 Diagnostic subtypes: A total of 309 cases of acute myeloid leukaemia were examined in this study. These were classified on the basis of morphological, cytochemical and immunophenotypic criteria as: (a) acute myeloblastic leukaemia (AML-M1/M2, n=125), (b) acute promyelocytic leukaemia (APL-M3, n=44), (c) acute myelomonocytic leukaemia (AMML-M4, n=89), and (d) acute monocytic leukaemia (AMoL-M5, n=51). The acute monocytic leukaemias comprised 27 cases of AMoL-M5a and 24 cases of AMoL-M5b morphological subtypes, and diagnostic sub-categorisation of the acute promyelocytic leukaemias defined 30 cases of the hypergranular variant (M3-Hyper) and 14 cases of the hypogranular form (M3-Hypo).

2.3.2.2 ANAE cytochemistry: Blast cells from the 309 cases of acute myeloid leukaemia were cytochemically stained for alpha-naphthyl acetate esterase (ANAE), using hexazotised pararosaniline as coupler, and their reaction patterns analysed with respect to diagnostic subtype. Preliminary analysis of this data indicated wide variability in staining reactions and strengths and, consequently, it was decided to reduce the observations to a simpler form and categorise the ANAE cytochemical reactions as:

Negative or weak diffuse: When the majority of blasts showed no significant reaction, or showed weak diffuse staining only (Figure 2.3), and where the proportion of blasts showing stronger ANAE staining (M-type) was <5%.

Moderate to strong diffuse: When a minimum of 20% blasts showed moderate to strong diffuse staining (referred to as M-type) and the remaining blasts showed weaker diffuse reactions of variable strength (Figure 2.4).

ANAE heterogeneity: When the majority of blasts showed weak diffuse staining with only 5% to 20% of the blasts showing typical M-type ANAE reactivity.

Granular or focal: Where a significant proportion of blasts showed a localised focal reaction (Figure 2.5), or granular staining which was generally coarse with deposits unevenly distributed in the cytoplasm (Figure 2.6).

Analysing the reaction patterns of the acute myeloid leukaemias in this way, it was found (Table 2.5) that blast cells in the majority (75%) of AML-M1/M2 cases were negative or only stained weakly for ANAE. A further 22% of cases exhibited atypical granular or focal reaction products. Moderate to strong diffuse M-type reactions were only occasionally seen (4/125) in the AML-M1/M2 diagnostic subgroup.

For the group of acute myelomonocytic leukaemias (AMML-M4), 47% of cases were either negative or weakly stained for ANAE; 15% exhibited ANAE staining heterogeneity with <20% of blasts showing M-type reactions; and the remaining 38% of cases were cytochemically characterised by the presence of >20% blasts with moderate to strong diffuse M-type ANAE staining, even though there was considerable inter-case variation in the reaction strengths of the blast cell population as a whole. When the blast cells in 39 of these AMML-M4 cases were additionally analysed for chloroacetate esterase (CAE) activity, four cytochemical subgroups became apparent; (i) those showing mixed

populations of CAE+ or ANAE+ (M-type reaction) blasts; (ii) those in which the blasts were uniformly ANAE negative but in which CAE+ blasts were seen; (iii) cases in which M-type ANAE+ blasts were noted in the absence of a CAE+ component; and (iv) cases in which the blasts were uniformly negative for both CAE and ANAE. The relative frequency of these four cytochemical subgroups of AMML-M4 were 20%, 20%, 23% and 37% respectively.

Examination of ANAE cytochemical patterns for the acute monocytic leukaemias showed that the majority (65%) of cases had significant proportions (exceeding 60% in most cases) of blasts with moderate/strong diffuse M-type ANAE reactions. However, a further 12% of AMoL-M5 cases showed ANAE staining heterogeneity and, of particular importance, blast cells in the remaining 23% of morphologically and immunologically classified M5 cases were characterised by negative or weak diffuse ANAE reactions. When the data was analysed for AMoL-M5a and AMoL-M5b morphological subtypes separately, no significant correlations with ANAE cytochemical patterns were found.

In contrast to the other acute myeloid leukaemia types, a significant proportion of acute promyelocytic leukaemias showed a distinctive ANAE reaction (Figure 2.7) pattern which was clearly different to that observed in the other diagnostic categories. Such cases, which were predominantly (20/30) associated with the hypergranular APL-M3 variant, showed a granular (occasionally seen as both granular and diffuse) type of staining which was evenly distributed throughout the cell cytoplasm. In such cases with significant, moderate to strong ANAE staining, the ANBE cytochemical reaction was present but was considerably weaker in strength. The type of granular reactivity in APL-M3 was in distinct contrast to the coarse granular reactivity found in some cases of AML-M1/M2. A further notable difference was that most of the leukaemic promyelocytes with ANAE staining simultaneously showed chloroacetate esterase activity (double esterase positivity).

2.3.2.3 Immunophenotypic characteristics: All cases of acute myeloid leukaemia studied showed unequivocal evidence of myeloid differentiation, as adjudged by cytoplasmic myeloperoxidase (MPO) staining and/or expression of membrane CD13 and/or CD33 determinants. Although the majority of myeloid leukaemias lacked detectable nuclear terminal deoxyribonucleotide transferase (TdT), which is a diagnostic marker for acute lymphoid proliferations, approximately 20% of cases were TdT-positive. However, in these cases there was no evidence for the expression of membrane T- or B-cell associated determinants (all cases of myeloid leukaemia considered to show bilineal or biphenotypic characteristics were specifically excluded from the study).

For the assessment of monocyte-associated membrane CD14 expression, a total of 89 cases of acute myelomonocytic leukaemia (AMML-M4) and 45 cases of acute monocytic leukaemia (AMoL-M5) were stained with a minimum of two monocyte-associated membrane CD14 determinants from a panel comprising FMC17, FMC32, Mo2, My4, LeuM3, UCHM1 and 61D3. CD14 expression was arbitrarily considered 'positive' if >20% of the leukaemic blasts were stained and, using this criterion, 66% (59/89) of the AMML-M4 cases and 89% (40/45) of the AMoL-M5 cases studied were defined as $CD14^+$ (Table 2.6). Although 20% $CD14^+$ blasts was considered to represent the lower limit of significance for expression of this particular antigen, it is important to note that in most $CD14^+$ AMoL-M5 cases, the proportions of positive blasts exceeded 60%. In addition, of the five AMoL-M5 cases that were adjudged to be $CD14^-$, it is perhaps relevant that four were of the AMoL-M5a morphological subtype.

As part of the investigation into normal and leukaemic cell ANAE cytochemistry, the acute myelomonocytic and monocytic leukaemias were additionally examined for the comparative expression of membrane monocyte-associated CD14 determinants and cytoplasmic ANAE. For this analysis, AMML-M4 and AMoL-M5 cases were grouped according to whether they were $CD14^+$ or $CD14^-$, as detailed above, and these groups were analysed with respect to their ANAE cytochemical staining patterns. The results

(Table 2.6) show that for the 89 cases of acute myelomonocytic leukaemia, 25% had a significant (>20%) monocytic component defined by both CD14 and ANAE expression ($CD14^+ANAE^+$); 13% of cases had a significant monocytic component defined by ANAE staining but not by CD14 expression ($^+$); 42% of cases had a significant monocytic component defined by CD14 expression but not by ANAE staining ($CD14^+ANAE^-$) and the remaining 20% of cases did not show an increased monocytic component by either immunological or ANAE cytochemical criteria (the diagnosis of these AMML-M4 cases was effectively based on morphological impressions and/or increased serum lysozyme concentrations [Bennett et al., 1985; Stark et al., 1987]).

For comparison, the acute monocytic leukaemias could be subdivided into those which were $^+$ (5/45), $CD14^+ANAE^-$ (16/45) or $CD14^+ANAE^+$ (24/45). Interestingly, 10/16 of the $CD14^+ANAE^-$ cases were completely unreactive for ANAE even though the monocytic component in most of them, as defined by CD14 expression, exceeded 60% of the blast cell population. As a result of the criteria applied for leukaemia case classification, no cases within the AMoL-M5 category were found to be $CD14^-ANAE^-$. Although occasional $CD14^-ANAE^-$ leukaemias with monocytoid features and increased serum lysozyme concentrations were noted during this investigation, their morphological features were not typically that of AMoL-M5 and, consequently, such cases were classified as AMML-M4.

2.3.2.4 Relationships between ANAE, CAE and MPO cytochemistries of monocytic AML variants: In addition to investigating their ANAE cytochemical patterns, the blast cells from 89 cases of AMML-M4 and 51 cases of AMoL-M5 were also stained for myeloperoxidase (MPO) and chloroacetate esterase (CAE). For this comparative analysis, these leukaemias were further subdivided on the basis of MPO cytochemistry into those with either 0-5% or >5% blasts with detectable cytoplasmic MPO staining (defined as MPO^- and MPO^+ respectively). Correlations between the MPO subgroups of both the AMML-M4 and AMoL-M5 groups with CAE and ANAE cytochemical staining

reactions are summarised in Table 2.7. The first observation of note is that the blast cells in all 16 MPO^- AMML-M4 cases were CAE^- (<5% positive blasts) and most (88%) of these were additionally $ANAE^-$ (<20% M-type positive blasts). In contrast, 47% (39/73) of the MPO^+ AMML-M4 cases were CAE^+ and 44% (32/73) had more than 20% M-type $ANAE^+$ blasts.

Of the AMoL-M5 cases studied, 33% (17/51) showed insignificant MPO and CAE staining but most (82%) of these had more than 20% M-type $ANAE^+$ cells. Comparison of the ANAE and CAE reactions, in relation to MPO staining, did not however reveal any definitive correlations.

2.3.3 Extraction of cellular esterases:

2.3.3.1 Optimisation of cellular esterase extraction procedure: Leucocyte esterases were extracted from a mixture of granulocytic and monocytic cells using six different extraction reagents: (a) 20mM MES buffer, (b) 0.25M potassium chloride (KCl), (c) 10mM CHAPS, (d) 10mg/ml IEGE, (e) 30mM n-octylglucoside, and (f) 10mg/ml Triton X-100 (Section 2.2.5.1). These extraction procedures provided an aqueous/detergent fraction, containing solubilised proteins, and a particulate suspension containing cellular debris and unsolubilised proteins. Quantitative analyses of solubilised cellular esterases, by measuring the relative concentrations of esterases in the extracted supernate and the resuspended cell pellet by fluorimetric assay with 4MB-Ac as substrate (Section 2.3.6.4), are shown in Table 2.8. From these results, it is seen that approximately 91% of leucocyte acetate esterase activity was extracted by aqueous MES buffer in the absence of any detergent. Increased acetate esterase extraction was obtained by the use of detergents, although the type of detergent appeared to be irrelevant, and the highest level (99.3%) of extraction was obtained with 0.25M potassium chloride.

Qualitative studies of the sonicate supernates by IEF ANAE isoenzyme analysis, are also summarised in Table 2.8. The ANAE isoenzyme distribution profiles were identical for

all the extraction buffers examined however, there were minor quantitative differences in the staining intensities of individual isoenzyme groups. Thus, while all ANAE isoenzymes could be extracted by aqueous 20mM MES alone, or 20mM MES containing 0.25M potassium chloride, better yields of isoenzymes within the pI range 5.5-6.2 were obtained when 20mM MES was supplemented with a detergent. As the type of detergent, zwitterionic or nonionic, did not appear to make any significant difference to the qualitative distribution of isoenzyme forms, it was decided to routinely incorporate the relatively inexpensive Triton X-100 in the initial extraction buffer.

2.3.3.2 Effect of different Triton X-100 concentrations on the apparent molecular weight (Mr) species of raw sonicate esterases: In order to determine whether the concentration of Triton X-100 used in the extraction procedure was important with respect to the presence of different molecular weight species, cellular esterases were extracted from ultrasonicated leukaemic AMML-M4 blasts with various different Triton X-100 concentrations, in 20mM MES (pH 6.4), ranging from 0% to 10.0%. This particular case of leukaemia was selected as it was known to contain all the major myeloid esterase isoenzyme forms as defined by IEF.

Fractionation of leucocyte acetate/butyrate esterases (as assessed by hydrolysis of fluorimetric substrates) by Superose-12 FPLC gel filtration chromatography (Section 2.2.5.2) resolved two major esterase forms which collectively represented >95% of the extractable cellular esterase activity (detailed analyses of myeloid esterase isoenzymes by gel filtration are given in Section 3.2). Furthermore, the relative concentrations of these two species, based on the hydrolysis of 4MB-Ac and 4MB-But in an equimolar (0.25mM) substrate mixture, indicated an approximate ratio of 1.75:1 for the lower and higher molecular weight forms. As this ratio was consistent throughout the range (0 - 10%) of Triton X-100 concentrations examined (Table 2.9 and Figure 2.8), the most important conclusions of these studies were therefore that; (i) the specific inclusion of Triton X-100 did not affect the relative distributions of the two esterase molecular weight

species; (ii) that the ratio of these two esterase forms was not affected by the actual detergent concentration; and (iii) that there was no evidence for the incorporation of esterases into detergent-micellar forms.

2.3.4 Isoelectric focusing (IEF) of myeloid cell esterases:

2.3.4.1 Extract preparation and rationale: Normal and leukaemic myeloid cases examined in this study were prepared and characterised as described in Sections 2.2.1 and 2.2.2. Only cases showing greater than 80% cell population homogeneity with viabilities in excess of 90% were selected for isoenzyme analysis, and cell extracts were prepared by ultrasonication and detergent solubilisation as described in Section 2.2.5.1. The solubilised esterases were subsequently examined by a combination of analytical IEF and alpha-naphthyl acetate esterase (ANAE) cytochemical staining in order to assess correlations between ANAE isoenzyme distributions, as defined by their isoelectric points (pI), cell-lineage and level of maturation. Studies of a large number of representative extracts also permitted a direct comparison of the ANAE isoenzyme expression with respect to normal and leukaemic myeloid differentiation, and additionally clarified the relationships between isoenzyme distributions, conventional ANAE cytochemistry and membrane CD14 expression.

2.3.4.2 ANAE isoenzyme patterns of normal myeloid cell fractions: ANAE isoenzyme analysis of normal granulocyte and monocyte extracts revealed three main isoenzyme pattern types which were designated G1, G2 and M (Figure 2.9). The G1 and G2 patterns were characterised by up to six ANAE isoenzyme forms within an approximate pI range of 6.7 - 7.9, with the G2 isoenzyme pattern differing only by the additional presence of a pair of weakly staining esterase isoenzymes with an approximate pI of 6.1; and the M esterase pattern showed high concentrations of four to six isoenzymes within a relatively narrow pI range of 5.5 - 6.2 (subsequently referred to as the MonEst species) in addition to the G1 myeloid ANAE isoenzyme forms. Of the 11 normal granulocyte extracts examined, four (36%) showed a G1 isoenzyme pattern and seven a G2 pattern

(Table 2.10). In contrast, all nine normal monocytic extracts showed an M type ANAE isoenzyme pattern. As the higher pI (6.7-7.9) esterase isoenzymes were seen in virtually all cell extracts analysed, these will be subsequently referred to as ComEst (common to both granulocytic and monocytic differentiation) forms irrespective of whether or not MonEst isoenzymes were present.

2.3.4.3 ANAE isoenzyme patterns of leukaemic myeloid cell fractions: All myeloid leukaemias examined in this study were classified by morphological, cytochemical and immunophenotypic criteria. Comparative analysis of IEF esterase zymograms revealed that the isoelectric points of individual ANAE isoforms in leukaemic cell extracts were identical to those observed in normal myeloid cell zymograms. In addition, although there was a greater degree of inter-case variation with respect to individual isoenzyme expression, leukaemic myeloid cell IEF patterns could also be grouped as G1, G2 or M (Figure 2.10). When the ANAE isoenzyme pattern types were analysed with respect to myeloid leukaemia subtypes, it was found that the non-monocytic (AML-M1/M2 and APL-M3) variants predominantly showed either a G1 or G2 pattern whereas M-type ANAE IEF patterns were invariably associated with monocytic (AMML-M4 and AMoL-M5) variants (Table 2.10). Although the observed pI and the relative staining intensities of individual ComEst isoenzyme forms was remarkably similar for all the myeloid extracts with G1 and G2 patterns, there was more variability regarding those cases with M-type ANAE isoenzyme patterns. For example, the occurrence of multiple (ranging from five to more than ten) MonEst isoenzyme components in the pI range 5.5 - 6.2, or a decrease in the relative concentrations of ComEst ANAE isoenzymes (Figure 2.11), was observed in a proportion of monocytic leukaemic extracts. Of the 96 AML-M1/M2 and APL-M3 fractions studied, 82% showed a G1 ANAE isoenzyme pattern, a further 17% showed a G2 pattern and only one case was found with significant concentrations of MonEst isoenzymes (Table 2.10). Of the monocytic myeloid leukaemia subtypes, 29/63 and 30/45 of the AMML-M4 and AMoL-M5 subtypes respectively showed M-type

ANAE isoenzyme patterns with the remaining cases showing either G1 or G2 type patterns.

2.3.4.4 Relationships between ANAE cytochemistry and isoenzyme patterns: The esterase isoenzyme patterns from 39 cases of AML-M1/M2, 40 cases of AMML-M4 and 45 cases of AMoL-M5 were additionally compared with respect to their ANAE cytochemistries (Table 2.11). Irrespective of diagnostic subtype, 66/69 cases with negative or weak diffuse ANAE cytochemistry showed G1 (n = 48) or G2 (n = 18) isoenzyme patterns, whilst MonEst isoenzymes were detected in only three cases (one AML-M1/M2 and two AMML-M4). Of the 16 cases with ANAE cytochemical heterogeneity, 13 showed significant levels of monocyte-associated ANAE isoenzymes (M-type pattern), as did 29/31 cases with moderate to strong blast cell ANAE staining. For comparison, none of the eight cases with granular or focal reaction products showed monocytic ANAE isoenzymes.

With respect to acute promyelocytic leukaemia, all 11 cases examined showed ANAE-IEF patterns of G1 or G2 type (Table 2.12). None of the APL-M3 cases were found to express monocyte-associated esterase isoenzymes even though the ANAE cytochemical (granular) staining reactions in two of these cases were of moderate to strong intensity.

2.3.4.5 Relationships between CD14 expression and ANAE isoenzyme patterns: The ANAE-IEF pattern types of 32 AMML-M4 and 42 AMoL-M5 (M5a and M5b subtypes analysed separately) leukaemias were further compared with membrane CD14 expression. This was achieved by arbitrarily defining cases with >20% of leukaemic blasts with detectable CD14 expression as $CD14^+$, and those with <20% as $CD14^-$. For the AMML-M4 cases, there appeared to be no significant relationship between ANAE-IEF pattern type and CD14 expression (Table 2.13). For comparison, the majority (71%) of AMoL-M5a cases showed M-type ANAE IEF patterns and there was again no apparent correlation between CD14 and ANAE isoenzyme expression; similar results were observed for AMoL-M5b cases which were all $CD14^+$.

2.3.4.6 Substrate specificity of esterase isoenzymes: Seven different myeloid leukaemia blast cell extracts, each of which expressed all the major myeloid esterase isoenzyme forms, were electrophoresed (IEF) in multiple lanes following which, individual tracks were immersed in staining solutions containing either alpha-naphthyl acetate, propionate or butyrate substrates (each at 5.0mM) with hexazotised pararosaniline as coupler at pH 6.3 for 30 min.

Consistent patterns of staining with the different substrates were observed for each extract studied, a representative analysis of which is shown in Figures 2.12 and 2.13. These studies clearly showed that the ComEst myeloid esterase isoenzyme forms preferentially hydrolysed substrates of low acyl-chain length (acetate), hydrolysed propionate with much lower efficiency, and showed no significant hydrolysis of the butyrate substrate. In contrast, the MonEst isoenzyme forms (pI 5.5 - 6.2), as well as the two weakly staining isoenzymes within the same pI range that characterised the G2 IEF pattern, hydrolysed all three substrates.

2.3.4.7 Inhibition studies of ANAE isoenzymes: A leukaemic (AMML-M4) blast cell extract, expressing both ComEst and MonEst isoenzymes, was electrophoresed (IEF) in multiple lanes. Following electrophoresis, individual tracks were stained in an alpha-naphthyl acetate esterase substrate reaction mixture containing various inhibitors at pre-determined concentrations. These procedures permitted direct and simultaneous assessments of the effect of these inhibitors on both the ComEst and MonEst isoenzyme forms, the results for which are summarised in Table 2.14.

Only two, sodium fluoride (NaF) and phenylmethylsulphonyl fluoride (PMSF), of the five compounds tested showed any inhibition of myeloid esterases. At the concentrations examined (0.1mg/ml NaF and 5.0mM PMSF), both these compounds significantly inhibited MonEst ANAE isoenzymes whilst showing only partial, or no inhibition of the ComEst species. However, when a range (up to 1.5 mg/ml) of NaF concentrations were

examined, it was evident that the inhibition of monocyte-associated isoenzyme forms by this compound was relative rather than absolute, as at higher NaF concentrations the ComEst isoenzyme components were also inhibited (Figure 2.14). A more detailed analysis of the relative staining intensities with respect to NaF concentration indicated an approximate 13 fold increased sensitivity of MonEst isoenzymes to NaF, when compared with the ComEst. In contrast, the inhibitory effects of PMSF (concentration ranging from 1.0 to 10.0mM) were found to be largely restricted to MonEst.

2.3.5 Optimisation of UV spectrophotometric esterase assays:

2.3.5.1 Effect of substrate concentration: A leukaemic AMML-M4 cell extract, containing all the major myeloid esterase isoenzyme forms, was diluted to 1:2 and 1:4, and examined for ANAE activity by UV spectrophotometric assay at 30°C with a range of substrate concentrations from 0.05mM to 0.75mM. The aim of this analysis was to determine a saturating substrate concentration (where the concentration of free substrate remained essentially unchanged over the reaction period) which could be used for routine assay purposes in this study. Alpha-naphthyl acetate was found to be soluble in 10mM MES pH 6.3 up to 0.75mM, above which the absorption at 235nm was unacceptably high. At both dilutions of cell extract examined, the rate of substrate hydrolysis increased in proportion to the increasing substrate concentration up to approximately 0.4mM (Figure 2.15). At this concentration and above, the rate of substrate hydrolysis only increased minimally with increasing substrate concentration (i.e. substrate concentration exceeded enzymic hydrolysis). On the basis of these findings a working substrate concentration of 0.5mM was chosen as being saturating for subsequent assays with ANAE activities up to 5.3mU (Section 2.3.5.4), where 1.0mU was defined as that amount of enzyme causing an increase in A235 of 0.023 in 1.0 min at pH 6.25 and at a temperature of 30°C.

2.3.5.2 Reaction rate linearity: The time course of the UV spectrophotometric ANAE assay, and the period over which the rate of substrate hydrolysis remained constant, was

established by investigating a leukaemic AMML-M4 blast cell extract which contained all the major myeloid isoenzyme forms. This extract was serially diluted to 1:8, and 20µl of each dilution was tested for ANAE activity at 30°C. The increase in absorbance at 235nm (A235) was measured at 0.5 min intervals over a period of 10.0 min. At a substrate concentration of 0.5mM, hydrolysis was found to be linear with respect to time over the 10 min period studied (Figure 2.16). However, as reaction rate linearity was more consistent in the first 5.0 min of the assay, this time period was chosen for subsequent determinations of ANAE activity by UV assay.

2.3.5.3 Kinetic characteristics of extract alpha-naphthyl acetate esterases: From the data obtained earlier (Figure 2.15), it was additionally possible to calculate the overall K_m and V_{max} values for the hydrolysis of alpha-naphthyl acetate by the mixture of myeloid esterase isoenzymes present in the leukaemic AMML-M4 blast cell extract. This was achieved by constructing a Lineweaver-Burk plot of 1/reaction velocity (1/v) against 1/substrate concentration (1/s). Straight lines were observed for both dilutions of cell extract, the lines intersecting the x-axis (abscissa) at a point corresponding to $-1/K_m$ and the y-axis (ordinate) at points corresponding to $1/V_{max}$ (Figure 2.17). The V_{max} values were calculated to be 0.03 and 0.015 A235units/min for the 1:2 and 1:4 diluted samples respectively and the K_m values were calculated to be 0.111mM for both dilutions of the extract. These results thus indicated that whilst the V_{max} values varied with the amount of enzyme in the sample, the K_m value was independent of enzyme concentration.

2.3.5.4 Effect of enzyme concentration: Cell extracts from four different myeloid leukaemias (two containing all the major myeloid esterase isoenzymes and two containing all the myeloid isoenzymes with the exception of those associated with monocytic differentiation) were serially diluted to 1:8 and each examined for their rates of substrate hydrolysis at 30°C with a standard 0.5mM solution of alpha-naphthyl acetate. The increase in A235 was monitored over a 5.0min period and the rate (per min) of substrate hydrolysis determined. Apparent ANAE activities of the four different undiluted

leukaemic cell extracts examined ranged from 2.0mU to 5.3mU with observed rates of change in A235 of 0.047 to 0.122/min. Subsequent analysis of dilutions of these cell extracts revealed (Figure 2.18) a linear correlation between the observed rate of ANA hydrolysis (increase in A235/min) and enzyme concentration (extract dilution). The minimum discernible linear rate of change in A235 in these experiments was found to be 0.007, which corresponded to a defined ANAE enzyme activity of 0.3mU.

2.3.5.5 Optimised UV spectrophotometric assay for ANAE: As a result of the above investigations, an optimised method for the assay of ANAE in cellular extracts was devised. A stock 50mM substrate solution of alpha-naphthyl acetate in 2-methoxyethanol (stored at -20°C) was prepared and a working substrate solution made by diluting this in 10mM MES pH 6.3 to a final concentration of 0.5mM. 20µl of test sample was added to 1.0ml of working substrate solution in a semiquartz cuvette and the rate of increase in A235 at 30°C measured (Pye Unicam 8800 UV/VIS Spectrophotometer) over a period of 5.0 min. Samples were re-analysed, in diluted form, if the reaction rate was found to be either non-linear or exceeded 0.14 (corresponding to the upper limit of activity shown to be linear in the above studies) absorbance units/min. By calculating the rate of substrate hydrolysis, based on the formation of alpha-naphthol, it was possible to quantitate the level of ANAE. As in previous studies [Mastropaolo & Yourno, 1981], one unit of ANAE activity was defined as that amount of enzyme required to hydrolyse 1.0umol of substrate in 1.0 min at a pH 6.3 at 30°C. Relating this to an absolute change in A235, the hydrolysis of 1.0nmol of substrate (i.e. by 1.0mU of ANAE) results in an increase in A235 of 0.023.

Similar evaluations were undertaken for alpha-naphthyl butyrate, and when using this substrate in a UV spectrophotometric assay, 1.0mU of enzyme (butyrate esterase) was defined as that which caused an increase in A235 of 0.024 under identical assay conditions to that outlined for alpha-naphthyl acetate esterase.

2.3.6 Optimisation of fluorimetric esterase assays:

2.3.6.1 Effect of substrate concentration: A leukaemic AMML-M4 blast cell extract, containing all the major myeloid esterase isoenzyme forms, was diluted to 1:2 and examined for acetate and butyrate activity by fluorimetric assay at 37°C with substrate concentrations ranging from 0.05mM to 1.0mM. As for the optimisation of the UV assay, the aim of this analysis was to determine a saturating substrate concentration which could be used routinely for subsequent studies. 4-methylumbelliferyl acetate (4MB-Ac) was found to be soluble in 50mM MES pH 6.3 up to a concentration of 1.0mM, whereas traces of the 4-methylumbelliferyl butyrate (4MB-But) substrate precipitated out of solution at concentrations exceeding 0.3mM. Despite this, the rate of hydrolysis of both substrates increased proportionately with increasing substrate concentration, although at substrate concentrations of >0.4mM the rate of hydrolysis became less pronounced (Figure 2.19). On the basis of these findings, a substrate concentration of 0.5mM was chosen as being near saturating for routine use with fluorimetric assays.

2.3.6.2 Reaction rate linearity: The time courses of the fluorimetric assays for cellular acetate and butyrate esterases were investigated with a leukaemic AMML-M4 blast cell extract, containing all the major myeloid esterase isoenzyme forms. The extract was serially diluted to 1:4, and 20µl of each dilution was incubated with 250µl of 0.5mM 4MB-Ac or 4MB-But at 37°C and the fluorescent emission at 460nm was measured at 10, 20, 35, 45, 55 and 65 min. The hydrolysis of both 4MB-Ac and 4MB-But was noted to be linear over the 65 min period examined (Figures 2.20a and 2.20b) and, on the basis of these findings, reaction periods up to a maximum of 60 min were chosen for subsequent fluorimetric assays.

2.3.6.3 Effect of enzyme concentration: Serial dilutions to 1:8 of blast cell extracts were prepared for two cases of myeloid leukaemias with monocytic involvement (one each of AMML-M4 and AMoL-M5), which contained all the major myeloid esterase isoenzyme forms, and two cases of AML-M1/M2 which contained all myeloid esterase isoenzyme

forms with the exception of those associated with monocytic differentiation. 20µl of each of the dilutions were assayed under standardised assay conditions of 37°C with 250µl of working 0.5mM substrate in 50mM MES pH 6.3. At 60 min, the fluorescent emission at 460nm was measured and corrected for inherent background fluorescence due to a small amount of autohydrolysis and (more importantly) from 'non-specific' interaction of proteins with the fluorigenic substrates. Subsequent analysis of the results showed a linear relationship between observed substrate hydrolysis and sample dilution (Figure 2.21).

2.3.6.4 Optimised fluorimetric assays for acetate and butyrate esterases: On the basis of the above findings, an optimised method for the measurement of solubilised esterases was devised. Stock 30mM substrate solutions of 4MB-Ac or 4MB-But (Koch-Light) were prepared in acetone and these solutions were stored at -20°C until required, with no significant deterioration of substrate. The working substrate solutions were prepared by diluting stock substrates in 50mM MES pH 6.3 to give a final substrate concentration of 0.5mM. A standard 20µl of cell extract (or 50-100µl of chromatography fraction) was added to 250µl of this working solution and the reaction mixture incubated at 37°C for a fixed period not exceeding 60 min. Following incubation, the reaction mixture was made up to 750µl with 50mM MES pH 6.3 and the fluorescent emission at 460nm measured, with an excitation wavelength of 400nm. Standard curves for 4-methylumbelliferone standards were also determined (data not shown) but, as the primary application of these fluorimetric assays were for measuring the relative hydrolysis (enzyme activities) of sequential fractions in chromatographic separation procedures, formal conversion of observed values to unit values were not considered necessary. Consequently, for the purposes of this study, acetate and butyrate esterase activities were reported as arbitrary fluorescent units under the standardised conditions outlined above.

2.3.7 Correlations between UV spectrophotometric and fluorimetric esterase assays:

2.3.7.1 Effect of substrate concentration: A leukaemic AMML-M4 blast cell extract was tested for acetate esterase activity by both optimised UV spectrophotometric (alpha-naphthyl acetate) and fluorimetric (4-methylumbelliferyl acetate) assays (Section 2.2.9). A standard 20µl of 1:2 extract was used throughout and the results revealed a linear relationship between the activities defined by both assay procedures over the substrate concentration range of 0.05 to 0.5mM examined (Figure 2.22).

2.3.7.2 Effect of enzyme concentration: Esterases extracted from leukaemic AMML-M4 and AML-M1/M2 blasts were examined for acetate esterase activity by both UV spectrophotometric and fluorimetric assay procedures. The extracts were serially diluted to 1:8, and 20µl of each dilution tested for acetate esterase activity by optimised UV spectrophotometric and fluorimetric assay procedures. A comparison of the rate of substrate hydrolysis by the two techniques revealed a linear relationship between the rate of hydrolysis of alpha-naphthyl acetate and 4MB-Ac for all the cell extract dilutions studied (Figure 2.23).

On the basis of these results, it was considered that the optimised UV spectrophotometric and fluorimetric assay procedures were likely to be measuring the same enzymes and that esterase activities measured by either assay were directly comparable.

2.3.8 Quantitation of cell extract esterase activities:

Acetate and butyrate esterase activities for a total of 56 acute myeloid leukaemias (AML-M1/M2, n=22; APL-M3, n=3; AMML-M4, n=19; and AMoL-M5, n=12) with G1 (n=24), G2 (n=18) or M (n=14) esterase isoenzyme IEF patterns were determined by UV spectrophotometric assay. As detailed earlier (Section 2.3.5.5), 1.0mU of acetate or butyrate esterase activity was defined as that amount of enzyme required to hydrolyse 1.0nmol of substrate in 1.0 min at a standardised pH (6.3) and temperature (30°C).

Relating this to an absolute change in absorption at 235nm, the hydrolysis of 1.0nmol of alpha-naphthyl acetate or butyrate substrate resulted in an increase in A235 of 0.023 and 0.024 respectively. Using this assay system, the acetate and butyrate esterase activities were initially calculated as mU/ml extract and this was converted, following measurement of the extract protein concentration, to mU/mg protein. When the acetate and butyrate esterase activities were examined in relation to the IEF pattern types (Figure 2.24), no obvious relationships were apparent even though cases with M-type patterns by IEF clearly had higher cellular esterase activities as assessed by conventional ANAE cytochemistry. Thus even though earlier cytochemical and electrophoretic analyses suggested that monocytic leukaemias with strong diffuse ANAE staining (and M-type IEF patterns) should have a higher ratio of butyrate to acetate esterase activity than myeloblastic leukaemias with insignificant or weak diffuse ANAE cytochemical staining (and G1 or G2 patterns), this was not reflected by their relative butyrate/acetate assays. These results therefore suggest that the esterase isoenzymes detected by IEF were not the only enzymes present in the myeloid cell extracts that could hydrolyse the substrates used in UV spectrophotometric and fluorimetric assays. Similarly, acetate and butyrate esterase activities (examined fluorimetrically as described in Section 2.3.6.4) of normal myeloid cell extracts (granulocyte, n=11 and monocyte, n=9) with G1 (n=4), G2 (n=7) and M-type (n=9) esterase isoenzyme patterns, also showed no apparent relationships between the IEF pattern types and enzyme activities (Figure 2.25). Indeed, the majority (17/20) of normal myeloid cell extracts studied had apparently similar levels of esterase activity irrespective of the IEF pattern type or the substrate (acetate or butyrate esters of 4-methylumbelliferone) examined. The remaining three cases which showed higher relative rates of butyrate hydrolysis, compared to acetate, all showed M-type IEF patterns.

2.4 DISCUSSION

This section of the study has extensively evaluated the cytochemical and electrophoretic characteristics of normal and leukaemic myeloid cell alpha-naphthyl acetate (ANAE) and butyrate (ANBE) esterases, and assessed differences in esterase isoenzyme distribution

associated with granulocytic and monocytic differentiation. Normal peripheral blood granulocytes and monocytes were investigated as representative models of mature myeloid differentiation and acute leukaemias, in common with previous studies [Griffin et al., 1981; Glasser 1983; Ferraro et al., 1983; Ball & Fanger 1983], as models of myeloid precursor differentiation. Acute leukaemias offer unique opportunities for examining immature cells characterised by variable degrees of maturation arrest. While leukaemogenesis may result in aberrant or asynchronous phenotypic expression [Scott et al., 1983; Smith et al., 1983], the blast cells in many cases of leukaemia are considered to retain lineage fidelity [Reinherz et al., 1980; Habeshaw et al., 1983; Greaves 1986] and express developmental markers appropriate to their normal maturational counterparts.

The first part of the investigation examined the ANAE cytochemical characteristics of normal and leukaemic myeloid cells. The acute leukaemias examined in this study were categorised by multiple morphological, cytochemical and immunophenotypic criteria (Section 2.3.2), into myeloblastic (AML-M1/M2), promyelocytic (APL-M3), myelomonocytic (AMML-M4) and monocytic (AMoL-M5) variants. Using alpha-naphthyl acetate as substrate at pH 6.0-6.3, ANAE activity has been reported as being very weak or negative in mature granulocytes, negative or weak granular in myeloblasts and moderate to strong diffuse in monocytic cells [Li et al., 1973; Kulenkampff et al., 1977; Gordon & Hubbard 1978; Hayhoe & Quaglino 1980; Flandrin & Daniel 1973, 1981; Scott et al., 1987]. Consequently, ANAE cytochemical activity has proved to be of value in defining cells of monocytic-lineage [Scott et al., 1983] although relatively little has been reported on the ANAE activity of non-monocytic myeloid cells.

This current study, where the ANAE cytochemical reactivities for a total of 309 acute myeloid leukaemias and 11 normal granulocyte and nine normal monocyte fractions were examined, confirms previous impressions that normal mature granulocytes and the immature blasts of AML-M1/M2 are essentially ANAE negative or show weak reactions only. For comparison, moderate to strong diffuse ANAE reactions were found to be

restricted to normal and leukaemic cells of monocytic-lineage. Although, the observation that blast cells in 22% (27/125) of the AML-M1/M2 and 45% (20/44) of APL-M3 cases examined, showed distinct granular or focal ANAE reactions is of potential diagnostic relevance as, in such cases, there may be a tendency to interpret the presence of significant ANAE activity as an indicator of possible monocytic involvement. However, it appears from the results of this and previous studies [Hayhoe & Quaglino 1980; Scott et al., 1983, 1984a, b, c] that blast cell esterase cytochemistry alone may not adequately differentiate between monocytic and non-monocytic AML variants and that their diagnostic delineation may be better achieved by immunological or electrophoretic studies [Scott et al., 1984a, b, c; Linch et al., 1984]. It is also apparent that the presence of significant ANAE cytochemical activity is not inconsistent with a diagnosis of AML, particularly when morphological and immunological impressions do not indicate monocytic involvement in the leukaemic process. For example, ANAE staining in APL-M3 was noted to be predominantly associated with hypergranular cases (with only 1/14 of the hypogranular cases showing weak ANAE activity) and confirms previously reported observations [Stavem et al., 1981; McKenna et al., 1982; Tomonaga et al., 1985]. This cytochemical reaction, which is atypical in that it is not seen in normal marrow promyelocytes [Scott et al., 1983; Tomonaga et al., 1985], has been interpreted to indicate 'bilineal' or 'mixed' monocytic-granulocytic differentiation [Tomonaga et al., 1985; Lemez, 1988] based on the tenuous assumption that significant cytoplasmic ANAE staining is a restricted 'marker' for cells of the monocyte-macrophage lineage. None of the $ANAE^{+}$ AML-M1/M2 or APL-M3 cases examined in this study however, showed monocytic differentiation as defined by membrane CD14 expression. Furthermore, where equivocal ANAE activity was observed in these cases, a study of ANAE isoenzyme distribution helped to clarify the lineage-commitment of these cells.

Acute myelomonocytic leukaemias (AMML-M4) are characterised by the presence of mixed populations of blasts showing monocytic and granulocytic differentiation, as adjudged by the presence of cytoplasmic ANAE and chloroacetate esterase (CAE)

activities [Gordon & Hubbard, 1978; Kass, 1979; Hayhoe & Quaglino, 1980; Flandrin & Daniel, 1981]. Monocytic involvement in AMML-M4 cases was found in previous studies [Flandrin & Daniel, 1981; Huhn & Twardizik, 1983; Ball & Fanger, 1983; Linch et al., 1984] to be variable as reflected by the heterogeneous expression of cytoplasmic ANAE activity and membrane CD14 determinants. However, these studies suffered an inherent disadvantage in that the cases were usually classified according to morphological features only. The consequent limitations of this type of classification are thus, to a certain extent, reflected by their heterogeneous cytochemical and immunological features. Taking these factors into consideration, this present study only investigated cases in which myelomonocytic classification was unequivocal, using multiple discriminating criteria as described in Section 2.3.2. Of the 89 AMML-M4 cases examined, 38% showed monocytic involvement as characterised by moderate to strong ANAE staining in >20% of the blast cell population. However, when examined for membrane CD14 expression, 66% (59/89) of the cases investigated showed apparent monocytic differentiation, as characterised by >20% antigen-positive blasts, with 63% of these 59 $CD14^+$ cases showing negative or equivocal cytoplasmic ANAE reactivity.

There are an increasing number of reports suggesting that apparent lineage commitment, characterised by the expression of granulocytic or monocyte-associated components, may not be irreversible and that there may be a transitional period in which changes in maturational direction can be affected by extracellular stimuli [Sachs, 1978; Rovera et al., 1979; Kirschner & Goldberg, 1981; Tarella et al., 1982; Fibach et al., 1982]. The presence of chloroacetate esterase (CAE) positive blasts in 40% of the AMML-M4 cases studied, together with the ANAE heterogeneity observed in these cases, may be viewed as being consistent with the hypothesis that AMML-M4 blasts represent an early and transitional stage of monocytic differentiation. It is proposed therefore, that the cytochemical heterogeneity of blasts in AMML-M4 represents distinct maturational stages which comprise, to varying degrees, (a) blasts showing early and potentially reversible granulocytic differentiation which are myeloperoxidase (MPO) positive, CAE-positive (or

negative) and ANAE-negative; (b) blasts showing early monocytic differentiation which are MPO-positive (or negative), CAE-negative and ANAE-negative (or weakly positive), these may represent cells not irreversibly committed to monocytic differentiation; and (c) irreversibly committed blasts of monocytic-lineage characterised by variable MPO activity, negative CAE and positive cytoplasmic ANAE. The findings of this, and previous studies [Flandrin & Daniel, 1981; Huhn & Twardizik, 1983; Ball & Fanger, 1983; Linch et al., 1984], that the blast cells of AMML are considerably more heterogeneous, perhaps because of their transitional nature, than the other variants of acute myeloid leukaemia is further reflected by their patterns of ANAE isoenzyme distribution (discussed below).

Examination of the ANAE cytochemistry of morphologically and immunophenotypically classified acute monocytic leukaemias (AMoL-M5) revealed that whilst the majority of the cases showed M-type reactivity, high proportions (>80%) of blasts in a significant number (35%) of cases showed no apparent ANAE staining. Indeed when the AMoL-M5 cases were further examined for the comparative expression of membrane monocyte-associated CD14 expression and cytoplasmic ANAE, it was found that 53% of cases showed expression of both CD14 and ANAE components ($ANAE^+$ $CD14^+$). A further 11% of cases showed typical M-type ANAE staining in the absence of CD14 expression ($ANAE^+$ $CD14^-$) whilst the remaining cases expressed membrane CD14 despite the absence of significant cytoplasmic ANAE ($ANAE^-$ $CD14^+$). It would appear therefore that, as with the AMML-M4 cases, a greater proportion of cases expressed membrane CD14, compared to cytoplasmic ANAE, and this is consistent (in the absence of leukaemia-associated defects in protein transcription and synthesis) with the view [Ferraro et al., 1983; Scott et al., 1984c, 1985] that expression of membrane CD14 precedes cytoplasmic ANAE synthesis.

Electron microscopy studies have shown that the intense diffuse esterase reactivity of monocytes is associated with a plasmalamella ectoenzyme [Bozdech & Bainton, 1977,

1981; Monahan et al., 1981]. Similarly, the granular or focal esterase reactivity of myeloblasts (conventional microscopy) and T-lymphocytes (electron microscopy) has been reported to be associated with clusters of smooth membrane vesicles in the cytoplasm found close to the Golgi apparatus [Bozdech & Bainton, 1981; Monahan et al., 1981; Marec et al., 1981; Zicca et al., 1981]. Indeed, the ANAE cytochemical pattern types, observed in cells of myeloid lineages, correlate well with the subcellular location of these enzymes as determined by ultrastructural studies.

The esterase polymorphism of haemopoietic cells, in view of their different cytochemical staining patterns and subcellular localisations, were further examined by zymogram studies, as advocated by Hunter and Burstone [1960]. The aims of these studies were primarily to clarify whether the above observations reflected differential subcellular distribution of the same isoenzymes or the existence of different esterase isoenzyme groups. In earlier studies, electrophoretic separation of esterase components was achieved using conventional polyacrylamide gel electrophoresis (PAGE) [Li et al., 1973; Kass & Peters 1978; Yourno & Mastropaolo, 1981]. For example, separation of myeloid esterase isoenzymes by PAGE at pH 4.0 [Li et al., 1973] defined nine main esterase components (designated 1 to 9). Four of these components (2, 4, 5 and 6), representing nonspecific esterases, were noted to hydrolyse acetate and/or butyrate esters of alpha-naphthol, while the remainder hydrolysed naphthol-AS-D chloroacetate and corresponded to chloroacetate esterases. Three nonspecific esterase components (2, 5 and 6) were additionally shown to be common to both granulocytes and monocytes, whereas component 4 was shown to be unique to cells of monocytic lineage. Moreover, inhibitor studies with 0.04M sodium fluoride (NaF) further indicated that isoenzymes 4 and 5 were NaF sensitive. Similarly, PAGE separation of leucocyte esterases at pH 9.5 [Yourno & Mastropaolo, 1981], resolved a total of 12 different isoenzyme components but again only four of these (designated A1, A2, AB1 and AB2) were noted to be expressed by cells of granulocytic and monocytic lineages. Two of these isoenzymes (A1 and A2) preferentially hydrolysed alpha-naphthyl acetate and were relatively NaF-resistant

whereas the other two species (AB1 and AB2) showed similar reactivity with both acetate and butyrate esters and were highly NaF-sensitive. However, despite differences in substrate and inhibitor properties all four esterase components were noted to be common to both granulocytes and monocytes.

While PAGE techniques have revealed differences between esterases from various types of haemopoietic cell, with the advent of isoelectric focusing (IEF) it became possible to demonstrate additional isoenzyme components [Kass & Munster, 1979a, 1979b] which were previously unresolved by PAGE. Separation of myeloid esterases by IEF [Kass & Munster, 1979a, 1979b; Radzun et al., 1980; Parwaresch et al., 1981; Scott et al., 1984a; Drexler et al., 1985; Cohn et al., 1987] resolved up to 18 esterase isoenzymes displaying different isoelectric points (pIs), and granulocyte preparations showed up to 11 esterase isoenzyme components within the pI range 6.3 to 7.9 [Radzun et al., 1980; Scott et al., 1984a; 1984b; Cohn et al., 1987]. In contrast, the strong diffuse cytochemical staining of normal and leukaemic monocytes was found to result from the presence of high cellular concentrations of 5-6 acidic isoenzymes within a narrow pI range of approximately 5.5 to 6.2 [Radzun et al., 1980; Scott et al., 1984a; 1984b; Drexler et al., 1985; Cohn et al., 1987]. These initial findings were considered to be sufficient to warrant the use of IEF, rather than conventional PAGE, for the characterisation of myeloid esterases in this study.

In this present study, esterase isoenzymes were extracted from purified myeloid cells by ultrasonication followed by membrane solubilisation using the nonionic detergent Triton X-100. Preliminary investigations showed that the solubilisation of cellular myeloid ANAE was partially facilitated by the incorporation of detergent in the extraction buffers and that the type of detergent used, nonionic or zwitterionic, made no significant qualitative or quantitative difference to the resulting esterase isoenzyme patterns. Consequently, the nonionic detergent Triton X-100, in agreement with previous studies [Yourno & Mastropaolo, 1981; Scott et al., 1983; Oertel & Kastner, 1984; Oertel et al.,

1985; Cohn et al., 1987], was chosen for the routine extraction of cellular ANAE. In addition, the use of this nonionic detergent permitted analyses of the extracted enzymes by means of biological activity and also allowed fractionation by ion-exchange and electrophoretic techniques. In this context, supplementary investigations showed that increasing Triton X-100 concentration (up to 10%) had no effect on the relative distribution of ANAE molecular forms when compared with ANAE extracted in absence of detergent. Therefore, the findings of this study indicate no evidence for the incorporation of ANAE into detergent-micellar forms which could lead to differences in molecular weight profiles of extracted proteins. However, the observations suggest that myeloid ANAE isoenzymes may be membrane-associated although, this and previous studies were unable to definitively elucidate the nature, intrinsic or extrinsic, of the enzyme-membrane association.

Composite IEF zymograms of esterase isoenzyme components for most normal blood myeloid elements have been investigated by independent groups [Radzun et al., 1980; Parwaresch et al., 1981; Cohn et al., 1987; Scott et al., 1989]. This present study, detailing the ANAE isoenzyme distributions of normal granulocytic and monocytic preparations, in addition to 204 acute myeloid leukaemias (representing different levels of maturation and lineage-commitment), extends these previous observations. Esterase zymograms of normal myeloid subtypes showed three main isoenzyme pattern types, designated G1, G2 and M (Figure 2.9). Normal granulocyte extracts expressed up to six ANAE isoenzyme components within a broad pI range of 6.3-7.9 (G1) and the monocytic extracts were characterised by the additional presence of a group of strongly staining isoenzymes within a narrow pI range of 5.5-6.2 (M). The third isoenzyme pattern type, seen with a significant proportion of the granulocyte extracts and designated G2, was identical to the G1 pattern with the exception that two weakly staining ANAE isoenzymes with pIs of 6.1-6.2 were seen in addition to the consistently observed isoforms with higher pIs.

Some variations in these composite pattern types have been reported in a small proportion of monocytic leukaemias [reviewed in Drexler et al., 1991a]. These include an over expression of monocyte-associated isoenzymes and/or reduced expression of isoenzymes within the pI range of 6.2-7.9. However, comparative analyses of IEF esterase zymograms in this current study indicate that the pI of individual ANAE isoenzymes and the distribution of the two main esterase isoenzyme groups (pI 6.3-7.9 and 5.5-6.2) in myeloid leukaemias were virtually identical with those seen in their normal counterparts. For example, the AML-M1/M2 and APL-M3 cases, representing early granulocytic differentiation, predominantly expressed ANAE isoenzymes within the pI range of 6.3 to 7.9 (i.e. G1 pattern), with a minor proportion of cases expressing the two additional isoenzymes characteristic of the G2 pattern. With regards to expression of monocyte-associated ANAE isoenzymes, only one of the 96 acute myeloblastic/promyelocytic cases studied showed the additional presence of these components; in retrospect, it is likely that this case was in fact a $CD14^-$ AMML-M4 which was incorrectly classified as AML-M2. The simultaneous expression of ANAE isoenzymes of pI range 6.3 to 7.9 and pI range 5.5 to 6.2 was closely associated with monocytic variants in which moderate to strong diffuse ANAE staining was seen. In acute myelomonocytic leukaemia (AMML-M4), IEF patterns appeared to reflect cytochemical heterogeneity in that 46% of cases showed the presence of significant monocyte-associated ANAE components, whilst the remainder only expressed ANAE isoforms with higher pIs. Of the 45 AMoL-M5 cases examined by IEF it was found, irrespective of morphological subtype (M5a and M5b) or membrane CD14 expression, that the majority (67%) of the cases expressed both groups of ANAE isoenzymes.

The true value of ANAE isoenzyme studies became apparent when the isoenzyme patterns were compared with the ANAE cytochemistry and CD14 expression. In addition to conclusively excluding monocytic involvement in leukaemias with atypical ANAE reaction products, these analyses were able to clarify the nature of myeloid leukaemias in which membrane CD14 expression was aberrant or where conventional ANAE

cytochemistry was equivocal and difficult to interpret. With respect to acute promyelocytic leukaemia, all cases examined showed a G1 or G2 ANAE-IEF pattern-type with no detectable monocyte-associated isoenzyme forms. The cases studied included five M3-hypergranular subtypes in which high proportions of the promyelocytes showed significant (weak to moderate/strong) ANAE staining, and indicates that the 'atypical' ANAE cytochemical staining patterns of hypergranular M3 cases are not due to the presence of monocytic esterase isoenzymes. It appears that the 'atypical' cytochemical patterns observed in these cases can be attributed to an over-expression of esterase components with pI 6.3 to 7.9, possibly as a result of leukaemogenesis, rather than the *de novo* synthesis of monocyte-associated isoenzymes. Therefore, the earlier interpretations of 'mixed' or 'bilineal' granulocytic/monocytic differentiation [Tomonaga et al., 1985; Lemez, 1988] in leukaemic promyelocytes are not confirmed by this current study of ANAE isoenzymes.

To broadly summarise these particular investigations, this current study has in common with previous reports [Scott et al., 1983, 1984a; Cohn et al., 1987] demonstrated by IEF two main groups of esterase isoenzymes in normal and leukaemic myeloid cells. The first group, with a pI range of 5.5 to 6.2, was found to be expressed exclusively by monocytoid cells and the second group (pI range 6.3 to 7.9) showed weaker staining intensities and was found to be expressed by both monocytic and granulocytic cells. The individual isoenzyme components comprising each of these esterase isoenzyme groups were found consistently to have the same pIs in all the normal and leukaemic myeloid cell extracts analysed. This suggested that the artefactual creation of secondary isoenzymes, subsequent to sonication/protein extraction, was not a significant factor. The differences in pI between the two isoenzyme groups clearly reflect differences in overall charge, which may be of genetic origin, making them true isoenzymes, or may result from post-translational modifications (i.e. secondary isoenzymes). Alternatively, differences in charge may well reflect the existence of distinct enzymes, which display different physiological roles *in vivo* but share the ability to hydrolyse the same non-physiological

substrate (alpha-naphthyl acetate) *in vitro*. For ease of discussion, the monocyte-associated ANAE isoenzymes of pI 5.5 to 6.2 will be hereafter referred to as 'MonEst' and the ANAE isoenzymes of pI 6.3 to 7.9 as 'ComEst' because of their common expression by both monocytic and granulocytic cells. Using standard biochemical nomenclature, MonEst are generally considered as being 'carboxylesterases' (EC 3.1.1.1) and are most commonly demonstrated cytochemically by a substrate-diazonium dye reaction using alpha-naphthyl acetate (ANA) as substrate and diazotised pararosaniline as coupler. Although, ComEst may also be demonstrated by a similar substrate-diazonium dye reaction, the nature of these esterases has (to date) not been described in terms of biochemical nomenclature.

Current concepts of myeloid differentiation are consistent with a common precursor for granulocytes and monocytes. With respect to esterase synthesis, this current study has shown that ComEst isoenzymes are synthesised by both normal and leukaemic myeloid elements, irrespective of lineage (granulocytic or monocytic) commitment and stage of maturation, and that myeloid cells committed to monocytic differentiation are characterised by the additional expression of MonEst isoenzymes, which are not observed in granulocytic differentiation. Thus the transition from precursor to committed myeloid cells is accompanied by the differentiation-linked expression of monocyte-specific esterases. Investigations to determine the level of myeloid maturation at which commitment to specific granulocyte or monocyte differentiation occurs have largely been based on the *in vitro* characterisation of cell lines such as HL60 (originally obtained from a patient with acute myeloblastic leukaemia [Collins et al., 1977]). HL60 cells have been demonstrated *in vitro* to differentiate either to mature granulocytes or monocytes following treatment with various chemical inducers. Granulocytic differentiation may be induced by treatment with dimethylsulphoxide (DMSO) or retinoic acid, and monocytic differentiation by 12-0-tetradecanoylphorbol-13-acetate (TPA). These cells therefore represent a possible model for studying alterations in biochemical characteristics associated with specific granulocytic or monocytic differentiation [Chiao et al., 1981;

Fibach et al., 1982; Ball & Fanger, 1983; Ferraro et al., 1983; Yourno et al., 1984]. The main conclusions drawn from these studies appear to indicate that commitment to granulocytic or monocytic differentiation occurs at the promyelocyte stage and that this process may not be irreversible as there appears to be a transitional period in which changes in maturational direction may be effected by alterations in extracellular stimuli [Rovera et al., 1979; Tarella et al., 1982; Fibach et al., 1982; Ball & Fanger, 1983]. Again, with respect to esterase synthesis, the expression of MonEst components appears to be restricted to HL60 cells induced to monocytic differentiation using TPA [Yourno et al., 1984], while non-induced and DMSO or retinoic acid induced cells are characterised by the expression of chloroacetate esterase (CAE). This hypothesis requires that both ANAE and CAE expression by committed myeloid cells is subject to differential gene activation, a possibility first suggested by Markert & Hunter [1959] and more recently by Yourno and Mastropaolo [1981].

Having established the lineage association of the two main myeloid esterase isoenzyme species, this study additionally undertook preliminary analyses of substrate specificities and inhibitor sensitivities in order to assess possible physicochemical differences between these two main esterase groups. Qualitative histochemical studies of the individual isoenzyme groups (following IEF fractionation), using substrates of increasing acyl chain length provided a definitive insight into the relative substrate affinities of the MonEst and ComEst species. These analyses revealed that whereas MonEst components were capable of hydrolysing equimolar acetate, propionate and butyrate esters, the ComEst components showed a markedly decreased staining with increasing acyl chain length and showed no significant hydrolysis of alpha-naphthyl butyrate. Esterase substrate specificities studies, following IEF fractionation of individual isoforms, have not previously been carried out and can be applied to explain earlier cytochemical observations where, for example, the finding that promyelocytes in APL-M3 with abnormally increased ANAE activity, which were unreactive with the butyrate substrate [Scott et al., 1989; Scott & Drexler, 1989], is entirely consistent with the absence of MonEst isoenzymes in these cells.

In order to further substantiate these observations, two different quantitative esterase enzyme assays were optimised. Using these procedures, the acetate and butyrate esterase activities of crude normal and leukaemic myeloid cell fractions were determined and related to esterase isoenzyme pattern types as defined by IEF. These studies however, showed no apparent correlations between the type of isoenzymes expressed and the rates of acetate or butyrate ester hydrolysis. Indeed, it was found that extracts with ComEst isoenzyme components only, hydrolysed butyrate esters at similar rates to those extracts containing both ComEst and MonEst isoenzyme components. These observations are not in accord with the above substrate studies, where the separated ComEst components consistently showed no significant hydrolysis of alpha-naphthyl butyrate.

The diversity of esterases from various tissues and the lack of specific differentiating characteristics has led to some confusion regarding the biochemical classification of myeloid esterase isoenzymes. Classification of enzymes with esterolytic activities have traditionally been based, in the absence of knowledge of physiological substrate, on criteria such as substrate specificity and inhibitor sensitivity. Histochemically and cytochemically important esterases, belonging to the carboxylic ester hydrolase (EC 3.1.1) group, include carboxylesterases (EC 3.1.1.1), arylesterases (EC 3.1.1.2), acetylesterases (EC 3.1.1.6), acetylcholinesterases (EC 3.1.1.7) and cholinesterases (EC 3.1.1.8). Carboxylesterases represent esterolytic enzymes which hydrolyse carboxylic esters and are inhibited by organophosphates (i.e. di-isopropyl fluorophosphate; DFP) but not eserine [Holmes & Masters, 1967; Krisch, 1971]. Arylesterases are classified on the basis of their restricted reactivity with aromatic esters, and their relative sensitivity to sulphydryl reagents (i.e. p-hydroxymercuribenzoic acid; HMBA) and resistance to organophosphates [Holmes & Masters, 1967]. Acetylesterases show restricted reactivity with aromatic esters of acetic acid and are not inhibited by organophosphates, sulphydryl reagents or eserine [Holmes & Masters, 1967]. Finally acetylcholinesterases and cholinesterases, as the nomenclature suggests, hydrolyse esters of acetylcholine and

choline respectively and are inhibited by organophosphates, neostigmine and/or eserine [Holmes & Masters, 1967].

Preliminary examination, undertaken in this current study, of inhibition characteristics of MonEst and ComEst isoenzymes revealed that sodium fluoride (NaF) and phenylmethylsulphonyl fluoride (PMSF) exerted inhibitory effects predominantly on the MonEst isoenzyme components. In agreement with previous studies [Scott et al., 1984a; Drexler et al., 1991a], the inhibition of MonEst by NaF was shown to be relative rather than specific, as inhibitor concentrations exceeding 1.5mg/ml (36mM) also inhibited ComEst components. Cohn et al., [1987] report, in their inhibition studies of myeloid esterases, that PMSF inhibits both groups of esterase isoenzymes and that the inhibition of MonEst is irreversible whilst that of ComEst is reversible. However, this is not substantiated in the current study, which demonstrated that PMSF specifically inhibits the MonEst species and shows no inhibitory activity against the ComEst components. PMSF and organophosphates irreversibly inactivate enzymes by covalently binding to a serine residue present in the active site of the enzyme, PMSF through sulphonylation and organophosphates through phosphorylation [Choudhury, 1971; 1972; Cohn et al., 1987]. Inhibition of the two isoenzyme groups by organophosphates (DFP or bis-(4-nitrophenyl)-phosphate; BNPP) have been reported by various authors [reviewed in Drexler et al., 1991a] and the findings consistently indicate that both esterase isoenzyme groups are inhibited by organophosphorous compounds, suggesting the presence of a serine residue at the active site of both isoenzyme groups [Choudhury, 1971; 1972; Drexler et al., 1991a]. Due to their highly toxic nature, organophosphorous compounds were not investigated in this present study although, a closer examination of recent publications reporting esterase inhibition with these compounds [Oertel & Kastner 1984; reviewed in Drexler et al., 1991a] reveals that where authors describe the inhibition of granulocyte-associated esterases these isoenzymes have been quoted as displaying pIs values of 4.5 to 5.0. Consequently, these isoenzyme components, in light of the findings of this and previous studies [Scott et al., 1983, 1984a; Cohn et al., 1987], almost

certainly do not represent the ComEst isoforms (pI 6.3-7.9) associated with cells of granulocytic and monocytic differentiation. Taking these points into account, it is proposed therefore, that MonEst isoenzymes probably have a serine active site whilst this is less likely for the ComEst species. Neostigmine, eserine and the sulphydryl reagent HMBA showed no observable inhibition of enzymatic activity of either enzyme group, thereby ruling out the possibility of either group being arylesterases (HMBA-sensitive), acetylcholinesterases or cholinesterases (neostigmine and/or eserine-sensitive).

In conclusion, the present study has demonstrated two distinct myeloid enzyme forms, showing esterolytic activity, with differing cytochemical, electrophoretic and catalytic characteristics. The MonEst components have been shown to be expressed in a lineage-restricted manner by cells of monocytic-lineage, whilst ComEst components have been demonstrated to be expressed by both monocytic and granulocytic cells. These observations, in conjunction with previous ultrastructural investigations [Bozdech & Bainton, 1977, 1981; Monahan et al., 1981; Marec et al., 1981; Zicca et al., 1981], which indicate differences in subcellular locations of the granulocytic and monocytic esterases, suggest that the ComEst and MonEst species could represent two distinct esterolytic enzymes which may be encoded by separate structural genes [Moss, 1986]. Indeed, it appears that the preliminary substrate specificity and inhibitor sensitivity studies undertaken here substantiate the general view that MonEst isoenzymes fulfil the rather loose criteria for categorisation as carboxylesterase (EC 3.1.1.1) and that the ComEst components, contrary to previous findings [Oertel & Kastner, 1984; Cohn et al., 1987], represent a separate and, as yet, uncharacterised esterase group. These initial observations and interpretations are further investigated in Chapters Three and Four.

CHAPTER 2

Figures 2.1a to 2.25 and Tables 2.1 to 2.14

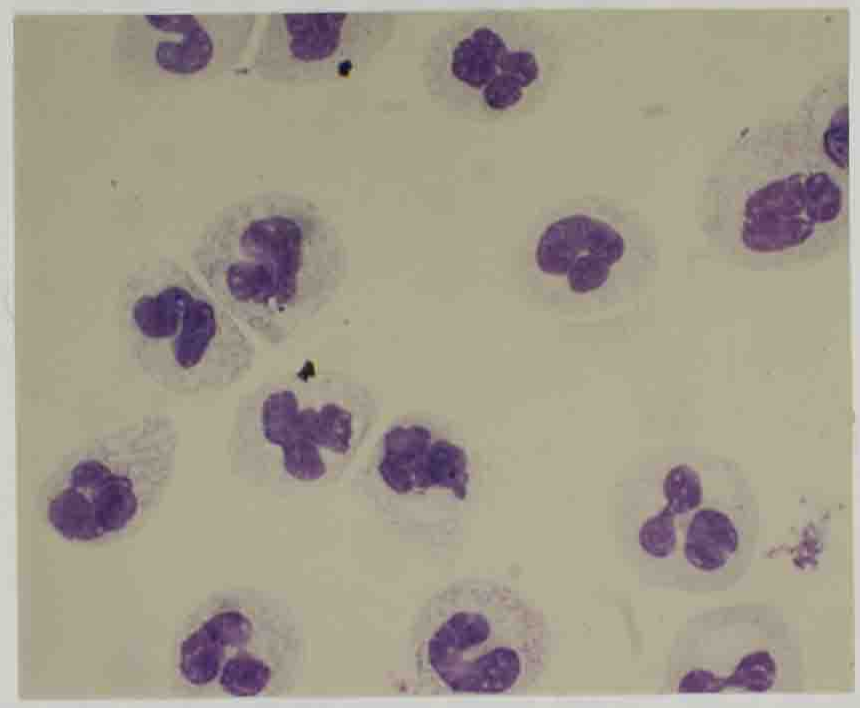

Figure 2.1a: *May-Grunwald Giemsa (Romanowsky) stained cytocentrifuge preparation of enriched peripheral blood neutrophils. Neutrophils were isolated from peripheral bloods of healthy donors by density sedimentation techniques (Section 2.2.1.1) with resulting purities of >98%, and viabilities in excess of 95%.*

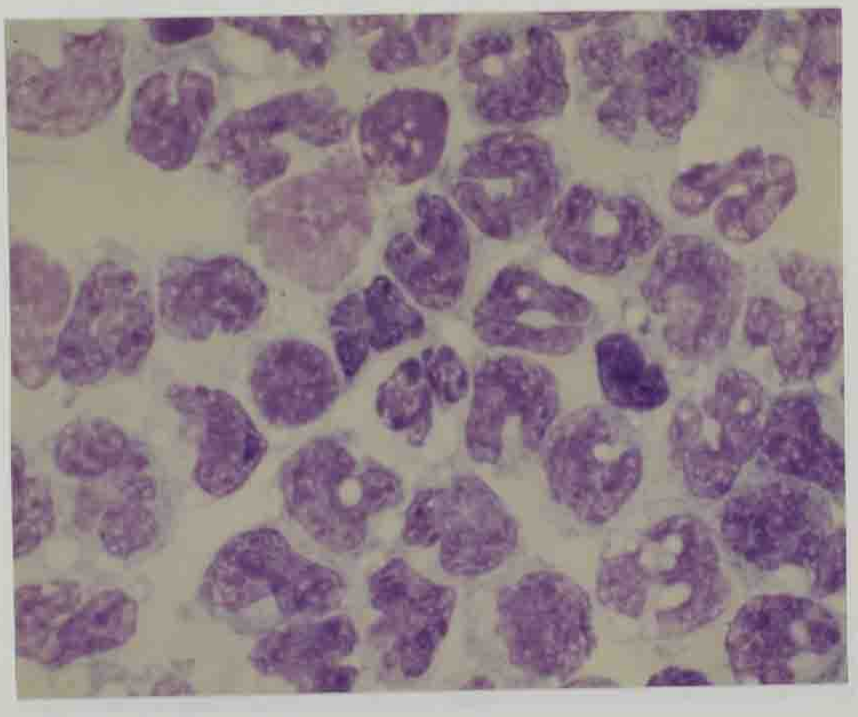

Figure 2.1b: *May-Grunwald Giemsa stained cytocentrifuge preparation of enriched peripheral blood monocytes isolated from the peripheral blood of healthy adult donors by a three-stage fractionation procedure (Section 2.2.1.2) to a mean purity of 77%, with viabilities in excess of 90%.*

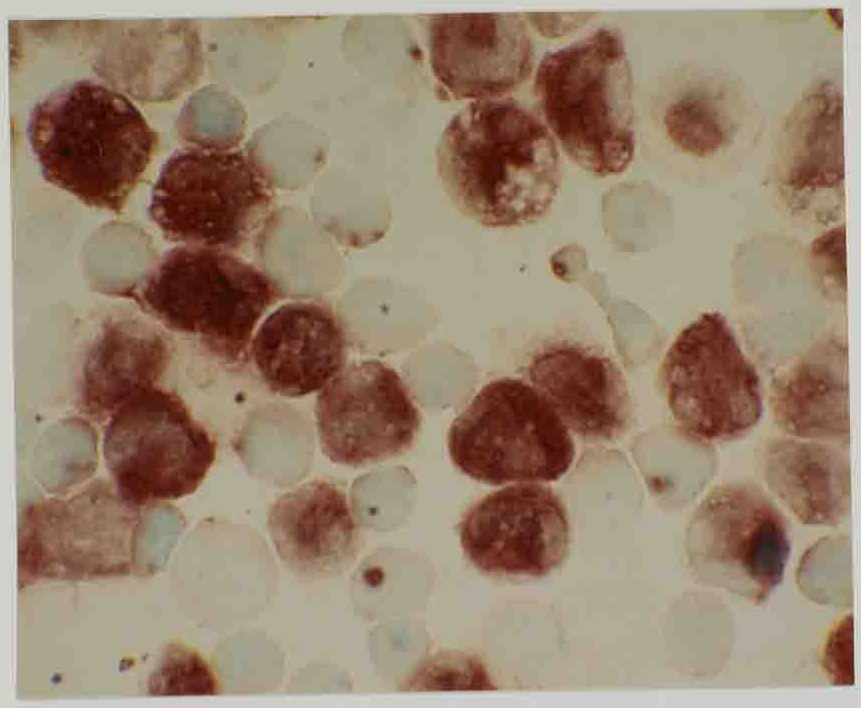

Figure 2.2a: *Alpha-naphthyl acetate esterase (ANAE) stained cytocentrifuge preparation of peripheral blood lymphocytes and monocytes fractionated by a single-step density sedimentation with Lymphoprep (Section 2.2.1.2). The monocytes show typical strong diffuse (M-type) reactivity whereas the lymphocytes show either negative or 'dot' type ANAE cytochemistries.*

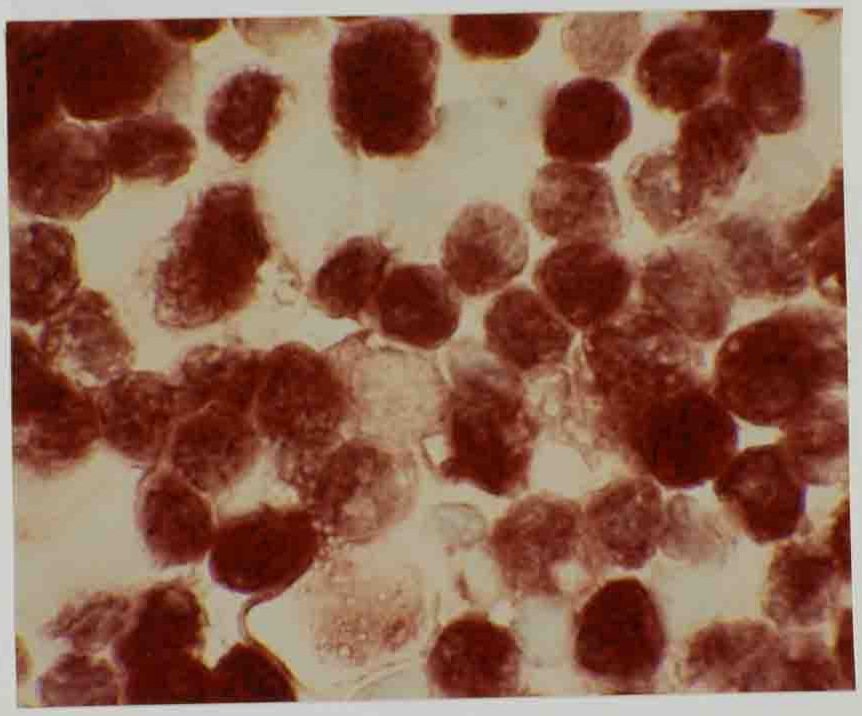

Figure 2.2b: *Alpha-naphthyl acetate esterase stained cytocentrifuge preparation of enriched peripheral blood monocytes isolated from the peripheral blood of healthy adult donors by a three-stage fractionation procedure (Section 2.2.1.2) to a mean purity of 77% with viabilities in excess of 90%. Note the strong diffuse staining (M-type) of virtually all cells in this particular fraction.*

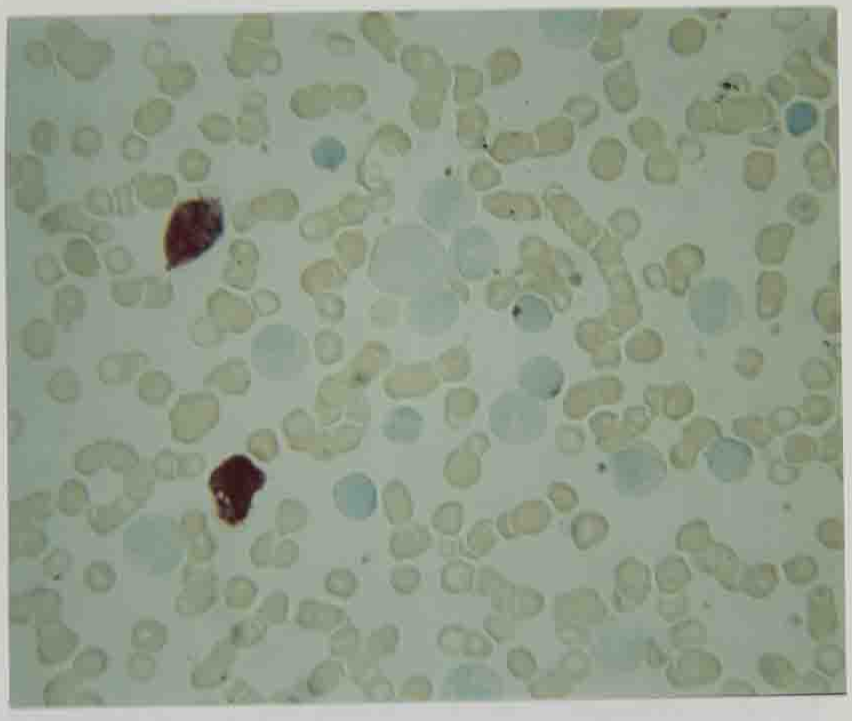

Figure 2.3: *Negative alpha-naphthyl acetate esterase (ANAE) blast cell cytochemistry in a case of acute myeloblastic leukaemia (AML-M1/M2). The majority of leukaemic blasts are unreactive for ANAE in contrast to the strong diffuse (M-type) reactivity of coexisting normal monocytes.*

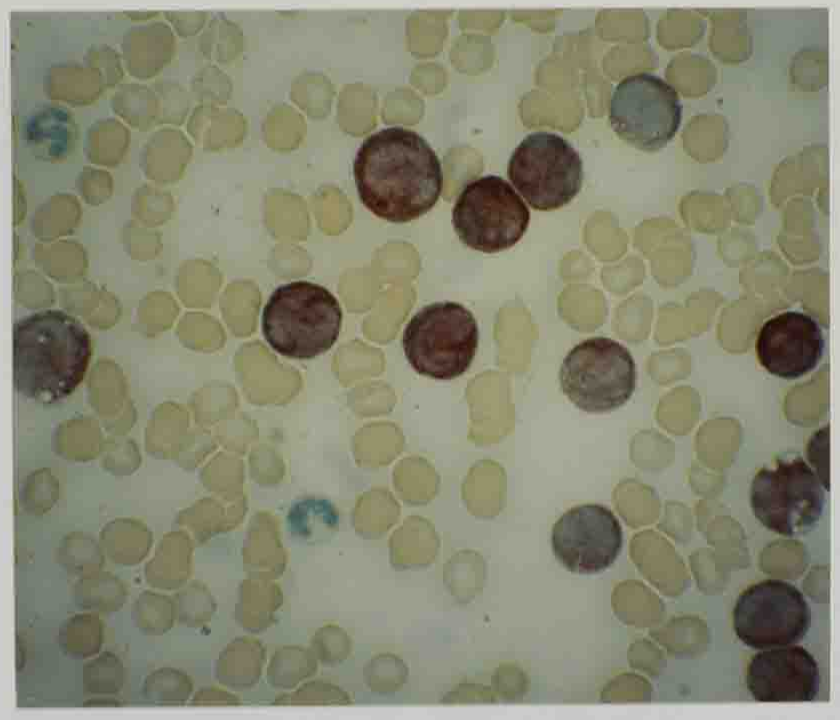

Figure 2.4: *Alpha-naphthyl acetate esterase (ANAE) blast cell cytochemistry in a typical case of acute monocytic leukaemia (AMoL-M5). The majority of leukaemic blasts show moderate to strong diffuse cytoplasmic (M-type) staining whereas the mature granulocyte forms are unreactive.*

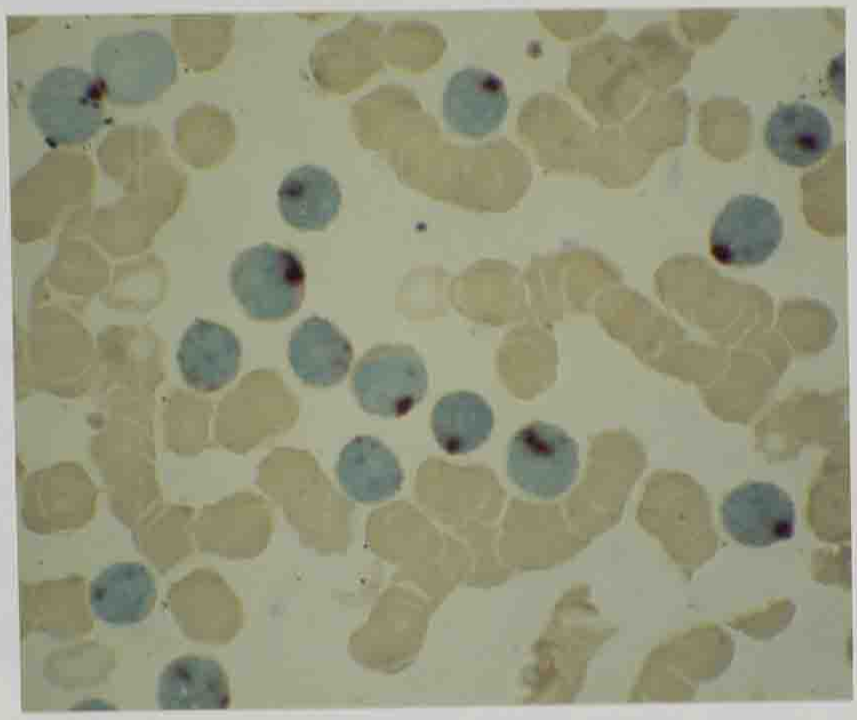

Figure 2.5: *Atypical alpha-naphthyl acetate esterase (ANAE) blast cell cytochemistry in a case of acute myeloblastic leukaemia (AML-M1/M2). The majority of leukaemic blasts show a moderate to strong localised (focal) ANAE staining pattern.*

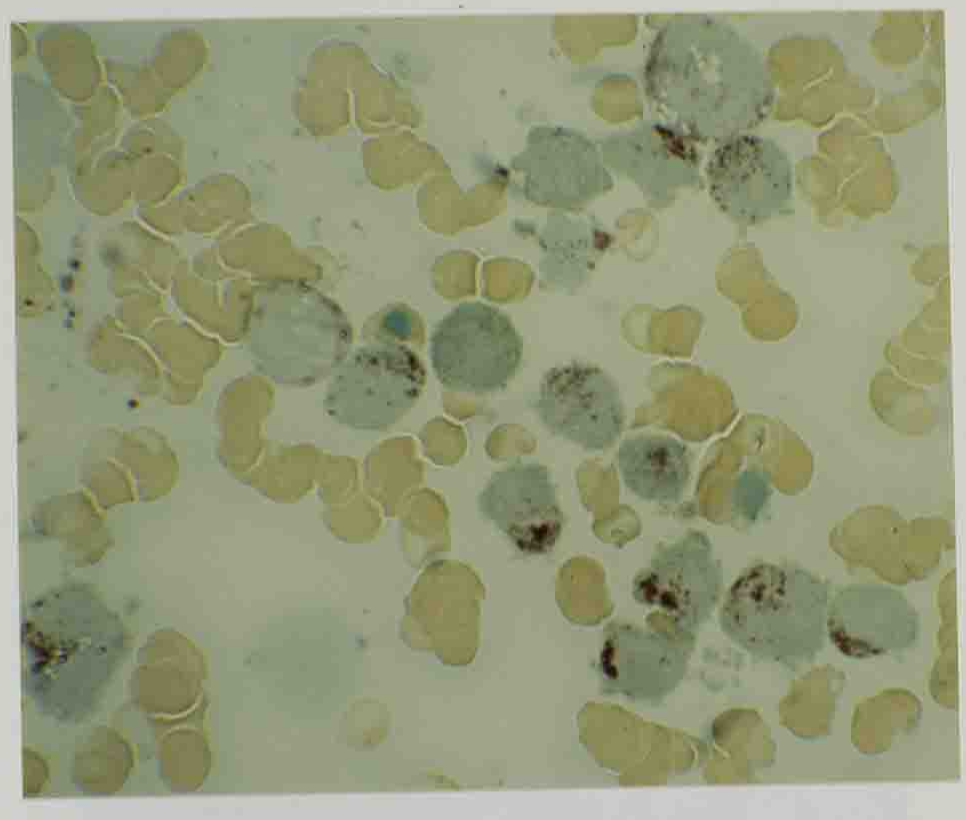

Figure 2.6: *Atypical alpha-naphthyl acetate esterase (ANAE) blast cell cytochemistry in a case of acute myeloblastic leukaemia (AML-M1/M2). The majority of leukaemic blasts show a localised coarse ANAE reaction which differs from the 'atypical granular reaction (AGR)' associated with granulocytes in some cases of MDS and hypergranular promyelocytic leukaemia (see Figure 2.7).*

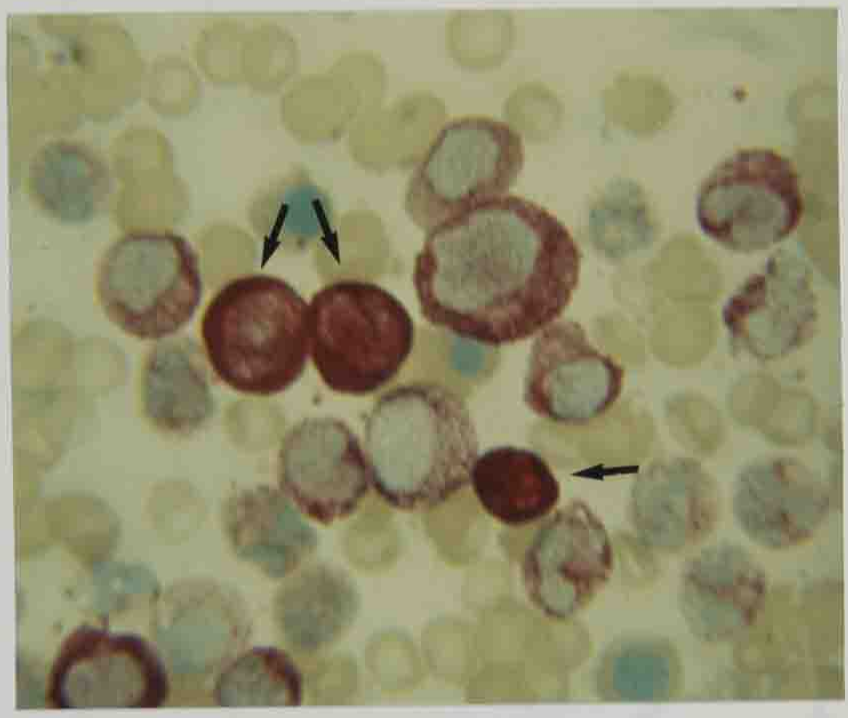

Figure 2.7: *Alpha-naphthyl acetate esterase (ANAE) cytochemical reaction in a case of myelodysplastic syndrome (MDS). ANAE-positive cells are predominantly of atypical granular (AGR) type and the reaction product is evenly distributed throughout the cytoplasm of both mature and immature granulocytes. This type of ANAE reactivity is also commonly seen in the hypergranular variant of acute promyelocytic leukaemia (APL: M3-hyper) and is in distinct contrast to typical M-type staining (arrows) and the coarse localised granular reaction occasionally seen in AML blasts (see Figure 2.6).*

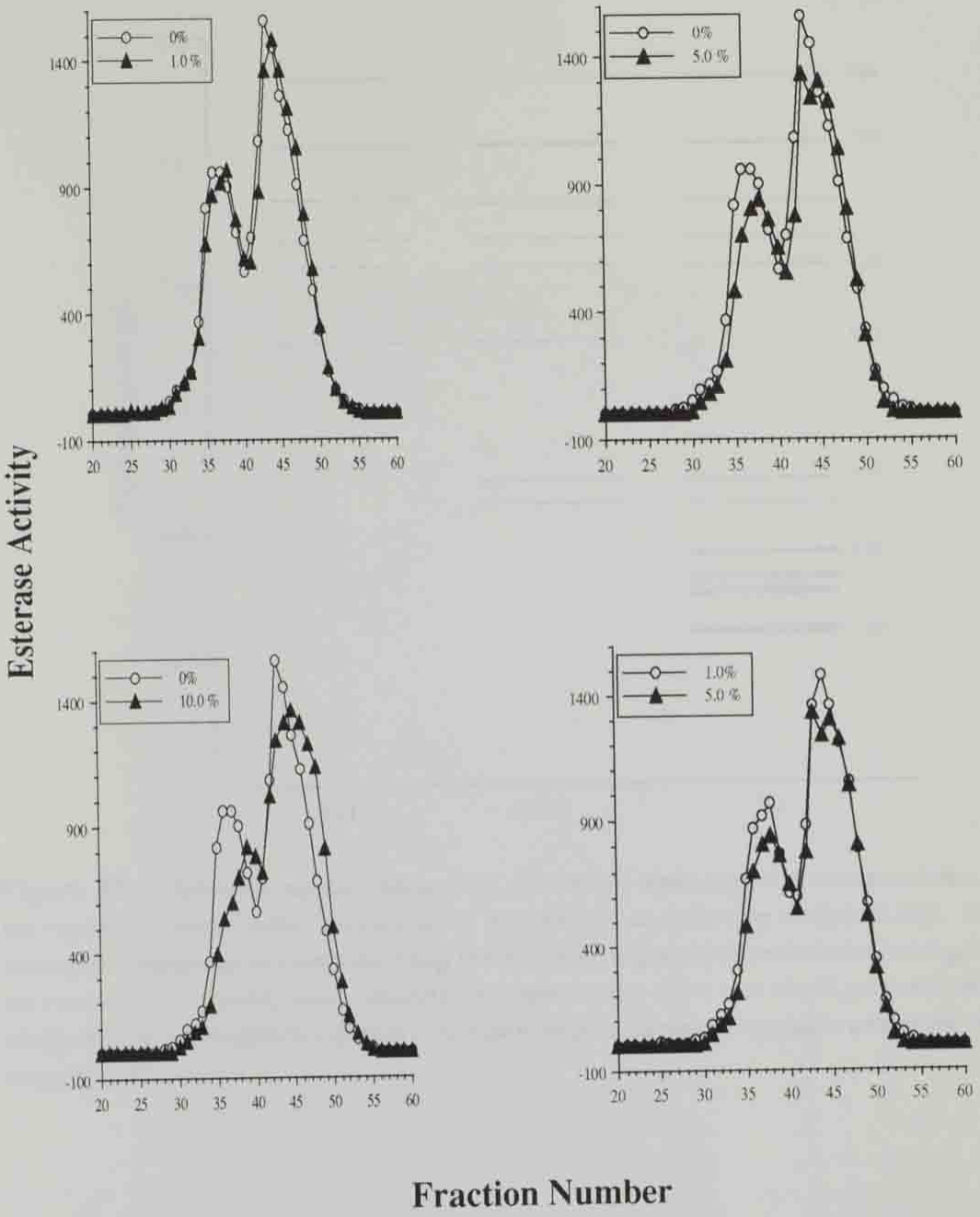

Figure 2.8: *Effect of incorporating different Triton X-100 detergent concentrations in the extraction buffer on the relative concentrations of esterase molecular weight species defined by Superose-12 FPLC gel filtration. Extraction procedures were carried out as detailed in Section 2.3.3.1. Column flow rates were 0.35ml/min, and esterase activities were determined by fluorimetric assay with an equimolar 4MB-Ac and 4MB-But substrate mixture (results expressed as fluorescent units/100ml fraction/hr at 37C). The four diagrams compare esterase molecular weight profiles obtained in the presence of 0%, 1.0%, 5.0% and 10.0% Triton X-100. Note that the relative proportions of the two esterase species are apparently unaffected by the different detergent concentrations used.*

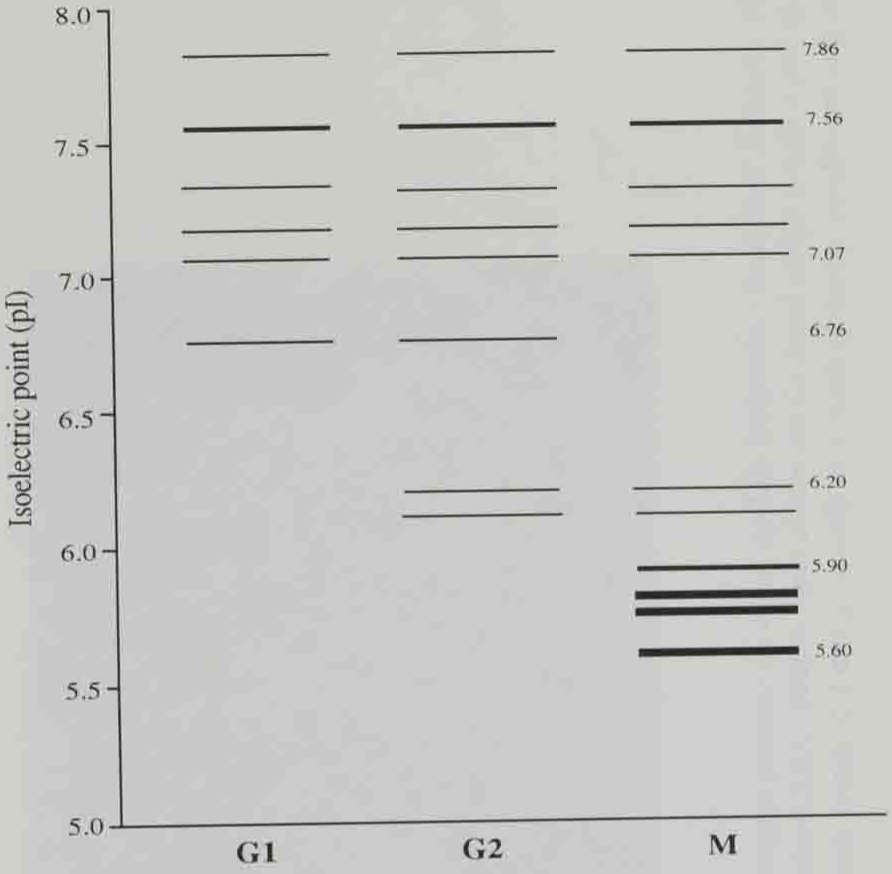

Figure 2.9: *Schematic representation of G1, G2 and M alpha-naphthyl acetate esterase (ANAE) isoenzyme patterns of normal and leukaemic myeloid cells, as defined by analytical IEF. Individual isoenzyme components are shown according to their isoelectric point (pI), and relative staining intensities are shown as —— weak, ▬ moderate, and ▬ strong. Note that the M pattern is characteristically defined by the presence of high concentrations of isoenzyme components within the narrow pI range 5.5 - 6.2.*

Figure 2.10: *Representative alpha-naphthyl acetate esterase (ANAE) isoenzyme patterns in acute myeloid leukaemia. Individual leukaemic blast cell extracts were electrophoresed (IEF) and stained for ANAE as described in Section 2.2.6.1. Pattern types shown are G1 (track e), G2 (tracks a and h), and M (tracks b and c). Cases in which ANAE isoenzyme components were undetectable (tracks d, f and g), usually as a result of having insufficient cells available for extract preparation, were excluded from the study.*

Figure 2.11: *Alpha-naphthyl acetate esterase (ANAE) isoenzyme pattern variations in acute monocytic leukaemia (AMoL-M5). Three representative cases are presented; (a) shows high concentrations of monocyte-associated (MonEst) isoenzymes (pI range 5.5-6.2) with relatively little expression of ComEst myeloid forms (pI range 6.3-7.9). In contrast, (b) and (c) both show 'common' isoenzyme forms in addition to those which are specifically monocyte-associated; note minor variations in the relative distributions of various isoenzyme components in these two cases.*

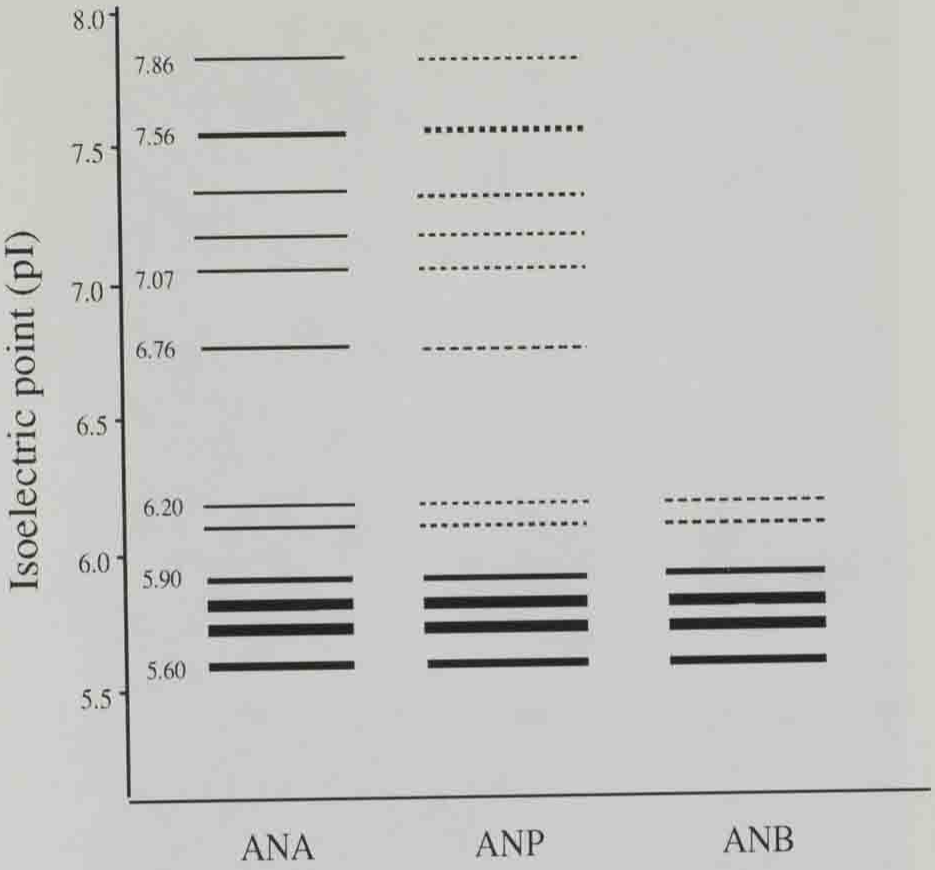

Figure 2.12: *Schematic representation indicating the relative hydrolysis of acetate (ANA), propionate (ANP) and butyrate (ANB) esters of alpha-naphthol by leukaemic cell esterase isoenzymes. Esterases extracted from leukaemic blasts were electrophoresed (IEF) and subsequently stained with staining solutions containing 5.0 mM ANA, ANP or ANB substrate concentrations and hexazotised pararosaniline as coupler. This Figure represents a summary of the results observed for a total of seven different leukaemias with M esterase IEF patterns (AMML-M4, n = 5; and AMoL-M5 n = 2).*

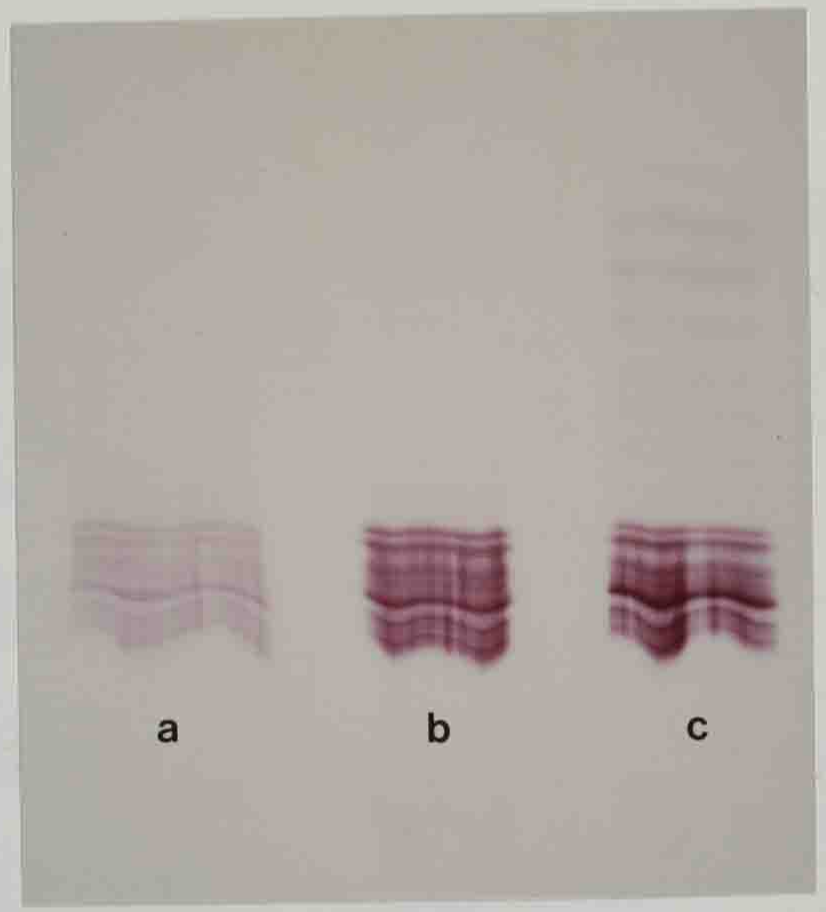

Figure 2.13: *Original electrophoretic gel comparing substrate specificities of esterase isoenzyme forms. A leukaemic blast cell extract containing all the major myeloid isoenzyme forms was electrophoresed (IEF) in three separate lanes, each of which was alternatively stained with butyrate (a), propionate (b), or acetate (c) substrates (Section 2.2.6.2). Note that the lower pI (monocyte-associated) esterase isoenzymes appear to be stained with all three substrates, although the reaction is weaker with alpha-naphthyl butyrate. For comparison, the higher pI ('common') isoenzymes preferentially stain with the alpha-naphthyl acetate substrate.*

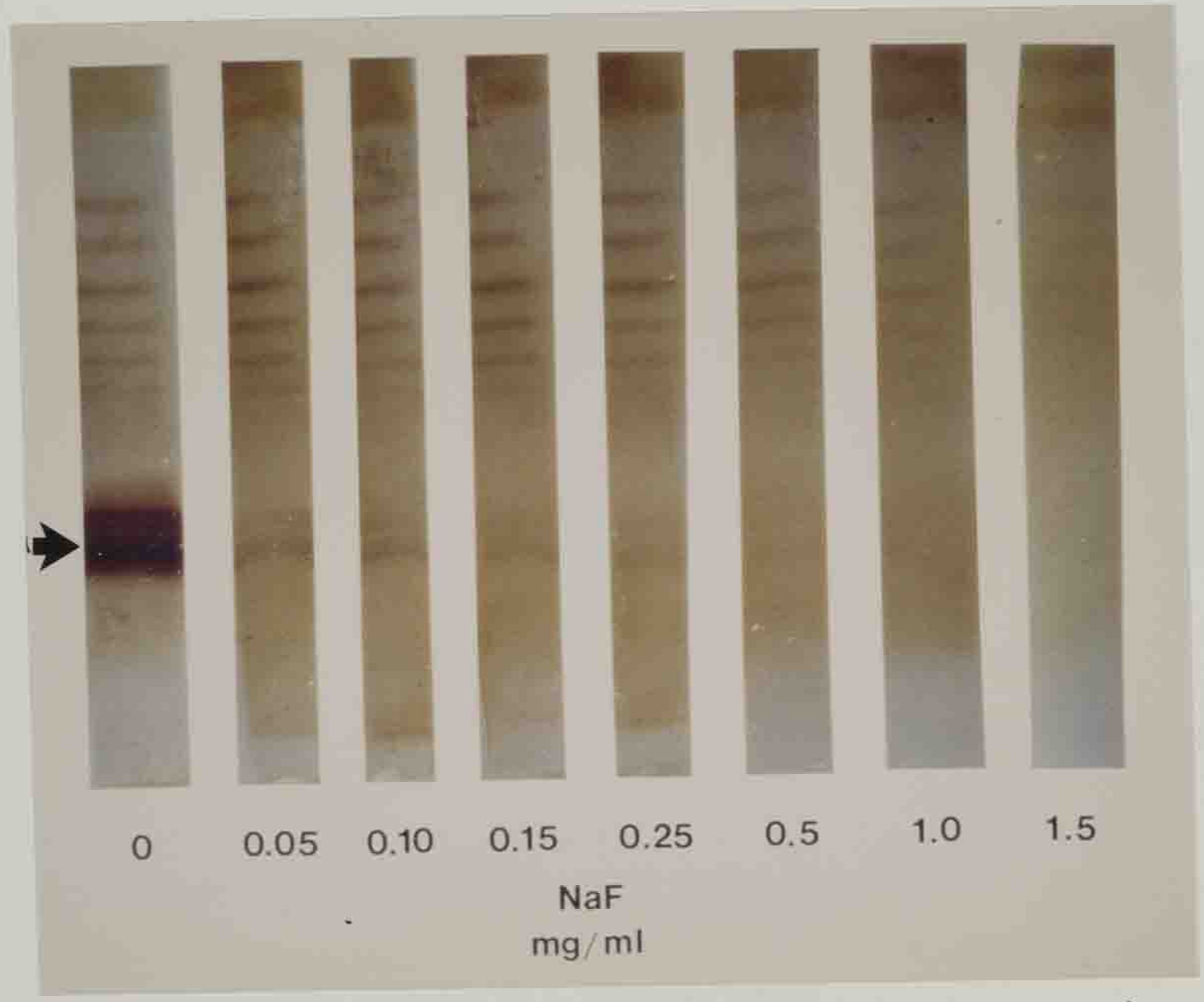

Figure 2.14: *Effect of sodium fluoride (NaF) on alpha-naphthyl acetate esterase (ANAE) isoenzyme activities. Replicate tracks of a single leukaemic cell extract, containing all the major myeloid esterase isoenzyme forms were electrophoresed (IEF) and stained for ANAE in the presence of increasing concentrations of NaF. Note that the staining of the monocyte-associated (MonEst) esterase isoenzyme forms (arrow) is markedly inhibited by low NaF concentrations whereas the higher pI (ComEst) isoenzyme forms are relatively resistant to NaF.*

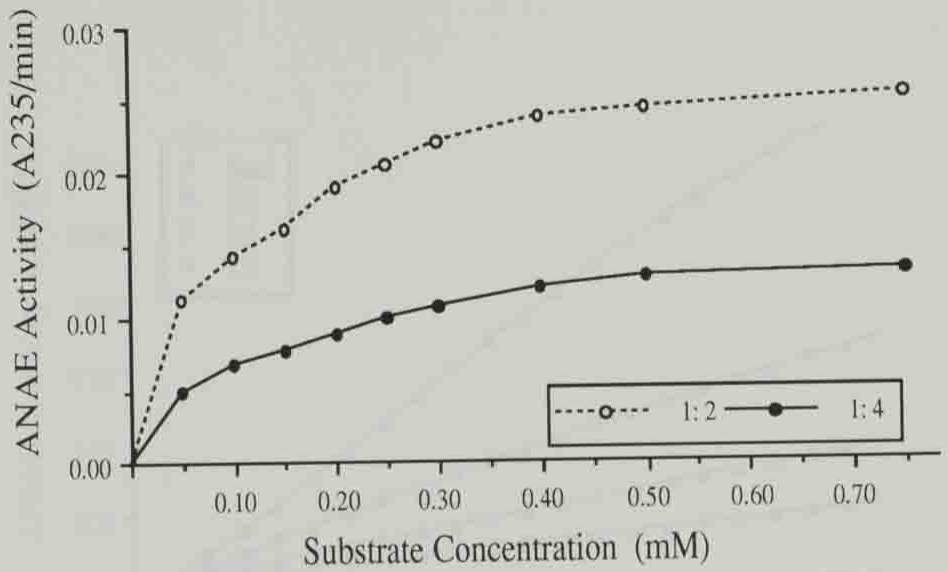

Figure 2.15: *Relationships between alpha-naphthyl acetate substrate concentration and observed reaction rates. A leukaemic AMML-M4 blast cell extract with an approximate ANAE activity of 110mU/ml was diluted 1:2 and 1:4, and the rate of substrate hydrolysis over 5.0 min determined for each of the substrate concentrations indicated. The reaction rates at each substrate concentration are shown as increases in absorbance at 235nm per min, with each data point representing a mean of duplicate determinations.*

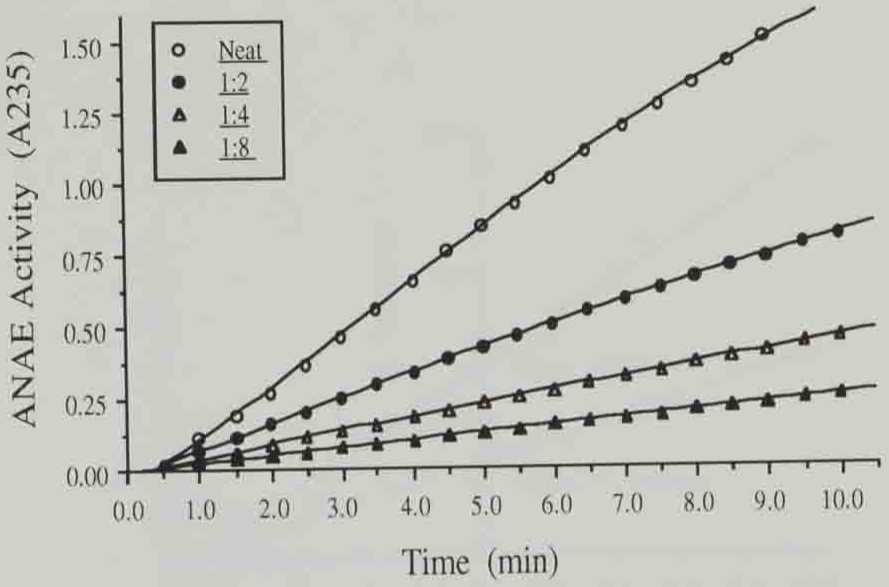

Figure 2.16: *Time course of the UV spectrophotometric assay for alpha-naphthyl acetate esterase (ANAE). A standard 0.5mM alpha-naphthyl acetate substrate concentration was used throughout and a single leukaemic AMML-M4 blast cell extract, containing all the major myeloid esterase isoenzyme forms, was tested at dilutions ranging from Neat to 1:8. The change in absorbance at 235nm (A235), for each extract dilution examined, was measured at 1.0 min intervals over a period of 10.0 min. The results are shown as the observed A235 values as a function of time.*

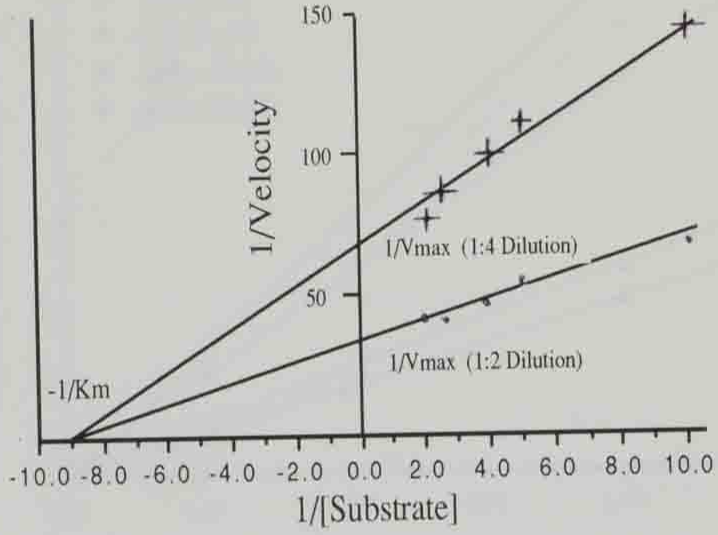

Figure 2.17: *Lineweaver-Burk plot for the estimation of Michaelis-Menten constant (K_m) and maximum velocities (V_{max}) for a mixture of alpha-naphthyl acetate esterases (ANAE) extracted from leukaemic AMML-M4 blasts. The data from Figure 2.15 was converted to inverse readings and the 1/velocity values plotted against 1/substrate (mM) concentration. The K_m value remained constant at 0.111mM for both dilutions of extract tested and the V_{max} values were calculated to be 0.03 and 0.015 A235/min for the 1:2 and 1:4 dilutions respectively.*

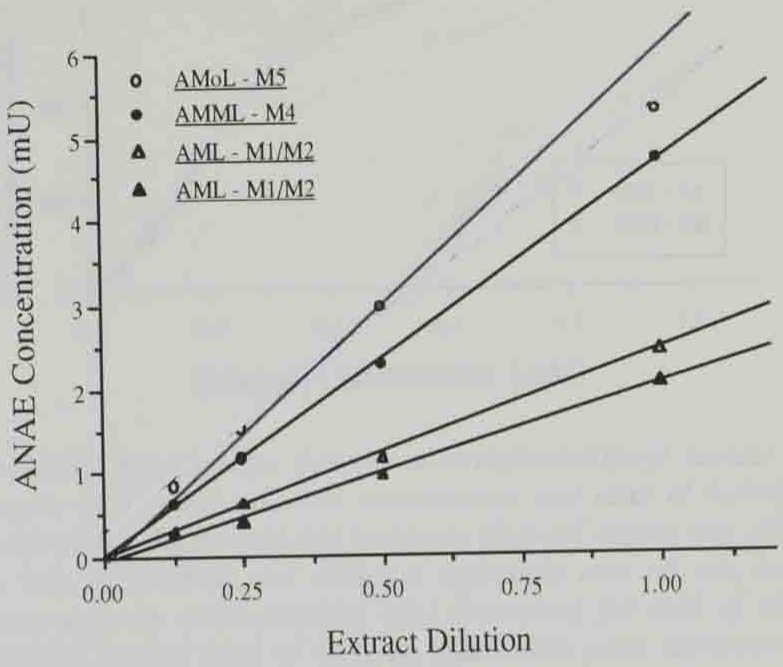

Figure 2.18: *Relationship between alpha-naphthyl acetate esterase (ANAE) concentration and reaction velocity. Serial dilutions, ranging from neat (1.0) to 1:8 (0.125), of two myelomonocytic leukaemia (AMML/AMoL-M4/M5) blast cell extracts (containing all the major myeloid isoenzyme forms) and two myeloblastic leukaemia (AML-M1/M2) blast cell extracts (containing all the major myeloid isoenzyme forms with the exception of those associated with monocytic differentiation) were analysed. The increase in the rate of substrate hydrolysis (standard 0.5mM alpha-naphthyl acetate concentration) for each extract dilution was determined over a period of 5.0 min at 30°C. Note the linear relationship between extract dilution and reaction rate within the range (0.25mU to 5.3mU) of ANAE activity studied (each data point representing a mean of duplicate determinations).*

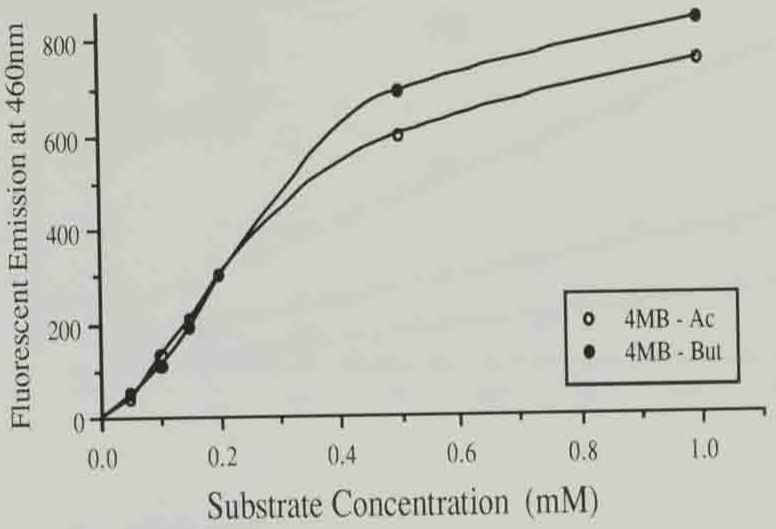

Figure 2.19: *Relationships between 4-methylumbelliferyl acetate (4MB-Ac) and butyrate (4MB-But) substrate concentration and rates of hydrolysis in the fluorimetric assay. An AMML-M4 leukaemic blast cell extract was diluted to 1:2 and the rate of 4MB-Ac and 4Mb-But hydrolysis over 60 min (expressed as fluorescent units at 460nm/20ul/60 min) determined for each of the substrate concentrations studied (0.05 to 1.0mM). Each data point represents a mean of duplicate determinations.*

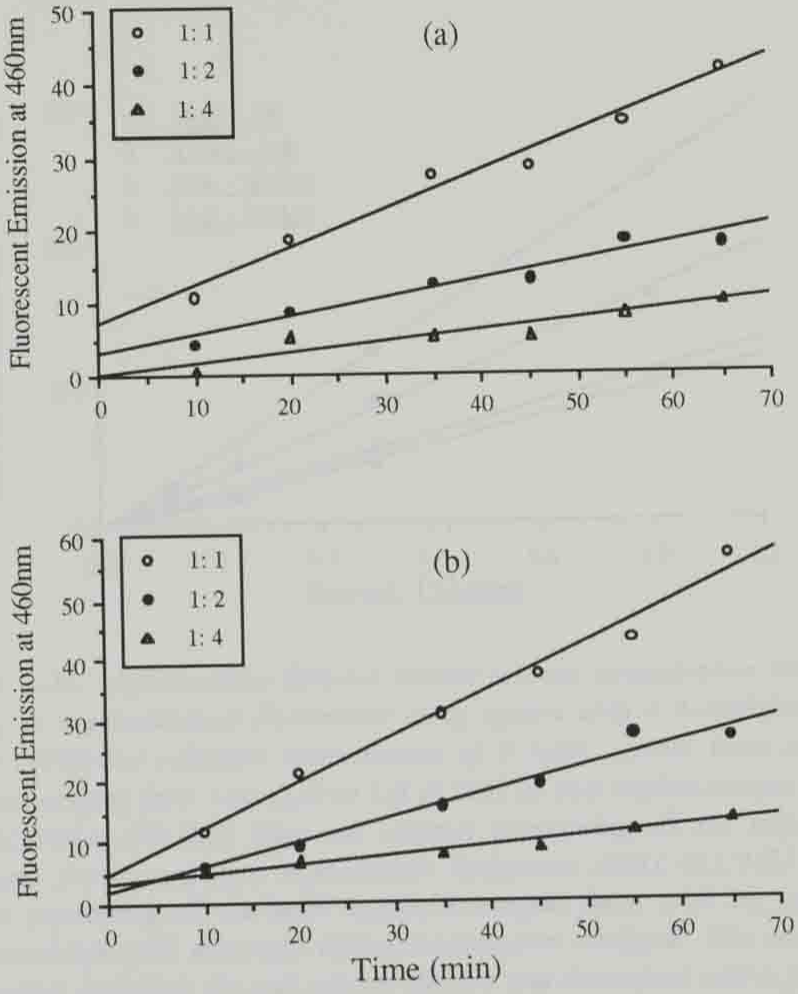

Figure 2.20: *Time course of the fluorimetric assay for acetate (a) and butyrate (b) esterases using standard 4-methylumbelliferyl acetate (4MB-Ac) and butyrate (4MB-But) substrate concentrations of 0.5mM. An AMML-M4 leukaemic blast cell extract, containing a mixture of all the major myeloid isoenzyme forms, was assayed for acetate esterase activity by fluorimetric procedures over a period of 65.0 min. The increase in fluorescent emission at 460nm resulting from enzymic hydrolysis of the 4MB-ester was determined in duplicate for undiluted, 1:2 and 1:4 dilutions of the leukaemic cell extract.*

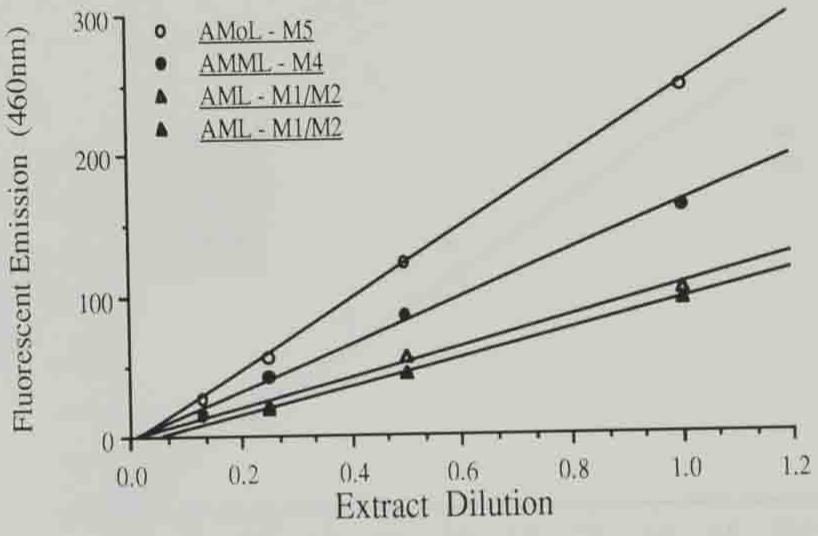

Figure 2.21: *Relationships between acetate esterase concentration and reaction velocity in a standardised fluorimetric assay system with a 4-methylumbelliferyl acetate (4MB-Ac) substrate concentration of 0.5mM. Serial blast cell extract dilutions, ranging from neat (1.0) to 1:8 (0.125), of two myelomonocytic leukaemia (AMML/AMoL-M4/M5) blast cell extracts (containing all the major myeloid isoenzyme forms) and two myeloblastic leukaemia (AML-M1/M2) blast cell extracts (containing all the major myeloid isoenzyme forms with the exception of those associated with monocytic differentiation) were analysed. The rate of 4MB-Ac substrate hydrolysis for each extract dilution was determined over a period of 60 min at 37°C (each data point representing a mean of duplicate determinations) and it is clearly seen that the reaction rate is linear throughout the range (20 to 240 units/20ul extract dilution/60 min) of acetate esterase activities studied.*

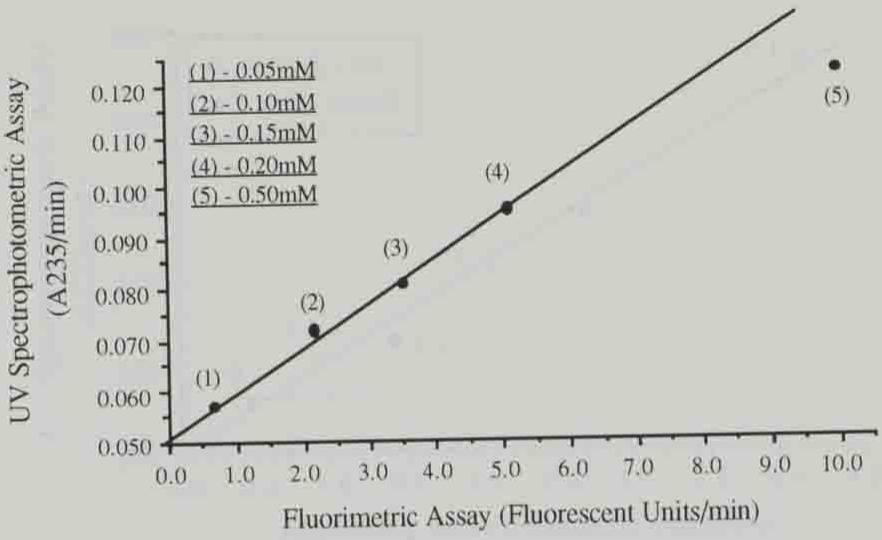

Figure 2.22: *Correlation between UV spectrophotometric (alpha-naphthyl acetate) and fluorimetric (4-methylumbelliferyl acetate) assays for the estimation of cellular extract esterase activities. A leukaemic AMML-M4 blast cell extract was diluted to 1:2 and assayed for acetate esterase activity by both assay procedures with a range (0.05 to 0.50mM) of substrate concentrations. A comparison of the rates (fluorescent or absorption units) of substrate hydrolysis reveals a linear relationship between the two assay procedures which is maintained throughout the range of substrate concentrations examined (each data point representing a mean of duplicate determinations).*

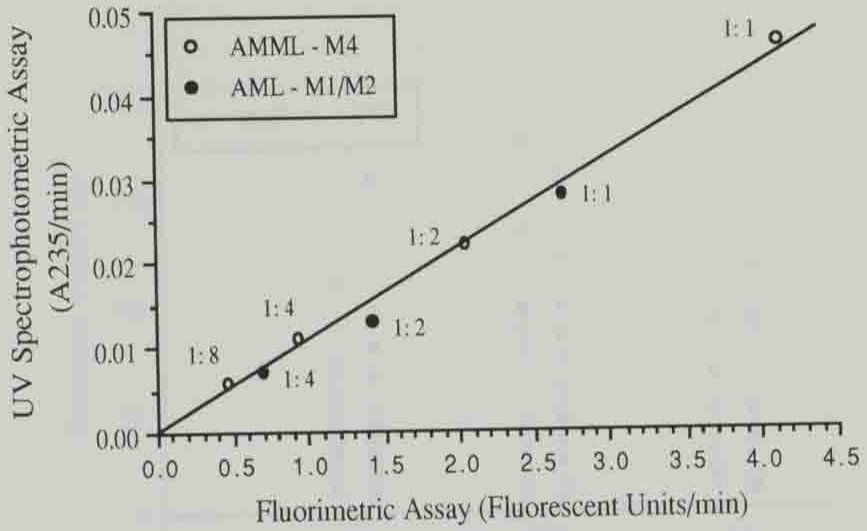

Figure 2.23: *Correlation between UV and fluorimetric acetate esterase assays. Serial dilutions from neat (1:1) to 1:8 of cell extracts from a case of acute myelomonocytic leukaemia (AMML-M4), containing all the major myeloid esterase isoenzyme forms, and neat to 1:4 from a case of acute myeloblastic leukaemia (AML-M1/M2), containing all the major myeloid esterase isoenzyme forms with the exception of those associated with monocytic differentiaion, were assayed for acetate esterase activity by both UV spectrophotometric and fluorimetric assay procedures. A comparison of the rate (absorption or fluorescent units/min) of alpha-naphthyl acetate (UV assay) and 4-methylumbelliferyl acetate (fluorimetric assay) substrate hydrolysis reveals a linear relationship between the two assay procedures throughout the range of extract dilutions examined (each data point representing a mean of duplicate determinations).*

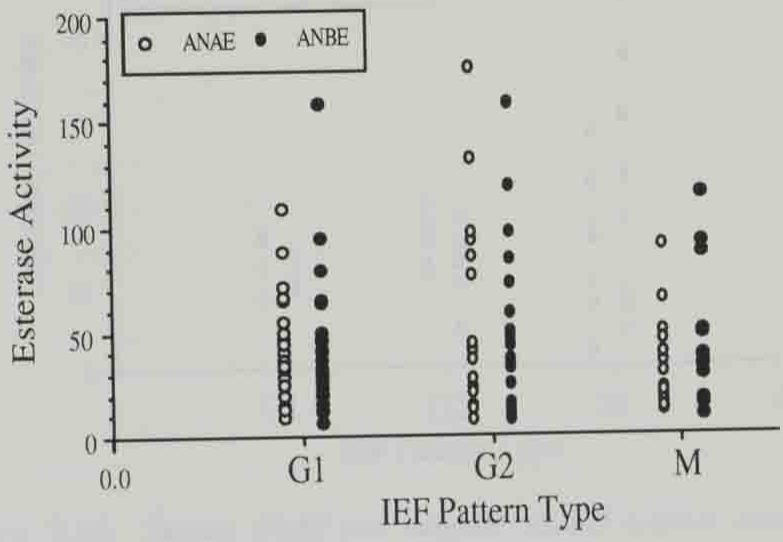

Figure 2.24: *Alpha-naphthyl acetate (ANAE) and butyrate (ANBE) esterase activities of 56 leukaemic myeloid cell extracts as determined by UV spectrophotometric assay. Results for individual leukaemic extracts, grouped according to alpha-naphthyl acetate esterase isoenzyme pattern types (G1, G2 or M), are given as mU acetate (or butyrate) activity per mg extract protein.*

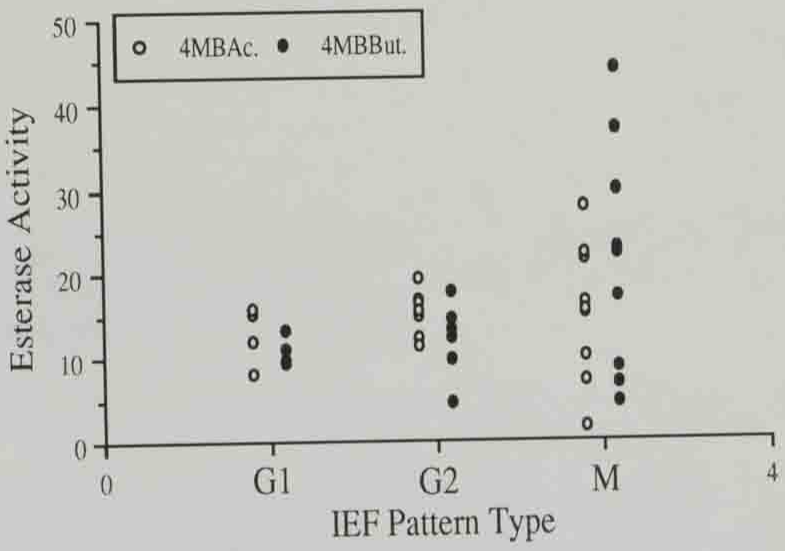

Figure 2.25: *Acetate (AcE) and butyrate (ButE) esterase activities of 20 normal granulocytic (n=11) and monocytic (n=9) cell extracts, as determined by fluorimetric assay. Results for individual extracts, grouped according to alpha-naphthyl acetate esterase isoenzyme pattern types (G1, G2 or M), are given as mU acetate (or butyrate) activity per mg extract protein.*

Table 2.1 Cytochemical reactions of blast cells in acute myeloid leukaemia [a]

Cytochemical stain	AML (M0)	AML (M1) [b]	AML (M2) [b]	APL (M3) [b]	AMML (M4)	AMoL (M5)
MPO	-	- or +	+	+	+	- or +
SBB	-	- or +	+	+	+	+
CAE	-	-	+	+	- or +	-
ANAE [c]	-	-	-	-	- or +	+

a Reactions indicate presence (+) or absence (-) of cytochemically positive blasts for MPO (myeloperoxidase), SBB (Sudan Black B), CAE (chloroacetate esterase) and ANAE (alpha-naphthyl acetate esterase).

b MPO, SBB and CAE reaction strengths increase with increasing maturity.

c ANAE positive blasts are occasionally observed in cases of M1, M2 and M3. However, the staining patterns in these are atypical as compared to the diffuse ANAE reactions seen in cells of monocytic lineage (discussed in Section 2.3.2.2).

Table 2.2 Monoclonal reagents used for the immunophenotypic classification of myeloid leukaemias by flow cytometry [a]

CD group	Clone	Primary specificity [b]	Source
3	T3	T	Dako
7	Leu19	T	Becton Dickinson
10	CALLA	Restricted B	Becton Dickinson
11c [c]	KB90	M, and Restricted B	Dako
13	My7	G, M	Coulter Electronics
14	Tuk4	M	Dako
19	HD37	B	Dako
33	My9	G, M	Coulter Electronics
-	HLA-Dr [d]	G, M, B	Becton Dickinson

a Leukaemic blast cell analyses carried out using FITC/PE two-colour combinations: CD3 with CD19; CD7 with CD13; CD10 with CD33; CD14 with HLA-Dr and; CD11c with CD19.

b Primary specificities: G, blasts of granulocytic lineage; M, blasts showing monocytic differentiation; B, blasts of immature B-lineage (i.e. ALL); T, blasts of immature T-lineage (i.e. T-ALL); Restricted B, reacts with B-cells at certain levels of B-differentiation only.

c Primarily employed for the differentiation of acute myeloid leukaemias of monocytic and non-monocytic type [Master et al., 1989].

d Primarily employed as a marker to assess relative levels of granulocytic differentiation.

Table 2.3 Purity and yield (recovery) of normal peripheral blood monocyte fractions prepared for esterase isoenzyme studies [a]

	Pre-fractionation [b]		Post-fractionation [b]		Yield [c]
	%	Total	%	Total	
	6.5	17.6	68	6.1	35
	13.0	25.9	75	7.9	30
	7.0	12.1	70	4.9	40
	7.0	14.3	69	8.9	62
	5.5	11.2	79	4.5	40
	7.5	20.1	81	5.3	26
	7.0	15.6	90	7.8	50
	3.0	13.6	83	4.5	33
	4.0	13.3	76	6.6	50
Mean	**6.7**	**16.0**	**77**	**6.3**	**41**

a Monocyte fractions prepared by a three-stage procedure as detailed in Materials and Methods (Section 2.2.1.2).

b Results expressed as percentages and total counts where the percentage indicates the proportion of each blood sample (pre-fractionation) or monocyte-enriched cell suspension (post-fractionation) defined as being of monocytic lineage (morphology, cytochemistry and/or membrane CD14 expression). The total represents the absolute number (x 10^9/l) of monocytes for the pre-fractionation blood sample (i.e. volume x leucocyte count x monocyte percentage) or for the post-fractionation monocyte-enriched cell suspension.

c Yield, expressed as a percentage, indicating the total number of monocytes in the post-fractionation suspension compared with the (starting) pre-fractionation monocyte number.

Table 2.4 Simultaneous analysis of monocyte-associated membrane CD14 determinants and cytoplasmic ANAE expression by normal peripheral blood monocytes [a]

CD14 Antibody	CD14/ANAE-defined monocyte subgroups [b]		
	$CD14^+ANAE^+$	$CD14^-ANAE^+$	$CD14^+ANAE^-$
LeuM3 (n=19)	91.5 (1.6)	3.8 (0.4)	4.7 (1.6)
61D3 (n=19)	91.9 (1.5)	3.0 (0.4)	5.1 (1.3)
My4 (n= 7)	93.4 (0.3)	2.2 (0.3)	4.4 (1.0)

a Peripheral blood monocytes were fractionated from EDTA-anticoagulated blood by density sedimentation on Lymphoprep to a purity of 10-25%, the contaminating cells being lymphocytes. Membrane CD14 expression was determined by an indirect immuno-rosetting technique (McKarill et al., 1987). Monocytes defined as CD14 positive were morphologically classified by the binding of at least three ox erythrocytes (oxE). ANAE cytochemistry was subsequently carried out on cytocentrifuged rosette preparations. ANAE positive cells with typical M-type reactions were defined as those with moderate-strong diffuse activity.

b Results are shown as the mean percentages (standard errors in parentheses), for all those cases examined, of each defined CD14/ANAE subpopulation.

Table 2.5 Alpha-naphthyl acetate esterase (ANAE) cytochemistry of blast cells in acute myeloid leukaemia subtypes [a]

Reaction Pattern [b]	AML M1/M2	APL M3-Hyper	APL M3-Hypo	AMML M4	AMoL M5a	AMoL M5b
Negative or weak diffuse	94/125	10/30	13/14	42/89	7/27	5/24
ANAE heterogeneity	-	-	-	13/89	2/27	4/24
Moderate to strong diffuse	4/125	-	-	34/89	18/27	15/24
Granular or focal	27/125	-	-	0/89	0/27	0/24
Other	0/125	20/30 [c]	1/14	0/89	0/27	0/24

a Acute leukaemia subtypes categorised according to combined morphological, cytochemical and immunophenotypic criteria (Section 2.2.2.2).

b Reaction patterns: Negative or weak diffuse - indicates that majority of blasts were unreactive or showed a weak diffuse staining reaction with only a minor proportion (<5%) of the remaining cells showing stronger staining; ANAE heterogeneity - indicates that whilst the majority of blasts showed weak diffuse staining, a proportion (5-20%) showed stronger M-type ANAE reactions; Moderate to strong diffuse - indicates that a minimum of 20% blasts were stained with good reaction intensity (i.e. M-type reaction) with the remaining blasts showing variable negative to moderate diffuse activity; Granular or focal - indicates that a minimum of 50% of blasts showed a reaction which was either of localised (focal) or granular type.

c Reaction patterns observed in M3-hypergranular (M3-Hyper) cases were either granular or diffuse and were associated with the concomitant presence of chloroacetate esterase activity (i.e. double esterase positive staining).

Table 2.6 Alpha-naphthyl acetate esterase (ANAE) cytochemistry and CD14 expression in acute myelomonocytic and monocytic leukaemias

ANAE reaction pattern [a]	CD14 expression [b]	
	Negative	Positive
Acute myelomonocytic leukaemia		
Negative or Weak diffuse (n = 42)	13/42	29/42
ANAE heterogeneity (n = 13)	5/13	8/13
Moderate to strong diffuse (n = 34)	12/34	22/34
Acute monocytic leukaemia		
Negative or weak diffuse (n = 10)	0/10	10/10
ANAE heterogeneity (n = 6)	0/6	6/6
Moderate to strong diffuse (n = 29)	5/29	24/29

a Reaction patterns: Negative or weak diffuse - indicates that majority of blasts were unreactive or showed weak diffuse staining reaction with only a minor proportion (<5%) of the remaining cells showing stronger staining; ANAE heterogeneity - indicates that whilst the majority of blasts showed weak diffuse staining, a proportion (5-20%) showed stronger M-type ANAE reactions; Moderate to strong diffuse - indicates that a minimum of 20% blasts were stained with good reaction intensity (i.e. M-type reaction) with the remaining blasts showing variable negative to moderate diffuse activity.

b CD14 expression was tested for with a minimum of two monocyte-associated antibodies (Section 2.2.2.2). Cases were defined as negative (<20% blasts positive) or positive (>20% blasts positive).

Table 2.7 Relationships between myeloperoxidase (MPO), chloroacetate esterase (CAE) and alpha-naphthyl acetate esterase (ANAE) cytochemistry in acute myelomonocytic leukaemia (M4) and acute monocytic leukaemia (M5) [a]

MPO	CAE		ANAE	
	<5%	*>5%*	*<20%*	*>20%*
AMML-M4 cases				
MPO^- (n = 16)	16/16	0/16	14/16	2/16
MPO^+ (n = 73)	34/73	39/73	41/73	32/73
AMoL-M5 cases				
MPO^- (n = 17)	17/17	0/17	3/17	14/17
MPO^+ (n = 34)	30/34	4/34 [b]	15/34	19/34

a Cases within each diagnostic group (AMML-M4 or AMoL-M5) were arbitrarily subdivided into those with 0 to 5% (MPO^-) and >5% MPO positive blasts (MPO^+). CAE cytochemistry indicates the proportions of MPO^- or MPO^+ cases that showed <5% or >5% CAE-positive blasts; ANAE cytochemistry indicates the proportions of MPO^- or MPO^+ cases with <20% or >20% blasts showing moderate to strong diffuse (M-type) reaction.

b CAE-positive blast cells in these four cases did not exceed 10% and these all showed >60% M-type ANAE or CD14 positive cells.

Table 2.8 Comparative solubilisation of myeloid leucocyte esterases with aqueous and detergent extraction reagents following ultrasonic cell disruption

Extraction Reagent [a]	% Esterase [b]	Isoenzymes groups [c]	
		pI 5.5-6.2 (MonEst)	pI 6.3-7.9 (ComEst)
20mM MES	90.7	++	++
0.25M KCl	99.3	++	++/+++
10mM CHAPS	97.1	++/+++	++/+++
10mg/ml IEGE	97.3	++/+++	++/+++
30mM n-octylglucoside	97.4	++/+++	++/+++
10mg/ml Triton X-100	95.8	++/+++	++/+++

[a] The extraction reagents, (a) potassium chloride (KCl); (b) zwitterionic detergent, 3-[(3-cholamidopropyl)dimethyl-ammonio]-1-propanesulphonate (CHAPS); (c) nonionic detergent isotridecylpoly (ethylene glycol ether) (IEGE); (d) nonionic detergent n-octylglucoside; and (e) nonionic detergent Triton X-100 were all dissolved in 20mM aqueous morpholino-ethane sulphonic acid (MES).

[b] Acetate esterase (ANAE) activity was determined by fluorimetric assay and the percentage enzyme extracted calculated.

[c] Esterase isoenzymes were fractionated by isoelectric focusing (IEF) and visualised by alpha-naphthyl acetate esterase (ANAE) cytochemical staining. The relative staining intensities of each defined pI (isoelectric point) group were graded as moderate (++) or moderate to strong (++/+++).

Table 2.9 Effect of different Triton X-100 concentrations on the relative distribution of esterase molecular weight (Mr) species [a]

Triton X-100	Acetate/butyrate esterase [b]		Ratio
(%)	HMW Species	LMW Species	LMW:HMW [c]
0.0	960	1560	1.6
0.25	800	1646	2.0
0.5	800	1390	1.7
1.0	970	1480	1.5
5.0	840	1330	1.6
10.0	820	1360	1.6

a Two molecular weight esterase species were defined by Superose-12 FPLC gel filtration (results detailed in Chapter 3 Section 3.2.2). For the purpose of this preliminary analysis, HMW represented a high molecular weight esterase form and LMW a low molecular weight form.

b Total acetate/butyrate esterase activities were measured by a fluorimetric assay using an equimolar (0.25mM) mixture of acetate and butyrate esters of 4-methylumbelliferone as substrates. The activities shown represent the observed modal values for each of the two molecular weight species.

c LMW:HMW ratios indicate the relative proportions of the low and high molecular weight acetate/butyrate esterases solubilised with increasing Triton X-100 concentrations.

Table 2.10 IEF pattern types of ANAE isoenzymes in normal and leukaemic myeloid cells

Myeloid cell type [a]	ANAE pattern type [b]		
	G1	G2	M
Normal neutrophils	4/11	7/11	0/11
Normal monocytes	0/9	0/9	9/9
Acute myeloblastic leukaemia (AML-M1/2)	76/85	8/85	1/85
Acute promyelocytic leukaemia (APL-M3)	3/11	8/11	0/11
Acute myelomonocytic leukaemia (AMML-M4)	12/63	22/63	29/63
Acute monocytic leukaemia (AMoL-M5)	13/45	2/45	30/45

a Myeloid leukaemic subtypes were defined by morphological, cytochemical and immunophenotypic criteria as outlined in Section 2.3.2.

b ANAE isoenzyme pattern types G1, G2 and M as defined by isoelectric focusing (Figure 2.9).

Table 2.11 Comparison of ANAE cytochemistry and isoenzyme patterns (IEF) in acute myeloid leukaemia. Cases are grouped according to ANAE cytochemical reaction independently of myeloid leukaemia subtype

ANAE cytochemistry [a]	IEF pattern [b]		
	G1	G2	M
Negative or weak diffuse (AML, n = 33; AMML, n = 24; AMoL, n = 12)	48/69	18/69	3/69
ANAE heterogeneity (AMML, n = 7; AMoL, n = 9)	0/16	3/16	13/16
Moderate to strong diffuse (AMML, n = 7; AMoL, n = 24)	0/31	2/31 [c]	29/31
Granular or focal (AML, n = 6; AMML, n = 2)	7/8	1/8	0/8

[a] Reaction patterns: Negative or weak diffuse - indicates that the majority of blasts were unreactive or showed a weak diffuse staining reaction with only a minor proportion (<5%) of the remaining cells showing M-type activity; ANAE heterogeneity - indicates that whilst the majority of blasts showed weak diffuse staining, a minor proportion (5-20%) showed stronger M-type ANAE reactions; Moderate to strong diffuse - indicates that a minimum of 20% blasts were stained with good reaction intensity (i.e. M-type reaction) with the remaining blasts showing variable negative to moderate diffuse activity; Granular or focal - where a significant proportion of blasts showed a localised (focal) reaction or a granular staining deposit which was generally coarse and unevenly distributed in the cytoplasm.

[b] ANAE isoenzyme patterns G1, G2 and M (Figure 2.9) as defined by analytical isoelectric focusing (IEF).

[c] Both these cases were AMML-M4.

Table 2.12 Comparison of ANAE cytochemistry, CD14 expression and IEF isoenzyme patterns in acute promyelocytic (APL-M3) leukaemia [a]

ANAE cytochemical reaction	CD14 [b]	IEF pattern [c]		
		G1	G2	M
Negative (M3-Hyper, n = 4; M3-Hypo, n = 2)	0/6	1/6	5/6	0/6
Weak diffuse/granular (M3-Hyper, n = 3; M3-Hypo, n = 0)	0/3	2/3	1/3	0/3
Moderate diffuse/granular (M3-Hyper, n = 2; M3-Hypo, n = 0)	0/2	0/2	2/2	0/2

[a] Acute promyelocytic leukaemia cases were further subdivided for this analysis into Hypergranular (M3-Hyper) and Hypogranular (M3-Hypo) variants [Bennett et al., 1980, 1985; Golomb et al., 1980].

[b] CD14 expression assessed using monoclonal antibodies LeuM3, My4, Mo2, UCHLM1 and FMC32.

[c] G1, G2 and M pattern types as defined by isoelectric focusing (Figure 2.9).

Table 2.13 Comparison of CD14 expression and ANAE isoenzyme patterns in acute myelomonocytic (AMML-M4) and monocytic leukaemias (AMoL-M5) [a]

CD14 expression [b]	IEF pattern [c]		
	G1	G2	M
AMML-M4 cases			
$CD14^+$ (n = 15)	5/15	5/15	5/15
$CD14^-$ (n = 17)	6/17	5/17	6/17
AMoL-M5a cases			
$CD14^+$ (n = 16)	4/16	1/16	11/16
$CD14^-$ (n = 5)	1/5	0/5	4/5
AMoL-M5b cases			
$CD14^+$ (n = 21)	7/21	1/21	13/21
$CD14^-$ (n = 0)	na	na	na

[a] Acute monocytic leukaemia subtypes defined in accordance with the French-American-British (FAB) classification [Bennett et al., 1976].

[b] CD14 expression was examined with a minimum of two monocyte-associated antibodies (Sections 2.2.2.2 and 2.2.4). Cases were graded as negative (<20% blasts positive) or positive (>20% blasts positive).

[c] ANAE isoenzyme patterns G1, G2 and M as defined by analytical isoelectric focusing (IEF) (Figure 2.9).

Table 2.14 Inhibition studies of myeloid alpha-naphthyl acetate esterase (ANAE) isoenzymes [a]

Inhibitor	Concentration mM	Isoenzyme Group [b] MonEst	ComEst
Phenylmethylsulphonyl fluoride (PMSF)	5.0	+	-
Sodium fluoride (NaF)	2.4	+	-
Neostigmine	10.0	-	-
Eserine	0.1	-	-
P-hydroxymercuribenzoic acid (HMBA)	1.0	-	-

a Leukaemic cell (AMML-M4) IEF zymograms were stained for ANAE in the presence of the above inhibitors as described in Section 2.2.6.3. The relative inhibition of the two main groups of myeloid cell ANAE isoenzymes with these inhibitors is shown as: (+) ANAE isoenzymes sensitive to inhibitor or (-) no significant inhibition with the inhibitor concentration tested.

b Two groups of myeloid esterases, as defined by isoelectric focusing, comprise isoenzymes with isoelectric point ranges of 5.5-6.2 (MonEst) and 6.3-7.9 (ComEst).

CHAPTER 3

EVALUATION OF ESTERASE PURIFICATION PROCEDURES

3.1 INTRODUCTION

Chromatography is the most widely accepted procedure for protein separation in modern biochemistry, and each methodological type exploits different physical and/or biological properties of the protein molecule as a basis for separation. Molecular characteristics that are frequently used for protein purification include size, hydrophobicity, solubility, charge, or specific binding affinity. In order to purify any given protein, some or all of these properties can be utilised, depending on the nature of the protein itself and the characteristics of coexisting contaminating solutes. Although the physical characteristics of a protein are important for the selection of chromatographic procedures, the preservation of biological activity also requires consideration, particularly for the purification of enzymes, where biological activity is invariably required for monitoring progress during the purification, and subsequently for the characterisation of the purified enzyme.

As a prelude to full-scale MonEst and ComEst purification, it was necessary to determine their chromatographic characteristics (i.e. their binding and elution profiles). Many of these studies were designed to evaluate buffer and pH parameters, and these data will not be shown. However, where an experimental procedure was found to be of direct importance to the understanding of the molecular characteristics of either species, these observations will be presented. With the exception of gel filtration and Benzamidine-Sepharose chromatography, where a representative number of different myeloid cell extracts were additionally analysed, all the methodological procedures were evaluated with a single cell extract (SA1) which was obtained from the leucophoresis of a patient with acute myelomonocytic leukaemia (AMML-M4). Analysis of ANAE isoenzyme components by IEF [Scott et al., 1984; Scott & Drexler, 1989] of this cell extract revealed the presence of moderate concentrations of MonEst isoenzymes together with significant staining of esterase isoenzyme components (ComEst) with a higher pI (>6.3) common to all myeloid cells [Scott et al., 1985, 1987, 1989]. Preparation of the protein

extract was achieved by ultrasonication (MSE Soniprep 150) of the cell-rich fluid at an amplitude of 18μ for one minute; 10% Triton X-100 was added to give a final detergent concentration of 1% and, after mixing at 4°C for 10 mins and subsequent high speed centrifugation, the solubilised protein supernate was removed from pelletted cell debris.

3.2 GEL FILTRATION CHROMATOGRAPHY

3.2.1 Introduction: Gel filtration or size exclusion chromatography provides a mild chromatographic method for the separation of bioactive molecules according to differences in their molecular size. The technique utilises the principle of molecular sieving, whereby different solutes are passed through a sponge-like matrix containing pores of uniform dimensions, and are eluted in order of decreasing molecular size. The matrices are generated by cross-linking dextran (e.g. Pharmacia Sephadex), polyacrylamide (Bio-Rad Biogels) or agarose (Pharmacia Sepharose) which are all hydrophilic compounds and swell to form insoluble porous gels.

Gel filtration columns are formed under conditions which permit uniform packing of the particles, thus forming the bed. The sample, containing a mixture of protein species differing in molecular sizes, is applied to the bed surface and subsequently passaged through the bed by eluent flow. As elution proceeds, molecules which are too large to enter the pores of the gel pass rapidly through the bed in the space surrounding the gel particles, and are eluted in a single void zone. Molecules which are capable of diffusing into the pores of the matrix are retarded in their migration through the bed, and the extent of retardation is inversely correlated to molecular size (i.e. the smallest molecules are retarded to the greatest extent and are thus the last to be eluted). The rate of passage of proteins through the bed is dependent on both the flow rate of the mobile phase and the partition coefficient between the porous stationary phase and the mobile phase. The molecular sieve processes may be viewed as a type of column liquid-liquid partition chromatography, where the liquid contained within the pores of the matrix forms the 'stationary' phase and the liquid surrounding the gel particles is the 'mobile' phase. The

partition coefficient of a solute is then determined by its ability to penetrate the pores of the matrix.

Gel filtration chromatography may be used analytically to determine molecular weights of unknown protein species. The elution profiles of known proteins (molecular weight standards) are determined and a standard curve constructed, from which the molecular weights of unknown proteins may be estimated. However, as migration through the gel matrix is also largely dependent on protein configuration, molecular weight determinations using gel filtration chromatography may only be regarded as estimates (i.e. relative molecular weights, Mr) rather than true values, which may be attained by more definitive electrophoretic techniques (described in Chapter Four).

3.2.2 Superose-12 FPLC gel filtration

3.2.2.1 Experimental procedures: The molecular weight distribution of esterase species of a representative number of 'crude' normal and leukaemic cell extracts was assessed using a 30 x 1.0cm Superose-12 high-resolution Fast Protein Liquid Chromatography (FPLC) column (MW range 10^3 to 3 x 10^5 kDa; Pharmacia). These extracts comprised normal blood granulocytes (n=6), normal blood monocytes (n=7), and blast cell fractions from 15 cases of acute myeloid leukaemia (AML-M1/M2, n=5; APL-M3, n=1; AMML-M4, n=4; and AMoL-M5, n=5). Enriched cell fractions were prepared as detailed in Section 2.2.1, and the extracts prepared by ultrasonication and detergent solubilisation (Section 2.2.5). The normal granulocyte extracts showed either G1 (n=3) or G2-type (n=3) ANAE-IEF isoenzyme patterns (Section 2.3.4.2); the normal blood monocyte-enriched fractions all showed an M-type ANAE isoenzyme pattern (Section 2.3.4.2); and the leukaemic myeloid blast cell extracts showed G1 (6/15), G2 (3/15) or M-type (6/15) ANAE isoenzyme patterns (Section 2.3.4.3).

Chromatographic separation was achieved by passage of 50µl of each extract through the Superose-12 column, pre-equilibrated with 50mM phosphate buffer pH 6.8 containing

0.15M NaCl, at an optimised flow rate of 0.35ml/min. The eluate was collected in 60 x 1.0min (0.35ml) sequential fractions and 50µl of each fraction was assayed for acetate and butyrate esterase activity by the standardised fluorimetric esterase (acetate and butyrate) assay procedures (Section 2.3.6.4). The relative molecular weights (Mr) of the observed esterase species for each 'crude' cell extract were estimated by comparison with a standard curve constructed from Ve/Vo (elution volume/void volume) versus the log molecular weights of protein standards.

3.2.2.2 Results: Fractionation of normal and leukaemic cell extracts by FPLC gel filtration chromatography revealed a diverse distribution of enzyme forms with esterolytic activity. Virtually all of the normal and leukaemic extracts examined showed two or three distinct peaks of esterase activity (Figures 3.1 and 3.2) which were designated P1, P2 and P3 in order of elution. The elution volume (Ve) of P1 was similar to the void volume (Vo) for the column and therefore esterase species eluted in P1 were considered to represent either high molecular weight enzyme-membrane complexes, enzyme-protein aggregates, or multimeric enzyme forms. Consequently, the analysis of esterase molecular weight distributions only considered the P2 and P3 components.

Representative gel filtration profiles of normal blood granulocyte and monocyte esterase forms are shown in Figure 3.1. The apparent molecular weights for the granulocyte and monocyte P2 species were 284-342 kDa (mean 313) and 135-236 kDa (mean 201) respectively (Table 3.1), and analysis of the relative rates of acetate and butyrate substrate hydrolysis showed that the P2 form preferentially hydrolysed acetate substrate in 4/6 of the granulocyte extracts and 2/7 of the monocyte extracts. For comparison, the granulocyte and monocyte P3 species showed apparent molecular weights of 53-77 kDa (mean 64) and 37-77 kDa (mean 63) respectively with a preferential rate of acetate substrate hydrolysis by 5/6 of the granulocyte cell extracts and 3/7 of the monocyte cell extracts. Additional analyses of the relative concentrations of the P2 and P3 molecular weight species with respect to cell type (i.e. granulocytic versus monocytic lineage) and

ANAE isoenzyme profile (G1 and G2 combined, versus M) revealed no apparent relationships.

Similarly, a total of 15 leukaemic blast cell extracts with G1/G2 (n=9) or M (n=6) type ANAE-IEF isoenzyme patterns were analysed by gel filtration chromatography. Esterase components analogous to normal granulocyte/monocyte peaks P1, P2 and P3 were also observed with the leukaemic samples (Figure 3.2) although species P2 was undetectable in two cases with G1/G2 IEF patterns (Table 3.2). The observed range of molecular weights for the P2 components were 100-333 kDa (mean 210) and 143-216 kDa (mean 173), for G1/G2 and M-type ANAE isoenzyme patterns respectively, with an apparent acetate substrate preference for most G1/G2 IEF extracts and a butyrate preference for most extracts with M-type IEF patterns. For comparison, the observed molecular weights of the P3 species were 24-38 kDa (mean 30) and 26-64 kDa (mean 43), for extracts with G1/G2 and M-type isoenzyme patterns, with no consistent pattern of substrate preference by individual extracts.

3.2.2.3 Conclusions: Superose-12 gel filtration of myeloid extracts resulted in the fractionation of two or three different molecular weight (Mr) enzyme species with esterolytic activity. Irrespective of cell-lineage commitment or type of esterase isoenzymes (ComEst or MonEst) expressed, these molecular weight species were present in virtually all of the normal and leukaemic myeloid cell extracts studied. The highest Mr form (designated P1) was eluted in the void volume of the gel filtration column, and was considered to represent either active enzyme aggregate or enzyme-membrane associated components, whereas the Mr of enzyme species P2 suggested that these components represented multiple subunit-associated forms of the P3 monomer. However, when molecular weights of the various P2 species (form normal and/or leukaemic myeloid extracts) were considered with respect to Mr of P3, apparent dimer, trimer, tetramer, pentamer and hexamer associations were implied for P2. It would therefore appear that the P2 species were unlikely to simply represent subunit-associated forms of P3, the

more likely explanation being that the various P2 and P3 species represented distinct enzymes with esterolytic activity.

The findings of this study suggest, that while gel filtration is able to fractionate esterolytic enzymes with different molecular weights, the accurate assessment of Mr (particularly of the high molecular weight form) or the subunit configuration of individual esterase forms may not be achieved when processing crude extracts. Gel filtration however, provides a means of separating proteins on the basis of molecular size and therefore its inclusion in the final protocol for the purification of esterase isoenzyme components, would allow the fractionation of esterase components from other contaminating proteins of differing Mr.

3.3 ION-EXCHANGE CHROMATOGRAPHY

3.3.1 Introduction: Proteins are amphoteric molecules which possess either a net positive or negative charge and are isoelectric (no net charge) at a pH referred to as the isoelectric point (pI) [Sluyterman & Wijdenes, 1977; 1978; Sluyterman & Elgersma, 1978]. The net molecular charge of a protein reflects the balance, at any given pH, of the ionisable amino ($-NH_2$) and carboxyl (-COOH) groups found on the side chains of basic and acidic amino acids. Principally, the amino groups of lysine (and its hydroxylated form) and arginine, and the carboxyl groups of aspartic and glutamic acids govern the differential charge characteristics of proteins. Other amino acid side chain groups (e.g. the hydroxyl groups of serine, threonine and tyrosine, the sulphydryl group of cysteine, and the imidazole ring of histidine) also contribute towards the net charge of proteins, but to a much lesser extent. At a pH above the pI of a protein, -COOH groups become ionised (-COOH ---> $-COO^-$) resulting in a net negatively charged protein. Conversely, at a pH below the pI, $-NH_2$ groups become ionised ($-NH_2$ ---> $-NH_3^+$) and the protein is thus positively charged. Electrophoretic titration curves are often used to predict the pH at which charge differences between contaminating proteins and the protein of interest are maximal, thus giving optimal separation by ion-exchange chromatography.

Protein retention on an ion-exchange column results from electrostatic interactions between the charged groups of the protein and oppositely charged groups of the matrix; the degree of retention being proportional to the charge density on both the ion-exchange matrix and the protein. In addition, the nature of the charged groups of the ion-exchanger and the distribution of charged amino acid groups on the protein also influence retention. Charged groups on ion-exchangers can be positive (anion exchange) or negative (cation exchange) and are covalently immobilised on insoluble cellulose, dextran or synthetic polymer-based matrices. Ion-exchangers may be broadly subdivided into; (a) strong, such as quaternary aminoethyl (QAE, anion) or sulphopropyl (SP, cation); or (b) weak, such as diethylaminoethyl (DEAE, anion) or carboxymethyl (CM, cation). In comparative terms, strong exchangers are completely ionised over a wide pH range and show little or no variation in ionic characteristics with changes in pH whereas, weak exchangers show marked variations in ionic state (and sample retention) with changes in pH. In practice, the charge distribution on the surface of protein molecules determines the number of charged groups available for interaction with the ion-exchanger, and thus govern their binding characteristics to ion-exchange media [Regnier, 1984].

For the successful separation of enzymes by ion-exchange chromatography, the technique can be viewed in two stages. Firstly, the enzyme of interest (and similarly charged proteins) is bound to the appropriate ion-exchanger using a low ionic strength buffer at a pre-determined pH. The appropriately charged proteins and buffer counterions (e.g. Cl^- for anion exchangers or Na^+ for cation exchangers) compete for binding sites on the exchanger whilst uncharged and oppositely charged proteins flow through the column in the void fraction. As the heterogeneous binding affinities exhibited by proteins are dependent on the ionic strength and pH of starting (binding) buffers, a gradually varying gradient based on either of these parameters may be used to differentially elute bound proteins. Ionic strength gradients at fixed pH are by far the most commonly used and the easiest to generate.

Desorption, and thereby elution, of proteins with ionic strength gradients is achieved by increasing the ionic strength (by continuous or step gradients) of the mobile phase. Counterions compete with bound proteins for the charged binding sites on the exchanger, resulting in desorption and elution of bound proteins which are generally displaced from the column in order of increasing charge (i.e. weakly charged proteins being eluted first). In addition, the pH of buffers used in ion-exchange separations should be at least 0.5 pH unit above (anion exchange) or below (cation exchange) the pI(s) of the component(s) of interest, and appropriate buffers should be selected to ensure that their pKa is within 0.3-0.5 units of the chosen pH. When separating bioactive molecules, this chosen pH also needs to be compatible with preservation of biological activity.

3.3.2 Anion-exchange chromatography:

3.3.2.1 Experimental procedures: In order to determine the binding and elution characteristics of MonEst and ComEst esterase isoenzyme species, anion-exchange chromatography was carried out using a quaternary aminoethyl-Sepharose (Q-Sepharose) exchanger (Pharmacia). For this preliminary evaluation, 5ml of the AMML leukaemic cell extract was dialysed against 25mM tris-HCl buffer pH 8.4 and then diluted to 25ml with the same. This was centrifuged at 10^4g for 20 min to remove particulate material and the resulting supernatant applied to a 15.0ml Q-Sepharose column, pre-equilibrated with tris buffer, at a flow rate of 1.0ml/min. Unbound proteins were collected, reduced in volume using an Amicon Ultrafiltration (10 kDa) cell, and the esterase activities (acetate and butyrate), protein concentration, and isoenzyme profile determined. Proteins binding to the column were eluted sequentially using the following 25mM buffers at 0.5 pH unit intervals; pH 7.0 tris-HCl, pH 6.5 bis-tris-HCl, pH 6.0 bis-tris-HCl, pH 5.5 piperazine-HCl, and pH 5.0 piperazine-HCl. Elutions were monitored using an in-line 280nm UV monitor and proteins desorbed at each pH interval were collected until baseline A280 readings were re-attained. Following the pH 5.0 elution, remaining bound proteins were eluted with 1.5M NaCl. The unbound fraction and all eluates were concentrated and analysed for protein, esterase activities and IEF esterase isoenzymes patterns.

3.3.2.2 Results: Analysis of the Q-Sepharose unbound and step pH eluates revealed that approximately 30% of starting protein did not bind to the column, and that this fraction contained most of the higher pI (7.3-7.9) ComEst isoenzymes (Table 3.3 and Figure 3.3). Further minor amounts of protein and ComEst esterase isoenzymes were eluted with the pH 7.0, 6.5 and 6.0 buffers, but the pH 5.5 piperazine eluate contained virtually all of the MonEst isoenzymes. A high proportion of the bound protein was subsequently eluted with the pH 5.0 and 1.0M NaCl buffers although these fractions did not contain any significant amounts of ComEst or MonEst isoenzymes. In addition, esterase activities determined by fluorimetric assays of the eluates did not show any apparent relationship with the relative staining intensities of myeloid esterase isoenzymes.

3.3.2.3 Conclusions: Quaternary aminoethyl (Q-Sepharose) anion-exchange chromatography using pH step elution, resulted in the complete separation of the two major myeloid esterase species. Q-Sepharose anion-exchange gel was chosen in preference to DEAE as it is capable of maintaining charge characteristics over a wide pH range. At a starting pH of 8.4, both the ComEst (pI range >6.3-7.9) and MonEst (pI range 5.5-6.2) esterase species should, in theory, be sufficiently negatively charged and bind to the anion-exchanger. However, the finding that the major ComEst components (pI 7.3-7.9) did not bind at this pH suggests that the negatively charged groups of these components were inaccessible (e.g. internal). In contrast, the MonEst species (together with a minor proportion of the lower pI ComEst isoenzymes) were sequentially eluted from the column at a pH lower than their pI. In summary, the use of a discontinuous step-pH elution enabled approximately 60% of the non-esterase extract proteins to be discarded, thus making this column procedure extremely useful as a preliminary step in the fractionation protocol.

3.3.3 Cation-exchange chromatography:

3.3.3.1 Experimental procedures: The unbound pH 8.4 Q-Sepharose fraction, containing enriched ComEst esterase species (pI range 7.3 to 7.9), was further examined by cation-exchange chromatography using a sulphopropyl-Sepharose gel (S-Sepharose, Pharmacia). A 5ml aliquot of this material was dialysed against 25mM morpholino-ethane sulphonic acid (MES) pH 6.0, diluted to 25ml with the same and passaged at a flow rate of 2.0ml/min through a 15ml pre-equilibrated S-Sepharose column. Unbound proteins were collected, reduced in volume by ultrafiltration and stored at 4°C. Proteins that bound to the column were eluted using a linear (0.0 to 0.5M) NaCl gradient in a total volume of 90ml. The eluate was collected as 2.0ml fractions, and 50µl of each was assayed for acetate and butyrate esterase activity by fluorimetric procedures. The NaCl concentration of each fraction was measured with a chloride meter (EEL-920 Corning), and fractions with significant enzyme activity were pooled as individual peaks, concentrated and analysed for protein, esterase activities and IEF isoenzyme patterns.

3.3.3.2 Results: The S-Sepharose elution profile of enriched ComEst species revealed two main peaks of esterolytic activity (Figure 3.4); peak (i) was eluted through a 40-140mM NaCl concentration range and found to show a marked preference for butyrate substrate hydrolysis; peak (ii) was eluted at a higher (120-220mM) NaCl concentration and appeared to preferentially hydrolyse the acetate substrate. Determination of specific enzyme activities showed that peak (i) had acetate and butyrate esterase concentrations respectively of 285 and 963 fluorescent units/ml/min/mg of extract protein compared to 446 and 169 for peak (ii). When these two fractions were further analysed by IEF, peak (i) showed relatively weak concentrations of ComEst isoenzymes within the pI range 6.1 to 7.2 whereas peak (ii) comprised high concentrations of the four major ComEst isoenzymes with a pI range of 7.3-7.9.

3.3.3.3 Conclusions: The cation-exchange binding/elution characteristics of MonEst isoenzymes were not investigated in this current study as the theoretical pH required to

promote their binding to a cation-exchange column (pH <5.0) was considered to be too low to maintain the structural integrity of this protein. The elution profile of ComEst components revealed two peaks of esterase activity, suggesting the presence of two distinct ionic forms. Elution of Peak (i) was achieved at a lower sodium chloride (NaCl) concentration than the elution of Peak (ii) and IEF analysis showed that the first of these fractions contained two to four weakly staining ComEst isoenzymes of lower pI (range 6.1-7.2) whereas the major ComEst components of higher pI (range 7.3-7.9) were associated with Peak (ii). Measurements of protein concentrations further indicated that the fractions which did not contain significant concentrations of ComEst (i.e. the unbound material and the fractions corresponding to Peak i) together represented approximately 40% of the starting protein. Consequently, the use of S-Sepharose chromatography for the purification of ComEst would allow the removal of a large proportion of contaminating non-esterase proteins whilst simultaneously providing a high binding capacity for the ComEst. Additionally, as S-Sepharose is able to withstand relatively high flow rates (up to 7ml/min), its inclusion in the early stages of ComEst fractionation would facilitate the processing of large starting extract volumes.

3.4 LECTIN AFFINITY CHROMATOGRAPHY

3.4.1 Introduction: Lectin affinity chromatography is a form of adsorption chromatography which is frequently used for fractionating glycoproteins. The proteins of interest specifically and reversibly interact with complementary lectin ligands immobilised on an insoluble matrix. Since the introduction of cyanogen bromide coupling of ligands to polysaccharide matrices [Axen et al., 1967], many novel procedures have been developed for the isolation of specific proteins from complex mixtures [reviewed in Wilchek et al., 1984]. Lectins are proteins which interact reversibly with specific sugar residues which, in effect, enables them to distinguish between polysaccharides, glycolipids and glycoproteins that display differences in their glycosidic contents. For glycoproteins, this binding is dependent on the type and extent of glycosylation of the polypeptide chains although, with respect to individual lectins, this affinity may also be

influenced by differences in the accessibility of sugar residues within the glycoprotein structure. Primary isoenzymes of a particular enzyme are not generally distinguishable using lectin chromatography however, secondary (post-translational) isoenzymes, which differ in their glycan constituents, are often resolved by employing lectin-affinity techniques [Swallow, 1977].

A potentially important means of fractionating leucocyte esterase enzymes in this current study was by the use of lectins immobilised on inert Sepharose or agarose matrices. An increasingly wide range of plant and animal lectins are available for protein separation although, Concanavalin-A (Con-A) is by far the most commonly used for enzyme purifications. The various types of lectin evaluated in this present study, together with their sugar affinities, are detailed in Table 3.6. Most lectins can be grouped into one of the following six categories, based on their carbohydrate-binding specificity (lectins used in this current study are shown in parentheses); mannose/glucose-binding lectins (Con-A, PSA, LCA); N-acetylgalactosamine/galactose-binding lectins (VVA B4, HPA, PNA, RCA I); N-acetylglucosamine-binding lectins (GSA II, WGA); L-fucose-binding lectins (UEA I); sialic acid-binding lectins and those with 'complex' specificities [Goldstein & Poretz, 1986].

3.4.2 Concanavalin A (Con-A) affinity chromatography:

3.4.2.1 Experimental procedures: Glycoproteins and glycolipids containing alpha-D-mannopyranosyl, alpha-D-glucopyranosyl or sterically related residues interact with Con-A in a reversible manner. The desorption of bound glycoproteins form Con-A is normally achieved with methyl alpha-D-mannopyranoside or methyl alpha-D-glucopyranoside, although desorption of proteins interacting through nonspecific ionic or hydrophobic associations may be achieved by sodium chloride or detergent. For glycoproteins binding through specific interactions the strength of binding, and consequently the ease of desorption, is generally proportional to the number of accessible sugar residues within the native structure. Binding of glycoproteins to Con-A is

additionally facilitated by the presence of low (0.5mM) concentrations of Mg^{2+}, Mn^{2+} and Ca^{2+} ions.

To analyse the binding characteristics of myeloid cell esterases, 1ml of the leukaemic AMML blast cell extract was dialysed overnight against 12.5mM succinic acid pH 6.8 containing 0.5mM each of magnesium, manganese and calcium chlorides. Following dialysis, the cell extract was further diluted to a final volume of 5ml in the same buffer and any particulate material, such as precipitated proteins and nucleic acids, were removed by centrifugation at 10^4g for 20 min. The resulting supernatant was applied to a pre-equilibrated 1.0 x 2.0cm Con-A Sepharose column (Pharmacia) at a flow rate of 1.0ml/min. Non-binding proteins were collected and concentrated by ultrafiltration (10 kDa membrane, Amicon) to the original starting volume, and proteins binding to the column through nonspecific-ionic interactions were eluted with a 30 minute linear (0.0 to 1.0M) NaCl gradient at a flow rate of 1.0ml/min; two minute (2ml) fractions were collected and 50µl of each assayed for acetate and butyrate esterase activity by fluorimetric procedures. Proteins remaining bound to the column were then eluted with 0.5M methyl alpha-D-mannopyranoside in 10mM phosphate buffer pH 7.0 and concentrated to 5ml.

The above elution procedure was repeated twice more with alternative linear (0.0 to 0.5M) gradients of alpha-D-glucopyranoside or alpha-D-mannopyranoside (total elution volumes of 30ml at a flow rate of 1.0ml/min). Fractions with significant esterase activities from each of the three gradients were individually pooled and concentrated prior to quantitative esterase assays and IEF-ANAE isoenzyme analysis. Electrophoresis of these fractions, as well as the unbound material from each experiment, was carried out as described in Section 2.2.6.1 and the gel was stained in a cytochemical reaction mixture containing 5.0mM alpha-naphthyl acetate and hexazotised pararosaniline (Section 2.2.3.4).

3.4.2.2 Results: The Con-A Sepharose binding characteristics of ComEst and MonEst were investigated using an AMML leukaemic cell extract expressing both isoenzyme forms. Acetate and butyrate esterase activities, for both the unbound fractions and column eluates, and IEF analyses are summarised in Table 3.4. Although the unbound fraction did not contain any significant amounts of ComEst or MonEst as determined by IEF, examination of the fluorimetric assay data for these, and other fractions lacking detectable ComEst or MonEst species, clearly indicated in all three experiments the presence of other enzymes/proteins capable of hydrolysing the fluorimetric esterase substrates.

Sodium chloride (NaCl) elution: Following the binding of leukaemic myeloid cell esterases to Con-A, a gradient (to 1.0M) of NaCl failed to elute either bound ComEst or MonEst. These esterases were however desorbed by the subsequent single-step elution with 0.5M alpha-D-mannopyranoside.

Methyl alpha-D-glucopyranoside elution: A minor proportion of the MonEst species was eluted by methyl alpha-D glucopyranoside concentrations of 0.1-0.18M and 0.19-0.28M. In contrast, majority of the ComEst was eluted with 0.19-0.28M glucopyranoside. No further ComEst or MonEst staining was seen in the higher (0.29-0.5M) glucopyranoside gradient fractions although residual bound ComEst and most of the bound MonEst was eluted with the subsequent 0.5M mannopyranoside wash.

Methyl alpha-D-mannopyranoside elution: Both ComEst and MonEst myeloid esterase species were completely eluted from the Con-A column at alpha-D-mannopyranoside concentrations of 0.1 to 0.38M. Again, although esterase activities were detectable by fluorimetric assay at higher mannopyranoside concentrations, no further ComEst or MonEst isoenzyme forms were detected by IEF.

3.4.2.3 Conclusions: Both ComEst and MonEst isoenzyme components bound to Con-A and were not eluted by increasing ionic strength (to 1.0M NaCl). Investigation of two

different sugar eluants showed that the main ComEst components were eluted by both alpha-D-glucopyranoside and alpha-D-mannopyranoside. In contrast, most of the bound MonEst, which was not desorbed by alpha-D-glucopyranoside, was eluted by alpha-D-mannopyranoside. Con-A exhibits a higher affinity for alpha-D-mannopyranoside than alpha-D-glucopyranoside [Goldstein & Poretz, 1986] thereby suggesting that ComEst binds to Con-A either through a specific interaction with alpha-D-glucopyranoside residues or through a weaker interaction with alpha-D-mannopyranoside residues. Conversely, MonEst isoenzymes are likely to predominantly bind to Con-A through specific interactions with alpha-D-mannopyranoside residues. These findings indicate that there are possible differences in the type and/or extent of glycosylation between the ComEst and MonEst isoenzyme components, and that the incorporation of this column in esterase fractionation procedures would be of potential value.

3.4.3 Supplementary Lectin affinity columns:

3.4.3.1 Experimental procedures: In addition to Con-A, nine further lectin affinity columns were evaluated in this study to determine the binding characteristics of leukaemic myeloid cell ComEst and MonEst esterase isoenzyme forms. The commercial sources for these gels and the types of matrices to which each lectin had been immobilised are shown in Table 3.5, their sugar specificities in Table 3.6, and the gel volumes and sugar eluants used for protein desorption in Table 3.7. For each lectin affinity gel studied, the chromatographic procedures followed were identical with respect to flow rates and column cross-sectional areas (10mm diameter, K10/10 column; Pharmacia).

The practical procedures for sample preparation, column loading and protein elution for each column were as follows: 1.0ml aliquots of the AMML cell extract were thawed and diluted to 15ml with lectin binding buffer (12.5mM succinic acid pH 6.9 containing 0.5mM each of calcium chloride, magnesium chloride and manganese chloride; and 0.15M sodium chloride). Large aggregates and cell debris were pelleted by centrifugation and the solubilised protein supernate was dialysed overnight against lectin binding buffer

at 4°C. Following dialysis and further centrifugation, the sample was passaged through the lectin column at 0.5ml/min. The absorbance at 280nm of unbound proteins was constantly monitored and after completion of sample loading, further buffer was passaged until the 280nm baseline was re-attained. The unbound protein fraction was then concentrated (Amicon Ultrafiltration Cell with a 10 kDa limiting membrane) to a final volume of 3-5ml and stored at 4°C. Proteins bound to the lectin were eluted with an appropriate group-specific desorption agent (Table 3.7) dissolved in the lectin binding buffer at a flow rate of 1.0ml/min. Eluted proteins were monitored at 280nm, collected on ice and then concentrated to approximately 1ml. The protein concentrations of bound and unbound fractions were determined with the bicinchoninic assay procedure (Section 2.2.7), and the esterase isoenzyme components of the bound fraction were analysed by IEF and histochemical staining of the gels in a reaction mixture comprising 5.0mM alpha-naphthyl acetate and hexazotised pararosaniline.

3.4.3.2 Results:

Enzyme and protein assays: By taking into account the volumes of the bound (eluted) and unbound fractions, it was possible from the enzyme and protein assays to determine the proportion (%) of extract acetate esterase, butyrate esterase and protein bound to each lectin column. Only minor (<2.0%) proportions of sonicate protein were bound to UEA I, HPA, and PNA lectins (Table 3.8 and Figure 3.5). Between 2.0% and 10.0% of extract protein bound to and was eluted from PSA, LCA, GSA II, VVA B4 and RCA I, whereas only WGA bound in excess of 10% extract protein. With respect to the relative amounts of bound esterase enzyme activity, the highest and lowest bound acetate esterase activities were obtained with the LCA (6.6%) and VVA B4 (0.1%) columns respectively, whereas the highest and lowest butyrate esterase activities were found with the LCA (14.2%) and HPA/PNA (both 0.3%) columns. In addition, although there was considerable variation in the actual amounts of bound enzyme, it was a consistent finding that the level of eluted butyrate esterase activity was always higher than acetate esterase activity.

Esterase isoenzyme analysis: Qualitative analyses of esterase isoenzymes was achieved by IEF. The esterase zymogram of the pre-column cell extract comprised both ComEst and MonEst myeloid isoenzyme species. Analysis of the binding characteristics of these isoenzyme groups to the lectin columns showed (Table 3.8) that the MonEst isoenzyme forms did not bind to any of the columns studied. In contrast, a minor proportion of ComEst isoenzyme forms were found to bind to LCA and PSA (Figure 3.6). Interestingly, even though the WGA and RCA I columns bound a significant proportion of cell extract esterase activity, as defined by the hydrolysis of 4-methylumbelliferyl butyrate, no ComEst or MonEst esterase isoenzymes were detected by IEF with either alpha-naphthyl acetate or butyrate substrates.

3.4.3.3 Conclusions: Of the nine supplementary lectin columns examined, only two (LCA and PSA) showed any significant binding of myeloid esterases. Both LCA and PSA, like Con-A, are categorised as mannose/glucose-binding lectins (Table 3.6) however, while Con-A showed strong binding of both ComEst and MonEst, LCA and PSA bound only a proportion of the ComEst species. Although collectively the lectin columns examined here appear to contribute only marginally, in terms of removing contaminating proteins from raw cell extracts, it is suggested that with repeated processing of the unbound material, their incorporation in a fractionation procedure would allow the removal of significant quantities of contaminating, non-ComEst/MonEst glycoproteins with esterolytic activity.

3.4.3.4 Glycan structures of ComEst and MonEst as determined by their lectin affinities: The lectin binding studies (Sections 3.4.2 and 3.4.3) showed that all MonEst bound to Con-A with no apparent affinity for any of the other lectins examined. In contrast, the ComEst species showed some affinity for PSA and LCA in addition to strong binding to Con-A. Carbohydrate structures of glycoproteins or proteoglycans are formed by the glycosidic linkage to an amino acid side-chain which may be N-glycosidic (through the amide nitrogen of an asparagine residue) or O-glycosidic (through the hydroxyl group of

serine, threonine, hydroxylysine or hydroxyproline). When interpreted in the context of the differential sugar specificities of these lectins, these observations reveal significant differences in the carbohydrate structure of these two myeloid esterase types. For example, the pattern of lectin binding for MonEst is compatible with the presence of oligomannosidic-type glycan(s) N-glycosidically linked to the protein (Figure 3.7a) whilst ComEst appears to contain approximately 80-90% biantennary N-acetyllactosamine-type (complex-type) asparagine-linked glycan(s) (Figure 3.7b) together with α-1,6-fucosylated biantennary N-acetyllactosamine-type glycan(s) (Figure 3.7c). The results of these lectin binding studies were partly interpreted by Dr H. Debray (Laboratoire de Chimie Biologique, Universite des Sciences et Techniques de Lille Flandres-Artois, France) who, in collaborative work, also assisted in proposal of the possible glycan structures for ComEst and MonEst enzymes denoted in Figure 3.7. These studies of 'crude' cell extracts for esterase-lectin affinities are further extended in Chapter 4, where analyses of purified MonEst and partially purified ComEst provide further confirmation of the proposed glycan structures for these myeloid esterase species.

3.5 NON-LECTIN AFFINITY CHROMATOGRAPHY

3.5.1 Introduction: Three non-lectin affinity columns were evaluated in this current study for the potential isolation of myeloid esterase species. Benzamidine-Sepharose (Pharmacia) is an affinity chromatography gel to which the synthetic trypsin inhibitor p-aminobenzamidine (PAB) is covalently coupled to an inert matrix. This allows the gel to specifically bind trypsin and other related proteins such as thrombin, urokinase and collagenase.

Arginine-Sepharose (Pharmacia) affinity chromatography has been used to isolate or remove various serine proteases from a wide range of materials. L-arginine residues are covalently coupled to a Sepharose matrix, predominantly through their alpha-amino groups, and affinity has been demonstrated in particular for enzymes which contain serine at their active sites and cleave proteins at the carboxyl group of arginine residues.

Arginine-Sepharose affinity chromatography may also be utilised to specifically adsorb molecules with biospecific or charge dependent affinity for L-arginine.

Blue-Sepharose (Pharmacia) is produced by the covalent attachment of Cibacron Blue F3G-A dye to a cross-linked agarose (Sepharose CL-6B). The dye itself is capable of binding many enzymes including kinases, dehydrogenases and most enzymes requiring adenyl-containing substances (e.g. NAD^+). A number of non-enzyme proteins such as albumin, lipoproteins, blood coagulation factors and interferon also bind this dye, thus making Blue-Sepharose chromatography particularly versatile with many potential applications.

3.5.2 Benzamidine-Sepharose chromatography:

3.5.2.1 Experimental procedures: In preliminary studies, it was found that optimal binding could be achieved by use of low ionic strength buffers, such as 25mM sodium phosphate pH 6.5 or 12.5mM succinic acid pH 6.8, and that elution of bound proteins could be facilitated by increasing the ionic strength (e.g. 0.3M sodium phosphate or molar sodium chloride). The following evaluations detail individual experimental aspects that are directly applicable to the design of a purification system for myeloid ComEst and MonEst species.

Experiment (a): A 5.0ml aliquot of the leukaemic AMML cell extract, containing both ComEst and MonEst myeloid esterase isoenzymes, was dialysed overnight against 25mM sodium phosphate buffer pH 6.5 containing 1% Triton X-100. Following dialysis, the sample was passaged through a 5ml Benzamidine-Sepharose column at 0.5ml/min and the unbound proteins collected. Proteins binding to the column were eluted with a linear sodium phosphate gradient to 0.3M at 1.0ml/min; one minute fractions were collected and assayed for butyrate esterase activity by fluorimetry. Fractions corresponding to distinct peaks of butyrate esterase activity were pooled, concentrated and analysed by IEF for the presence of ComEst and MonEst species.

Experiment (b): 0.5ml aliquots of nine different leukaemic cell extracts, variously expressing G1, G2 or M-type ANAE-IEF patterns, were dialysed overnight into 25mM sodium phosphate buffer pH 6.5 containing 1% Triton X-100. These dialysed samples were individually passaged at 0.5ml/min through a 5ml Benzamidine-Sepharose column and, in each case, bound proteins were single-step eluted with 0.3M sodium phosphate buffer. Unbound and eluted proteins (after concentrating to approximately 0.5ml) were assayed for acetate and butyrate esterase activity by fluorimetry using standard procedures, and analysed by IEF for the presence of ComEst and MonEst species. The relative proportions of total acetate and butyrate esterase activity in the unbound and bound fractions were then calculated.

Experiment (c): A 5.0ml aliquot of the leukaemic AMML cell extract, expressing both ComEst and MonEst myeloid esterase isoenzymes, was dialysed overnight against 25mM sodium phosphate buffer pH 6.5 containing 1% Triton X-100. Following dialysis, the sample was passaged through a 5ml Benzamidine-Sepharose column at 0.5ml/min and the unbound proteins collected; proteins binding to the column were single-step eluted with 0.3M sodium phosphate buffer and concentrated to 5.0ml. The pre-column sample, as well as the unbound and bound (eluted) fractions were then investigated by IEF for the presence of ComEst and MonEst species and additionally analysed by Superose-12 FPLC gel filtration chromatography for esterase molecular weight species (Section 3.2.2).

3.5.2.2 Results:

Experiment (a): Gradient elution (to 0.3M sodium phosphate) of proteins binding to the Benzamidine-Sepharose column resolved (Figure 3.8) two distinct peaks of butyrate esterase activity which eluted at approximate phosphate concentrations of 55mM (peak i) and 145mM (peak ii). The relative rates of acetate and butyrate hydrolysis (Ac:But ratio) for these two peaks were both 0.4, although the highest total activity was associated with peak ii. However, IEF studies showed that neither peak contained demonstrable ComEst or MonEst myeloid esterase isoenzyme species and, furthermore, analysis of the unbound

fraction confirmed that there had been no apparent binding of these particular myeloid esterase isoenzymes to the column. This lack of binding was unequivocal, despite evidence that significant amounts of proteins with esterolytic activity were eluted from the column. Interestingly, even though there was apparent differential binding, the Ac:But ratios of the pre- and post-column fractions remained essentially unchanged at 1.04:1.

Experiment (b): The results for this particular investigation showed that, irrespective of the ANAE-IEF pattern type (i.e. G1, G2 or M), means of 39% (range 24-60%) and 25% (17-35%) of the total leukaemic cell extract acetate and butyrate esterase activities were bound to the Benzamidine column (Table 3.9). Esterolytic activities of these bound protein fractions were not however associated with the presence of detectable ComEst or MonEst isoenzymes when analysed by IEF.

Experiment (c): In this part of the study, comparative studies of the ANAE-IEF isoenzyme pattern types and the Superose-12 gel filtration profiles of enzymes with esterolytic activities were undertaken for (i) the pre-Benzamidine column cell extract, (ii) the post-Benzamidine (unbound) column fraction, and (iii) the protein fraction eluted from the Benzamidine column with 0.3M sodium phosphate pH 6.5. In this way it was found that the pre-column extract, which contained both ComEst and MonEst isoenzyme components, showed two distinct molecular weight species (with approximate elution times of 36 and 43 mins), and that both of these showed higher relative rates of butyrate substrate hydrolysis (Figure 3.9). Following passage of this sample through the Benzamidine column, a high proportion of the high molecular weight species (Ve, 36) was removed but the ANAE-IEF pattern of this unbound fraction did not appear to have been affected. Moreover, proteins eluted from the Benzamidine column contained significant concentrations of the high molecular weight species (with marked preference for butyrate substrate hydrolysis) but no ComEst or MonEst isoenzymes were demonstrable by IEF.

3.5.2.3 Conclusions: The results for the three experiments showed that neither ComEst nor MonEst isoenzymes bound to Benzamidine-Sepharose even though significant concentrations of esterolytic enzymes were clearly present in the eluate. As Benzamidine-Sepharose binds trypsin and closely related serine proteases, these observations suggest that the contaminating enzymes with esterolytic activity are likely to be trypsin-like proteases. However, as many proteolytic enzymes are known to catalyse the hydrolysis of ester substrates (due to the similarities between ester and peptide bonds), these findings are perhaps not unexpected and illustrate the requirement of more than one analytical approach to monitor ComEst and MonEst activities during fractionation. Furthermore, gel filtration studies of the pre- and post-Benzamidine-Sepharose fractions suggest that the proteolytic enzymes with esterolytic activity, which bound to the column, have a similar Mr to the high molecular weight esterase species (P2) present in many cell extracts (Section 3.2.2).

It is concluded that Benzamidine-Sepharose affinity chromatography is an important step in the purification of esterase isoenzyme components, as it provides a means of removing a large proportion of non-MonEst/ComEst esterolytic activity whilst simultaneously removing potentially harmful digestive proteases from the extract.

3.5.3 Arginine-Sepharose chromatography:

3.5.3.1 Experimental procedures: To evaluate the application of this affinity column to esterase fractionation, 5.0ml of leukaemic AMML cell extract was dialysed overnight against 12.5mM succinic acid buffer pH 6.8 containing 0.5mM each of magnesium, manganese and calcium chlorides. Following dialysis, the sample was diluted to 25ml in the same buffer and passaged through a pre-equilibrated 10.0 x 1.1cm Arginine-Sepharose (Pharmacia) column at 1.0ml/min. The unbound material was collected and following re-attainment of baseline A280, bound proteins were eluted with a single-step passage of molar sodium chloride in succinic acid (pH 6.8). The unbound and eluate fractions were then dialysed into 50mM morpholino-ethane sulphonic acid (MES) pH 6.4

and concentrated by ultrafiltration to approximately 5.0ml. These concentrated fractions were then analysed by IEF, for the presence of ComEst and MonEst esterase isoenzymes, and assayed for protein concentrations and (fluorimetric) acetate/butyrate esterase activities.

3.5.3.2 Results: The column performance characteristics for Arginine-Sepharose are shown in Table 3.10. These show that 32% of the total extract protein and approximately 40% of the acetate/butyrate esterase activities were associated with the proteins eluted from the column. Interestingly, IEF analysis clearly revealed that despite the relatively high esterase activities of the eluate, neither ComEst nor MonEst species bound to the column.

3.5.3.3 Conclusions: Arginine-Sepharose is known to bind serine proteases, particularly those cleaving proteins at the carboxyl group of arginine residues. As with Benzamidine-Sepharose chromatography, significant esterolytic activity was demonstrated, in the absence of demonstrable ComEst or MonEst isoenzyme components, in the Arginine-Sepharose eluate. The conclusions drawn for this column are therefore similar to those for Benzamidine-Sepharose in that Arginine-Sepharose chromatography provides an additional means of removing potentially harmful contaminating proteases with esterolytic activity.

3.5.4 Blue-Sepharose chromatography:

3.5.4.1 Experimental procedures: To evaluate this affinity column for esterase fractionation, 5.0ml of leukaemic AMML cell extract was dialysed overnight against 12.5mM succinic acid buffer pH 6.8 containing 0.5mM each of magnesium, manganese and calcium chlorides. Following dialysis, the sample was diluted to 25ml in the same buffer and passaged through a pre-equilibrated 10.0 x 1.1cm Blue-Sepharose (Pharmacia) column at 1.0ml/min. The unbound material was collected and following re-attainment of baseline A280, bound proteins were eluted with a single-step passage of

molar sodium chloride in succinic acid (pH 6.8). The unbound and eluate fractions were then dialysed into 50mM MES pH 6.4 and concentrated by ultrafiltration to approximately 5.0ml. These concentrated fractions were subsequently analysed by IEF, for the presence of ComEst and MonEst esterase isoenzymes, and assayed for protein concentrations and (fluorimetric) acetate/butyrate esterase activities.

3.5.4.2 Results: The column performance characteristics for Blue-Sepharose are summarised in Table 3.10. These show that 35% of the total extract protein, including 34% and 52% of the acetate and butyrate esterase activities respectively, were associated with proteins eluted from the column. IEF analysis again revealed that there was no significant binding of the MonEst species to Blue-Sepharose and that, apart from a minor proportion of ComEst within the pI range 6.3 - 7.2, most of the ComEst isoenzymes (particularly within the pI range 7.3-7.9) failed to show significant binding.

3.5.4.3 Conclusions: Blue-Sepharose binds a large number of proteins including kinases, dehydrogenases, albumin, lipoproteins, blood coagulation factors, interferon and most enzymes requiring adenyl-containing compounds. The results for these preliminary evaluations indicate that although esterolytic activity could be demonstrated in the eluate, very little of the ComEst and none of the MonEst isoenzyme components bound to this gel. Therefore, as with the Benzamidine- and Arginine-Sepharose columns, Blue-Sepharose chromatography provides yet another method for removing potentially harmful proteases displaying esterolytic activity. Additionally, when the starting material is contaminated with serum proteins, the use of Blue-Sepharose would facilitate the specific removal of albumin and blood coagulation factors which are present in relatively high concentrations in human serum.

3.6 HYDROPHOBIC INTERACTION CHROMATOGRAPHY (HIC)

3.6.1 Introduction: Hydrophobic interaction chromatography separates biomolecules on the basis of varying degrees of hydrophobicity. Most proteins contain amino acid residues with hydrophobic side chains and many of these groups are sufficiently exposed in their native state to allow interaction with an HIC gel. Two commercially available HIC gels in general use are octyl- and phenyl-Sepharose. However, as strongly hydrophobic proteins are not easily eluted from octyl-Sepharose, and because the hydrophobicities of ComEst and MonEst components were unknown, phenyl-Sepharose was used in this current study. HIC gels are equilibrated using high ionic strength buffers (i.e. under conditions favouring hydrophobic binding) and, following sample application, elution is usually achieved by means of a decreasing ionic strength gradient.

3.6.2 Phenyl-Sepharose HIC:

3.6.2.1 Experimental procedures: The binding and elution characteristics of MonEst and ComEst species were evaluated by two different procedures. For both, 5.0ml of the leukaemic AMML cell extract was dialysed overnight against 50mM phosphate buffer pH 7.0 containing 1.0M ammonium sulphate. The sample was then diluted to 25ml with the same buffer and passaged through a 5.0 x 1.6cm phenyl-Sepharose column at a flow rate of 1.0ml/min. For the first experimental procedure, unbound proteins were collected and proteins binding to the column were eluted using a linear descending (1.0 to 0.0M) ammonium sulphate gradient over a period of 60mins at a flow rate of 1.0ml/min. Fractions were collected at 4min intervals and each dialysed against 50mM MES pH 6.4 prior to fluorimetric assay for acetate and butyrate esterase activities.

For the second experiment, unbound proteins were again collected and proteins binding to the column were step-eluted using 50mM phosphate buffers pH 7.0 containing decreasing ammonium sulphate concentrations of 0.8, 0.6, 0.4, 0.2 and 0.0M. Protein desorption was monitored with an in-line 280nm UV monitor and proteins eluted at each ammonium sulphate concentration were collected until baseline A280 readings were re-

attained. Following passage of the final phosphate buffer eluant (0.0M ammonium sulphate), the remaining strongly-binding proteins were eluted with 6.0M urea. Unbound and eluate fractions were then dialysed against 50mM MES pH 6.4 and concentrated (Amicon Ultrafiltration cell with a 10 kDa limiting membrane) to approximately 5.0ml prior to ANAE-IEF isoenzyme analysis, determination of protein concentrations, and fluorimetric acetate and butyrate esterase assays.

In addition to these studies, a pH 5.5 piperazine eluate from the Q-Sepharose anion exchanger (Section 3.3.2.2), which contained relatively high concentrations of MonEst isoenzymes but no detectable ComEst, was processed instead of the raw leukaemic cell extract. For this experiment, 25ml of the piperazine Q-Sepharose eluate was dialysed overnight into 50mM phosphate buffer pH 7.0 containing 1.0M ammonium sulphate, and passaged through a pre-equilibrated 5.0 x 1.6cm phenyl-Sepharose column at a flow rate of 2.0ml/min. Ammonium sulphate/urea step-elutions were carried out in exactly the same way as for the whole leukaemic cell extract and, following the final 6M urea elution of strongly-bound proteins, unbound and eluate fractions were dialysed into 50mM MES pH 6.4, and concentrated prior to esterase isoenzyme analysis, protein and esterase assays.

3.6.2.2 Results: Following passage of the leukaemic cell extract through the HIC column, desorption of bound proteins was initially evaluated using a linear (1.0 to 0.0M) descending ammonium sulphate gradient. The results for this experiment (Figure 3.10) revealed that acetate and butyrate esterase activity was eluted over a wide ammonium sulphate concentration range with no evidence for differences in the elution of individual esterase HIC species.

For the step-gradient elution of bound proteins from the passaged whole leukaemic cell extract, the acetate/butyrate esterase activities, protein concentrations and myeloid esterase isoenzyme (ComEst and MonEst) profiles of the unbound and eluate fractions (decreasing 0.2M ammonium sulphate intervals) are summarised in Table 3.11. These analyses

revealed that a total of 56% of the original extract protein, as well as 44% of the acetate esterase and 17% of the butyrate esterase activities, were associated with the 1.0M (unbound), 0.8, 0.6 and 0.4M ammonium sulphate eluates combined even though ComEst or MonEst myeloid esterase isoenzymes were not detectable by IEF. Although some staining of these isoenzymes was observed in the 0.2M ammonium sulphate eluate, the highest concentrations of ComEst and MonEst isoenzymes were associated with the 0.0M ammonium sulphate and 6M urea eluates. Together, these two fractions represented approximately 30% of the original protein, and 47% and 75% of the original acetate and butyrate esterase activities respectively.

Examining the step elution characteristics of the partially-processed MonEst-enriched sample (Q-Sepharose pH 5.5 piperazine eluate) in the same way, it was again found (Table 3.12 and Figure 3.11) that the presence of demonstrable MonEst isoenzymes was associated with the 0.4-0.0M ammonium sulphate and 6.0M urea eluates. Protein and esterase assays further revealed that 55% of the protein processed did not bind to the column, whereas the eluates containing MonEst together represented 32% of the protein and approximately 70% of the acetate/butyrate esterase activities.

3.6.2.3 Conclusions: Hydrophobic interaction chromatography (HIC) was investigated by both linear and step descending gradients of ammonium sulphate. The results show that, whereas the linear elution of proteins bound to the phenyl-Sepharose column did not result in any great degree of resolution, with respect to ComEst and MonEst components, stepped elution clearly showed that these myeloid esterases were only successfully desorbed in the latter part of the gradient. This suggests that both these esterase species are very hydrophobic, and that the procedure provides a means of removing a high proportion of relatively lower hydrophobic contaminating proteins. Furthermore, as phenyl-Sepharose is an intermediate-capacity column, which is able to withstand relatively high flow rates (up to 7ml/min), its inclusion in the early stages of fractionation would facilitate the processing of large starting extract volumes.

3.7 DISCUSSION

A primary consideration for the purification of any enzyme is the maintenance of biological activity throughout the fractionation procedure. Consequently, the chromatographic techniques proposed for the full-scale purification of myeloid esterases utilise both mild buffer conditions and, where possible, a pH close to that of physiological fluids. The preliminary analyses detailed in this Chapter, of the binding affinities of esterolytic enzymes in crude cellular extracts, showed that a high proportion of the total esterase activity, defined by assays with esters of alpha-naphthol and 4-methylumbelliferone, could not be demonstrated by standard azo-dye histochemical zymogram procedures. This suggested that these particular enzymes (including trypsin and chymotrypsin-like enzymes), which were present in addition to the histochemically demonstrable ComEst and MonEst isoenzyme species, hydrolysed the substrates in such a way as to make subsequent and spontaneous coupling with the diazonium salt impossible. Although further studies of these particular enzymes were not undertaken, as they were not the subject of these present studies, the recognition of their presence effectively meant that (a) the column procedure designed for the final chromatographic purification of MonEst and ComEst had to ensure their removal, (b) that the monitoring of column efficiencies and their ability to successfully resolve these myeloid esterase species could only be reliably achieved by histochemical zymogram analysis, and (c) that the conventional expression of enzyme purification and column yields (i.e. reporting enzyme activities relative to protein concentration) would be meaningless. It was also concluded from these initial studies that because of the diverse chromatographic characteristics of co-existing esterolytic enzymes, and because the deteriorated nature of the starting material precluded the technically less demanding processing of a cell membrane extract, that an extensive series of columns would be required to achieve an acceptable degree of resolution for the myeloid esterases. Consequently, a wide range of chromatographic columns were required for fractionating the ComEst and MonEst and, where possible, these columns were specifically arranged to minimise the requirement of

time-consuming buffer changes and loss of enzymatic activity through prolonged exposure to non-optimal conditions.

For the first stage of both MonEst and ComEst fractionation, Q-Sepharose anion-exchange chromatography would be used because of its high protein binding capacity and ability to withstand relatively high (up to 7.0ml/min) flow rates. Furthermore, preliminary column binding studies indicated that both MonEst and ComEst could be separated at a starting pH of 8.4 and, as the use of NaCl gradients from 0.0 to 1.0M did not appear to offer significant advantages (preliminary analyses, data not shown) in terms of specific ComEst/MonEst resolution, a series of pH step elutions would be used. The major ComEst components (pI 7.3-7.9) were found in the unbound fraction and using pH step elutions, the MonEst components were subsequently eluted by pH 5.5 (piperazine) elution. These studies also indicated that in addition to the separation of MonEst and ComEst, Q-Sepharose also significantly reduced the total starting volume and protein concentration and allowed the removal of a large proportion of contaminating lipid and nucleic acid (DNA and RNA) components.

MonEst Fractionation:

Following anion-exchange chromatography, phenyl-Sepharose hydrophobic interaction chromatography (HIC) was considered the most suitable for the next stage of MonEst fractionation. This particular column was considered to be more appropriate than the S-Sepharose cation-exchanger, as the pH required (< or = 5.0) to bind MonEst to this latter column could potentially affect its native structure and biological activity. Furthermore, the preliminary studies detailed in this Chapter indicated that the use of HIC could considerably reduce the sample volume and total protein. As with Q-Sepharose, the use of a linear descending ammonium sulphate gradient from 1.0 to 0.0M for HIC desorption did not appear to increase MonEst resolution and, therefore, a series of step elutions would be used. MonEst was found to avidly bind to phenyl-Sepharose, as evidenced by its presence in the low ammonium sulphate and 6M Urea eluates, and could thus be separated from other less hydrophobic proteins. Indeed, in view of the hydrophobic

nature of MonEst, which can induce irreversible protein aggregation when concentrated, a principle of negative enrichment was employed for subsequent chromatographic procedures. Consequently, the next stages of chromatographic fractionation were specifically designed to take account of this and to allow the MonEst components to pass through in the unbound fraction.

The Benzamidine-, Arginine- and Blue-Sepharose non-lectin affinity columns, which predominantly bind trypsin, chymotrypsin and other related proteolytic enzymes, were chosen to precede the lectin columns because of their ability to remove potentially degradative proteases. Furthermore, these particular columns were shown in the preliminary evaluations to bind significant amounts of proteins with esterolytic activity, with no apparent affinity for MonEst. Consequently, these three columns were connected in series and incorporated into the purification scheme. It was also considered necessary to introduce two additional non-lectin affinity columns (Protein-A and Protein-G) at this stage of MonEst purification to remove any residual contaminating serum IgG present in the starting material.

A series of lectin columns were selected to follow Protein-A/Protein-G. These were ordered according to the proportions of starting protein they bound; i.e. lectins which bound the least amount of protein preceded those binding higher proportions of the starting protein. However, in practice the actual order of the lectin series was not an important factor as unbound proteins would be re-passaged through the series until no further binding was detected. These lectin columns provided an important means of removing various contaminating glycoproteins that might not otherwise be resolved by conventional chromatographic procedures.

Con-A lectin chromatography was then chosen to precede the final Superose-12 gel filtration stage, as preliminary analyses indicated that all MonEst components bound to Con-A and would therefore be concentrated prior to use of the low-capacity Superose-12 gel filtration column. Con-A binds glycolipids and glycoproteins containing alpha-D-

mannopyranosyl, alpha-D-glucopyranosyl or sterically related residues as part of their glycan structure and consequently provides a means of separating MonEst components from non-glycoproteins or other glycoproteins with different glycan structures. Again, as the use of linear (glucopyranoside or mannopyranoside) elution gradients did not appear to offer significant advantages in terms of specific MonEst resolution, a single step elution with mannopyranoside would be used to desorb bound components. The final stage of the proposed purification procedure would then utilise Superose-12 gel filtration to separate MonEst from residual contaminating proteins with differing relative molecular weights (Mr).

ComEst Fractionation:

The rationale followed for the purification of ComEst was essentially the same as that for the MonEst in that, the columns were specifically organised to allow a rapid fractionation with the least number of buffer exchanges required. The primary consideration was to reduce the rather large starting sample volume using high- and intermediate capacity columns thus leaving manageable volumes for the low capacity column procedures.

S-Sepharose cation-exchange column was chosen to further process the Q-Sepharose unbound fraction containing the major ComEst components. Its high protein binding capacity and ability to withstand relatively high flow through rates would permit the processing of large volumes of starting material. For this, a starting pH of 6.35 was chosen, which allowed the binding of ComEst components and their separation from many of the contaminating proteins by a single-step elution with 0.25M NaCl. Benzamidine- and Arginine-Sepharose non-lectin affinity columns would subsequently be employed to further remove a high proportion of potentially degradative proteases from the ComEst-enriched post S-Sepharose fraction followed by the lectin affinity columns (with the exception of *Lens culinaris agglutinin* and *Pisum sativum agglutinin),* which would be arranged as described for the MonEst fractionation. As for MonEst, Con-A and Superose-12 chromatography procedures were chosen for the final purification stages of ComEst.

In summary, the investigations detailed in this Chapter provide a basis for constructing fractionation schemes for the independent purification of MonEst and ComEst myeloid esterase species. In the next Chapter, these proposed procedures will be applied to the full-scale purification of these enzymes, and the resulting fractionated esterases will be extensively investigated to compare their molecular and biochemical characteristics.

CHAPTER 3

Figures 3.1 to 3.11 and Tables 3.1 to 3.12

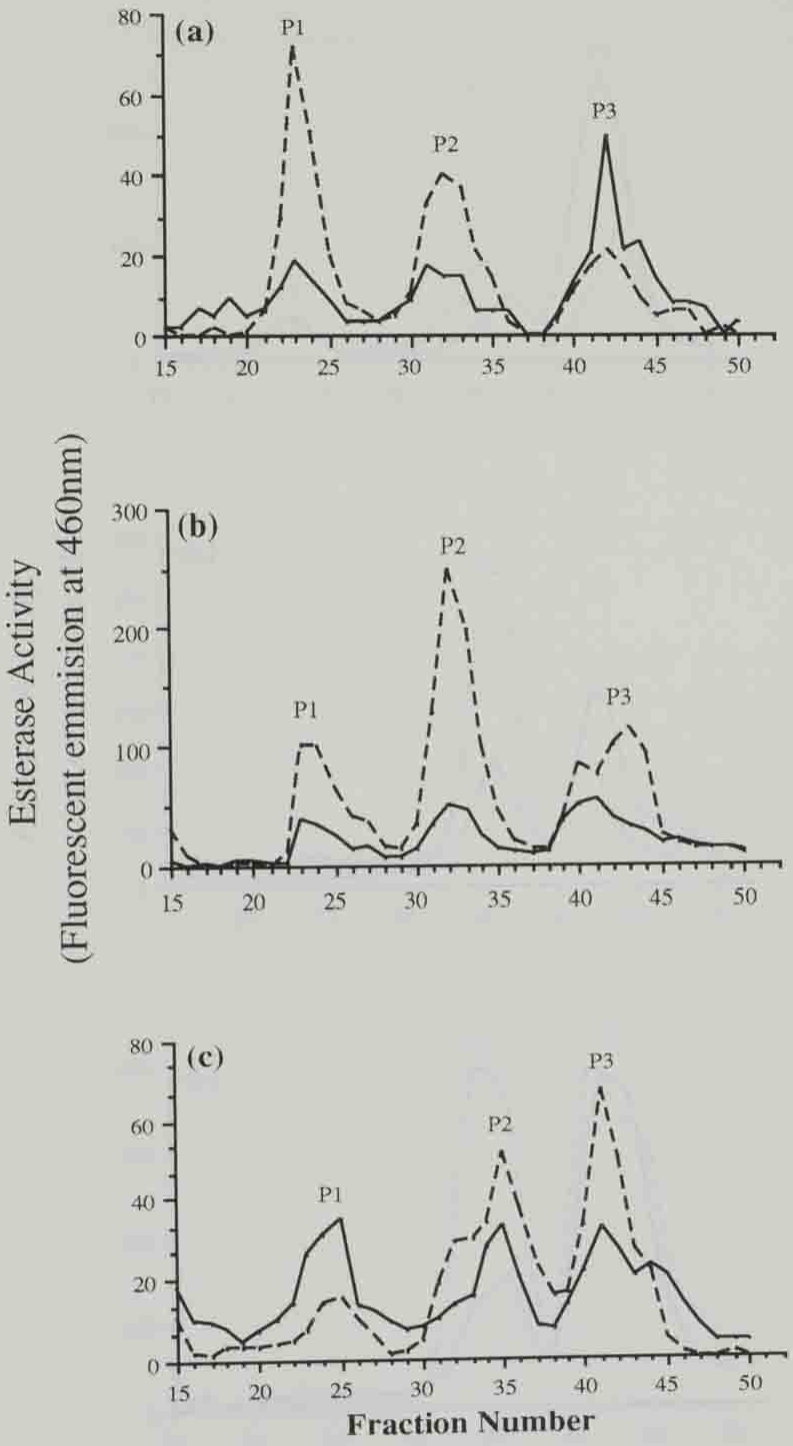

Figure 3.1: *Representative Superose-12 FPLC gel filtration profiles of esterase molecular weight species of normal mature myeloid cells (granulocytes/monocytes). Diagrams (a), (b) and (c) illustrate the observed esterase molecular weight species for cell extracts with G1, G2 and M-type IEF isoenzyme patterns respectively (detailed in Section 2.3.4.2); the continuous lines indicate acetate esterase activities of the gel filtration fractions, and the broken lines butyrate esterase activities. Note that the P1 species was eluted at Vo, suggesting that this represented high molecular weight aggregates or enzyme-protein complexes.*

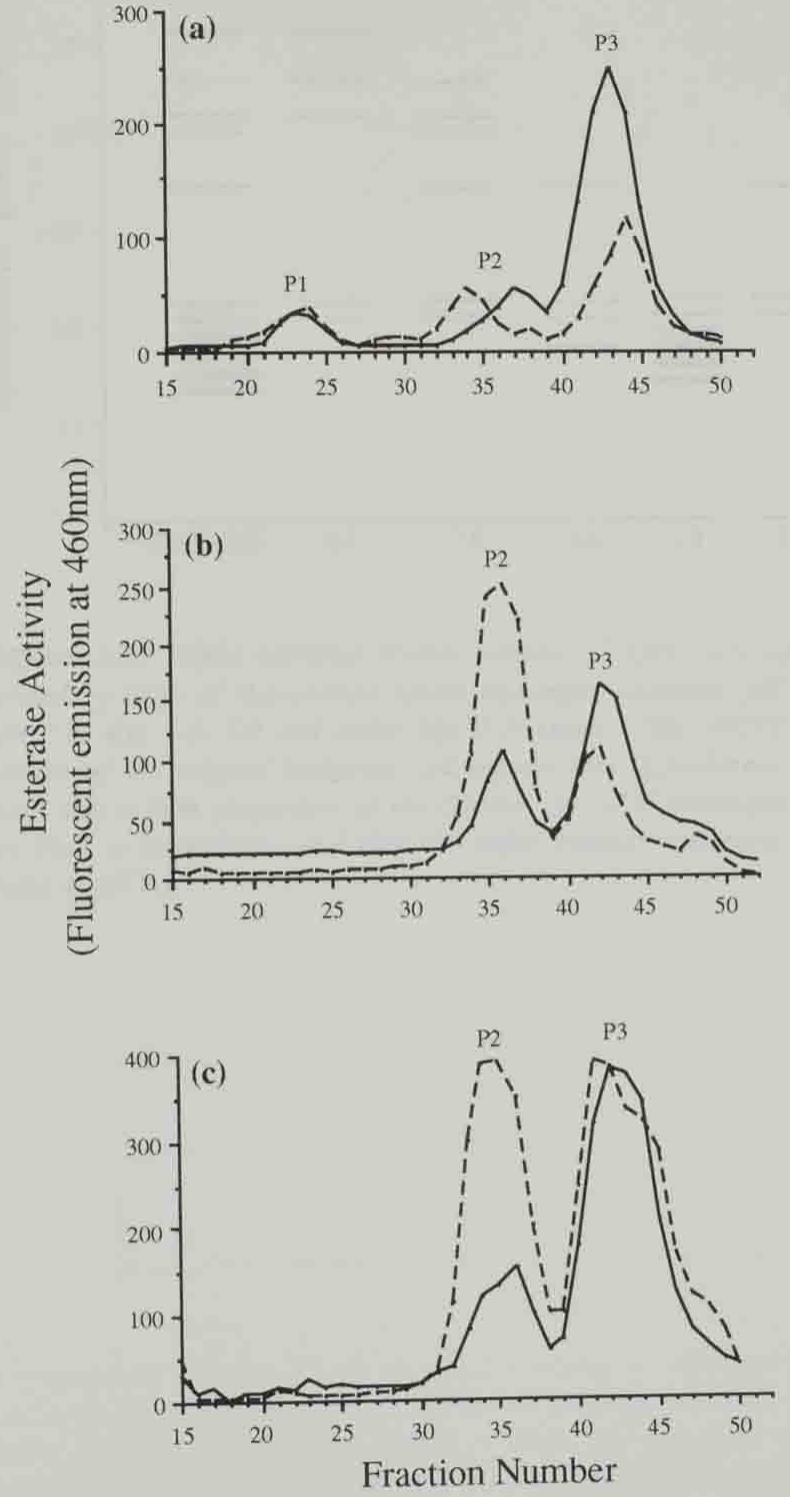

Figure 3.2: *Representative Superose-12 FPLC gel filtration profiles of esterase molecular weight species of leukaemic myeloid blasts. Diagrams (a), (b) and (c) illustrate the observed esterase molecular weight species for leukaemic cell extracts with G1, G2 and M-type IEF isoenzyme patterns respectively (detailed in Section 2.3.4.3); the continuous lines indicate acetate esterase activities of the gel filtration fractions, and the broken lines butyrate esterase activities. Note that the P1 species was eluted at Vo, suggesting that this represented high molecular weight aggregates or enzyme-protein complexes.*

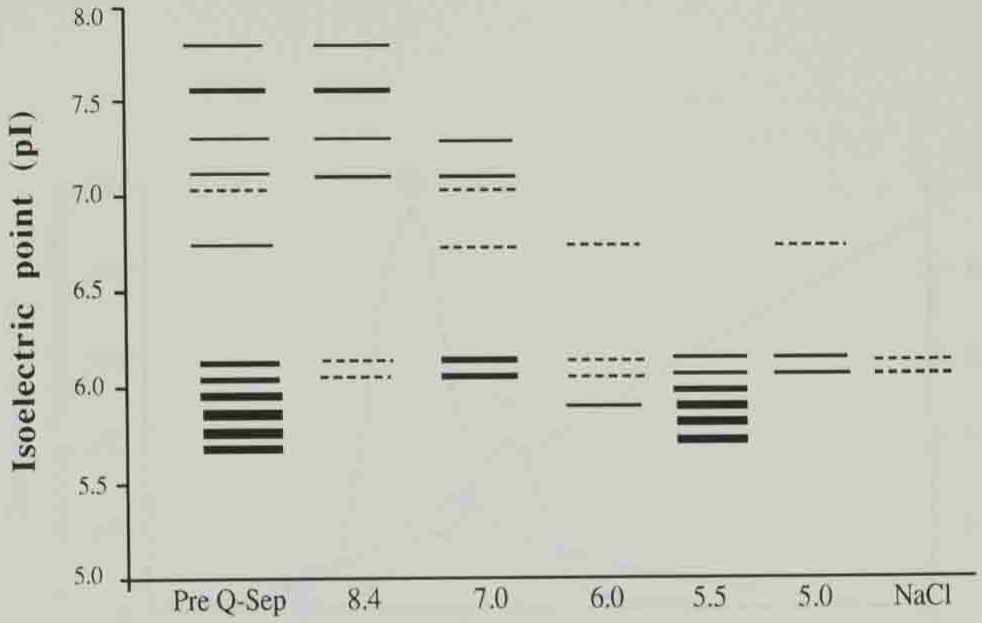

Figure 3.3: *Alpha-naphthyl acetate esterase (ANAE) isoenzyme profiles, as defined by IEF, of Q-Sepharose (anion exchanger) unbound (pH 8.4) and eluate (pH 7.0, 6.0, 5.5, 5.0 and molar NaCl) fractions. The ANAE-IEF isoenzyme pattern of the original leukaemic cell extract (Pre Q-Sepharose) is also shown. Note that a high proportion of the ComEst (pI >7.0) isoenzyme components do not bind to Q-Sepharose and that the major MonEst isoenzyme components are eluted at pH 5.5.*

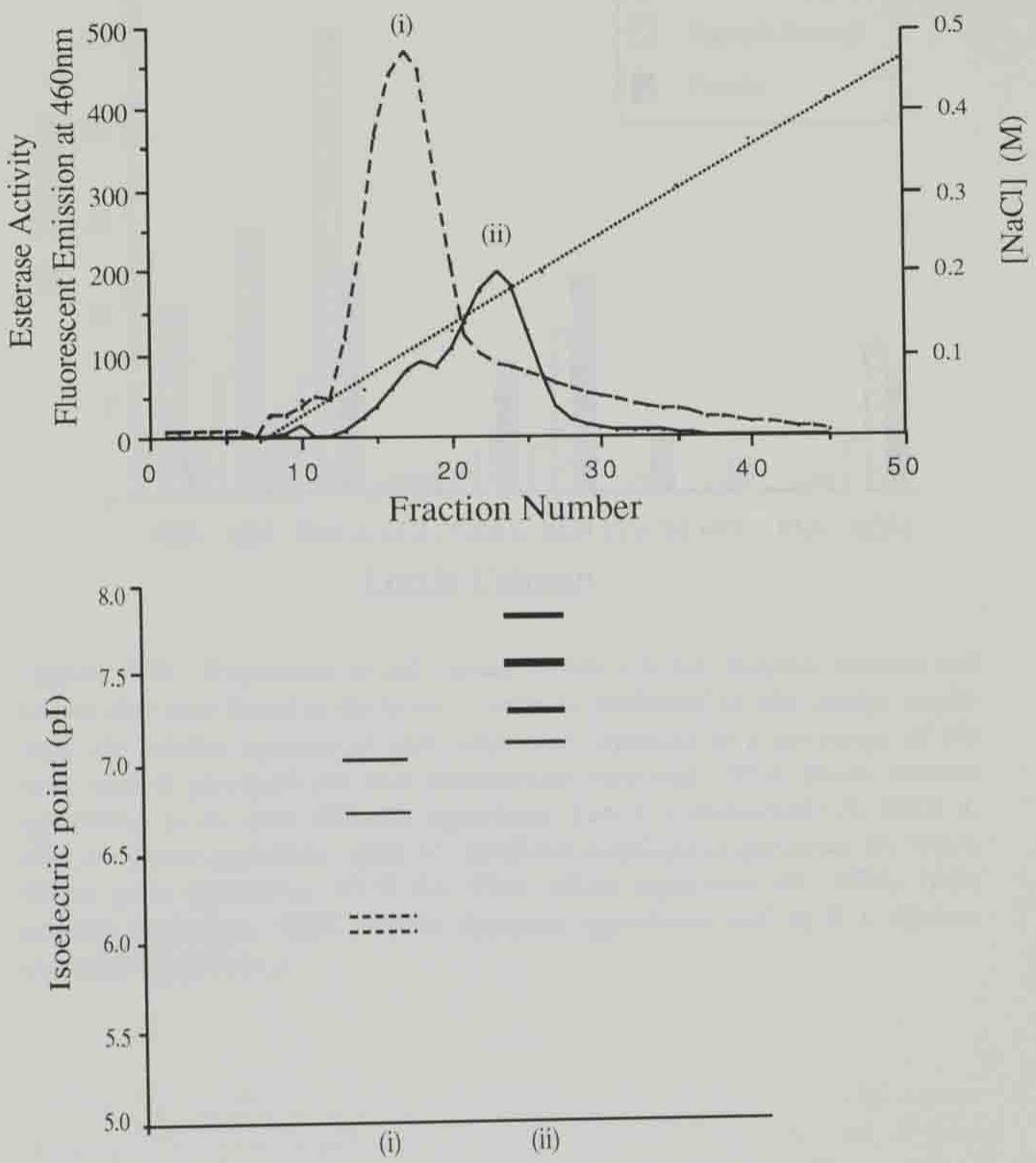

Figure 3.4: *Elution profile of ComEst isoenzyme components binding to S-Sepharose cation exchanger at pH 6.0. Bound proteins were desorbed with a 0.0 to 0.5M linear sodium chloride (NaCl) gradient, and fractions were assayed for acetate (continuous line) and butyrate esterase (broken line) activities. The upper diagram reveals that two distinct peaks of esterase activity were eluted under these conditions; the first of these (i) shows a marked preference for butyrate substrate hydrolysis, but when analysed by IEF (lower diagram), shows low levels of ComEst (pI range 6.2-7.2) isoenzyme staining; peak (ii) in contrast shows an acetate substrate preference with high levels of ComEst isoenzyme (pI range 7.3-7.9) staining.*

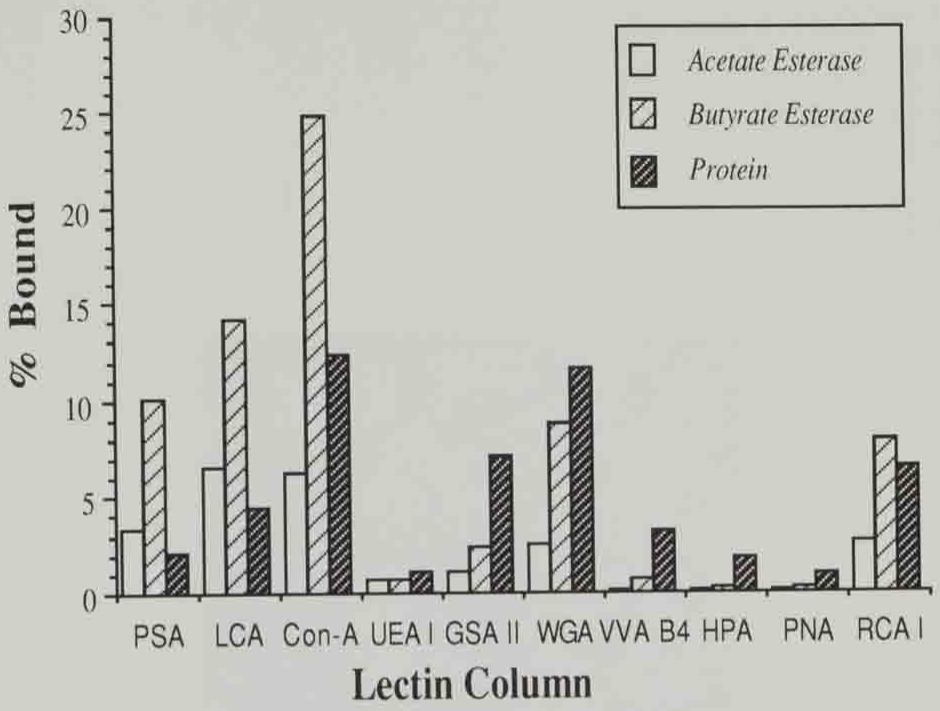

Figure 3.5: *Proportions of cell extract acetate esterase, butyrate esterase and protein that were bound to the lectin columns evaluated in this study; results show the relative amounts of each component, expressed as a percentage of the total amount processed, for each characteristic measured. PSA, Pisum sativum agglutinin; LCA, Lens culinaris agglutinin; Con-A, Concanavalin-A; UEA I, Ulex europaeus agglutinin; GSA II, Griffonia simplicifocia agglutinin II; WGA, Wheat germ agglutinin; VVA B4, Vicia villosa agglutinin B4; HPA, Helix pomatia agglutinin; PNA, Arachis hypogaea agglutinin; and RCA I, Ricinus communis agglutinin I.*

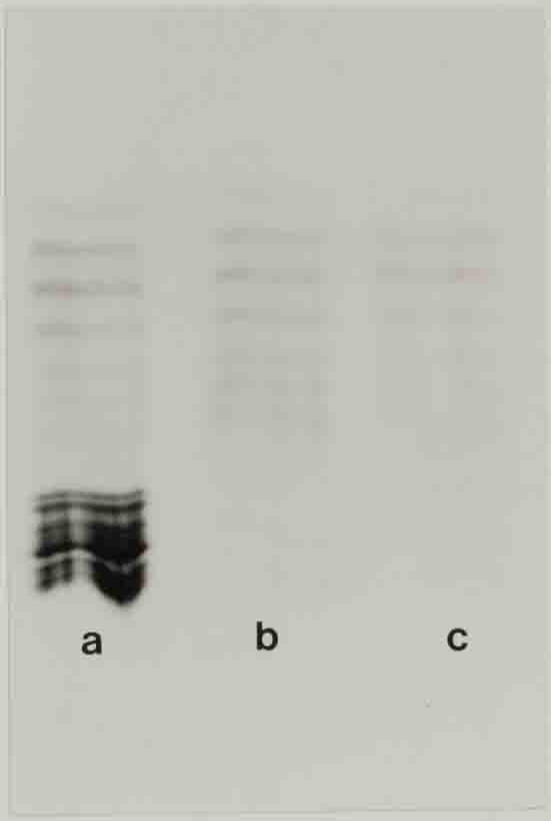

Figure 3.6: *Isoelectric focusing (IEF) of myeloid cell alpha-naphthyl acetate esterase (ANAE) isoenzymes eluted from Concanavalin A (Con-A; track a), Lens culinaris agglutinin (LCA; track b) and Pisum sativum agglutinin (PSA; track c). Note that Con-A binds both the MonEst and ComEst species while PSA and LCA bind minor proportions of ComEst only.*

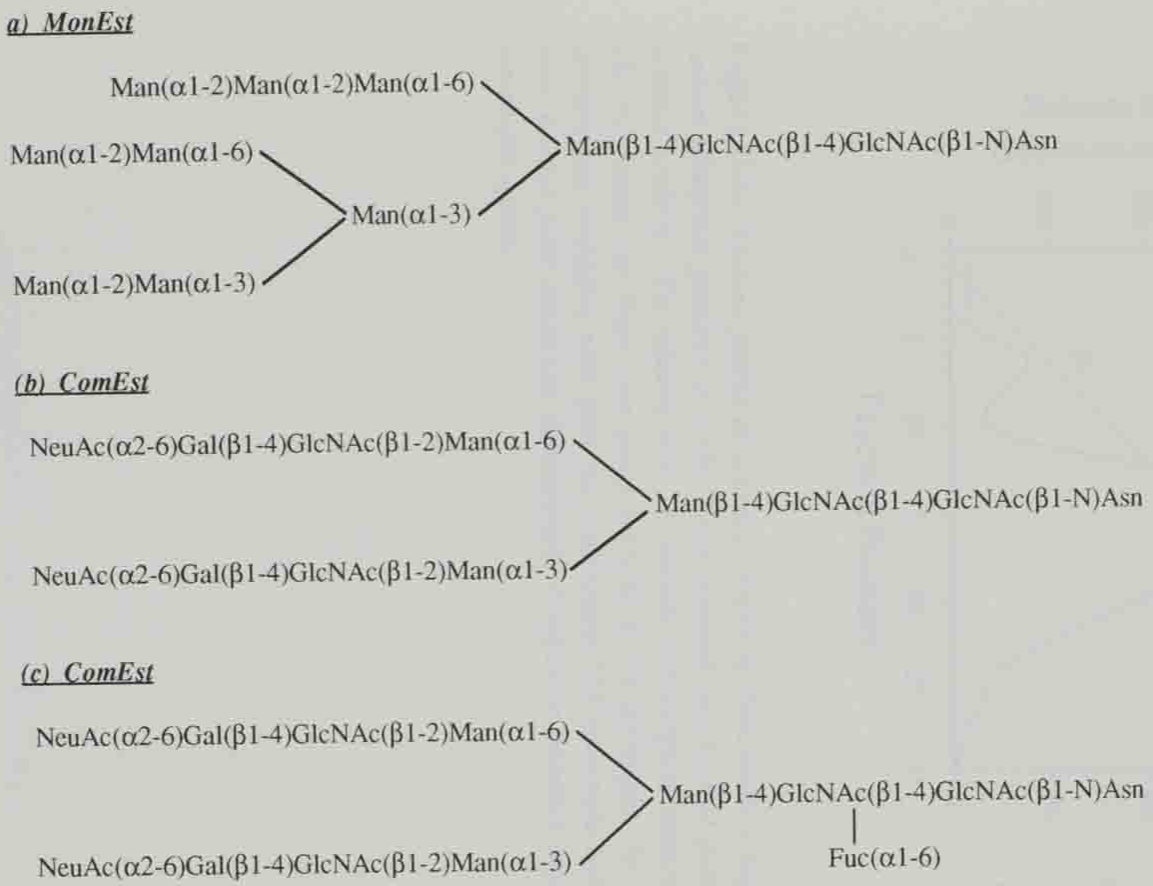

Figure 3.7: *Proposed glycosydic structures of MonEst and ComEst myeloid esterase species as assessed by lectin binding studies. Binding of MonEst to Con-A, but not to any of the other lectins, is compatible with the presence of oligomannosidic-type glycan(s) N-glycosydically linked to an asparagine residue (a). In contrast, the binding of ComEst to Con-A and to a lesser extent to PSA and LCA, suggests that this species contains biantennary N-acetyllactosamine-type (complex) asapragine-linked glycan(s) (b) together with a 1,6-fucosylated biantennary N-acetyllactosamine-type glycan(s) (c).*

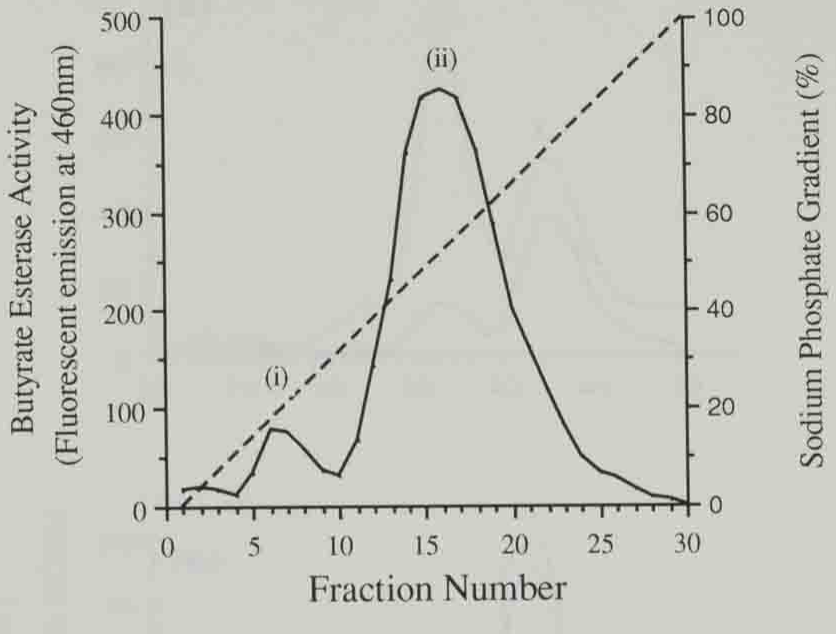

Figure 3.8: *Elution profile of esterases bound to Benzamidine-Sepharose. The sodium phosphate gradient to 0.3M (100%) resolved two distinct peaks of butyrate esterase activity. The relative rates of acetate and butyrate substrate hydrolysis (Ac:But ratio) for these two peaks were both 0.4 although, the highest total activity was associated with peak (ii). Subsequent IEF isoenzyme analysis revealed that neither peak contained demonstrable ComEst or MonEst myeloid esterase isoenzyme species.*

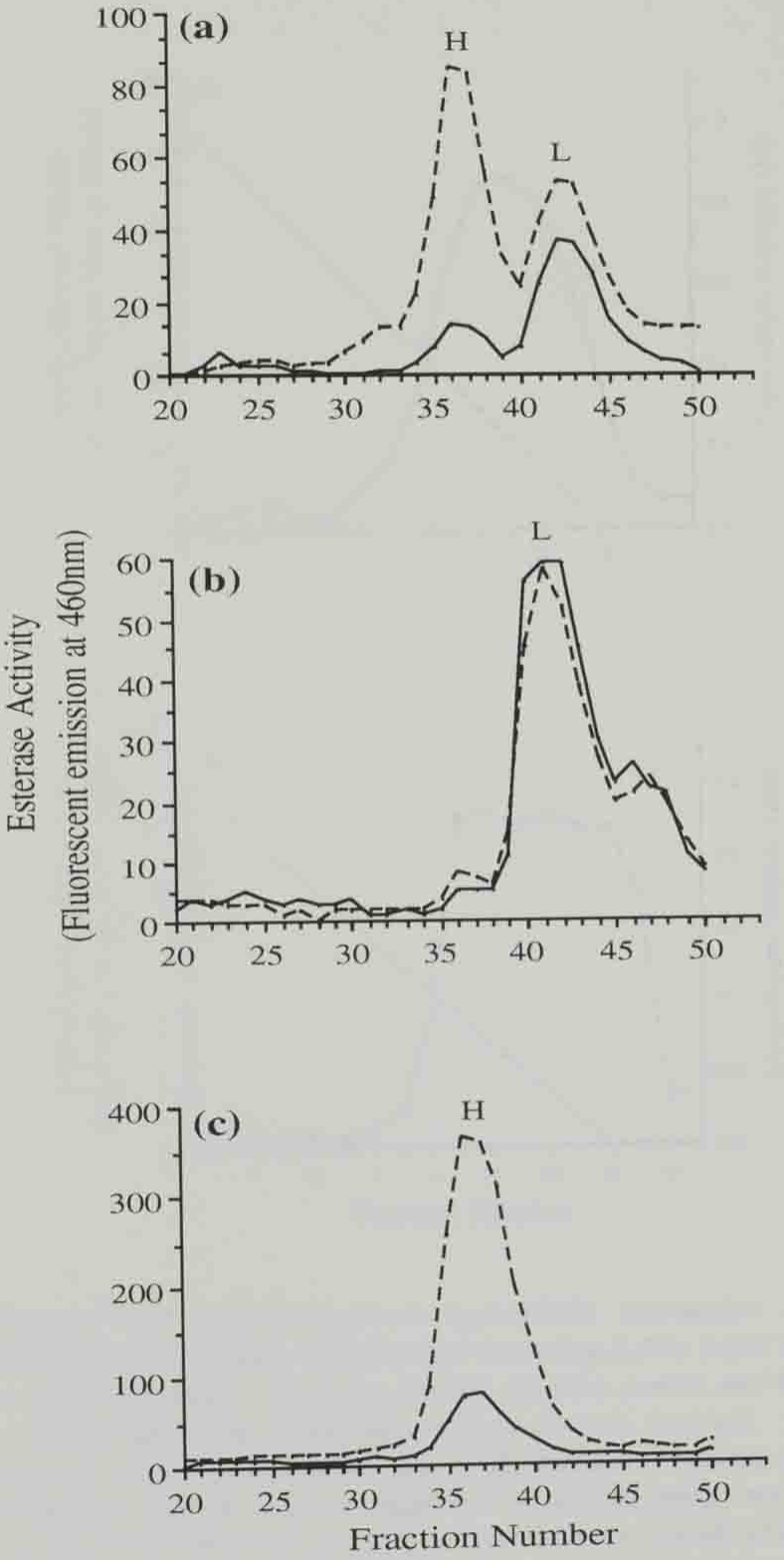

Figure 3.9: *Superose-12 FPLC gel filtration profiles of esterase molecular weight species; (a) pre-Benzamidine column leukaemic cell extract SA1; (b) post-column (unbound) protein fraction; (c) protein eluate from Benzamidine-Sepharose. Note that a great proportion of the high molecular weight species (H), is removed from the leukaemic cell extract by passage through this column and that this constitutes the majority of esterases in the eluate fraction.*

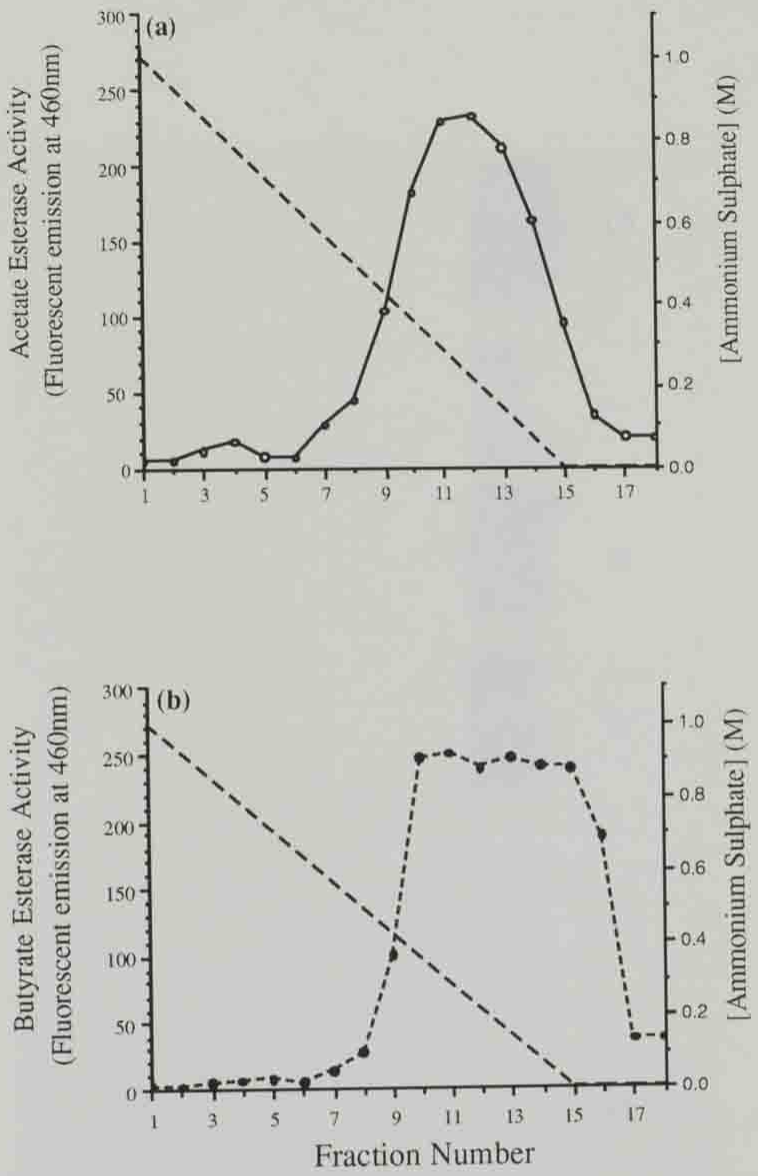

Figure 3.10: *Phenyl-Sepharose hydrophobic interaction chromatography (HIC) profile of esterases eluted with a descending (1.0 to 0.0M) linear gradient of ammonium sulphate. Diagrams (a) and (b) show acetate and butyrate esterase activities respectively of fractions assayed at 4min intervals. Note that there appears to be a continuous elution of esterases from approximately 0.5M ammonium sulphate, with no suggestion of distinct peaks, and that elution of proteins with esterolytic activity continues for a considerable time at 0.0M ammonium sulphate, suggesting their relatively high hydrophobicity.*

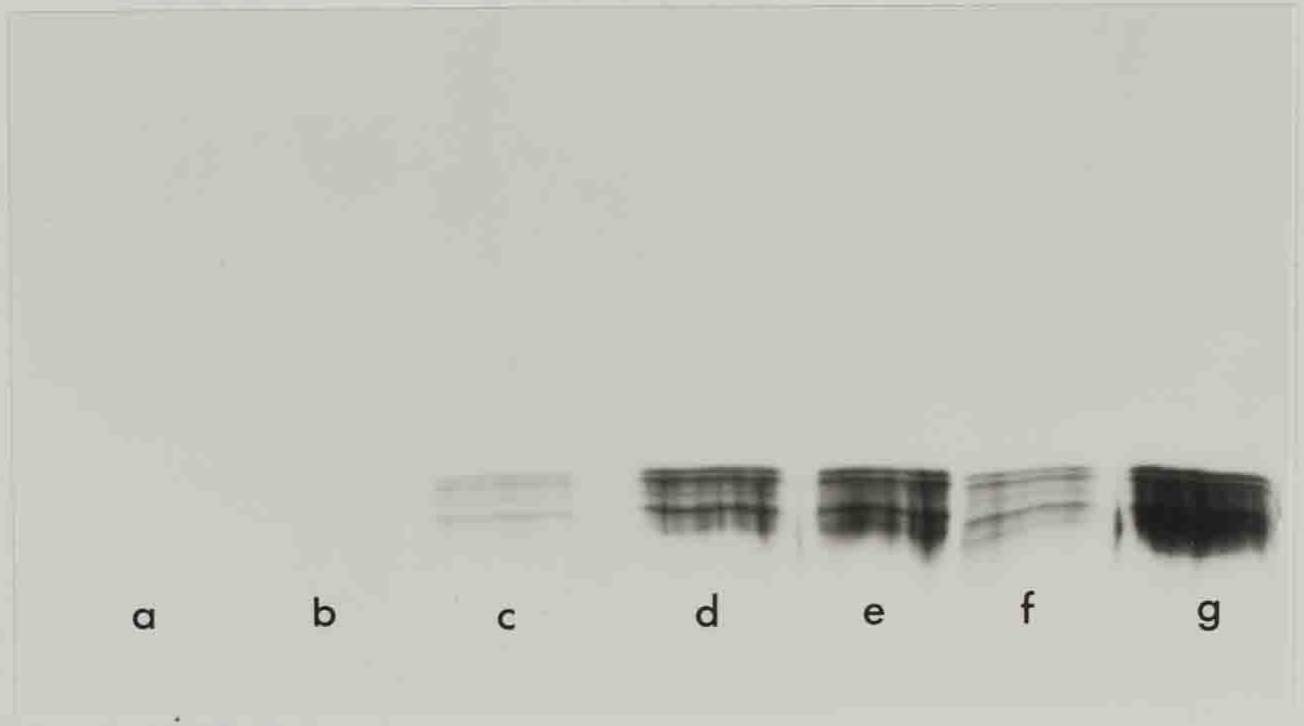

Figure 3.11: *Phenyl-Sepharose hydrophobic interaction chromatography (HIC) isoenzyme profiles of MonEst isoenzyme components, as defined by IEF, eluted using a descending (1.0 to 0.0M; lanes a-f) stepped gradient of ammonium sulphate and a final elution with 6M urea (lane g). Note that the majority of MonEst isoenzyme components are present in the 0.4-0.2M ammonium sulphate and 6M urea eluates, with the 0.6 and 0.0M ammonium sulphate eluates showing minor MonEst components only.*

Table 3.1 Analysis of normal myeloid cell (granulocyte/monocyte) esterase molecular weight species by Superose-12 FPLC gel filtration [a]

Cell type	IEF Pattern	Peak P2 Mr	Peak P2 Ac:But	Peak P3 Mr	Peak P3 Ac:But
Granulocytes	G1	342	1.6	64	2.3
Granulocytes	G1	342	1.6	64	2.7
Granulocytes	G1	284	1.2	64	4.0
Granulocytes	G2	284	1.0	64	2.4
Granulocytes	G2	284	0.9	77	1.9
Granulocytes	G2	342	0.7	53	0.7
Mean (G1 & G2):		**313**	**1.2**	**64**	**2.3**
Monocytes	M	236	0.5	64	0.6
Monocytes	M	236	0.9	53	0.9
Monocytes	M	236	0.4	77	0.2
Monocytes	M	236	0.6	77	1.6
Monocytes	M	135	1.4	77	1.7
Monocytes	M	196	1.6	53	2.3
Monocytes	M	135	0.7	37	0.7
Mean (M):		**201**	**0.8**	**63**	**1.1**

[a] Apparent molecular weights (Mr) were determined by comparison of Ve/Vo (elution volume/void volume) values obtained using protein standards of known molecular weight. The relative hydrolysis of acetate and butyrate substrates (Ac:But ratios) were determined using standard fluorimetric assay conditions (Section 2.3.6.4), and IEF pattern types G1, G2 and M are as defined in Section 2.3.4.2.

Table 3.2 Analysis of leukaemic myeloid cell esterase molecular weight species by Superose-12 FPLC gel filtration [a]

Diagnostic Category	IEF Pattern	Peak P2 Mr	Peak P2 Ac:But	Peak P3 Mr	Peak P3 Ac:But
M1/M2	G1	-	-	38	0.1
M1/M2	G1	100	0.2	26	1.6
M1/M2	G1	262	6.9	30	0.7
M3	G1	143	3.7	34	0.3
M4	G1	333	2.4	30	0.3
M4	G1	206	5.5	30	0.6
M4	G2	162	1.1	24	1.6
M1/M2	G2	262	1.1	26	0.3
M1/M2	G2	-	-	34	0.6
Mean (G1 & G2):		**210**	**2.9**	**30**	**0.7**
M4	M	196	0.3	64	1.4
M5	M	216	0.3	64	1.1
M5	M	162	0.1	26	0.5
M5	M	143	0.5	38	1.5
M5	M	162	2.9	34	1.2
M5	M	162	2.1	34	0.5
Mean (M):		**173**	**0.8**	**43**	**1.0**

[a] Cases grouped according to IEF pattern type. Apparent molecular weights (Mr) were determined by comparison of Ve/Vo (elution volume/void volume) values obtained using protein standards of known molecular weight. The relative hydrolysis of acetate and butyrate substrates (Ac:But ratios) were determined using standard fluorimetric assay conditions (Section 2.3.6.4), and IEF pattern types G1, G2 and M are as defined in Section 2.3.4.2.

Table 3.3 Anion-exchange (Q-Sepharose) binding characteristics of myeloid cell ComEst and MonEst esterase isoenzyme species [a]

Q-Sepharose Fraction	Protein [b]	Esterase Activity [b]		IEF Pattern [c]		
		Acetate	Butyrate	MonEst	ComEst-1	ComEst-2
Unbound (pH 8.4)	32	75	33	-	-	+++
pH 7.0 eluate	15	7	12	-	+	-
pH 6.5 eluate	1	6	10	-	-	-
pH 6.0 eluate	1	1	5	-	+	-
pH 5.5 eluate	11	4	14	+++	-	-
pH 5.0 eluate	30	3	16	-	+	-
1M NaCl eluate	10	4	10	-	+	-

a A leukaemic blast cell extract, containing all the major myeloid esterase isoenzyme components, was passaged through a Q-Sepharose column (starting pH 8.4) and the bound proteins eluted with buffers at 0.5 pH unit intervals.

b Protein concentrations determined by the bicinchoninic method (Section 2.2.7); esterase (acetate and butyrate) activities were assayed by standard fluorimetric procedures (Section 2.3.6.4). Results are expressed as percentages of the total protein or esterase activities that are associated with each designated fraction.

c Esterase isoenzyme components grouped according to their isoelectric points (pI); MonEst monocyte-associated esterases (pI range 5.5-6.2), ComEst-1, common myeloid esterases within the pI range range 6.3-7.2; and ComEst-2, common myeloid esterases within the pI range 7.3-7.9. Relative staining intensities are shown as negative to strong (- to +++).

Table 3.4 Fractionation of myeloid esterase isoenzymes by *Concanavalin-A* (Con-A) lectin affinity chromatography. Comparison of elutions using sodium chloride (NaCl), alpha-D-glucopyranoside and alpha-D-mannopyranoside [a]

	Esterase activity (%) [b]		IEF patterns [c]	
	Acetate	Butyrate	ComEst	MonEst
(a) NaCl eluant				
Unbound	89	73	-	-
NaCl: 0 - 1.0M	4	10	-	-
0.5M Mannopyranoside wash	7	17	+/++	++/+++
(b) Glucopyranoside eluant				
Unbound	86	65	-	-
Eluant: 0.10 - 0.18M	2	5	-	+/++
0.19 - 0.28M	3	8	+/++	+/++
0.29 - 0.38M	3	7	-	-
0.39 - 0.48M	3	7	-	-
0.49 - 0.50M	2	5	-	-
0.5M Mannopyranoside wash	1	3	+	++/+++
(c) Mannopyranoside eluant				
Unbound	79	56	-	-
Eluant: 0.10 - 0.18M	5	14	+/++	+++
0.19 - 0.28M	5	10	+/++	++/+++
0.29 - 0.38M	3	6	+	+/++
0.39 - 0.48M	4	7	-	-
0.49 - 0.50M	4	7	-	-

[a] A leukaemic blast cell extract, conatining both the major myeloid esterase isoenzyme components, was passaged through a Con-A column (Section 3.4.2.1) and eluted using linear gradients of: (a) 0.0 to 1.0M NaCl; (b) 0.0 to 0.5M methyl alpha-D-glucopyranoside, and (c) 0.0 to 0.5M methyl alpha-D-mannopyranoside.

[b] Acetate and butyrate esterase activities were determined by standardised fluorimetric assay (Section 2.3.6.4). Results are expressed as the percentage (%) of total acetate or butyrate esterase activity within each defined fraction of the chromatographic procedure.

[c] Esterase ComEst and MonEst isoenzyme components as defined in Section 2.3.4.2. Reaction strengths graded from negative (-) to strong (+++).

Table 3.5 Lectin affinity column matrices and sources

Lectin	Lectin Support	Source [a]
Pisum sativum agglutinin (PSA)	4% Beaded Agarose	Sigma
Lens culinaris agglutinin (LCA)	Sepharose 4B	Pharmacia
Concanavalin A (Con-A)	Sepharose 4B	Pharmacia
Ulex europaeus agglutinin I (UEA I)	4% Beaded Agarose	Sigma
Griffonia simplicifocia agglutinin II (GSA II)	4% Beaded Agarose	Sigma
Wheat germ agglutinin (WGA)	Sepharose 4B	Pharmacia
Vicia villosa agglutinin B4 (VVA B4)	4% Beaded Agarose	Sigma
Helix pomatia agglutinin (HPA)	4% Beaded Agarose	Sigma
Arachis hypogaea (Peanut) agglutinin (PNA)	4% Beaded Agarose	Sigma
Ricinus communis agglutinin I (RCA I)	4% Beaded Agarose	Sigma

[a] Sigma Chemical Co., Poole, Dorset, England; Pharmacia LKB Biotechnology, Milton Keynes, England

Table 3.6 Sugar specificities of immobilised lectins evaluated in this current study

Lectin	Sugar Specificity [a]
Pisum sativum agglutinin (PSA)	α-D-man > α-D-glc = GlcNAc
Lents culinaris agglutinin (LCA)	α-D-man > α-D-glc > GlcNAc
Concanavalin A (Con-A)	α-D-man > α-D-glc > GlcNAc
Ulex europaeus agglutinin I (UEA I)	α-L-Fuc
Griffonia simplifocia agglutinin II (GSA II)	β-D-GlcNAc = α-D-GlcNAc
Wheat germ agglutinin (WGA)	β-D-GlcNAc
Vicia villosa agglutinin B4 (VVA B4)	α-D-GalNAc
Helix pomatia agglutinin (HPA)	α-D-GalNAc
Arachis hypogaea (Peanut) agglutinin (PNA)	β-D-gal(1-3)-D-GalNAc > α-D-Gal = β-D-Gal
Ricinus communis agglutinin I (RCA I)	β-D-Gal > α-D-Gal

[a] Indicates primary sugar specificity, spatial relationships with other sugar groups may be important: D-man, D-mannose; D-Gal, D-galactose; D-glc, D-glucose; D-GalNAc, D-N-acetyl galactosamine; D-GlcNAc, D-N-acetyl glucosamine; L-Fuc, L-fucose.

Table 3.7 Lectin affinity column volumes and eluants

Lectin	Volume	Eluant
Pisum sativum agglutinin (PSA)	1.0ml	200mM methyl α-D mannopyranoside
Lens culinaris agglutinin (LCA)	8.0ml	200mM methyl α-D mannopyranoside
Concanavalin A (Con-A)	5.0ml	200mM methyl α-D mannopyranoside
Ulex europaeus agglutinin I (UEA I)	1.0ml	50mM α–L-fucose
Griffonia simplicifocia agglutinin II (GSA II)	1.0ml	100mM N-acetyl-D-glucosamine
Wheat germ agglutinin (WGA)	6.0ml	100mM N-acetyl-D-glucosamine
Vicia villosa agglutinin B4 (VVA B4)	2.5ml	20mM N-acetyl-D-galactosamine
Helix pomatia agglutinin (HPA)	2.5ml	20mM N-acetyl-D-galactosamine
Arachis hypogaea (Peanut) agglutinin (PNA)	2.0ml	50mM D-galactose
Ricinus communis agglutinin I (RCA I)	2.5ml	50mM D-galactose

Table 3.8 Protein concentrations, enzyme assays and isoenzymatic characteristics of sonicate extract proteins with lectin affinities [a]

Lectin	Esterase Activity			Esterase Isoenzymes [b]	
	Acetate	Butyrate	Protein	MonEst	ComEst
Ulex europaeus agglutinin I (UEA I)					
Bound	0.7	0.6	1.2	-	-
Unbound	99.3	99.4	98.8	+++	+++
Arachis hypogaea agglutinin (PNA)					
Bound	0.2	0.3	1.3	-	-
Unbound	99.8	99.7	98.7	+++	+++
Helix pomatia agglutinin (HPA)					
Bound	0.1	0.3	1.8	-	-
Unbound	99.9	99.7	98.2	+++	+++
Pisum sativum agglutinin (PSA)					
Bound	3.3	10.1	2.2	-	+
Unbound	96.7	89.1	97.8	+++	++
Vicia villosa agglutinin B4 (VVA B4)					
Bound	0.1	0.6	3.2	-	-
Unbound	99.9	99.4	96.8	+++	+++
Lens culinaris agglutinin (LCA)					
Bound	6.6	14.2	4.5	-	+
Unbound	93.4	85.8	95.5	+++	++
Ricinus communis agglutinin I (RCA I)					
Bound	2.7	8.0	6.7	-	-
Unbound	97.3	92.0	93.3	+++	+++
Griffonia simplifocia agglutinin II (GSA II)					
Bound	1.1	2.4	7.1	-	-
Unbound	98.9	97.6	92.1	+++	+++
Wheat germ agglutinin (WGA)					
Bound	2.5	8.9	11.6	-	-
Unbound	97.5	91.1	88.4	+++	+++

[a] Unbound and eluted sonicate fractions were concentrated to approximate volumes of 4ml and 1ml respectively. These were assayed for acetate esterase activity, butyrate esterase activity, and protein concentrations as described in Section 3.4.3. Results are shown as the proportions (%) of the total extract esterase or protein that does not bind (unbound) or binds to (eluate) each column. These data are graphically presented in Figure 3.5.

[b] IEF patterns were determined for both the unbound and bound fractions. Results are shown as the relative staining intensities of monocyte-associated (MonEst; pI range 5.6 - 6.1) and 'common' (ComEst; pI >6.2) myeloid esterase isoenzymes; - indicates no isoenzymes detected in the (bound or unbound) fraction; +++ indicates high isoenzyme concentrations; and + or ++ indicates intermediate levels.

Table 3.9 Analysis of Benzamidine-Sepharose for the binding of ComEst and MonEst myeloid esterase species [a]

Sample	Pre-Column IEF	Benzamidine Eluate			Benzamidine Unbound		
		Acetate	Butyrate	IEF	Acetate	Butyrate	IEF
1	G1	60%	26%	-	40%	74%	G1
2	G1	40%	31%	-	60%	69%	G1
3	G1	40%	22%	-	60%	78%	G1
4	G1	49%	27%	-	51%	73%	G1
5	G2	36%	19%	-	64%	81%	G2
6	G2	38%	21%	-	62%	79%	G2
7	G2	30%	27%	-	70%	73%	G2
8	M	24%	35%	-	76%	65%	M
9	M	30%	17%	-	70%	83%	M

[a] Results indicate the proportions of the total acetate and butyrate activities that are associated with the unbound and eluate fractions, together with their ANAE-IEF patterns (G1, G2 or M as defined in Section 2.3.4.2; - indicates complete absence of demonstrable ComEst and MonEst species).

Table 3.10 Evaluation of Arginine-Sepharose and Blue-Sepharose columns for the fractionation of myeloid esterase isoenzyme species [a]

Column	Protein [b]	Esterase Activity [b] Acetate	Butyrate	Esterase Isoenzymes [c] MonEst	ComEst
Arginine-Sepharose					
Unbound	68%	58%	51%	+++	++
Molar NaCl eluate	32%	42%	49%	-	-
Blue-Sepharose					
Unbound	65%	66%	48%	+++	++ [d]
Molar NaCl eluate	35%	34%	52%	-	+ [d]

a Leukaemic AMoL-M5 blast cell extract SA1 was passaged through Arginine-Sepharose or Blue-Sepharose columns; unbound proteins were collected, bound proteins were single step-eluted with molar sodium chloride (NaCl).

b Protein concentrations and esterase activities (acetate and butyrate) of unbound and eluate fractions were determined by standard procedures (Sections 2.2.7 and 2.3.6.4) and each expressed as proportions of the total processed.

c Analysis of esterase isoenzyme components by isoelectric focusing (IEF); the relative staining intensities of MonEst and ComEst myeloid isoenzyme species are shown as - (negative) to +++ (strong).

d ComEst isoenzymes which did not bind to the Blue-Sepharose column (i.e. unbound fraction) were characteristically of higher pI (range 7.3 - 7.9); ComEst components binding to and being eluted from this column were restricted to a lower pI range (6.3 - 7.2) and of weaker staining intensity.

Table 3.11 Evaluation of hydrophobic interaction chromatography (HIC) for the fractionation of esterase isoenzymes: The binding and elution characteristics of MonEst and ComEst myeloid esterase isoenzyme species to phenyl-Sepharose [a]

Fraction	Protein [b]	Esterase Activity [b]		Esterase Isoenzymes [c]	
		Acetate	Butyrate	MonEst	ComEst
1.0M ammonium sulphate	3	4	2	-	-
0.8M ammonium sulphate	15	14	5	-	-
0.6M ammonium sulphate	18	11	4	-	-
0.4M ammonium sulphate	20	15	6	-	-
0.2M ammonium sulphate	12	9	8	+	+
0.0M ammonium sulphate	10	20	39	+++	+++
6M urea eluate	22	27	36	+/++	+/++

[a] Leukaemic AMoL-M5 blast cell extract SA1 was passaged through the phenyl-Sepharose column in the presence of 1.0M ammonium sulphate. Unbound proteins were collected; bound proteins were step-eluted with decreasing (0.8 to 0.0M) ammonium sulphate concentrations at intervals of 0.2M, followed by elution with 6M urea.

[b] Protein concentrations and esterase activities (acetate and butyrate) of unbound and eluate fractions were determined by standard procedures (Sections 2.2.7 and 2.3.6.4) and each expressed as proportions of the total processed.

[c] Analysis of esterase isoenzyme components by isoelectric focusing (IEF); the relative staining intensities of MonEst and ComEst myeloid isoenzyme species are shown as - (negative) to +++ (strong).

Table 3.12 Evaluation of Phenyl-Sepharose hydrophobic interaction chromatography (HIC) for the fractionation of MonEst isoenzymes [a]

Fraction	Protein [b]	Esterase Activity [b]		MonEst [c]
		Acetate	Butyrate	
1.0M ammonium sulphate	55	29	19	-
0.8M ammonium sulphate	6	2	2	-
0.6M ammonium sulphate	7	2	6	-
0.4M ammonium sulphate	9	8	15	+/++
0.2M ammonium sulphate	8	16	16	+++
0.0M ammonium sulphate	7	20	20	+++
6M urea eluate	8	23	22	+++

a Partially-purified Q-Sepharose pH 5.5 piperazine eluate conatining high concentrations of MonEst, but not ComEst (Section 3.3.2.2), was passaged through the phenyl-Sepharose column in the presence of 1.0M ammonium sulphate. Unbound proteins were collected; bound proteins were step-eluted with decreasing (0.8 to 0.0M) ammonium sulphate concentrations at intervals of 0.2M, followed by elution with 6M urea.

b Protein concentrations and esterase activities (acetate and butyrate) of unbound and eluate fractions were determined by standard procedures (Sections 2.2.7 and 2.3.6.4) and each expressed as proportions of the total processed.

c Analysis of esterase isoenzyme components by isoelectric focusing (IEF); the relative staining intensities of the MonEst myeloid isoenzyme species are shown as - (negative) to +++ (strong).

CHAPTER 4

MYELOID CELL ESTERASE ISOENZYMES: PURIFICATION, MOLECULAR AND BIOCHEMICAL CHARACTERISATION

4.1 INTRODUCTION

This Chapter describes the purification, and subsequent molecular and biochemical characterisation of the two major myeloid esterase enzyme forms (MonEst and ComEst). The earlier findings of this study (Chapter 2) showed that MonEst is expressed in a lineage-restricted manner, by cells of the monocytic lineage, whilst ComEst components are commonly expressed by both monocytic and granulocytic cells. These observations, together with previous ultrastructural studies [Bozdech & Bainton, 1977, 1981; Monahan et al., 1981; Marec et al., 1981; Zicca et al., 1981] which indicated differences in the subcellular localisation of esterases in granulocytes and monocytes, suggest that the ComEst and MonEst species may represent distinct esterolytic enzymes. In order to further clarify their relationships, it was considered necessary to purify both enzyme forms and compare their molecular and biochemical characteristics.

Individual isoenzymes of any given enzyme often display differences in catalytic and physicochemical properties. Differing isoenzyme forms can originate at the genetic level (i.e. representing different gene products and corresponding to primary or true isoenzymes) or as a consequence of post-translational modification (secondary isoenzymes). Primary isoenzymes can additionally differ in that they may be encoded by either multiple genetic loci, common to all members of the species, or they may result from the existence of allelic variation at the enzyme-determining structural locus (allozymes). Some generalisations can be made, with greater or lesser degrees of certainty, about isoenzymes determined by multiple gene loci. They usually show differences in catalytic properties, such as substrate affinity or sensitivity to inhibitors, they can be expected to be antigenically distinct, (reflecting differences in the amino acid sequences encoded by distinct structural genes), and they are likely to differ in stability. By comparison, the nature of 'allozymes' is much less predictable as they are derived from individual random mutations, although monoclonal antibodies are generally capable of distinguishing between different allozymic forms of an enzyme molecule. In contrast, the catalytic properties of post-translational variants are often similar, there may be only

minor changes in stability, and the modified form may be antigenically identical even though differences in size and net molecular charge may be seen [reviewed in Moss, 1986].

Based on their gel binding characteristics, individual procedures were devised for the fractionation and purification of ComEst and MonEst myeloid esterase species (Section 3.7). In view of the large amount of serum/cellular extract protein used for these fractionations, and taking into account the fact that many different types of columns were included as part of these chromatographic sequences, it was considered that wherever possible a single buffer system should be employed (to obviate the need for time-consuming buffer changes) and that the columns should be primarily employed to remove contaminating proteins (i.e. negative enrichment of ComEst and MonEst). This latter point is particularly important as the high hydrophobicity of esterases (clearly evidenced by the results from the phenyl-Sepharose evaluation; Section 3.6.2.2) tends to lead to significant aggregation when these enzymes are concentrated, and because chromatographic procedures which utilise the principles of negative enrichment permit the sequential linkage of different columns in series. Furthermore, the preliminary column evaluations indicated that a high proportion of the total esterase activity defined by assays with esters of alpha-naphthol and 4-methylumbelliferone could not be demonstrated by standard azo-dye histochemical zymogram procedures. This suggested that these particular enzymes (including trypsin and chymotrypsin-like enzymes), which were present in addition to the histochemically demonstrable ComEst and MonEst isoenzyme species, hydrolysed the substrates in such as way as to make subsequent and spontaneous coupling with the diazonium salt impossible. Although further studies of these particular enzymes were not undertaken, as they were not the subject of these present studies, the recognition of their existence effectively meant that (a) the column procedure designed for the final chromatographic purifications of ComEst and MonEst had to ensure their complete removal, (b) that the monitoring of column efficiencies and their ability to successfully resolve individual esterase species could only be reliably

achieved by histochemical zymogram analysis, and (c) that the conventional expression of enzyme purification and column yields (i.e. reporting enzyme activities relative to protein concentration) would be meaningless. It was also concluded from these initial studies that because of the diverse chromatographic characteristics of these co-existing esterolytic enzymes, and because the nature of the starting material for MonEst in particular precluded the technically less demanding processing of a cell membrane extract, that an extensive series of columns would be required to achieve acceptable enzyme resolution. Thus, all the columns used in the final purification procedure (detailed in Table 4.1) were found to positively contribute towards this aim, with the final sequence being designed to maximise the removal of unwanted proteins and esterolytic enzymes and to minimise the loss of the myeloid esterase species.

4.2 MATERIALS AND METHODS

4.2.1 Purification of myeloid cell esterases:

4.2.1.1 Purification of monocyte-specific esterase (MonEst) isoenzymes: The raw material used for the full-scale fractionation of MonEst was obtained, at post-mortem, from the chest cavity of a patient who died with untreated acute monocytic leukaemia (AMoL-M5). Because there was evidence of significant cellular deterioration, with relatively high concentrations of MonEst in the extracellular fluid, it was decided to process all of the post-mortem material, rather then the cells alone. The intact cellular components were disrupted by ultrasonication (MSE Soniprep 150) at an amplitude of 18μ for one minute and Triton X-100 was added to give a final detergent concentration of 1%. After mixing at 25°C for 10 mins and subsequent high speed centrifugation, the solubilised protein supernate was recovered from pelletted cell debris.

Individual 150-200 ml aliquots (total volume, 950ml) of the solubilised protein extract were dialysed overnight against 25mM tris-HCl buffer pH 8.4 containing 5% betaine (TBB), diluted to 400ml in the same, centrifuged and then filtered using 1.0μm nitrocellulose membranes (Millipore). This material was then passaged at a flow rate of

4.0ml/min through a 26.0 x 2.5cm Fast Flow Q-Sepharose anion exchange column which had been pre-equilibrated with TBB. Sample loading was monitored at 280nm and the unbound protein fraction, which was confirmed by IEF analysis to lack detectable MonEst, was discarded. After re-attainment of the A280 baseline, desorption of bound proteins with approximate pIs of >6.5 was achieved by passaging 25mM bis-tris-HCl (containing 5% betaine) pH 6.3 at a flow rate of 4.0ml/min. MonEst isoenzymes were then eluted with 25mM piperazine-HCl (containing 5% betaine) pH 5.5, and this was followed by elution from the column of remaining bound proteins with molar NaCl. A substantial proportion (approximately 35%) of the processed serum/cell extract protein remained tightly bound to the column and this could only be eluted by 2M NaOH. Although this particular eluate could not be analysed for the presence of active esterase isoenzymes there was no suggestion, from comparing the IEF profiles of pre-column sample with that of the pH 5.5 piperazine (MonEst) eluate, that there had been any significant loss of MonEst components.

Individual Q-Sepharose piperazine (pH 5.5) eluates were pooled and dialysed overnight at 4°C against 1.0M ammonium sulphate in 50mM phosphate buffer pH 7.0 and, following centrifugation to remove protein aggregates, passaged through a 17.5 x 2.5cm phenyl-Sepharose column at a flow rate of 4.0ml/min. The unbound protein material, which did not contain any esterase isoenzyme forms as defined by IEF, was discarded. Following re-attainment of the A280 baseline, desorption of bound proteins was achieved by sequential passage of decreasing 0.2M ammonium sulphate steps (i.e. 0.8, 0.6, 0.4, 0.2 and 0.0M) in 12.5mM succinic acid pH 6.8 containing 0.5mM each of magnesium, manganese and calcium chlorides (Affinity Buffer A) at a flow rate of 4.0ml/min. A further desorption from phenyl-Sepharose of tightly-bound proteins was then carried out with 6M urea in Affinity Buffer A. The eluates from phenyl-Sepharose were then dialysed against Affinity buffer A and analysed by IEF for the presence of esterase isoenzymes; eluates (see Results Section 4.3.1.1) containing significant concentrations of MonEst were then pooled prior to further chromatographic processing.

The dialysed MonEst enriched eluate from phenyl-Sepharose was passaged through 20.0 x 1.6cm Benzamidine-Sepharose 6B, 6.0 x 2.5cm Arginine-Sepharose 4B and 23.0 x 2.5cm Blue-Sepharose CL-6B columns connected in series at a flow rate of 3.0ml/min. The unbound material, containing majority of the MonEst was collected at 4°C; proteins (including a minor proportion of the total MonEst) binding to this column series were eluted with molar NaCl and discarded. Following re-equilibration of these affinity columns, the procedure was repeated until no further binding (as adjudged by elution profiles monitored by A280) was seen. The unbound material was then dialysed against fresh Affinity Buffer A for 4hrs at 25°C prior to further processing.

The dialysed unbound proteins from the Benzamidine/Arginine/Blue-Sepharose column series were passaged at a flow rate of 3.0ml/min through 10ml Protein-A Sepharose CL-4B and 6ml Protein-G Sepharose CL-4B columns connected in series. Bound proteins were eluted with molar acetic acid and the unbound material was, following re-equilibration of the columns with Affinity Buffer A, re-passaged until no further binding was seen. At this stage, the unbound material was dialysed overnight at 4°C against Affinity Buffer A containing 0.15M NaCl (Affinity Buffer B).

The dialysed unbound proteins from Protein-A/Protein-G were then passaged at a flow rate of 1.0ml/min through a sequential series of previously evaluated lectin columns comprising *Ulex europaeus agglutinin* (UEA I), *Arachis hypogaea agglutinin* (PNA), *Helix pomatia agglutinin* (HPA), *Pisum sativum agglutinin* (PSA), *Vicia villosa agglutinin Isolectin B4* (VVA B4), *Lens culinaris agglutinin* (LCA, Pharmacia), *Ricinus communis agglutinin I* (RCA-I), *Griffonia simplifocia agglutinin II* (GSA II) and *Wheatgerm agglutinin* (WGA, Pharmacia). Bound proteins were eluted from this column series with a sugar solution comprising a mixture of 200mM methyl α-D mannopyranoside, 50mM α-L-fucose, 100mM N-acetyl-D-glucosamine, 20mM N-acetyl-D-galactosamine and 50mM D-galactose, and the columns were re-equilibrated with Affinity Buffer B. The unbound material, containing MonEst, was then re-passaged

and the process repeated until no further protein binding was seen. The unbound proteins were then passaged through a *Concanavalin-A* Sepharose column (Con-A) at a flow rate of 2.0ml/min. Bound proteins, including virtually all detectable MonEst, were eluted with Affinity Buffer B containing 200mM methyl α-D mannopyranoside. The MonEst-enriched eluate was then mixed with glycerol to a concentration of 20% (final approximate volume 40ml). The unbound material, representing 69% of the total protein passaged through Con-A, was discarded.

The final stage of MonEst purification was achieved by Superose-12 FPLC gel filtration chromatography; ten 500µl aliquots of the Con-A eluate were separately chromatographed at a flow rate of 0.35ml/min in 50mM phosphate buffer pH 7.0 containing 0.15M NaCl and 20% glycerol. Fractions were collected at one minute intervals and assayed for acetate and butyrate esterase activity by fluorimetry. Those fractions containing esterase activity, from all ten runs, were pooled and concentrated to approximately 0.7ml with Centricon 10 microconcentrators (Amicon) and glycerol added to 50% v/v prior to storage at -20°C.

4.2.1.2 Purification of common myeloid esterase (ComEst) isoenzymes: The starting material used for ComEst fractionation was obtained from the leucophoresis of a patient with acute myelomonocytic leukaemia (AMML-M4). Cells obtained from this patient, which were used for the preliminary evaluations of chromatographic procedures (Chapter 3) and were shown by IEF analysis to express both ComEst and MonEst esterase isoenzymes, were pelletted and stored at -20°C prior to processing. Individual 100 ml aliquots (total volume, 400ml) of the cell pellets were disrupted by ultrasonication (MSE Soniprep 150) at an amplitude of 18µ for one minute; Triton X-100 was added to give a final detergent concentration of 1% and, after mixing at 25°C for 10 mins and subsequent high speed centrifugation, the solubilised protein supernate was recovered from pelletted cell debris. The solubilised proteins were then dialysed overnight against 25mM tris-HCl buffer pH 8.4 containing 5% betaine (TBB), diluted to 400ml in the same and centrifuged

to remove protein aggregate. The supernatant was then filtered using 1.0µm nitrocellulose membranes (Millipore). This material was then passaged at a flow rate of 4.0ml/min through a 26.0 x 2.5cm Fast Flow Q-Sepharose anion exchange column which had been pre-equilibrated with TBB. Sample loading was monitored at 280nm and the unbound protein fraction, containing the main group of non-specific or 'common' myeloid (ComEst) esterase isoenzymes (pI range 7.3-7.9), was concentrated by ultrafiltration (Amicon) to 500ml. Proteins remaining bound to the gel were desorbed with 1M NaCl and an absence of additional ComEst species confirmed by IEF. The pH 8.4 Q-Sepharose unbound (ComEst-rich) proteins, from all four original 100ml aliquots, were pooled and extensively dialysed against 25mM morpholino-ethane sulphonic acid (MES) pH 6.2. This was then passaged as four individual 500ml aliquots (total protein, 2100mg) through a pre-equilibrated Fast Flow S-Sepharose cation exchange column at a flow rate of 4.0ml/min. Unbound proteins were collected, concentrated and discarded after confirming an absence of ComEst isoenzymes. Preliminary experiments had shown that esterase isoenzymes which bound to S-Sepharose could be eluted at relatively low NaCl concentrations. A single-step elution with 0.25M NaCl was therefore carried out and following re-attainment of the A280 baseline, the remaining bound proteins were eluted with 1.0M NaCl. Both eluates were concentrated, and IEF isoenzyme analyses undertaken to confirm that all the ComEst isoenzymes were confined to the 0.25M NaCl fraction. The 0.25M NaCl eluates from the four original 500ml aliquots were then pooled, concentrated to approximately 300ml and dialysed overnight at 4°C against Affinity Buffer A.

The dialysed material eluted from S-Sepharose was passaged through Benzamidine-Sepharose and Arginine-Sepharose columns connected in series at a flow rate of 2.0ml/min. Bound proteins were eluted with molar NaCl and discarded; unbound proteins were collected and re-passaged following column re-equilibration. After repeating this process until no further protein binding occurred, the unbound material was dialysed overnight at 4°C against Affinity Buffer B.

The unbound material from the Benzamidine/Arginine-Sepharose columns was then passaged at a flow rate of 1.0ml/min through a sequential series of lectin columns comprising UEA I, PNA, HPA, VVA B4, RCA-I, GSA-II, and WGA. The columns were re-equilibrated after eluting bound proteins with a sugar solution, as detailed above for MonEst purification, and the process repeated until no further binding was seen. The unbound material was then passaged through a pre-equilibrated Con-A column at a flow rate of 1.0ml/min. The bound material, containing all detectable ComEst isoenzyme activity, was desorbed with an eluent comprising 200mM methyl α-D mannopyranoside in Affinity Buffer B, and concentrated to 2.0ml. The unbound material was discarded after confirming by IEF an absence of ComEst isoenzymes.

As for MonEst, the final stage of ComEst purification was achieved by Superose-12 FPLC gel filtration (four 500µl aliquots) of the concentrated Con-A eluate at a flow rate of 0.35ml/min. One minute fractions were collected and esterase activities determined by fluorimetric assays using 4MB-Ac and 4MB-But as substrates. Fractions showing acetate esterase activity were pooled and concentrated to approximately 0.3ml with Centricon 10 microconcentrators. Glycerol was then added to a final concentration of 50% and this was stored at -20°C prior to further analysis.

4.2.2 Protein and enzyme activities of purified MonEst and ComEst:

Protein concentrations of chromatography fractions and purified esterase species were determined by the bicinchoninic acid method (Pierce Chemicals), and esterase enzyme assays were performed either by UV spectrophotometric (alpha-naphthyl acetate or butyrate) or fluorimetric (4-methylumbelliferyl acetate or butyrate; 4MB-Ac, 4MB-But) procedures as previously described (Sections 2.3.5.5 and 2.3.6.4). For the UV spectrophotometric method, one unit of acetate or butyrate esterase activity was defined as the amount of enzyme required to hydrolyse 1µmol of substrate in one minute at 30°C, where in a total assay volume of 1.0ml, the enzymatic hydrolysis of 1.0nmol of alpha-

naphthyl ester results in an increase in A_{235} of 0.023 and 0.024 absorbance units respectively for alpha-naphthyl acetate and butyrate [Mastropaolo & Yourno, 1981]. Purified, post gel filtration, MonEst was also examined with an extensive range of peptidase substrates by standard fluorimetric, spectrophotometric, radiometric or HPLC techniques (this work was carried out by Professor AJ Turner; Department of Biochemistry, University of Leeds).

4.2.3 Electrophoretic and chromatographic studies:

4.2.3.1 Electrophoretic analyses of MonEst and ComEst:

Introduction: Gel electrophoresis in polyacrylamide gels provide rapid and reproducible methods for studying the molecular characteristics of proteins. Although the detailed theory of gel electrophoresis is complicated, the fundamental concepts are easily understood. During electrophoretic separation, charged particles (proteins) migrate under the influence of an externally applied electric field toward the electrode of opposite polarity, with the degree of mobility (electrophoretic migration) being largely dependent on the charge density of the molecule being examined. Electrophoretic migration is however, also influenced by interactions between the charged protein and the surrounding gel matrix, which acts as a molecular sieve (gradient-PAGE) [reviewed in Garfin, 1990]. Consequently, proteins are separated during gel electrophoresis on the basis of their net charge and additionally their size and conformation. Many electrophoretic systems have been developed which variously involve the use of continuous or discontinuous buffer systems, but no attempt will be made to summarise these here. This brief introductory review will however summarise the essential features of the most important electrophoretic techniques in relation to this present study.

Of the various electrophoretic methods that are available, Native-PAGE is of particular value for the characterisation of biologically active molecules as it allows the molecule of interest to be demonstrated by its specific activity. Systems designed to separate proteins under non-denaturing conditions are unable to distinguish between the differential effects

of size, shape, and charge on electrophoretic migration. Consequently, proteins with differing molecular size may often be seen to have identical electrophoretic mobilities. Thus, while PAGE methods for native proteins are valuable for fractionating protein mixtures and for the visualisation of enzymes using specific enzyme stains, they are of little value in assessing purity of preparation or the molecular weight of unknown proteins.

In contrast, SDS-PAGE is frequently used for the estimation of molecular weights of unknown proteins. This technique additionally provides an excellent means of monitoring protein purification and is particularly useful for the assessment of protein homogeneity. SDS-PAGE overcomes the limitations of native-PAGE by imposing uniform charge characteristics on all proteins in a sample mixture. This anionic detergent (SDS) binds to most proteins at an approximate ratio of 1.4:1 and results in negatively charged protein-detergent complexes. Furthermore, the interaction with SDS disrupts non-covalent protein associations and generally causes proteins to assume uniform tertiary configurations. Concomitant treatment with disulphide-reducing agents, such as 2-mercaptoethanol or dithiothreitol (DTT), results in the denaturation of covalent bonds and consequently the proteins are reduced to their constituent subunits. Migration of SDS derivatives is toward the anode at rates inversely proportional to the logarithms of their molecular weights. This allows the molecular weight of an SDS-treated protein to be estimated from its relative mobility in a calibrated SDS-PAGE gel, and a single band in such a gel is often regarded as a useful indication of protein homogeneity. A major disadvantage of this technique is that SDS treatment generally results in the denaturation of native protein conformation and consequently, proteins often lose biological activity.

Gradient gels for Native- and SDS-PAGE enhance resolution, sharpen the protein bands and allow complex mixtures of proteins, with wide variations in molecular weights, to be separated on a single gel. Electrophoresis, using gradient gels, utilises the sieving properties of a gel which results from a three-dimensional network of fibres and pores

formed by the cross-linking of bisacrylamide with adjacent polyacrylamide chains. The pore size, as a rule, decreases with increasing acrylamide concentration and the effective pore size of the gel governs its sieving properties.

Native-PAGE analysis: Conventional polyacrylamide gel electrophoresis was carried out at pH 8.8 on 8-25% polyacrylamide gradient PHASTgels (molecular weight range for native proteins of 50 to 750 kDa), using a PHAST electrophoresis system (Pharmacia). One microlitre aliquots of the purified MonEst and ComEst esterase fractions were electrophoresed for 268 volt hours (Vhrs) at a constant current of 10mA and the gels were subsequently stained for ANAE activity. In addition, the effects of four different glycosidases on the relative distribution of MonEst and ComEst charged species was also examined. This was achieved by incubating the purified esterase forms, for 20hrs at 37°C, with neuraminidase (Sigma), alpha-mannosidase (Sigma), alpha-L-fucosidase (Boehringer) or endoglycosidase-H (Genzyme) at final supplier-recommended concentrations of 0.8mU/ml, 66U/ml, 4U/ml and 0.2U/ml respectively, prior to Native-PAGE analysis.

SDS-PAGE analysis: SDS-PAGE was carried out at pH 8.6 on 8-25% polyacrylamide gradient PHASTgels (molecular weight range for SDS-treated proteins of 6 to 300 kDa). 10µl aliquots of purified MonEst and ComEst were each incubated with equal volumes of 20mM tris-HCl pH 8.6 containing 1.0mM EDTA, 0.01% bromophenol blue and 2.0% SDS at 25°C for 5 mins. One microlitre of these SDS-treated samples were electrophoresed for 75 Vhrs at a constant current of 10mA, using the PHAST electrophoresis system, and the gels were subsequently silver stained for proteins or for alpha-naphthyl acetate esterase by standard azo-dye histochemistry. Purified MonEst was additionally analysed by reduced SDS-PAGE, following treatment with 5% 2-mercaptoethanol, 1% dithiothreitol (DTT), and also subsequent to endoglycosidase-H treatment (as described above for Native-PAGE).

Isoelectric focusing (IEF): The principles of IEF are fully described in Section 2.1.2. Methodologically, the IEF analysis was carried out by fractionation of purified ComEst and MonEst on 5% polyacrylamide gels containing 2.4% ampholine (Pharmacia/LKB) for 1500 Vhrs (LKB Multiphor 2103) as previously described (Section 2.2.6.1). Plates were stained for alpha-naphthyl acetate esterase (ANAE) activity following electrophoresis, by standard histochemical procedures (Section 2.2.3.4), and the isoelectric points (pI) of individual isoenzyme components determined by reference to the pH gradient measured using a surface pH electrode. The effect of neuraminidase treatment on the relative distribution of MonEst and ComEst charge species was also examined by IEF. In order to achieve this, 200µl of the purified esterase species were incubated for 20hrs at 37°C with 0.8mU/ml of *Vibrio cholera* neuraminidase (Sigma). Following this, the samples were re-analysed by IEF to determine whether or not neuraminidase treatment induced any changes in the observed pI of the individual esterase isoenzyme species.

4.2.3.2 Chromatographic analyses of MonEst and ComEst:

Gel filtration chromatography: The relative molecular weights (Mr) of purified MonEst and ComEst species were assessed with a 30 x 1.0cm Superose-12 high-resolution FPLC analytical gel filtration column. Chromatographic separation was achieved by the passage of 50µl aliquots of each species, at an optimised flow rate of 0.35ml/min, through the gel filtration column, pre-equilibrated with 50mM phosphate buffer pH 6.8 containing 0.15M NaCl and 20% glycerol. Apparent molecular weights of the esterase species were estimated by comparison with a standard curve constructed from the Ve/Vo (elution volume/column void volume) versus the log molecular weights of protein standards.

Further investigations of MonEst were also undertaken to determine the possible existence of subunits and, if present, to assess the nature (electrostatic or hydrophobic) of this subunit association. In order to achieve this, purified MonEst was pre-incubated (30 mins at 25°C) and chromatographed in the presence of four different reagents (5M urea,

4M guanidine hydrochloride, 1% Triton X-100 and 1% SDS), all of which promote subunit dissociation. Chromatography was carried out under identical column conditions, one minute (0.35ml) fractions were collected and esterase activities determined by fluorimetric assays using 4MB-Ac and 4MB-But as substrates.

Concanavalin A (Con-A) affinity chromatography: As described previously (Section 3.4), the lectin binding of MonEst isoenzymes was restricted to Con-A, in contrast to ComEst which additionally showed some affinity for LCA and PSA. Further clarification of the mechanisms of lectin binding was obtained by analysing the purified (post Con-A eluates; Section 4.3.1) MonEst and ComEst enzymes. This was carried out by dialysing 400µl of MonEst or ComEst (stored at -20°C in 20% glycerol) for three hours against lectin binding buffer (12mM succinic acid pH 6.9, containing 0.15M sodium chloride and 0.5mM each of calcium, magnesium and manganese chlorides). Following dialysis, 200µl aliquots of each esterase species were taken into glass vials with 25µl of endoglycosidase H (endo H, Genzyme; 400U/ml) or 25µl of buffer (control). These vials were then sealed and incubated at 37°C for 20hrs. Following incubation the samples were diluted to 1.5ml with the lectin binding buffer and in turn, the endo H treated and untreated (control) esterase samples were passaged through a 2ml pre-equilibrated Con-A column at 1.0ml/min. For each sample, the unbound fraction was collected and the bound protein eluted with 200mM methyl alpha-D mannopyranoside. The volumes of both unbound and bound fractions were measured and their acetate and/or butyrate esterase activities determined by fluorimetric assay (Section 2.3.6.4). Following this, the remaining samples were reduced in volume to 100µl with Centricon 10 microconcentrators (Amicon) and then analysed by IEF.

4.2.4 Kinetic studies:

4.2.4.1 Influence of pH on MonEst and ComEst activities: As part of the assay standardisation, preliminary analyses were undertaken to determine the relationships between pH and reaction rates. This was carried out by standardised UV

spectrophotometric assays (Section 2.3.5.5) in 10mM buffers at 0.5 pH unit intervals through the range 4.0-9.0 (lactic acid, pH 4.0 and 4.5; piperazine, pH 5.0; MES, pH 5.5-6.5; phosphate, pH 7.0 and 7.5; and tris, pH 8.0 and 9.0). The initial rates of hydrolysis of alpha-naphthyl acetate or butyrate, were determined at a constant substrate concentration of 0.3mM, over the pH range examined. Immediately prior to assay, the concentrations of purified MonEst and ComEst esterase preparations were adjusted to a level which ensured linear rates of enzyme hydrolysis with 0.3mM substrates over a two minute period; in practice, this was equivalent to an approximate maximum mean rate of change in absorbance at 235nm (A_{235}) of 0.40/min. 10µl of esterase fraction was then added to 1.0ml of pre-warmed (30°C) substrate solution in a semimicro quartz cuvette, the contents were mixed and the rate of increase in A_{235} measured at 12 second intervals over a period of 2.0 min (Pye Unicam 8800 UV/VIS spectrophotometer).

4.2.4.2 MonEst and ComEst enzyme kinetics: Relationships between rates of hydrolysis and substrate concentrations were determined for both the MonEst and ComEst myeloid isoenzyme species by standardised UV spectrophotometric assays (Section 2.3.5.5). The initial rates of hydrolysis of each ester of alpha-naphthol studied (acetate, propionate and butyrate; Sigma) were determined at various substrate concentrations within the range 0.0125mM to 0.60mM. These were prepared by diluting stock 25mM substrate solutions (in 2-methoxyethanol; Sigma) with 10mM MES pH 6.3. Immediately prior to assay, the concentrations of purified MonEst and ComEst were adjusted as described above (Section 4.2.4.1) and 10µl of each fraction was then added to 1.0ml of pre-warmed (30°C) substrate solution in a semimicro quartz cuvette; the contents were rapidly mixed and the rate of increase in A_{235} measured at 12 second intervals over a period of 2.0 min. The specific enzymatic turnover rates, at each substrate concentration studied, were corrected for the observed rates of change from spontaneous substrate hydrolysis (in the absence of enzyme). Lineweaver-Burk plots of 1/initial reaction velocity (1/v) against 1/substrate concentration (1/[s]) and Eadie-Hofstee plots of v against v/[s] were constructed and used

to estimate V_{max} and K_m values for the hydrolysis of acetate, propionate and butyrate substrates individually by both MonEst and ComEst species.

4.2.4.3 MonEst and ComEst inhibitor studies: The nature of enzyme-substrate interactions, and the possibility that ComEst and MonEst esterase species differed in their reaction mechanisms, was initially evaluated by incorporating a range of enzyme inhibitors in the standardised alpha-naphthyl acetate UV spectrophotometric assay (Section 2.3.5.5). 10µl aliquots of chromatographically purified ComEst and MonEst fractions (adjusted as described in Section 4.2.4.1) were pre-incubated for 5 mins at 30°C, in semimicro glass cuvettes, with 1.0ml of 10mM morpholino-ethane sulphonic acid (MES) pH 6.3 containing pre-defined concentrations of various inhibitors (detailed in Results Section 4.3.4.4). At this time, the enzyme reactions were initiated by the addition of (10µl) 50mM stock alpha-naphthyl acetate substrate (in 2-methoxyethanol), to give final working concentration of 0.5mM. After mixing, the rate of increase in absorption at 235nm (A_{235}) was measured at 12 second intervals over a period of 2.0 min. Enzyme reaction rates, both in the presence and absence of inhibitor, were corrected for spontaneous substrate hydrolysis and the results expressed as:

$$\frac{\text{Reaction rate (A235/min) in the presence of inhibitor}}{\text{Reaction rate (A235/min) in the absence of inhibitor}}$$

The type of inhibitory action (reversible or irreversible) exerted was further investigated by gel filtration and/or isoelectric focusing (IEF) for those compounds causing significant decrease in ComEst and/or MonEst activity. This involved pre-incubation of MonEst and ComEst with inhibitor followed by (a) passage through an FPLC Superose-12 gel filtration column (Pharmacia) and fluorimetric esterase assay of eluted components, or (b) IEF electrophoresis and subsequent histochemical (alpha-naphthyl acetate esterase) staining of gels. Reversible inhibition was indicated by regeneration of enzymatic activity following chromatographic or electrophoretic separation and irreversible inhibition by irrecoverable loss of enzymatic activity.

4.2.4.4 MonEst and ComEst inhibition kinetics: Sodium fluoride (NaF) is commonly incorporated in histochemical staining procedures for the delineation of monocytic esterases. However, the inhibition of monocyte-associated esterase (MonEst) by this compound appears to be, compared to ComEst, relative rather than specific [Drexler et al., 1991a; Chapter 2 of this study]. Additionally, although the inhibition of esterases by NaF is extensively documented, the mechanisms of esterase inactivation remain unknown. In order to further investigate this, the effect of NaF (as well as phenylmethylsulphonyl fluoride, PMSF; and diethyl pyrocarbonate, DEPC) on enzyme reaction rates was analysed; studies which were primarily designed to compare their inhibitory actions (competitive, non-competitive, uncompetitive, irreversible etc) on the ComEst and MonEst species. In practical terms, the inhibition constants (K_i) for both myeloid esterase species could be determined by measuring the effect of increasing the inhibitor concentration on substrate hydrolysis, at different substrate concentrations.

Experimentally this was achieved by pre-incubating 10µl of ComEst (or MonEst) fraction (adjusted as described in Section 4.2.4.1) with 1.0ml of 10mM MES pH 6.3 containing differing concentrations of NaF, PMSF or DEPC in a semimicro quartz cuvette for 5 mins at 30°C. At this time 4, 8, 12 or 16µl of stock 25mM alpha-naphthyl acetate solution (in 2-methoxyethanol) were added to the cuvette contents (final substrate concentrations, 0.1 to 0.4mM) and the rate of increase in absorption at 235nm (A_{235}) measured at 12 second intervals over a two minute period. At each inhibitor concentration studied, a Lineweaver-Burk plot of 1/initial reaction velocity (1/v) against 1/substrate concentration (1/[s]) was constructed and the maximum velocity in the presence of inhibitor (Vmax') estimated. Secondary plots of the reciprocal Vmax' values (1/Vmax'), at the different inhibitor concentrations examined, against inhibitor concentration were constructed for each inhibitor and the inhibition constant K_i determined. In this present study, the NaF inhibitor concentrations examined ranged from 0.0 to 1.0mM; PMSF concentrations (prepared immediately prior to assay from

stock PMSF solutions in 2-isopropanol) ranged from 0.0 to 0.2mM; and DEPC concentrations from 0.0 to 0.1mM.

4.2.5 N-terminal amino acid sequencing: A 100µl sample (at a concentration of 0.5mg/ml) of purified MonEst was diluted 1:2 with 0.2M 4-methylmorpholine/0.1% SDS and dried in 50µl aliquots onto a Sequelon-diisothiocyanate membrane disc at 56°C. The disc was washed in 1ml water, redried and the diisothiocyanate-coupled polypeptide then subjected to automated solid-phase Edman degradation on a MilliGen/Biosearch 6600 ProSequencer (this work was carried out by Dr J. Keen, Department of Biochemistry, University of Leeds).

4.3 RESULTS

4.3.1 Column performance characteristics:

4.3.1.1 MonEst fractionation: The first part of this fractionation involved the use of a Q-Sepharose anion exchanger at pH 8.4 and was designed to remove the maximum amount of non-esterase protein with the minimum loss of MonEst. As the use of NaCl gradients from 0.0 to 1.0M did not appear to offer significant advantages in terms of specific MonEst resolution, a series of pH step elutions were used. Protein estimations and IEF analyses of esterase isoenzyme components for the different eluates showed that the Q-Sepharose unbound, bis-tris (pH 6.3), piperazine (pH 5.5) and molar NaCl fractions represented 22.3%, 8.7%, 17.2% and 17.0% of the total applied (26125mg) protein respectively (Figure 4.1 and Table 4.2a); esterase isoenzyme (IEF) studies of these same fractions confirmed that all detectable MonEst was restricted to the piperazine eluate. Subsequent fractionation of this eluate by phenyl-Sepharose hydrophobic interaction chromatography showed that most of the MonEst activity was associated with the 0.2M/0.0M ammonium sulphate and 6M urea eluates and that together, these comprised 2.8% of the starting protein and 20.1% of the original acetate esterase activity. Subsequent processing of the pooled MonEst-rich phenyl-Sepharose eluates through the series of Benzamidine, Arginine and Blue-Sepharose columns similarly showed that

virtually all of the MonEst remained unbound, and passage of this through Protein-A and Protein-G columns removed a further 59% of protein (predominantly IgG). The following series of nine lectin columns removed an additional 25% of the remaining protein, with no significant loss of MonEst activity, and the Con-A column used to concentrate the MonEst component prior to gel filtration chromatography resulted in the removal of a further 69% of contaminating protein and 44% of acetate esterase activity that was not associated with MonEst as adjudged by IEF analysis. Prior to the final stage of FPLC gel filtration, the Con-A eluate containing high concentrations of MonEst represented 0.2% of the starting solubilised extract protein and 2.1% of the original acetate esterase activity. With this final chromatographic procedure, a single peak of esterase activity was identified which was collected and stored at -20°C in 50% glycerol prior to further analysis (elution characteristics of FPLC gel filtration are further detailed in Section 4.3.3.2).

4.3.1.2 ComEst fractionation: The starting material for ComEst fractionation consisted of an ultrasonicated detergent extract of leukaemic blasts from a patient with myelomonocytic leukaemia (AMML-M4), known to express high concentrations of both ComEst and MonEst enzyme forms. Following centrifugation and dialysis, the total amount of starting protein passaged (as four separate aliquots) through the Q-Sepharose anion exchanger at pH 8.4 was 8107mg. The unbound material from the pH 8.4 Q-Sepharose anion exchanger, which constituted 26% of the starting protein (Figure 4.2 and Table 4.2b) and contained majority of the extract ComEst, was pooled from all four original aliquots and extensively dialysed against 25mM morpholino-ethane sulphonic acid (MES) pH 6.2. A further 44% of the starting protein was desorbed from the anion exchanger with 1M NaCl and shown by IEF analysis to lack ComEst isoenzymes (but did contain the MonEst species), with the remaining tightly bound proteins being eluted with 2M NaOH (the observed concentration of ComEst isoenzymes obtained in the unbound fraction tended to exclude the possibility that this latter fraction contained significant amounts of this species). The dialysed unbound material was then passaged in MES

through a S-Sepharose cation exchange column at pH 6.2. Step elutions of bound proteins and IEF analysis showed that all detectable ComEst was associated with the 0.25M NaCl eluate whereas the 1.0M NaCl and unbound S-Sepharose fractions, representing 1.5% and 20% of starting protein respectively, had no ComEst activity and were discarded. Subsequent passage of this 0.25M NaCl eluate through Benzamidine/Arginine columns removed a further 8.4% of protein with little loss of acetate esterase activity. The protein concentration of the unbound material from the Benzamidine/Arginine columns was reduced by a further 52% by passage through a series of seven lectin columns, and the final pre-gel filtration binding of ComEst to Con-A reduced the level of contaminating proteins by a further 91%. Prior to the final stage of FPLC gel filtration, the Con-A eluate protein represented 0.11% of the original raw extract protein and 4.1% of the starting acetate esterase activity. With this final chromatographic procedure, a single peak of esterase activity was identified which was collected and stored at -20°C in 50% glycerol prior to further analysis (elution characteristics of FPLC gel filtration are further detailed in Section 4.3.3.2).

4.3.2 Protein and enzyme activities of purified MonEst and ComEst:

Further purification and characterisation of ComEst and MonEst species (eluted from Con-A Sepharose) by Superose-12 FPLC gel filtration revealed significant differences in their apparent molecular weights (Mr). The purified MonEst eluted from the gel filtration column as a single species with a higher Mr than the ComEst which also eluted as a single Mr form (the Mr values and further differences in molecular characteristics between these two species are examined in Section 4.3.3.2). With respect to the MonEst eluted from Con-A, the additional gel filtration step did not, because of its relative homogeneity, result in any further significant purification apart from the removal of a minor proportion of low Mr contaminants. However, the 9mg of Con-A enriched ComEst, when passaged through the Superose-12 gel filtration column, was further purified by the removal of approximately 85% of contaminating proteins. The protein concentrations of the final ComEst and MonEst fractions were 1.29mg/ml and 1.04mg/ml respectively, and UV

spectrophotometric esterase assays revealed distinct differences in substrate affinities between the two esterase forms. The ComEst species showed a marked preference (>40:1) for alpha-naphthyl acetate substrate hydrolysis (1.21U/ml, see Section 4.2.2 for enzyme Unit definitions), compared to alpha-naphthyl butyrate (0.03U/ml). In contrast, MonEst showed similar rates of butyrate (2.46U/ml) and acetate (2.74U/ml) hydrolysis although, at the substrate concentration (0.5mM) used in the assay, there is evidence that apparent butyrate esterase activities may be underestimates (Section 4.3.4.2), i.e. alpha-naphthyl butyrate appears to inhibit esterase activity at concentrations exceeding 0.4mM. Additional studies demonstrated that purified MonEst did not show peptidase activity and was incapable of hydrolysing a series of aminopeptidase, angiotensin converting enzyme, carboxypeptidase, dipeptidylpeptidase, endopeptidase, microsomal dipeptidase, or glutamyltransferase substrates (Table 4.3).

4.3.3 Electrophoretic and chromatographic studies

4.3.3.1 Electrophoretic analyses of MonEst and ComEst:

Native-PAGE electrophoresis: Native-PAGE analyses revealed four different charge forms for both MonEst and ComEst; MonEst comprised one major charge species and three additional minor species with greater anodic migration, and ComEst comprised four main individual charge species with a pattern of migration distinct from that of MonEst (Figures 4.3a & 4.3b). As FPLC gel filtration (Section 4.3.2) revealed only one Mr form for each species, it is concluded that under the electrophoretic conditions used in this particular study (0.88M L-alanine and 0.25M tris at pH of 8.8) that MonEst and ComEst components both migrated as charge species rather than Mr forms. Indeed, because of their relative pIs, it was predicted and confirmed that anodic migration of the MonEst species would be greater than the ComEst forms.

Additional studies, undertaken to assess whether the various charge isoforms of the MonEst resulted from differential post-translational glycosylation, revealed that the treatment of MonEst with α-mannosidase, α-L-fucosidase or endoglycosidase-H had no

effect on either the apparent enzymatic activity or the relative distribution of the individual charge isoforms (Figure 4.4). These studies were not possible for the ComEst because of presence of protein contaminants in this preparation and also due to limited sample availability.

SDS-PAGE electrophoresis: SDS-PAGE followed by silver (protein) staining, confirmed that MonEst had been purified to homogeneity and migrated as a single protein band with an apparent molecular weight of 63 kDa, under both reducing and non-reducing conditions (Figures 4.5a and 4.5b). Attempts to histochemical stain this band for alpha-naphthyl acetate/butyrate esterase failed. This observation indicates that SDS has a denaturing effect on MonEst activity, either because of the protein-SDS association or possibly as a result of subunit dissociation. Additional studies also showed that the treatment of purified MonEst with endoglycosidase-H resulted in its deglycosylation and the generation of a protein band with a lower Mr of 60.1 kDa (Figure 4.5b).

For comparison, silver staining of the purified ComEst species revealed a small number of contaminating proteins and, consequently, it was not possible to directly determine its molecular weight by this method. However in contrast to MonEst, SDS had no apparent effect on ComEst enzymatic activity and, because this myeloid esterase species also migrated in the presence of SDS as a single band (Figure 4.5a), it was possible to estimate its apparent molecular weight (68 kDa) following ANAE staining.

An additional finding of interest (discussed in Section 4.3.4.4) was that treatment of ComEst with SDS (i.e. generating SDS-ComEst complexes) could be reversed by gel filtration, as adjudged by the regeneration of the four active charge isoforms (Native-PAGE), whereas this could not be demonstrated for SDS-treated MonEst, which remained enzymatically inactive following gel filtration.

Isoelectric focusing (IEF): Analysis of ComEst and MonEst species by IEF demonstrated that the chromatographic purification procedures employed in this study had

successfully resolved the two enzyme groups and that the pI distribution of individual isoforms had not been affected by the fractionation techniques (Figure 4.6). Alpha-naphthyl acetate esterase (ANAE) histochemical staining of IEF plates revealed no significant cross-contamination of the two esterase groups, and MonEst was confirmed as representing a series of isoenzymes within a narrow pI range of 5.5-6.2, compared to the ComEst species which comprised four main isoenzymes within the pI range 7.3-7.9. Staining of IEF plates with alpha-naphthyl butyrate showed that MonEst, but not ComEst, hydrolysed this substrate, although its staining intensity was significantly less than that seen with alpha-naphthyl acetate. Further studies were also undertaken to ascertain whether the individual isoenzymes of ComEst and MonEst resulted from post-translational differences in glycosidic structures of these species. However, these experiments revealed no effect on the observed pIs of ComEst isoforms following neuraminidase treatment (Figure 4.7), or on the distribution of MonEst isoenzymes following α-mannosidase, α-L-fucosidase or endoglycosidase-H treatment (Figure 4.4).

4.3.3.2 Chromatographic analyses of MonEst and ComEst:

Gel filtration chromatography: The relative molecular weights (Mr) of the native MonEst and ComEst enzyme species were examined using an analytical gel filtration (Superose-12) column. Chromatographic separation was achieved by the repassage of 50µl aliquots of each purified species, at an optimised flow rate of 0.35ml/min, through the gel filtration column pre-equilibrated with 50mM phosphate buffer pH 6.8 containing 0.15M NaCl and 20% glycerol. Estimation of the Mr was achieved by comparison of elution volumes (Ve/Vo) of esterase species with a molecular weight curve obtained by chromatography of standard proteins under identical conditions. ComEst myeloid esterase isoforms eluted as a single molecular weight species with an approximate Mr of 53.2 kDa (Figure 4.8 a); fluorimetric assays further indicating that substrate hydrolysis of 4MB-Ac by ComEst esterases was considerably in excess of 4MB-But. For comparison, MonEst eluted with a higher Mr of 157.0 kDa (Figure 4.8 b) and hydrolysed 4MB-But at approximately 1.9 times the rate of 4MB-Ac. Further studies, undertaken to assess the

nature of subunit association of the MonEst enzyme, revealed that gel filtration in the presence of 5M urea and 1% Triton X-100 had no effect on the Ve of MonEst or on its enzymic activity, and that MonEst activity was completely absent when gel filtration was carried out in the presence of 4M guanidine hydrochloride (Figure 4.8 c) or 1% SDS (Section 4.3.4.4).

Concanavalin A (Con-A) affinity chromatography: As described previously (Chapter 3, Section 3.4), the lectin binding of monocyte-specific MonEst isoenzymes was restricted to Con-A whereas the ComEst species showed some affinity for LCA and PSA in addition to Con-A. The mechanisms of Con-A binding were investigated for purified MonEst and partially purified ComEst subsequent to endoglycosidase H (endo H) treatment. Fluorimetric (acetate and butyrate) esterase assays of untreated (control) MonEst and ComEst species showed that >90% of the total activity was bound to and eluted from Con-A. However, following endo H treatment, the proportion of total MonEst activity binding to Con-A was significantly reduced to 25% whereas, in marked contrast, the binding of ComEst to Con-A was not affected by endo H treatment. A more definitive and specific analysis for the presence of MonEst and ComEst in the endo H treated and untreated fractions was obtained by IEF which conclusively demonstrated (Figure 4.9) that the treatment of MonEst with endo H abolished its binding to Con-A, but had no influence on ComEst binding. These particular observations substantiate the earlier interpretation (Chapter 3, Section 3.4.3.4) of lectin-binding studies of unfractionated myeloid esterases, with respect to their glycan structures, and indicate the presence of N-glycosidically linked oligomannosidic-type glycan(s) for MonEst and a mixture of (80-90%) biantennary N-acetyllactosamine-type (complex-type) asparagine-linked glycan(s) and α-1,6-fucosylated biantennary N-acetyllactosamine-type glycan(s) for the ComEst species.

4.3.4 Kinetic studies:

4.3.4.1 Influence of pH on MonEst and ComEst activities: Preliminary studies to examine relationships between pH and substrate hydrolysis rates by purified ComEst and MonEst showed (Figure 4.10) that both esterase species hydrolysed alpha-naphthyl acetate with only marginal differences through a pH range of 5.5-7.5 and that the observed increases in reaction rates above this pH were insignificant. Hydrolysis of alpha-naphthyl butyrate by the MonEst species (ComEst caused no detectable hydrolysis of this substrate) showed marginal differences in the reaction rates within the pH range 6.0-7.0 but, because of a marked increase in spontaneous substrate breakdown above pH 7.0, further analysis at higher pH was not possible. The UV spectrophotometric esterase assay used in this study, which measures the generation of alpha-naphthol at 235nm, was originally standardised in morpholino-ethane sulphonic acid (MES) at pH 6.3. As the histochemical demonstration of ComEst and MonEst are also typically carried out at an acidic pH, and in order to standardise the pH for both acetate and butyrate substrates, a pH of 6.3 was used for all the kinetic analyses in this present study.

4.3.4.2 Enzyme kinetic studies: Initial IEF studies of unfractionated myeloid esterases, and subsequent histochemical staining with alpha-naphthyl acetate, propionate or butyrate (Chapter 2, Section 2.3.4.6) indicated that the ComEst species were primarily acetate specific, in contrast to MonEst isoenzymes which could hydrolyse all three substrates, albeit with apparent decreasing staining intensities with increasing acyl chain length (Figure 4.11). In order to further substantiate these observations, the hydrolysis of acetate, propionate and butyrate esters of alpha-naphthol was determined by quantitative UV spectrophotometric assays, for both purified MonEst and ComEst myeloid isoenzyme species. The initial reaction rates were determined for all three substrates at differing concentrations (Figure 4.12) and V_{max} and K_m values were calculated by constructing Lineweaver-Burk (1/v against 1/[s]) and Eadie-Hofstee (v against v/[s]) plots. These analyses clearly demonstrate that the ComEst species preferentially hydrolysed alpha-naphthyl acetate, showed minimal hydrolysis of the propionate ester and no significant

hydrolysis of the butyrate ester (Figures 4.12 and 4.13). In contrast, the kinetic results for the MonEst isoenzyme species differed considerably in that esters of higher acyl chain lengths (butyrate > propionate > acetate) were preferentially hydrolysed (Figures 4.12 and 4.14). An additional observation of some relevance to histochemical practice was that the kinetic characterisation of MonEst provides some evidence that the butyrate substrate caused reaction inhibition at concentrations exceeding 0.4mM (Figure 4.15).

Examination of the V_{max} and K_m values for each group of esterase isoenzymes (Table 4.4) suggest that although ComEst have similar affinities for acetate and propionate substrates, the turnover rate for the propionate substrate is considerably lower. In addition, as the turnover rate of ComEst for alpha-naphthyl butyrate was below the limit of accurate detection, these results are completely in accord with the preliminary observations for histochemical staining of esterases fractionated by IEF (Section 2.3.4.6). For comparison, examination of the data for the MonEst species revealed decreasing K_m values with increasing acyl chain length, suggesting a considerably higher binding affinity of these isoenzymes for the butyrate substrate. Furthermore, the V_{max} values for the hydrolysis of these substrate esters by MonEst indicated an increasing rate of catalysis with increasing acyl chain length. These observations therefore indicate that (a) the binding site of MonEst enzyme recognises butyrate and propionate acyl groups more readily than the acetate group, and (b) that the catalytic mechanism of MonEst hydrolyses the butyrate substrate with greater efficiency than propionate and acetate esters.

4.3.4.3 Enzyme kinetics of deglycosylated MonEst: Studies to determine the effect of endo H treatment on the hydrolysis of alpha-naphthyl acetate (ANA) by MonEst, showed (Figure 4.16) that the deglycosylated form of this esterase species had a higher affinity for ANA (K_m = 0.08mM) than the untreated control (K_m = 0.16mM) and that the substrate turnover rates did not appear to be significantly different (V_{max} deglycosylated, $0.17A_{235}$/min; control, $0.22A_{235}$/min). These findings suggest that the glycan structure

of MonEst may be closely associated, in the native tertiary/quaternary conformation, with the substrate binding site. However, it is noted that this observation may only be validated by detailed conformational studies, which were not undertaken in this current study.

4.3.4.4 Enzyme inhibitor studies: This study additionally examined the effects of a wide range of enzyme inhibitors on the purified ComEst and MonEst esterases. To allow a direct comparison of the effects of the various inhibitors on these two esterase species, the substrate used throughout these analyses was alpha-naphthyl acetate. The inhibitors examined, their concentrations and effects are summarised in Table 4.5 and, based upon arbitrary definitions of significant and partial inhibition as <50% and 50-80% of control (no inhibitor) activity respectively, only two compounds (1% SDS; and 1.0mM diethyl pyrocarbonate; DEPC) exerted significant inhibitory effects on both ComEst and MonEst. Phenylmethylsulphonyl fluoride (PMSF, 0.2mM), 3-4, dichloroisocoumarin (DCIC, 5.0 x 10^{-3}mM), and N-tosyl-L-phenylalanine chloromethyl ketone (TPCK, 0.1mM) inhibited MonEst but not ComEst; sodium fluoride (NaF, 1.0mM) was shown to significantly inhibit MonEst while partially inhibiting ComEst; and iodoacetamide (2.0mM) induced partial inhibition of ComEst but not MonEst. All the other inhibitors examined failed to cause any significant inhibitory effects on either of the esterase isoenzyme species.

Chromatographic and electrophoretic (IEF) studies, which were undertaken to investigate the mechanisms of inhibition, revealed that higher concentrations of PMSF (5.0mM) and DCIC (0.05mM) irreversibly inhibited MonEst isoenzyme activity whilst showing no significant inhibition of ComEst forms (Table 4.6). For comparison, 20mM NaF inhibited both esterase species in a reversible manner, in that ComEst and MonEst activities were restored following electrophoretic or chromatographic separation. A further finding of note was that 1% SDS, which inhibited both ComEst and MonEst isoenzyme activities in the UV spectrophotometric assay, was shown to inhibit ComEst in a reversible fashion whereas, in distinct contrast, SDS treatment of MonEst isoenzymes

resulted in irreversible inhibition. Finally, DEPC was shown to reversibly inhibit both ComEst and MonEst species, compared to TPCK which inhibited MonEst irreversibly with little significant effect on ComEst activity.

4.3.4.5 Inhibition kinetics: A series of kinetic studies were undertaken in order to further characterise the mechanisms involved in the inhibition of ComEst and MonEst isoenzyme species by NaF, PMSF and DEPC. The type of inhibition (competitive, uncompetitive, noncompetitive, or mixed) exerted by these three compounds was assessed by investigating the effects of varying the inhibitor concentration on K_m and/or V_{max} values for substrate hydrolysis, and their inhibition constants were determined by constructing secondary plots of altered V_{max} values against the inhibitor concentration.

Lineweaver-Burk plots of 1/v against 1/[s] for the inhibition of ComEst and MonEst isoenzymes by NaF clearly showed that both esterase species were inhibited in a noncompetitive manner (Figures 4.17 and 4.18). Furthermore, the K_i values calculated for NaF inhibition of purified myeloid esterases, indicated that the MonEst isoenzymes were approximately 130 times more sensitive to NaF inhibition than the ComEst (Table 4.7). Similarly, the reversible inhibition of esterase species by DEPC was shown to be noncompetitive for ComEst and MonEst (Figures 4.19 and 4.20) although, in contrast to NaF, the K_i values for both species were found to be similar (Table 4.7). For comparison, PMSF which was shown by electrophoretic analyses to irreversibly inhibit MonEst isoenzymes at high concentration (5.0mM), appeared to show 'mixed competitive-noncompetitive' inhibition at lower concentrations (Figure 4.21). Determination of the inhibition constants for the mixed competitive/noncompetitive revealed that the value of competitive inhibition constant (K_I = 0.11mM) was identical to the observed affinity (K_m) of MonEst species for alpha-naphthyl propionate (Table 4.4) and that the K_i (noncompetitive inhibition) value of 0.01mM was identical to that found for NaF inhibition of MonEst (Table 4.7). Consequently, it is concluded that the binding site of MonEst 'recognises' alpha-naphthyl propionate and PMSF with similar affinities

and that the reversible noncompetitive inhibition, which is apparent at lower PMSF concentrations, is likely to result from interactions between the MonEst active site and the fluoride ion of PMSF.

4.3.5 N-terminal amino acid sequencing:

The N-terminal amino acid sequence of purified MonEst is shown in Table 4.8. Approximately one half of the protein was found to start with histidine (residue 2 in sequence), giving rise to an overlapping sequence of residues. This did not however present any difficulties in elucidating the sequence, and this heterogeneity would have had no effect on pI or other characteristics employed in the purification of the protein in this study. Although it is not possible to comment on whether the missing glycine residue is an artefact of isolation, or due to variable processing of the signal sequence, but it is interesting to note that rat and rabbit liver esterases also start at the histidine residue.

The average repetitive sequencing yield was 93% and the theoretical initial sequencing yield was calculated to be approximately 150pmol. This value for the amount of protein sequencing at the start of the run confirmed that the major component of the sample was being sequenced rather than a minor contaminant. The amount of protein used for attachment to the sequencing disc (50µl, 800pmol) would more than saturate the binding capacity of the membrane (200-300pmol), thus the 150pmol sequencing represents about one half of the material attached to the disc, a figure normally encountered in sequence analysis.

The observed amino acid sequence shows a high degree of sequence identity to the recently described human alveolar macrophage esterase [Munger et al., 1991] and differs only in a Val --- Thr substitution at position 12. It is of some interest that rat liver esterase shows the same substitution whereas rabbit liver microsomal esterase shows the same Thr as human MonEst. Furthermore, when compared to the two non-human esterase N-terminal sequences, human MonEst and alveolar macrophage esterase both

show His substituting for Lys at position 13; and Ile instead of Val at positions 32 and 36.

4.4 DISCUSSION

The diversity of esterases from various tissues and the lack of specific differentiating characteristics has led to considerable confusion regarding their biochemical classification. Categorisation of enzymes with esterolytic activity have traditionally been based, in the absence of knowledge of physiological substrate, on criteria such as substrate specificities and inhibitor sensitivities. Histochemically and cytochemically important esterases, belonging to the carboxylic ester hydrolase (EC 3.1.1) group, include carboxylesterases (EC 3.1.1.1), arylesterases (EC 3.1.1.2), acetylesterases (EC 3.1.1.6), acetylcholinesterases (EC 3.1.1.7) and cholinesterases (EC 3.1.1.8). Carboxylesterases represent esterolytic enzymes which hydrolyse carboxylic esters and are inhibited by organophosphates (e.g. di-isopropyl fluorophosphate; DFP) but not eserine [Holmes & Masters, 1967; Krisch, 1971]. Arylesterases are defined on the basis of their primary reactivity with aromatic esters, their relative sensitivity to sulphydryl reagents (e.g. p-hydroxymercuribenzoic acid; HMBA), and their resistance to organophosphates [Holmes & Masters, 1967]. Acetylesterases show restricted reactivity with esters of acetic acid and are not inhibited by organophosphates, sulphydryl reagents or eserine [Holmes & Masters, 1967]. Finally, acetylcholinesterases and cholinesterases hydrolyse esters of acetylcholine and choline respectively and are inhibited by organophosphates, neostigmine and/or eserine [Holmes & Masters, 1967].

Leucocyte-associated carboxylesterases in their strictest sense catalyse the hydrolysis of uncharged carboxylic esters [Krisch, 1971] but may also act upon esters of fatty acids or glycerol [Nachlas & Seligmann, 1949], a property usually associated with lipases. Despite these overlaps in substrate specificity, carboxylesterases may be regarded in general terms as enzymes which act on esters of short-chain glyceryl and simple monoesters (acyl chain-lengths of C2-C4) whereas lipases act on longer chain (fatty acid)

esters (C8 or more) [Khoo & Steinberg, 1975]. The most widely used substrates for the cytochemical demonstration of leucocyte esterases are alpha-naphthyl acetate, propionate and butyrate which show progressive increases in acyl chain length of C2, ($CO.CH_3$), C3, ($CO.C_2H_5$) and C4, ($CO.C_3H_7$) respectively.

Normal and leukaemic myeloid cells express two main groups of alpha-naphthyl acetate esterase (ANAE) isoenzymes, as defined by isoelectric focusing (IEF). The first of these (ComEst), expressed by cells of both granulocytic and monocytic lineage, comprise a series of isoenzymes with isoelectric points ranging from 6.3 to 7.9. In contrast, expression of the second myeloid esterase group, which comprises a series of isoenzymes (MonEst) within a narrow pI range of 5.5-6.2, is restricted to cells of monocytic lineage.

It is crucial to the study of any enzyme that molecular and biochemical analyses are undertaken in the absence of contaminating proteins. This is particularly true when contaminants have molecular and/or biochemical characteristics that are similar to those of the enzyme of interest. Preliminary chromatographic studies (Chapter 3) indicated that there were many proteins in the starting cellular extracts which displayed esterolytic activity and showed similarities in molecular features (such as charge, hydrophobicity, lectin affinity and relative molecular weight) to the MonEst and ComEst myeloid esterase species. Also, because the MonEst starting material contained contaminating serum protein, in addition to extracted cellular proteins (discussed in Section 4.2.1.1), an extensive range of chromatographic procedures were utilised for the final purification protocols. Whilst these chromatographic procedures are far more complex than the relatively simple fractionation steps previously described (detailed later in this Discussion) for the purification of monocyte-associated esterases [Lam et al., 1978; Yourno, 1986; Saboori & Newcombe, 1990], the nature of the starting material necessitated their use. Indeed, despite the extremely complex nature of the raw materials, the success of the purification schemes was evidenced by the isolation of approximately 40mg of

homogeneous MonEst and 1.3mg of enriched ComEst. Isoelectric focusing of the purified MonEst and partially purified ComEst revealed that the isoenzyme distributions and pI values were identical to those of the unprocessed serum/cell extracts, thus confirming that modifications in the charge characteristics of these two myeloid esterase species had not occurred as a result of the purification procedures. Furthermore, in terms of esterolytic activity and accepting the difficulty of estimating non-specific losses, the results of this current study also suggested that when related to the original total esterase activities of the serum/cell extract used for the purification of MonEst, only 2.1% of the starting acetate esterase and 2.2% of the butyrate esterase activities corresponded to the recovered purified MonEst enzyme. For comparison, the resulting partially-purified ComEst fraction constituted 4.1% of the total original cell extract acetate esterase activity. While there is little doubt that the extensive purification schemes used in this study led to a non-specific loss of the enzymes of interest, the results nevertheless indicate that a high proportion of the esterolytic activity present in the starting materials was not of ComEst or MonEst type. This conclusion is entirely consistent with the observations detailed in Chapter 3.

Early comparisons of carboxylesterases (EC 3.1.1.1) from diverse sources revealed a number of common structural and chemical features [reviewed in Heymann, 1980]. Many of these esterases were reported to be trimeric proteins in their native state, possessing three identical subunits each capable of enzymatic activity. Molecular weights in the region of 162-168 kDa were recorded for mammalian (including human) liver carboxylesterases [reviewed in Krisch, 1971], with some evidence of reversible dissociation to active subunits in presence of dilute acid or salt concentrations exceeding 0.5 molar [Barker & Jencks, 1969]. More recent reports [Heymann et al., 1971; Harano et al., 1988; Ozols, 1987; 1989] however, suggest that the true molecular weight of hepatic carboxylesterases is considerably lower (50-60 kDa) and that an apparent higher Mr species results from *in vitro* generation of polymeric enzyme forms during extraction and purification. Although these observations may provide broad insights into the

general nature of esterases, it is nevertheless important not to assume that these characteristics are a common feature of all esterases, including the ComEst and MonEst species which are the subject of this study.

The results of this current study have considerably clarified the molecular weight characteristics of myeloid esterase enzyme forms. The purified MonEst enzyme eluted from an analytical gel filtration column as a single enzymatically active peak with an approximate Mr of 157 kDa. When examined by SDS-PAGE (reduced and non-reduced) this esterase species migrated as a single enzymatically inactive protein band with an apparent molecular weight of 63 kDa. For comparison, partially purified ComEst eluted from an analytical gel filtration column as a single lower (53 kDa) Mr form, which migrated as a single enzymatically active band, with a Mr of 68 kDa, following SDS-PAGE. These findings therefore suggest that the enzymatic activity of MonEst enzyme is predominantly associated with a trimeric protein and that, contrary to previous reports [Lam et al., 1978; Yourno, 1986; Yourno et al., 1986; Cohn et al., 1987; Saboori & Newcombe, 1990], no significant enzymatic activity is associated with its monomeric constituents. In contrast to MonEst, the ComEst species showed no apparent subunit association as adjudged by reduced and non-reduced SDS-PAGE.

With regards to subunit association of the MonEst enzyme, repeated gel filtration of the 157 kDa species following treatment with 5M urea or 4M guanidine hydrochloride (reagents which cause dissociation of polymeric proteins) revealed that, whilst degradation of enzymatic activity was observed with guanidine hydrochloride, neither compound induced the generation of active monomeric enzyme forms. Furthermore, because native MonEst could be fully dissociated to its monomeric constituents by an anionic (SDS) rather than nonionic detergents (Triton X-100 and CHAPS), it was considered that MonEst subunit associations are ionic rather than hydrophobic in nature, a view shared by Yourno [1986]. The possibility of covalent (disulphide) associations were ruled out because the trimeric protein was fully dissociated by SDS under non-

reducing conditions and showed no further alteration in monomer Mr following reduction with β-mercaptoethanol or dithiothreitol (DTT). Furthermore, and in distinct contrast to MonEst, the inhibition of ComEst activity by SDS could be reversed following its removal by gel filtration. These findings are particularly relevant because they clearly indicate fundamental differences between the molecular characteristics of the two enzyme forms. In this respect, it is suggested that SDS binding to MonEst results in the disruption of its quaternary structure (i.e. dissociation of subunits) which is essential for enzymatic activity. By comparison, it is postulated that the association of SDS with ComEst protein disrupts the tertiary structure, inducing a change in the active site and resulting in a loss of enzymatic activity in the presence of SDS however, because quaternary structure in not involved, SDS inactivation may be reversed by its removal (i.e. ComEst protein is able to re-establish its tertiary conformation following SDS removal).

As little is known of the carbohydrate side-chain expression by the MonEst and ComEst species, this study also analysed their binding characteristics to a range of immobilised lectins. Preliminary studies were undertaken in Chapter 3 (Section 3.4) using an unprocessed solubilised cell extract from a case of myeloid leukaemia, which was known to express high concentrations of both esterase species. These investigations showed that all detectable MonEst bound to *Concanavalin A* (Con-A) and that this esterase species had no apparent affinity for any of the other lectins examined. In contrast, the ComEst species showed partial affinity for *Pisum sativum agglutinin* (PSA) and *Lens culinaris agglutinin* (LCA) in addition to strong binding to Con-A. Carbohydrate structures of glycoproteins or proteoglycans are formed by the glycosidic linkage to an amino acid side-chain which may be N-glycosidic (through the amide nitrogen of an asparagine residue) or O-glycosidic (through the hydroxyl group of serine, threonine, hydroxylysine or hydroxyproline). When interpreted in the context of the differential sugar specificities of these lectins, these observations indicate significant differences in the carbohydrate structure of these two myeloid esterase types. Thus, the pattern of lectin binding for

MonEst is compatible with the presence of oligomannosidic-type glycan(s), N-glycosidically linked to the protein, whilst ComEst appears to contain approximately 80-90% biantennary N-acetyllactosamine-type (complex-type) asparagine-linked glycan(s) together with an α-1,6-fucosylated biantennary N-acetyllactosamine-type glycan(s). Further confirmation of the interpretation for the MonEst species was obtained in this part of the study by treatment of purified enzyme with endo H, which acts on oligosaccharide units of the high-mannose or hybrid type, which completely abrogated its binding to Con-A. In addition, deglycosylation of MonEst with endoglycosidase H (endo H) increased its affinity for alpha-naphthyl acetate (discussed in detail below) but had little effect on either the rate of substrate turnover or the isoelectric points (pI) of the various molecular isoforms which constitute this enzyme species. This latter observation is important in that it not only excludes the possibility that the carbohydrate components of this enzyme contribute to the pI, but confirms previous studies [Scott et al., 1984c] that treatment of MonEst with neuraminidase also has no discernible effect on pI (i.e. consistent with the lack of terminal N-acetyl neuraminic acid groups). In distinct contrast, endo H treatment of partially purified ComEst did not affect its binding to Con-A, further supporting the interpretation for the presence of biantennary N-acetyllactosamine-type glycan(s). One difficulty with this interpretation however is that attempts to increase the binding of ComEst to an immobilised *Ricinus communis agglutinin I* (RCA I) column, or to modify the pI of individual ComEst components [Scott et al., 1984c], by *clostridium perfringens* neuraminidase treatment failed. RCA I column specificity was checked and confirmed with neuraminidase-treated and untreated purified human serotransferrin.

In this context, it is also important to note that glycoproteins which bind to any given lectin share only that property; a common factor which itself does not necessarily imply the identical nature of their oligosaccharide structures. Furthermore, although it has been suggested that molecular variants of individual glycoproteins display glycan uniformity, there is some evidence that this may not always be the case [Lis & Sharon, 1986]. Such interpretations of lectin binding studies may also be limited by oligosaccharide side-chain

modifications induced by contaminating glycosidases in complex (unfractionated) biological mixtures and the occasional cross-reactivity between lectins and the amino acid constituents of a protein. However, providing these factors are taken into account, it is nevertheless possible to ascertain the basic oligosaccharide structure for any glycoprotein based on its lectin binding profile; analyses that are particularly relevant to the functional analysis of cloned esterase proteins where variations in glycosylation may have a considerable influence on biological properties.

Having excluded a possible contribution of the glycan structures to the variable pIs of the different MonEst and ComEst isoforms, it is concluded that these are likely to result from either other post-translational modifications, such as deamidation (glutamine and asparagine), sulphydryl oxidation or acetylation of the terminal amino groups of lysine or arginine, or may originate at the genetic level (i.e. true isoenzymes) through differences in constituent amino acid sequences. Primary modifications of this nature could adequately explain charge differences in that substitution of basic amino acid residues with acidic residues could lead to differences in charge in the absence of alteration in Mr, and could also explain the apparent ionic association of the MonEst monomeric subunits to form the active trimeric protein.

Examination of charge species by pH 8.8 Native-PAGE electrophoresis in this study revealed that both MonEst and ComEst each comprised four different Native-PAGE isoforms. MonEst consisted of one major species, with three minor charge components, while the four ComEst Native-PAGE isoforms appeared to directly correspond to the four pI forms resolved by IEF. For comparison, two previous independent investigations reported the existence of nine different haemopoietic cell (myeloid, lymphoid and platelet) esterase isoforms as defined by Native-PAGE at pH 4.0 [Li et al., 1973], and 12 different isoforms at pH 9.5 [Yourno & Mastropaolo, 1981]. At pH 4.0, two main esterase components (designated isoforms 4 and 5) were found to be associated with both monocytes and platelets. PAGE isoform 5 was seen to preferentially hydrolyse alpha-

naphthyl acetate (ANA) whereas PAGE isoform 4 showed a high affinity for alpha-naphthyl butyrate (ANB); the first of these (isoform 5) was however not considered lineage-specific, due to its high concentration in platelets. In contrast, normal and leukaemic granulocytes only expressed small amounts of PAGE isoforms 5 and 6. The second study of PAGE isoforms at pH 9.5 described two granulocytic esterase components, designated A1 and A2, which preferentially hydrolysed acetate esters and were relatively fluoride-resistant. For comparison, the monocytic esterase components (designated AB1 and AB2) hydrolysed both acetate and butyrate esters and were fluoride-sensitive. Comparison of these findings with those of this current study indicate that PAGE isoforms 5/6, A1 and A2 correspond to the ComEst species whereas isoforms 4, AB1 and AB2 almost certainly represent the MonEst species.

As it was not possible to fractionate sufficient amounts of each ComEst/MonEst isoform, many of which differed only by a minor charge or pI differences, the kinetic analyses were carried out, in this current study, under the assumption that individual isoforms of each species would not show major variations in substrate affinities or inhibitor characteristics. This assumption was substantiated to some extent by the observed linear reaction kinetics for both ComEst and MonEst, which allowed the calculation of K_m and K_i values and would not have been possible had the individual isoforms shown significant differences in substrate and inhibitor reactivities.

The enzymatic catalysis of a given single substrate reaction, generally speaking, takes place via a two stage process: *(a) Enzyme + Substrate <=> Enzyme/substrate complex; and (b) Enzyme/substrate complex <=> Enzyme + Product(s).* Under equilibrium conditions, the Michaelis-Menten constant (K_m) derived from the Michaelis-Menten equation: $v = V_{max}[s]/[s] + K_m$, (where v = initial reaction velocity, [s] = initial substrate concentration and V_{max} = maximal initial velocity at the enzyme concentration being examined) represents the enzyme/substrate dissociation constant and can therefore be interpreted as being a reciprocal measure of the affinity of the enzyme for its substrate.

Conversely, the V_{max} value is a direct measure of the rate of breakdown of the enzyme/substrate complex and for single substrate reactions, indicates the capacity of the enzyme to catalyse the hydrolysis of its substrate. Consequently, the lower the K_m value the greater the affinity of the enzyme for its substrate, and the higher the V_{max} value the higher the capacity of the enzyme to catalyse the hydrolysis of that substrate. Numerous methods have been described for the calculation of K_m and V_{max} values although the most frequently reported interpretations are those derived from Lineweaver-Burk or Eadie-Hofstee plots [Dixon & Webb, 1979a; Palmer, 1985]. As the Lineweaver-Burk method has been criticised for placing greater emphasis on enzymatic activity at low substrate concentrations, this current study calculated K_m and V_{max} values by both the Lineweaver-Burk and Eadie-Hofstee linear regression methods. Computerised data processing, based on the 'least-squares' approach to curve fitting, have also been used more recently for the determination of K_m and V_{max} values, although values calculated by such procedures are not entirely reliable because they fail to discriminate between true linear graphs and those which deviate slightly from linearity [Palmer, 1985].

As the pH can significantly influence substrate hydrolysis by any given enzyme, preliminary studies were carried out to determine the reaction rates of both myeloid esterase species over a wide pH range. These results showed that the assay of MonEst species for butyrate esterase activity was not possible above pH 7.0, because of high rates of spontaneous substrate hydrolysis, and that in the pH range 6.0-7.0 there appeared to be little significant difference in the reaction rate. Although acetate esterase activities could be measured at a higher pH, the reaction rates were generally similar throughout the pH range of 6.0-9.0. The UV spectrophotometric esterase assay used in this study, which measures the generation of alpha-naphthol at 235nm, was originally standardised in morpholino-ethane sulphonic acid (MES) at pH 6.3. As the histochemical demonstration of ComEst and MonEst are also typically carried out at an acidic pH, and in order to standardise the pH for both acetate and butyrate substrates, a pH of 6.3 was used for all the kinetic analyses in this present study.

It was evident from the kinetic studies, which examined the ability of purified myeloid esterases to hydrolyse alpha-naphthol esters of increasing acyl chain length, that ComEst and MonEst showed distinctly different substrate reactivities. The K_m and V_{max} values conclusively showed that the ComEst species had higher affinities and turnover rates for the shorter acyl chain esters (acetate > propionate), with negligible turnover of the butyrate ester. Furthermore, although the ComEst species had similar binding affinities for both the acetate and propionate substrates, the turnover rate for the acetate substrate was four-fold higher than that of propionate. These quantitative findings confirm earlier qualitative analyses obtained by IEF zymogram studies (Section 2.3.4.6), and strongly suggest that the ComEst species should be biochemically classified as an acetylesterase (EC 3.1.1.6) rather than a carboxylesterase (3.1.1.1). In marked contrast, the MonEst species showed considerably higher affinities and turnover rates for the longer acyl chain esters (butyrate > propionate > acetate) even though preliminary qualitative studies (Section 2.3.4.6) also showed that the staining intensity of MonEst with this substrate was considerably weaker than with ANA. Additional studies, undertaken to clarify this discrepancy, revealed that MonEst was inhibited by ANB concentrations exceeding 0.4mM and, as the ANB concentration routinely used for histochemical techniques often exceeds 5 mM, it was concluded that the apparent lower reactivity with ANB in the two-stage histochemical procedure used for staining IEF gels was due to reaction inhibition by high substrate concentrations.

Inhibition reactivities and substrate specificities are particularly informative in discerning the mechanism of enzyme action and ultimately the physiological role of the enzyme. Inhibitors in biological systems serve as control mechanisms and many xenobiotic drugs and toxic agents are effective through their ability to act as enzyme inhibitors. Enzyme inhibition can either be a reversible or irreversible process; reversible inhibition results from a loose, usually electrostatic, association between an inhibitor and enzyme whereas with irreversible inhibition, the inhibitor usually becomes covalently linked to the

enzyme. In this study, purified MonEst and ComEst preparations were extensively investigated to determine their inhibitor sensitivities with respect to types of inhibition (competitive, uncompetitive, non-competitive, reversible or irreversible). For this, representative carboxyl ester hydrolase (EC 3.1.1) and protease inhibitors (Table 4.5) were used, firstly to elucidate differences between the MonEst and ComEst enzymes, and secondly to provide further insights into the nature of their active sites. Of the 17 inhibitors examined, only two (sodium dodecyl sulphate, SDS; and diethyl pyrocarbonate, DEPC) showed significant (>50%) inhibition of the ComEst species, and a further two (sodium fluoride, NaF; and iodoacetamide) exerted partial (20-50%) inhibition. For comparison, a total of six compounds (NaF; phenylmethylsulphonyl fluoride, PMSF; 3-4 dichloroisocoumarin, DCIC; N-tosyl-L-phenylalanine chloromethyl ketone, TPCK; DEPC and SDS) caused significant (>50%) inhibition of the monocyte-restricted MonEst species. NaF, SDS and DEPC were therefore the only compounds to show common inhibition of both esterase species.

NaF is traditionally incorporated in cytochemical procedures to differentiate monocyte-associated esterases from other myeloid esterases [Wachstein & Wolf, 1958; Braunstein, 1959; Yam et al., 1971; Li et al., 1973; Dulac & Yang, 1991]. More recent analysis of esterase isoenzymes [Scott et al., 1984c; Drexler et al., 1991a] have indicated that the inhibition of MonEst species by NaF is a relative rather than specific phenomenon, in that the ComEst species is also inhibited at higher NaF concentrations (exceeding 1.0mg/ml). Although much work has been carried out to assess the inhibition of esterases by NaF, the mechanisms of its inhibitory action are unknown [Drexler et al., 1991a]. This current study reveals for the first time that the NaF inhibition of both MonEst and ComEst species is reversible and further shows that this inhibition is noncompetitive for both enzymes. The most commonly observed mode of NaF inhibition is through fluoride ion binding to essential metal ions (e.g. Ca^{2+}, Mg^{2+}, Mn^{2+} etc.) although examination of the effects of metal chelating agents (described below) suggest that neither myeloid esterase contain metal prosthetic groups and are therefore unlikely to be inhibited by this means.

Furthermore, as NaCl does not exert any inhibitory effects on either MonEst or ComEst, inhibition of enzymatic activity must result through a specific fluoride ion interaction rather than through monovalent Na^+ ion. These observations therefore suggest that the fluoride ion interacts with the enzyme at a position which is distinct from the substrate binding site (i.e. K_m values remain unaltered and therefore substrate affinity is not affected by increasing NaF concentration), and that inhibition is achieved through decreasing the turnover rate of the enzyme rather than by diminishing the proportion of enzyme molecules (i.e. the V_{max} decreases with increasing NaF concentration). Although the type of inhibition exerted by NaF was similar for both esterase species, the magnitude of inhibition was quite different, in that MonEst showed a 128-fold increased sensitivity to NaF compared to ComEst (K_i values of 0.01mM and 1.28mM respectively). In a previous study [Scott et al., 1984c], a thirteen-fold increased sensitivity to NaF inhibition was reported for MonEst when compared to ComEst, although these findings were obtained from analysis of raw cell extracts with only semi-quantitative comparisons of cytochemically stained IEF plates. In an earlier study [Lam et al., 1978], NaF inhibition of monocytic esterases was reported as being competitive rather than noncompetitive although the study did not document how this conclusion was reached.

The presence of essential serine residues, in the active site of an enzyme, is commonly assessed by investigating the inhibitory effects of di-isopropyl fluorophosphate (DFP) and other related organophosphates. Although organophosphate inhibition of esterases has been widely interpreted as evidence for the presence of an active serine residue, alternative studies [Choudhury, 1972; Li et al., 1973] have suggested that these inhibitors may react with a group (possibly imidazole) near the active centre and that the actual inhibition results from steric hindrance rather than through a specific inactivation of the active serine. However, in subsequent studies [reviewed in Dixon & Webb, 1979b] organophosphates have been shown to react with a single unique serine residue in the enzyme active site. Furthermore, peptide sequence analyses, of the active sites of a

number of mammalian enzymes (Table 4.9), have shown a high degree of conservation around an active serine residue. Although these enzymes differ considerably with respect to their physiological roles, they all share a common ability to catalyse the hydrolysis of esters. DFP and related organophosphates are thought to act as active-site-directed inhibitors; i.e. they gain access to the enzyme active site by mimicking the substrate and subsequently covalently modify, through phosphorylation, the essential serine residue causing irreversible inhibition.

A recent study [Saboori et al., 1991] which investigated the effects of several different organophosphorous compounds on human monocyte carboxylesterase activity found that the molecular bulk and charge of these particular inhibitors plays a significant role in determining their effectiveness on esterase activity. These authors further demonstrated that charged organophosphates, such as diphenyl phosphate, are far less effective inhibitors of esterase activity than uncharged (diphenyl methyl phosphate) compounds, and that increasing the molecular bulk of phosphorous compounds results in decreased esterase inhibition. These findings are particularly relevant to the physiological role(s) of esterases which are discussed in more detail in Chapter 5.

Two compounds used in this current study (PMSF and DCIC) are known to show similar inhibitor characteristics to DFP. Both these compounds, which are are considerably less toxic than organophosphates, were shown to specifically inactivate the MonEst isoenzymes whilst causing no significant inhibition of ComEst. PMSF and DCIC irreversibly inactivate enzymes containing active serine residues by sulphonylation and acylation respectively. These observations therefore clearly indicate a significant difference between the two myeloid esterases in that the presence of a serine residue in the active site is strongly indicated for MonEst but not for ComEst. These findings are in accord with earlier observations [reviewed in Drexler et al., 1991a] postulating the existence of a serine active site for monocytic esterases. Further characterisation of the effects of lower (0.05-0.20mM) PMSF concentrations on MonEst, revealed that PMSF

inhibits MonEst activity through mixed competitive-noncompetitive inhibition. The competitive inhibition provides significant evidence that this compound mimics the substrate to gain access to MonEst active site. It is therefore proposed that at higher PMSF concentrations (>0.2mM), when the equilibrium is shifted to the right (i.e. *Enzyme + PMSF >> Enzyme-PMSF*), PMSF sulphonylates an essential serine residue and causes irreversible inhibition. In contrast, the noncompetitive inhibition of MonEst is almost certainly fluoride-induced and this conclusion is supported by the finding that the K_i values for the noncompetitive inhibition of MonEst by both PMSF and NaF are identical.

Assessing the presence of essential histidine residues within the enzyme active site is generally achieved by examining the inhibitory effects of diethyl pyrocarbonate (DEPC), N-tosyl-L-phenylalanine chloromethyl ketone (TPCK), or N-alpha-p-tosyl-L-lysine chloromethyl ketone (TLCK) [Palmer, 1985]. All three compounds mimic the enzyme substrate to gain access to the active site and subsequently alkylate an essential histidine residue causing irreversible inactivation. Any differences in the sensitivity of individual enzymes to these compounds therefore reflect the ability of their binding sites to recognise these particular inhibitors as potential substrates.

DEPC reversibly inhibited both ComEst and MonEst species in a noncompetitive manner; the mechanism of inhibition for both enzymes being similar to that exhibited by NaF. In this situation, DEPC reversibly binds to an amino acid residue close to the active site which, although not preventing substrate binding, does cause sufficient conformational change to the catalytic machinery to result in diminished substrate hydrolysis. Although DEPC inhibits both esterase forms, the presence of an essential histidine residue is not (because of its reversible action) suggested for either ComEst or MonEst. In contrast, TPCK irreversibly inhibits MonEst activity and shows no inhibitory effect against ComEst. As TPCK also irreversibly inactivates chymotrypsin, the results suggest a degree of similarity between the active site configuration of this proteolytic enzyme and

MonEst. More fundamentally, the findings indicate additional differences between ComEst and MonEst, with regards to requirement of histidine residues for enzymatic activity. The finding that MonEst isoenzymes are specifically and irreversibly inactivated by TPCK suggest the possible involvement of histidine residues in the active site of these esterase forms. Although, as mentioned above, inhibition could also result from steric hindrance, where the binding of TPCK to a histidine residue close to, but not part of the active centre, may physically prevent access of a substrate to the active site and therefore X-ray crystallography or further studies involving specific amino acid modifications would be required in order to confirm the role of histidine in the catalytic mechanism of MonEst. The inability of TLCK to exert significant inhibition of either esterase species further suggests that the active sites of both ComEst and MonEst differ from that of trypsin.

Iodoacetamide inactivates enzymes containing essential cysteine residues by alkylation [Palmer, 1985]. Consequently, the lack of significant inhibition of either esterase isoenzyme species by this compound tends to rule out the involvement of cysteine residues within their active centres. This interpretation is further substantiated by the failure of p-hydroxymercuribenzoic acid (HMBA), which inactivates enzymes containing -SH groups in a non-specific manner, to show any significant effect. This is in accord with previous conclusions [Kirsch, 1971] that sulphydryl groups are not essential for acetylesterase and carboxylesterase activity.

Analysis of two metal-chelating reagents (1-10, phenanthroline and EDTA) further confirmed that MonEst and ComEst are not metalloproteins, and this substantiates earlier conclusions that the NaF inhibition of myeloid esterases does not occur through conventional inactivation of metal prosthetic groups. Neostigmine, a potent cholinesterase inhibitor and the chymotrypsin inhibitor 2-nitro-4-carboxyphenyl N,N-diphenyl-carbamate (NCDC) also failed to significantly inhibit MonEst or ComEst activities.

This study also analysed the N-terminal amino acid sequence of purified MonEst. The derivation of this sequence not only confirmed homogeneity of the preparation but also revealed a high degree of homology with carboxylesterases from at least two other non-human sources (Table 4.8) and with carboxylesterases from human alveolar macrophages [Munger et al., 1991]. Only one difference, an apparent Val --- Thr substitution at position 12, was noted when the first 39 N-terminal amino acid residues of MonEst were compared with that of human alveolar macrophage esterase. As the MonEst purified in this study was obtained from a single individual, the possibility that this difference is due to sequence polymorphism cannot be excluded. Further investigation of this possibility presents many practical difficulties, not least in obtaining sufficient cellular material from a representative number of different subjects. Longer term amino acid sequencing studies of proteolytic fragments of MonEst, generated by chemical and enzymatic cleavage of the protein, should provide sequence information from various parts of the molecule and should clarify whether or not there are more significant differences between human MonEst and macrophage carboxylesterase.

The complete amino acid sequence of monocyte/macrophage-associated carboxylesterase species has recently been reported by two separate groups [Munger et al., 1991; Zschunke et al., 1991]. As there is virtual identity between the N-terminal sequence of MonEst and the cDNA-derived sequence of Munger and colleagues [1991], together with distinct similarities in their esterase cytochemistries and undoubted ontogenic relationships, this suggests that MonEst is very similar if not identical (not withstanding possible minor amino acid substitutions) to alveolar macrophage carboxylesterase. Similarly, excluding the first 47 amino acid residues, which were not sequenced by Zschunke and colleagues [1991], an overlay of the remaining sequence indicates almost absolute identity between normal macrophage carboxylesterase and that expressed by the myelomonocytic U937 cell line.

Amino acid sequencing is frequently useful in determining the targeting of newly synthesised proteins. This is particularly the case when assessing whether the synthesised protein is retained within the cell, transported to specific subcellular organelles (e.g. mitochondria, endoplasmic reticulum, Golgi apparatus, lysosomes, plasma membrane or other cellular membranes), or secreted from the cell. For example, the COOH-terminal tetrapeptide sequence of His-Ile-Gly-Leu (HIEL) found for monocyte/macrophage esterases [Munger et al., 1991; Zschunke et al., 1991] is very important. This sequence is analogous to the Lys-Asp-Glu-Leu (KDEL) retention sequence, which is now widely accepted as being expressed by proteins retained in the endoplasmic reticulum of cells [Andres et al., 1990; Munger et al., 1991; Haugejorden et al., 1991]. In a separate study Robbi and Beaufay [1991], who reviewed the COOH terminal sequences of several wild-type and mutant liver carboxylesterase species, found that KDEL and related tetrapeptide carboxyl terminal sequences (including HIEL) play an important role in determining whether liver esterase is retained or secreted. They conclude that the presence of KDEL-like carboxyl terminal sequence is strongly indicative of retention of protein in the ER. However, Zschunke and colleagues [1991] postulate from hydropathy calculations of their amino acid sequence, that monocyte esterase is an integral membrane protein with a membrane-spanning hydrophobic portion comprising amino acid residues 352 to 367. Munger and colleagues [1991] also reported that macrophage carboxylesterase was insoluble in the absence of solubilising reagents, a view not shared by either this current study or that of Zschunke et al., [1991]. Furthermore, these same investigators suggest that macrophage carboxylesterase may also be a secretory protein. Both these observations would appear to contradict what is currently understood about proteins expressing KDEL-like carboxyl terminal tetrapeptides, in that these proteins are generally soluble and rarely secreted [Andres et al., 1990; Haugejorden et al., 1991; Robbi & Beaufay, 1991]. However, it is pertinent to point out that the degree of retention of protein in the endoplasmic reticulum, whilst requiring the presence of a retention signal, is also influenced by the remaining sequence.

As such, it may well be that although MonEst expresses the retention tetrapeptide, the remaining sequence may not be conducive to its retention in the endoplasmic reticulum.

Furthermore, analysis of the N-terminal amino acid sequence of MonEst reveals insufficient consecutive hydrophobic amino acids to suggest that the enzyme is anchored to the membrane in this region. Although not conclusive, it is generally accepted that most integral membrane ectoenzymes express hydrophobic portions at either the NH_2- or COOH-terminal (i.e. the bulk of the enzyme, including active site, is invariably on either the external or the internal face of the membrane and not equally distributed between both [Kenney & Turner 1987]). Consequently, the interpretation by Zschunke et al., [1991] that monocyte esterase is associated with the plasma membrane through hydrophobic amino acids (residues 352-367) in the sequence could only be explained if MonEst had an unconventional membrane-association.

In conclusion, this study has successfully purified MonEst to homogeneity and ComEst to a highly enriched state. The availability of these myeloid esterase species in purified form further provided an opportunity to compare their molecular and biochemical characteristics. Before summarising the similarities and differences between the MonEst and ComEst species, it is perhaps pertinent to consider the findings of other investigators who have claimed the apparent purification of human MonEst (there are no reports to date of ComEst purification). For example, Saboori & Newcombe [1990] recently described a four column procedure which provided a single 60 kDa protein that, under non-denaturing conditions, formed a trimer with a relative Mr of 200 kDa. However, the apparent pIs of this purified protein ranged from 7.5-7.8 and are clearly more in keeping with the ComEst species rather than the widely accepted pI range of 5.5-6.2 for MonEst [Radzun et al., 1980; Cohn et al., 1987; Scott & Drexler, 1989]. Furthermore, examination of the kinetic results reveal discrepancies in the K_m (ANA, 0.16mM; ANB, 0.33mM) and V_{max} (ANA, 7.5 U/mg protein; ANB, 14.0 U/mg protein) values of their purified esterase in that these data indicate a two-fold higher binding affinity for ANA

even though the butyrate ester was hydrolysed at twice the rate of ANA. Although it is conceivable that the acetate substrate inhibits purified MonEst, thereby giving a low K_m value accompanied by an unexpectedly low V_{max}, the results of this current study clearly indicate the converse to be the case (i.e. ANB causes MonEst inhibition and not ANA). In an earlier study [Lam et al., 1978], a carboxylic ester hydrolase isolated from myelomonocytic leukaemia blasts was shown to have a molecular weight of approximately 70 kDa but its substrate specificities suggested that this was an acetylhydrolase and kinetically inconsistent with MonEst. In a separate study of the myelomonocytic cell line ML-1 [Yourno, 1986], monocyte-associated esterase isoenzymes fractionated by sequential DEAE-52 anion exchange, Sephadex G-200 gel filtration and hydroxyapatite adsorption chromatography were shown to exist as 68 kDa monomers and 205 kDa trimers. In addition, IEF analysis of these species demonstrated that their pIs were similar (monomer, range 6.2-6.3; trimer, range 5.8-6.1) and that electrophoresis of dissociated forms in the presence of SDS indicated the esterase molecule to be a single chain structure with an apparent molecular weight of 62 kDa. However, whereas this fractionated enzyme appeared to be a single protein with the molecular and pI characteristics of MonEst, enzyme homogeneity was not confirmed by more precise techniques such as amino acid sequencing. Furthermore, although the two molecular weight forms were shown in the same report to correspond to previously defined pH Native-PAGE isoforms AB1 (monomer) and AB2 (trimer), the MonEst purified to homogeneity in this present study revealed only one major Mr form (157 kDa) with no evidence for the existence of an active monomer. One immediate explanation reconciling the presence of an active monomer in this earlier study, and the failure to convincingly demonstrate it in this, is that the active monomeric AB1 esterase described by Yourno [1986] is in fact not monocyte-specific and corresponds to the pair of higher pI (monomeric) ComEst isoenzymes which are characteristically found in both monocytic and granulocytic cells (associated with the G2-type IEF pattern; detailed in Chapter 2) and share many of the chromatographic features of MonEst.

Although the genetic mechanisms which control the synthesis and expression of MonEst and ComEst myeloid esterases are unknown, it has been suggested [Yourno et al., 1984] that monocyte-specific esterase isoenzymes (MonEst) may be derived from pre-formed (ComEst) esterases with a higher pI, with 'conversion' resulting from commitment to monocytic differentiation. This concept is clearly based on the assumption that the ComEst and MonEst species are subject to related genetic mechanisms. However, the results presented in this part of the study provide strong evidence that these are in fact distinct enzymes which share a common ability to hydrolyse simple non-physiological esters. In this context, it is considered that MonEst should be biochemically classified as a carboxylesterase (EC 3.1.1.1) whereas ComEst has characteristics that are more in keeping with acetylesterases (EC 3.1.1.6).

CHAPTER 4

Figures 4.1 to 4.21 and Tables 4.1 to 4.9

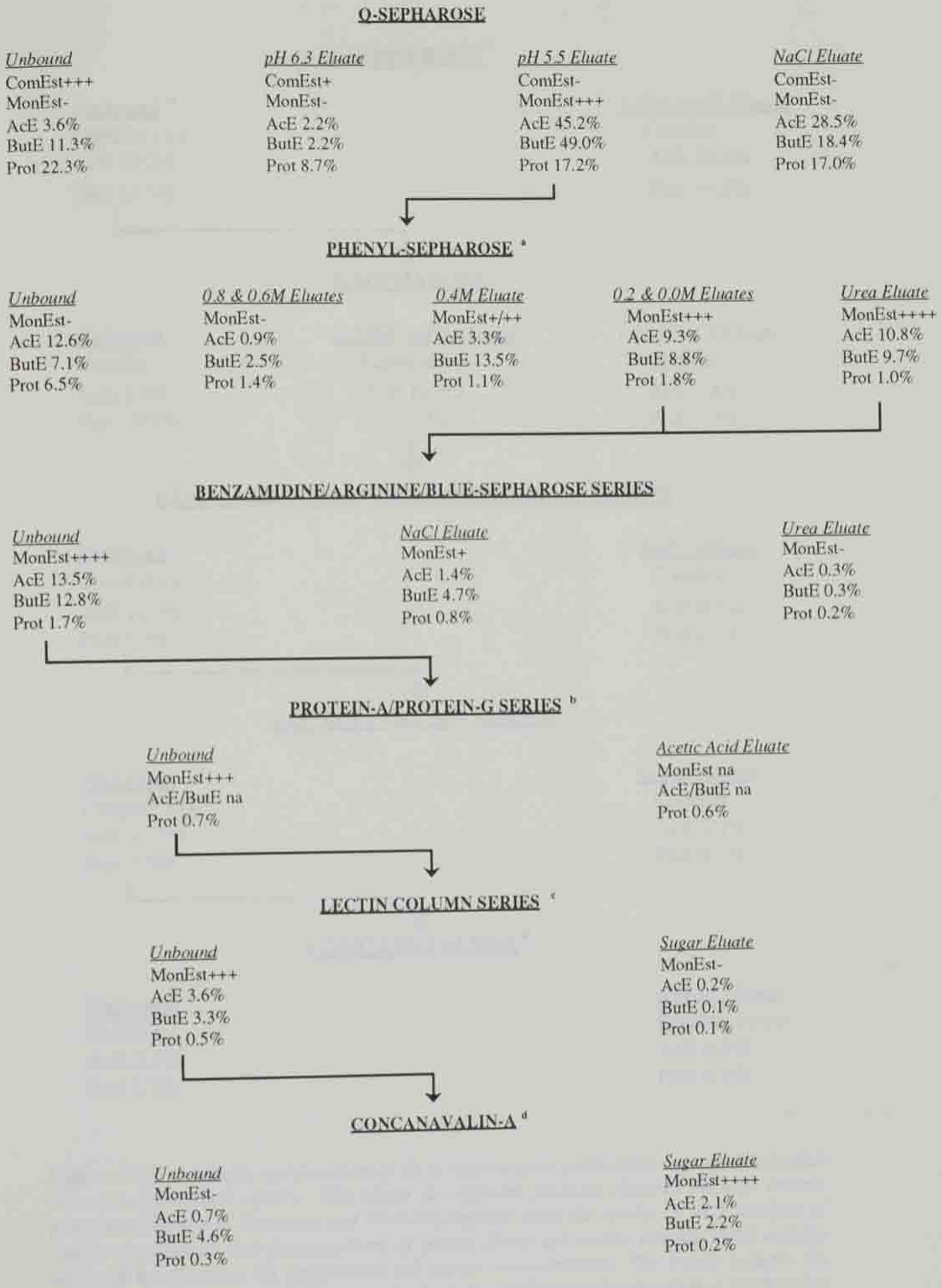

Figure 4.1: *Schematic representation of the chromatographic purification sequence for MonEst from a leukaemic cell extract. This shows the different fractions obtained for each column procedure (detailed in Materials and Methods) together with the results for IEF analysis of ComEst and MonEst isoenzymes, and the proportions of protein (Prot) and acetate esterase (AcE) activity expressed in relation to the original raw cell extract concentrations. The arrows indicate the fractions which were sequentially passaged; a) indicates that the 0.2M, 0.0M and 6M urea eluates were pooled prior to benzamidine/arginine/Blue-Sepharose chromatography; b) indicates that these columns were used for the removal of immunoglobulins (predominantly IgG) from the crude cell extract; c) refers to the series of lectin columns comprising Ulex europaeus agglutinin, Arachis hypogaea agglutinin, Vicia villosa agglutinin Isolectin B4, Helix pomatia agglutinin, Ricinus communis agglutinin, Griffonia simplifocia agglutinin II, Wheatgerm agglutinin, Pisum sativum agglutinin and Lens culinaris agglutinin, and the use of a sugar mixture comprising 200mM methyl α-D mannopyranoside, 50mM α-L-fucose, 100mM N-acetyl-D-glucosamine, 20mM N-acetyl-D-galactosamine and 50mM D-galactose for column elution; and d) indicates specific desorption from Con-A with 200mM methyl α-D mannopyranoside.*

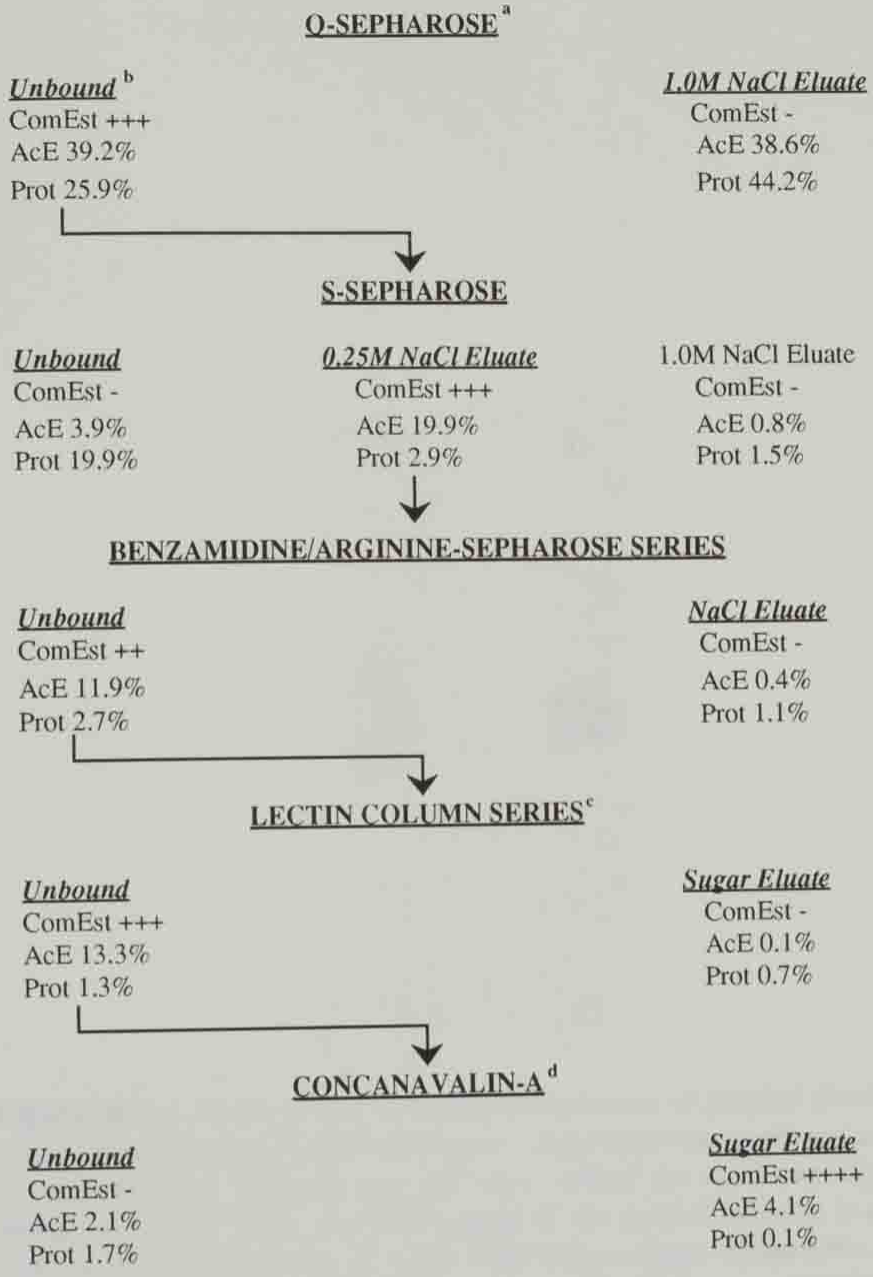

Figure 4.2: *Schematic representation of the chromatographic purification sequence for ComEst from a leukaemic cell extract. This shows the different fractions obtained for each column procedure (detailed in Materials and Methods) together with the results for IEF analysis of ComEst isoenzymes, and the proportions of protein (Prot) and acetate esterase (AcE) activity expressed in relation to the original raw cell extract concentrations. The arrows indicate the fractions which were sequentially passaged; a) indicates preliminary fractionation of the MonEst and ComEst species by Q-Sepharose chromatography; b) the unbound protein from Q-Sepharose was precipitated with 80% ammonium sulphate prior to further fractionation by S-Sepharose; c) refers to the series of lectin columns comprising Ulex europaeus agglutinin, Arachis hypogaea agglutinin, Vicia villosa agglutinin Isolectin B4, Helix pomatia agglutinin, Ricinus communis agglutinin, Griffonia simplifocia agglu-tinin II and Wheatgerm agglutinin and the use of a sugar mixture comprising 200mM methyl α-D mannopyranoside, 50mM α-L-fucose, 100mM N-acetyl-D-glucosamine, 20mM N-acetyl-D-galactosamine and 50mM D-galactose for column elution; and d) indicates specific desorption from Con-A with 200mM methyl α-D mannopyranoside.*

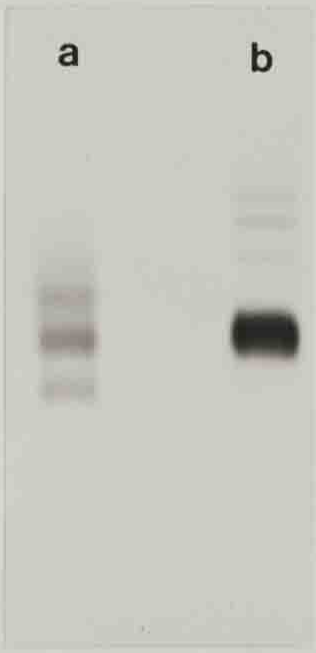

Figure 4.3a: *Native-PAGE (pH 8.8) electrophoresis of purified ComEst (track a) and MonEst (track b) myeloid esterases. Separation was performed as detailed in Materials and Methods, and gels were stained for alpha-naphthyl acetate esterase (ANAE). Note that the majority of the purified MonEst is associated with a single charge species, with three minor charge components, whereas ComEst comprises three/four charge species that appear to correspond to the individual ComEst pI forms defined by IEF.*

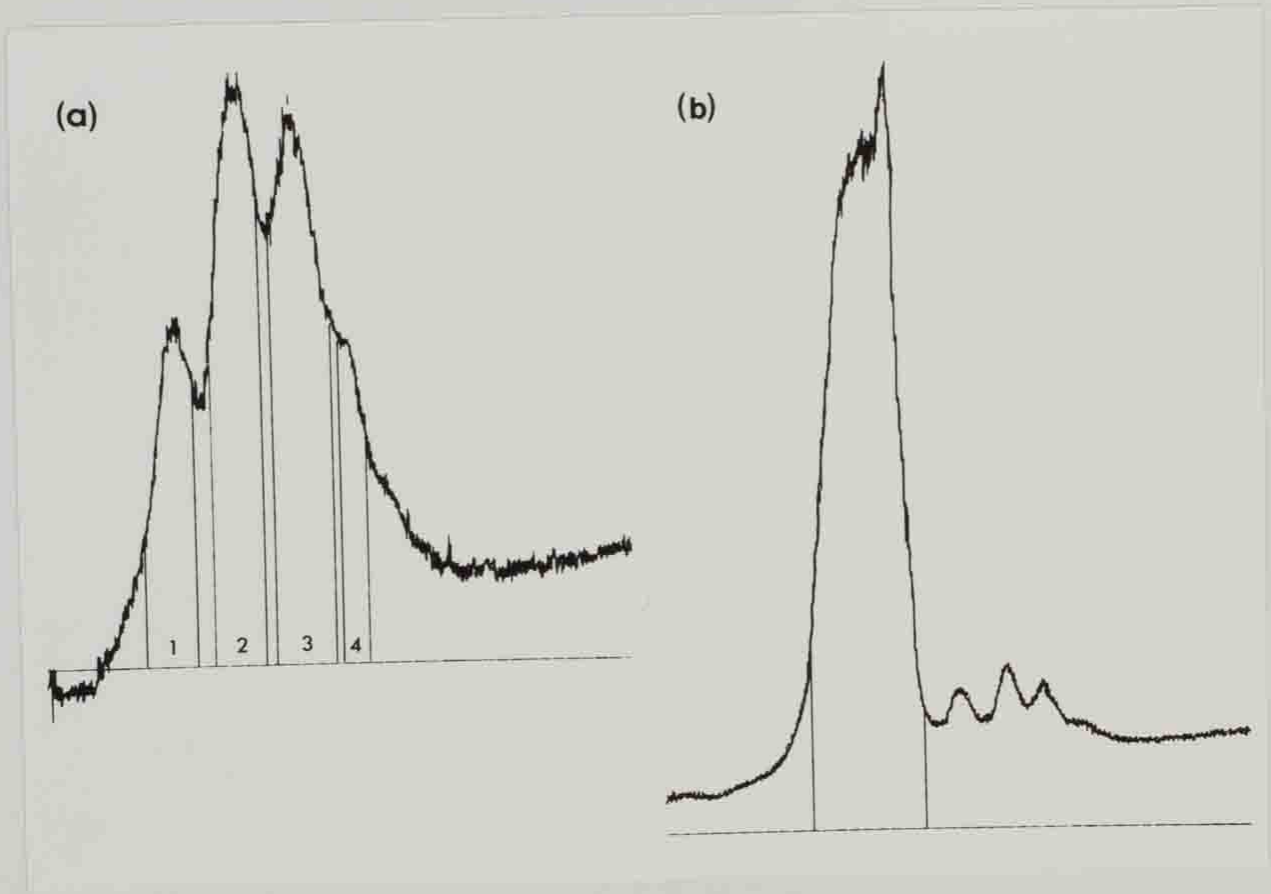

Figure 4.3b: *Densitometric scans of purified (a) ComEst and (b) MonEst myeloid esterase charge species separated by pH 8.8 Native-PAGE electrophoresis (see also Figure 4.3a). Note that ComEst comprises four individual charge forms with an evenly spaced distribution whereas, in contrast, most of the purified MonEst is associated with a single charge species.*

Figure 4.4: *Native-PAGE (pH 8.8) electrophoresis of purified MonEst following treatment with alpha-mannosidase (track b), alpha-L-fucosidase (track c) and endoglycosidase H (track d) as detailed in Materials and Methods. The gels were stained for alpha-naphthyl acetate esterase (ANAE). Note that the distribution of the various charge species remains identical to the untreated MonEst (track a). The minor retardations noted in track c resulted from presence of high glycerol concentrations in the alpha-L-fucosidase solution.*

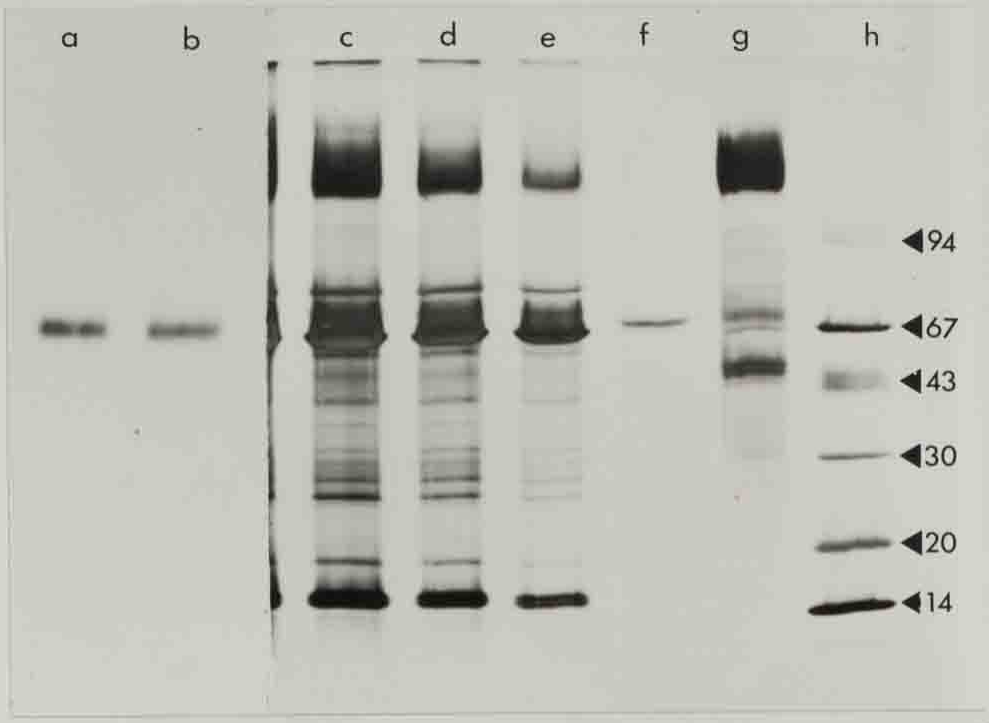

Figure 4.5a: *SDS-PAGE electrophoresis (pH 8.6) of chromatographically purified MonEst and ComEst myeloid esterase fractions; tracks (a) and (b) show the partially purified ComEst fraction stained for alpha-naphthyl acetate esterase (ANAE); (c) - (e) show Neat, 1:2 and 1:4 dilutions of the original serum/cell extract used for the MonEst purification (silver stain); (f) and (g) show purified MonEst and partially purified ComEst respectively (silver stain); and track (h) shows molecular weight standards (silver stain). It is evident from these observations that (i) the fractionated MonEst is homogeneous and migrates with an apparent molecular weight of approximately 63KDa, and (ii) that the ComEst fraction is contaminated with a number of other proteins (predominantly aggregate) but is homogeneous (68KDa) with respect to ANAE staining.*

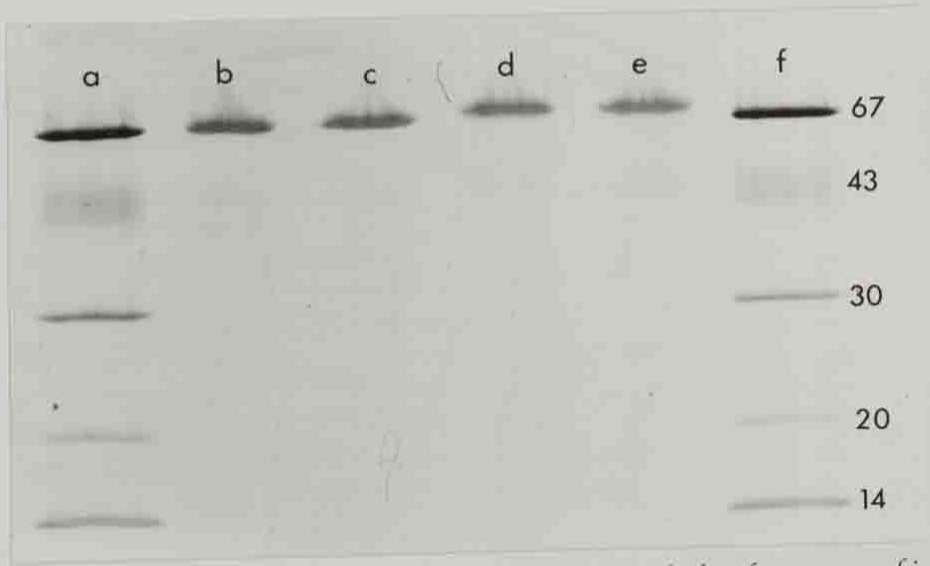

Figure 4.5b: *SDS-PAGE electrophoresis (pH 8.6) of the chromatographically purified MonEst myeloid esterase fraction; tracks (a) and (f) show molecular weight standards (silver stain); (d) and (e) show non-reduced and reduced MonEst (Mr of 63.4) purified to homogeneity as described in Section 4.2.1.1; (b) and (c) show non-reduced and reduced MonEst subsequent to digestion by endoglycosidase-H (Mr of 60.1KDa).*

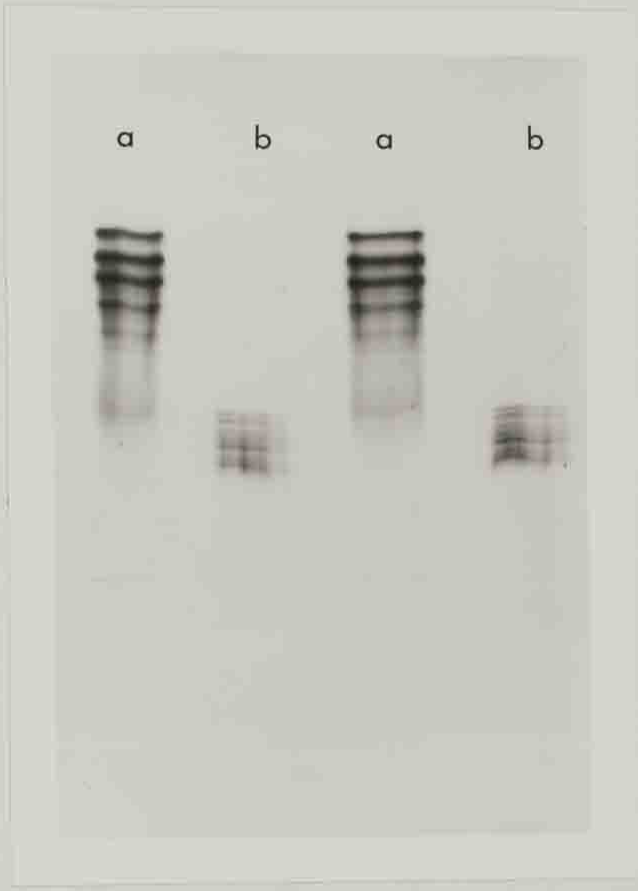

Figure 4.6: *IEF profiles of purified ComEst (a) and MonEst (b) esterase isoenzyme species confirming that the isoelectric point characteristics of individual isoenzyme components were unaffected by the chromatographic fractionation procedures.*

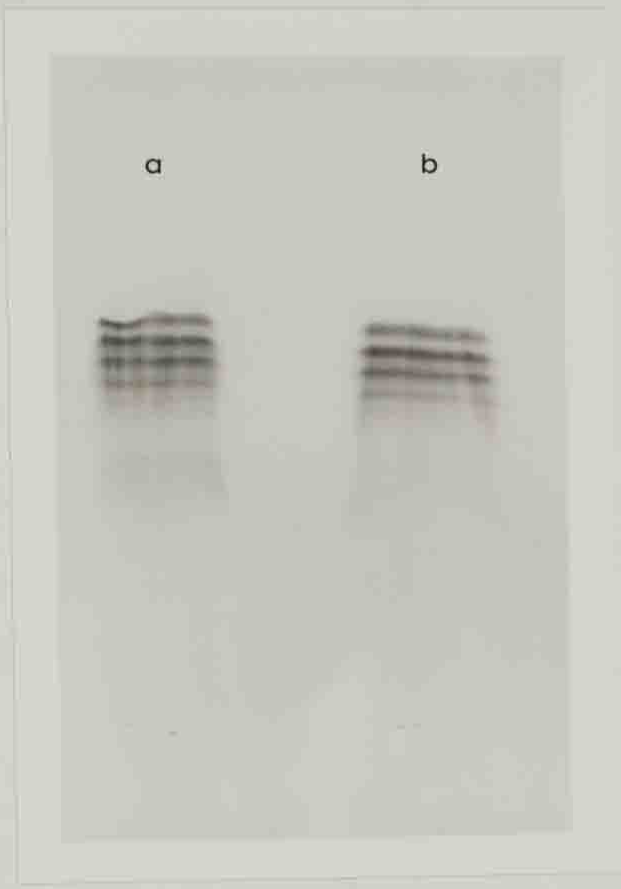

Figure 4.7: *IEF profiles of purified ComEst esterase isoenzyme species, untreated (a) and following digestion with neuraminadase (b). Note that there is no alteration in the apparent charge distribution of individual ComEst isoenzymes as a result of desialyation.*

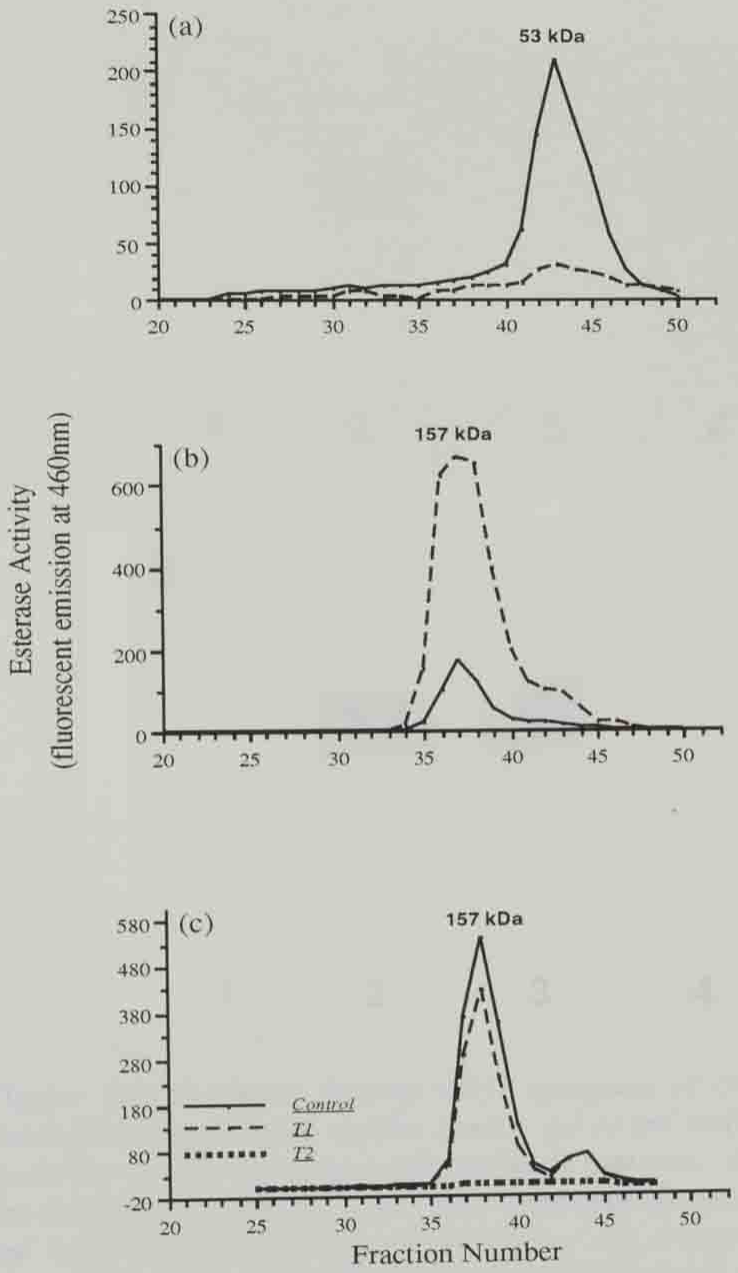

Figure 4.8: *Superose-12 FPLC gel filtration profiles of purified myeloid cell esterase species. Diagrams (a) and (b) show the elution volumes (fraction number) and enzymatic activities (acetate esterase, unbroken lines; butyrate esterase, broken lines) of ComEst and MonEst respectively. The main points of note are that ComEst has an apparent molecular weight (Mr) of 53kDa and shows a marked preference for acetate substrate hydrolysis, in contrast to MonEst which has a predominant Mr of 153kDa (with only a minor proportion of activity being associated with the 53kDa species) and preferentially hydrolyses the butyrate substrate. Figure (c) shows the effect of 5M urea (T1) and 4M guanidine hydrochloride (T2) on the Mr and enzymatic (acetate/butyrate combined esterase) of purified MonEst. Note: Urea displays no apparent effect on either the Mr or enzymatic activity whereas guanidine hydrochloride completely abrogates MonEst activity.*

Figure 4.9: *Isoelectric focusing (IEF) zymograms of Con-A unbound and eluted fractions of partially purified ComEst (gel A) and purified MonEst (gel B) species prior to and following endoglycosidase H treatment. Tracks 1 and 2 show Con-A binding characteristics, where track 1 indicates the Con-A unbound fraction and track 2 the Con-A eluted fraction; tracks 3 and 4 show the Con-A binding characteristics of endo H treated ComEst and MonEst, where track 3 indicates the Con-A unbound fraction and track 4 the Con-A eluted fraction. Note that untreated ComEst and MonEst species both show complete binding to Con-A but, following exposure to endo H, MonEst fails to bind Con-A whereas ComEst binding is unaffected.*

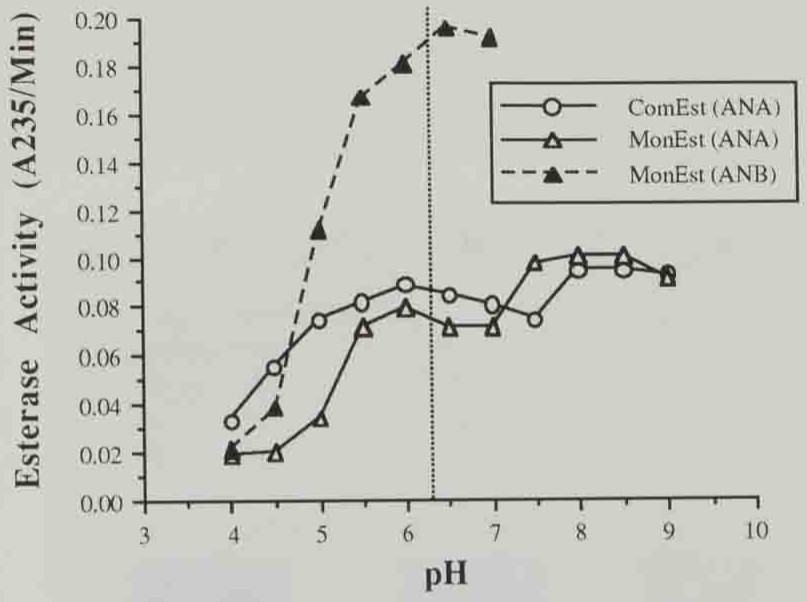

Figure 4.10: *Effect of pH on ComEst and MonEst reaction rates. Enzyme activities at 30°C of ComEst and MonEst with 0.3mM alpha-naphthyl acetate (ANA) and alpha-naphthyl butyrate (ANB) substrates were determined at 0.5 pH intervals between the range of 4.0 and 9.0. Note that ComEst did not cause significant hydrolysis of ANB and that because of a high degree of spontaneous ANB hydrolysis above pH 7.0, the reaction rates for MonEst with this substrate were not evaluable. The pH at which inhibition kinetics were carried out is shown by the vertical dotted line.*

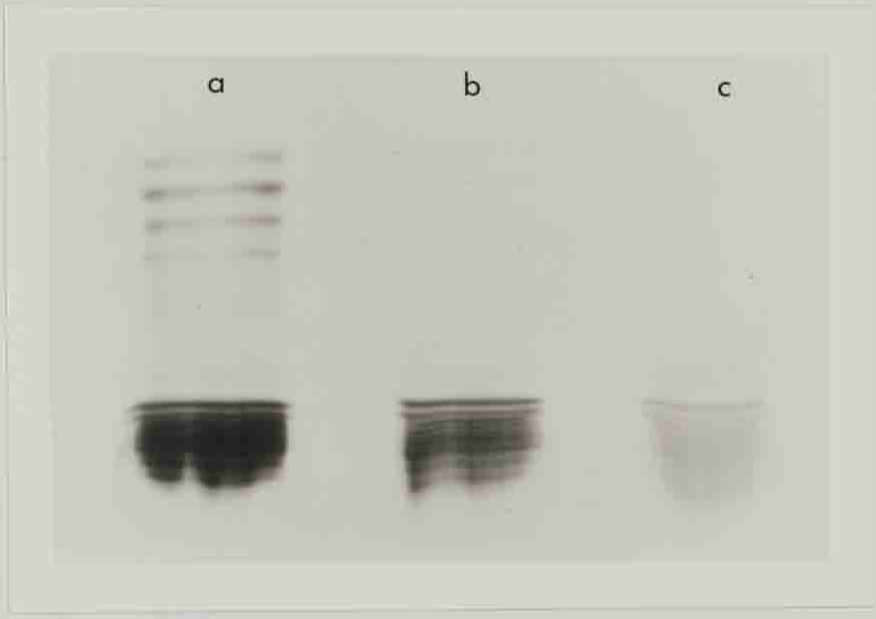

Figure 4.11: *Histochemical staining of MonEst and ComEst myeloid esterase species using alpha-naphthyl acetate (ANA, track a), propionate (ANP, track b) and butyrate (ANB, track c) as substrates. Note that the ComEst esterase isoenzymes only appear to hydrolyse ANA, with little or no staining in the presence of ANP or ANB. In contrast, the lower pI MonEst species hydrolyse all three substrates but the histochemical staining with ANB is considerably weaker than that seen with ANA and ANP.*

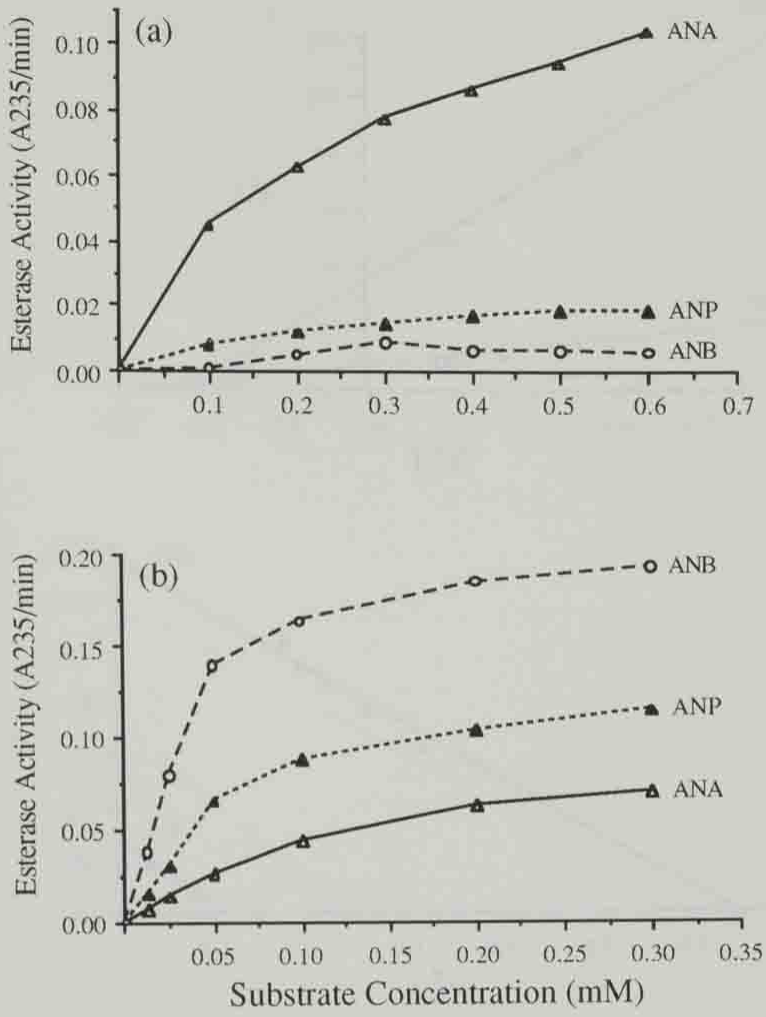

Figure 4.12: *Relationships between alpha-naphthyl acetate (ANA), propionate (ANP) and butyrate (ANB) substrate concentrations and observed reaction rates of purified ComEst (diagram a) and MonEst (diagram b) species. The rate of hydrolysis of acetate, propionate and butyrate esters was determined by UV spectrophotometry [Mastropaolo & Yourno, 1981] at each substrate concentration as detailed in Materials and Methods. Reaction rates are shown as increases in absorbance at 235nm/min, with each data point representing a mean of duplicate determinations.*

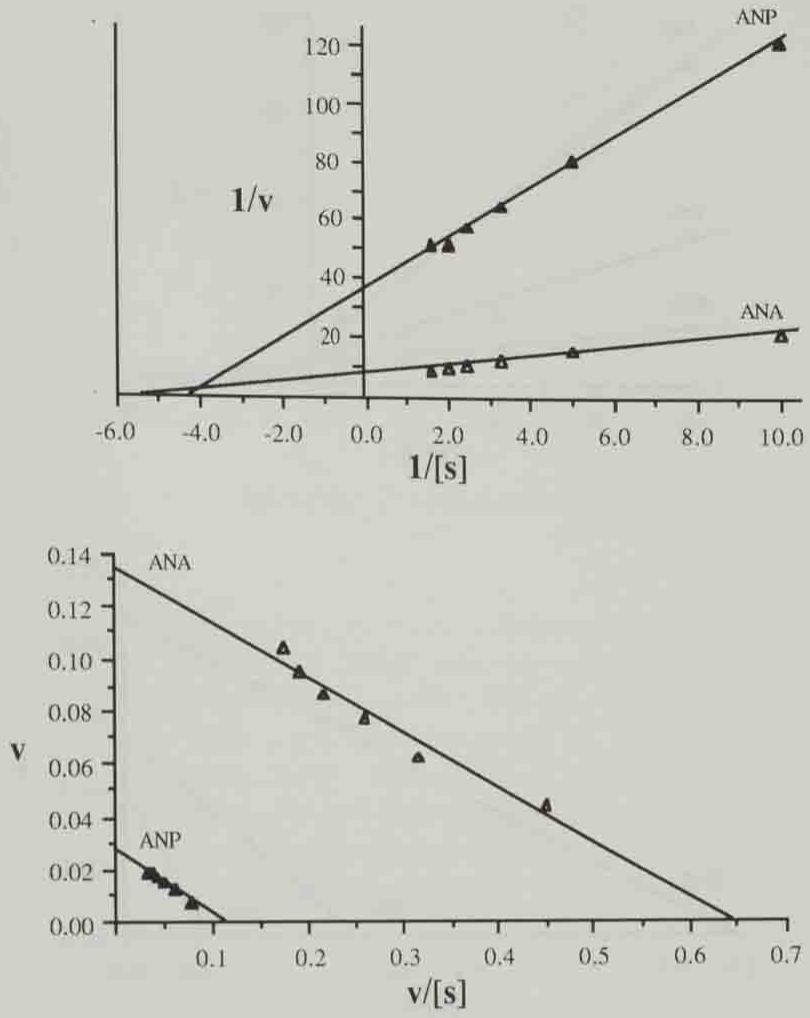

Figure 4.13: *Lineweaver-Burk (upper diagram) and Eadie-Hofstee (lower diagram) plots for the determination of Michaelis-Menten constants (Km) and maximum velocities (Vmax) for the purified ComEst species with alpha-naphthyl acetate (ANA) and propionate (ANP) substrates. ComEst did not cause any significant hydrolysis of alpha-naphthyl butyrate. The data from Figure 4.12 was converted to inverse readings and the 1/velocity values plotted against 1/substrate concentration.*

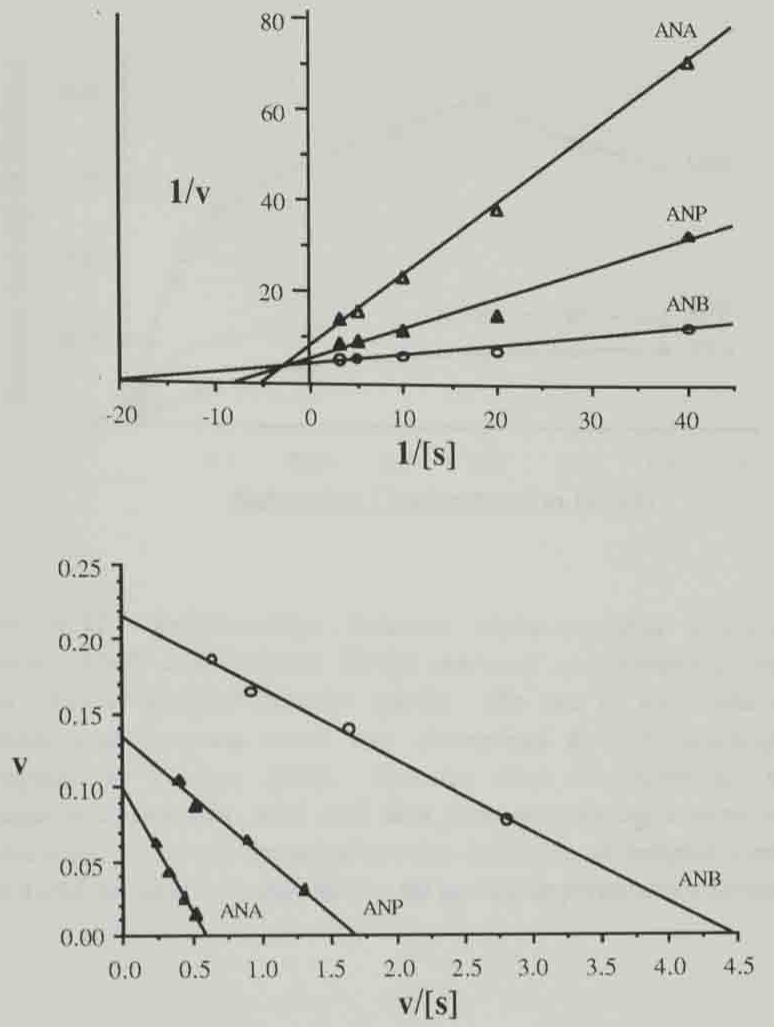

Figure 4.14: *Lineweaver-Burk (upper diagram) and Eadie-Hofstee (lower diagram) plots for the determination of Michaelis-Menten constants (Km) and maximum velocities (Vmax) for the purified MonEst species with alpha-naphthyl acetate (ANA), propionate (ANP) and butyrate (ANB) substrates. The data from Figure 4.12 was converted to inverse readings and the 1/velocity values plotted against 1/substrate concentration.*

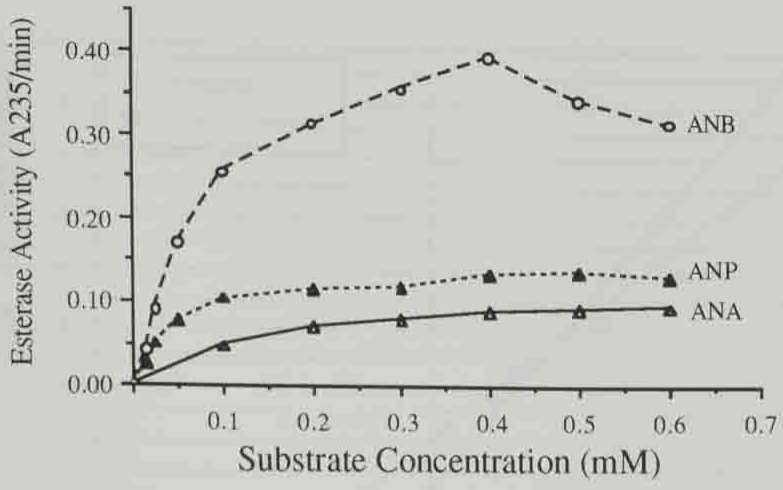

Figure 4.15: *Relationships between alpha-naphthyl acetate (ANA), propionate (ANP) and butyrate (ANB) substrate concentrations and observed reaction rates of purified MonEst species. The rate of hydrolysis of acetate, propionate and butyrate esters was determined by UV spectrophotometry [Mastropaolo & Yourno, 1981]. Reaction rates are shown as increases in absorbance at 235nm/min, with each data point representing a mean of duplicate determinations. Note the apparent reaction inhibition at butyrate concentrations above 0.4mM which is not observed for the acetate or propionate substrates.*

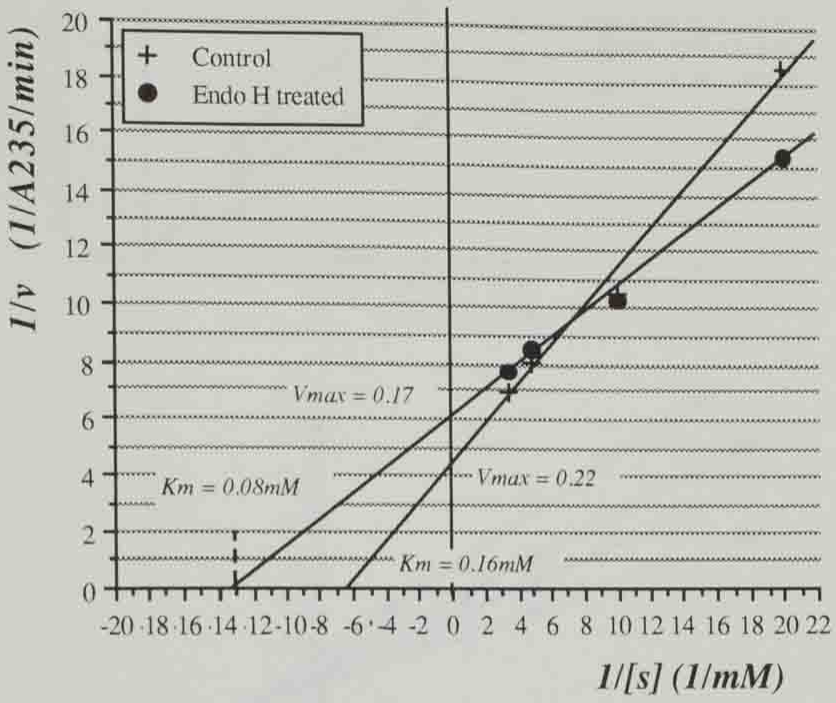

Figure 4.16: *Lineweaver-Burk kinetic plot (1/v against 1/[s]) for the analysis of Michealis-Menten constant (Km) and maximum velocity (Vmax) of Endo H treated and untreated purified MonEst. Note that deglycosylation of MonEst appears to significantly increase its affinity for alpha-naphthyl acetate (i.e. km value of 0.08mM as compared to 0.16mM for the untreated form), whilst showing only a marginal decrease in the Vmax value.*

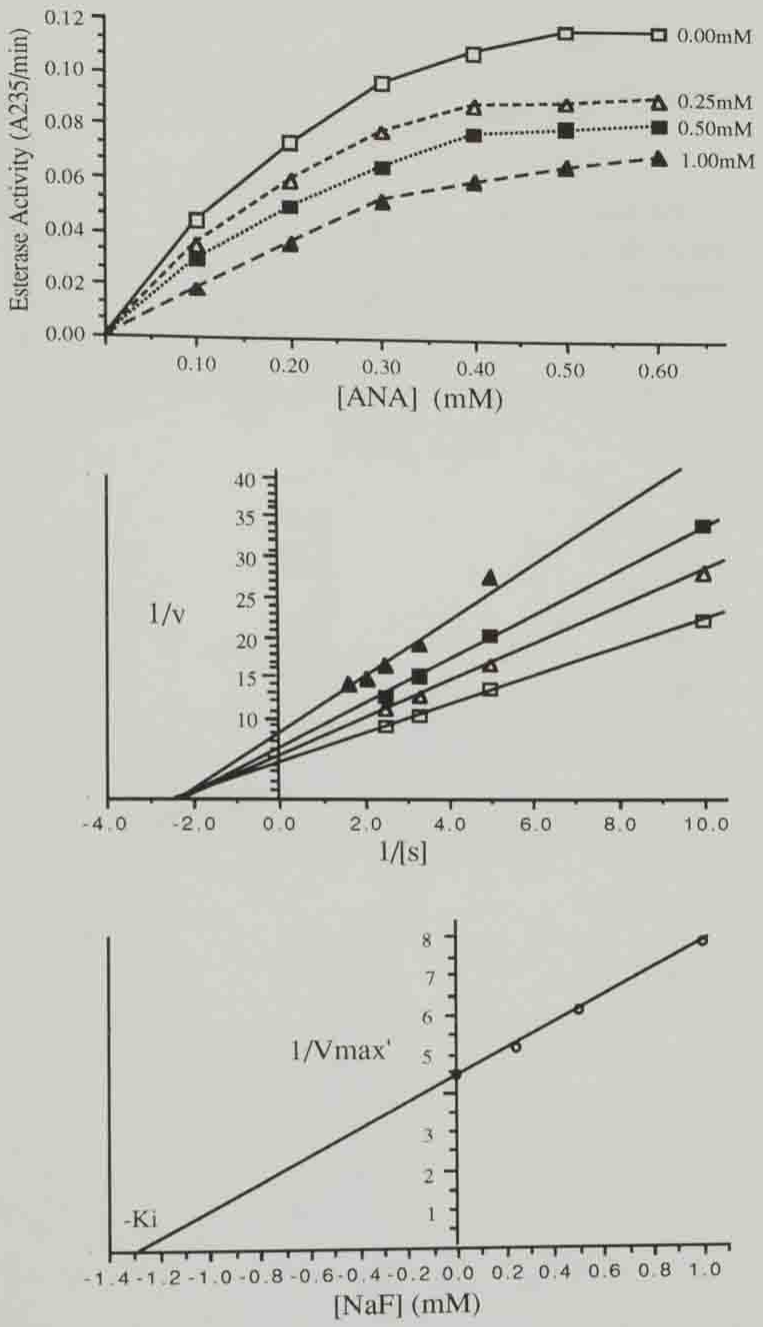

Figure 4.17: *Effect of sodium fluoride (NaF) on the observed rate of alpha-naphthyl acetate (ANA) hydrolysis by the purified ComEst species. The rate of ANA hydrolysis was determined by UV spectrophotometry [Mastropaolo & Yourno, 1981] at each inhibitor concentration as detailed in Materials and Methods; reaction rates are shown as increases in A235/min with each data point representing a mean of duplicate determinations. The upper diagram shows the ComEst rates of ANA hydrolysis with a range (0.1 to 0.6mM) of substrate concentrations in the presence of increasing NaF (0.0 to 1.0mM). The Lineweaver-Burk plot (centre diagram) indicates that NaF exerts noncompetitive inhibition on ComEst; a secondary plot of altered 1/Vmax (1/Vmax') values versus inhibitor concentration (lower diagram) was used to determine the inhibition constant (Ki) for inhibition of ComEst by NaF.*

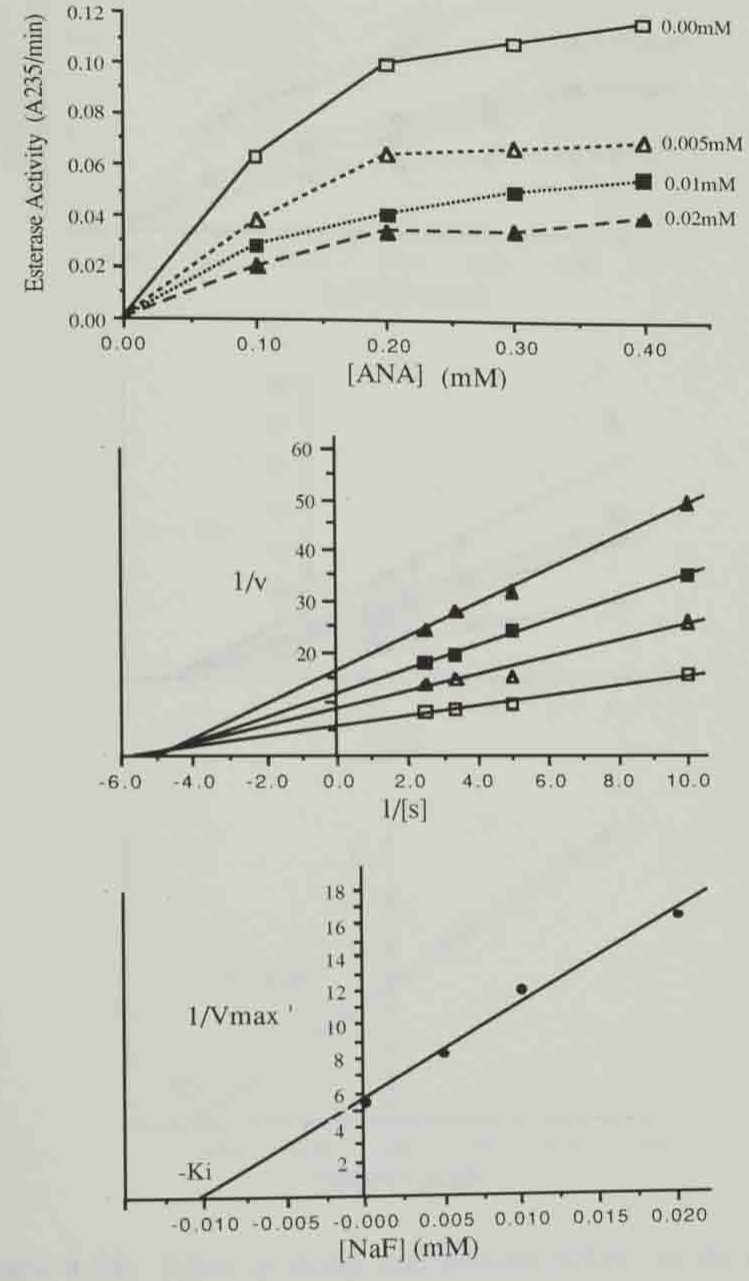

Figure 4.18: *Effect of sodium fluoride (NaF) on the observed rate of alpha-naphthyl acetate (ANA) hydrolysis by the purified MonEst species. The rate of ANA hydrolysis was determined by UV spectrophotometry [Mastropaolo & Yourno, 1981] at each inhibitor concentration as detailed in Materials and Methods; reaction rates are shown as increases in A235/min with each data point representing a mean of duplicate determinations. The upper diagram shows the MonEst rates of ANA hydrolysis with a range (0.1 to 0.4mM) of substrate concentrations in the presence of increasing NaF (0.0 to 0.02mM). The Lineweaver-Burk plot (centre diagram) indicates that NaF exerts noncompetitive inhibition on MonEst; a secondary plot of altered 1/Vmax (1/Vmax') values versus inhibitor concentration (lower diagram) was used to determine the inhibition constant (Ki) for inhibition of MonEst by NaF.*

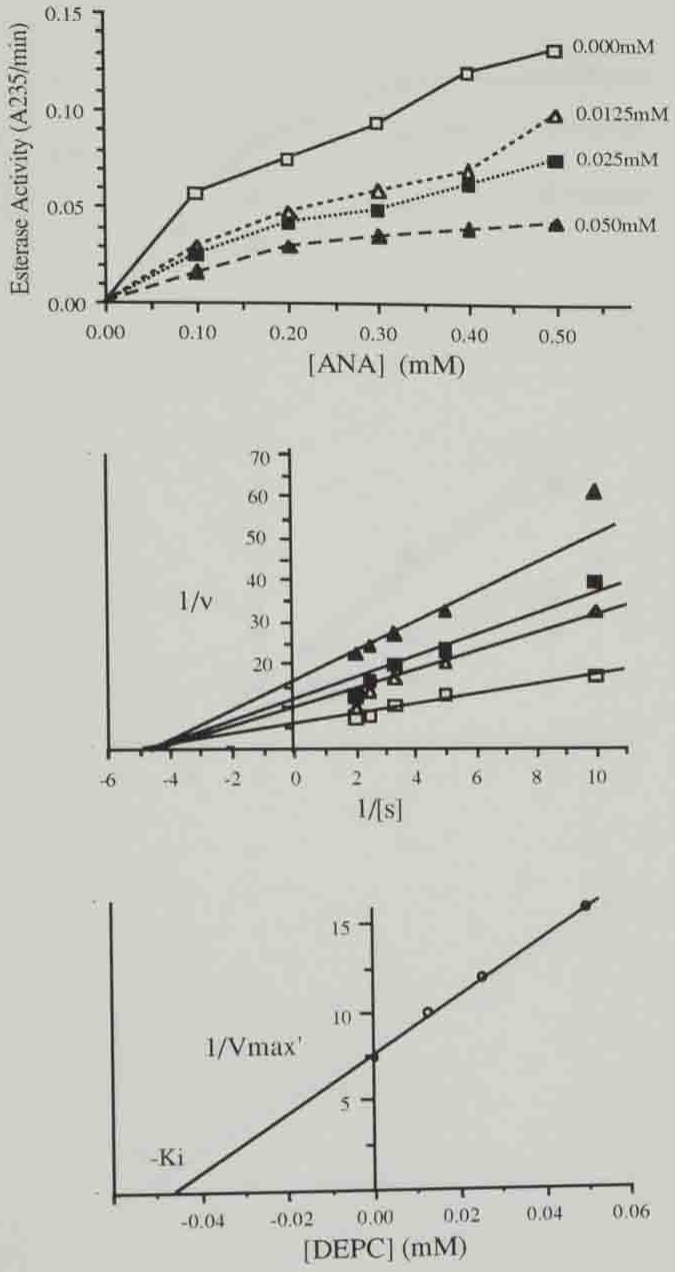

Figure 4.19: *Effect of diethyl polycarbonate (DEPC) on the observed rate of alpha-naphthyl acetate (ANA) hydrolysis by the purified ComEst species. The rate of ANA hydrolysis was determined by UV spectrophotometry [Mastropaolo & Yourno, 1981] at each inhibitor concentration as detailed in Materials and Methods; reaction rates are shown as increases in A235/min with each data point representing a mean of duplicate determinations. The upper diagram shows the ComEst rates of ANA hydrolysis with a range (0.1 to 0.5mM) of substrate concentrations in the presence of increasing DEPC (0.0 to 0.05mM). The Lineweaver-Burk plot (centre diagram) indicates that DEPC exerts noncompetitive inhibition on ComEst; a secondary plot of altered 1/Vmax (1/Vmax') values versus inhibitor concentration (lower diagram) was used to determine the inhibition constant (Ki) for inhibition of ComEst by DEPC.*

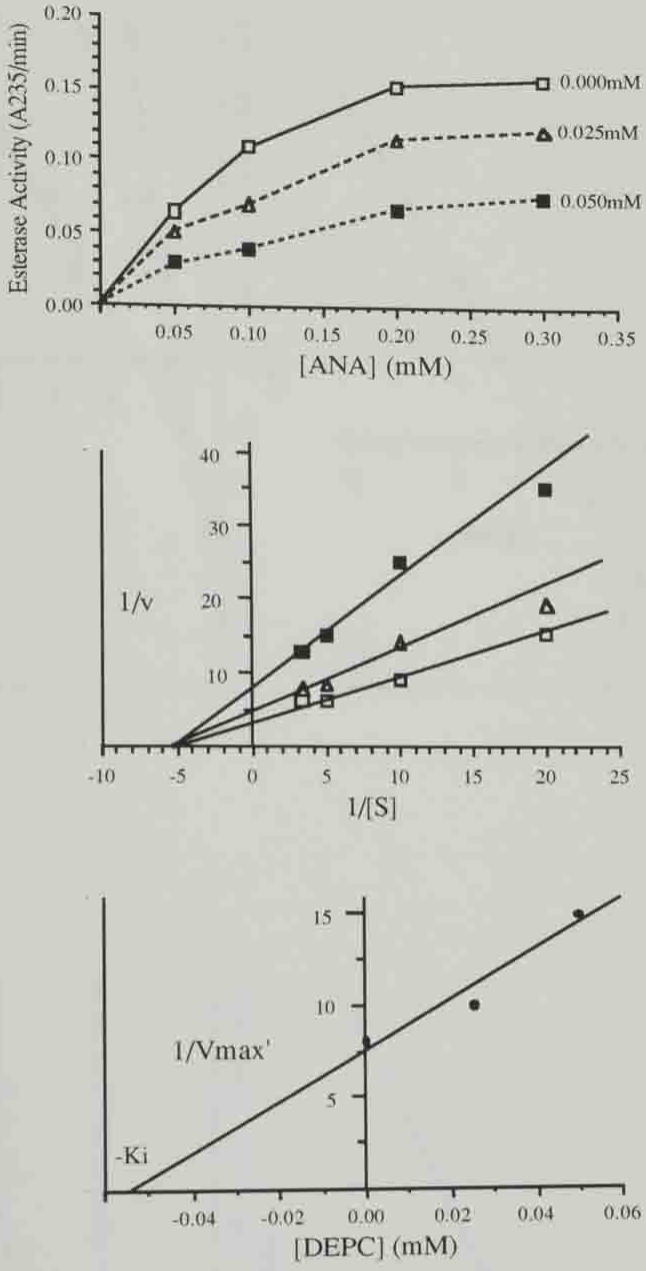

Figure 4.20: *Effect of diethyl polycarbonate (DEPC) on the observed rate of alpha-naphthyl acetate (ANA) hydrolysis by the purified MonEst species. The rate of ANA hydrolysis was determined by UV spectrophotometry [Mastropaolo & Yourno, 1981] at each inhibitor concentration as detailed in Materials and Methods; reaction rates are shown as increases in A235/min with each data point representing a mean of duplicate determinations. The upper diagram shows the ComEst rates of ANA hydrolysis with a range (0.05 to 0.3mM) of substrate concentrations in the presence of increasing DEPC (0.0 to 0.05mM). The Lineweaver-Burk plot (centre diagram) indicates that DEPC exerts noncompetitive inhibition on MonEst; a secondary plot of altered 1/Vmax (1/Vmax') values versus inhibitor concentration (lower diagram) was used to determine the inhibition constant (Ki) for inhibition of MonEst by DEPC.*

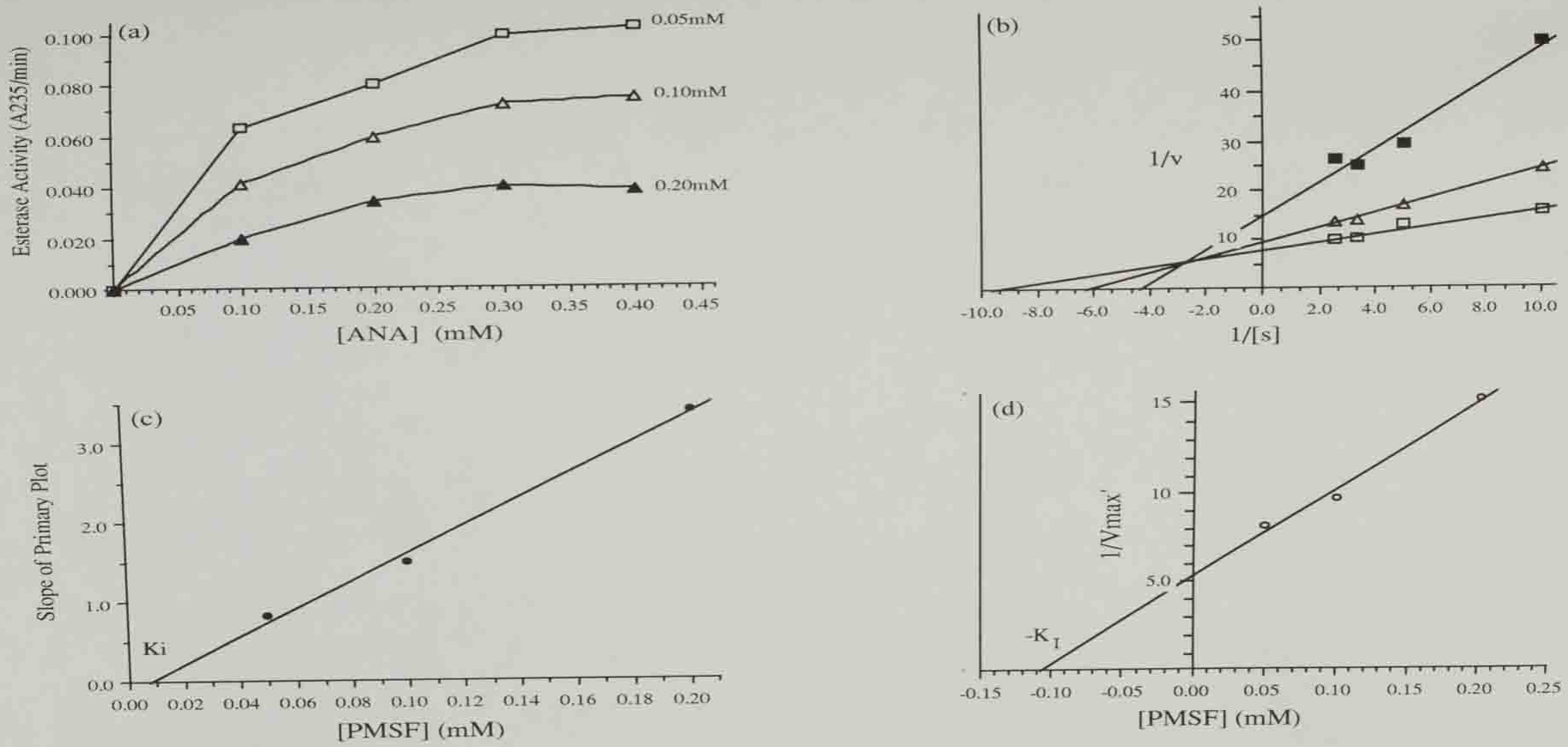

Figure 4.21: *Relationships between phenylmethylsulphonyl fluoride (PMSF) inhibitor concentrations and observed rates of alpha-naphthyl acetate (ANA) hydrolysis by the purified MonEst species (diagram a). The rate of ANA hydrolysis was determined by UV spectrophotometry [Mastropaolo & Yourno, 1981] at each inhibitor concentration as detailed in Materials and Methods; reaction rates are shown as increases in A235/min with each data point representing a mean of duplicate determinations. The Lineweaver-Burk plot (diagram b) indicates that PMSF exerts mixed competitive/noncompetitive inhibition on MonEst. Secondary plots of slope of primary plot (b) and altered 1/Vmax (1/Vmax') values versus inhibitor concentration were used to determine inhibition constants for the noncompetitive (Ki, diagram c) and competitive (KI, diagram d) inhibition respectively of MonEst by PMSF.*

Table 4.1 Chromatography columns used for the purification of MonEst and ComEst myeloid esterase species [a]

Column	MonEst	ComEst	Dimensions	Source
Fast Flow Q-Sepharose	*	*	26.0 x 2.5cm	Pharmacia
Phenyl-Sepharose	*		17.5 x 2.5cm	Pharmacia
Fast Flow S-Sepharose		*	11.5 x 2.5cm	Pharmacia
Benzamidine-Sepharose 6B	*	*	20.0 x 1.6cm	Pharmacia
Arginine-Sepharose 4B	*	*	6.0 x 2.5cm	Pharmacia
Blue-Sepharose CL-6B	*		23.0 x 2.5cm	Pharmacia
Protein-A Sepharose CL-4B	*		10.0ml	Pharmacia
Protein-G Sepharose CL-4B	*		6.0ml	Sigma
Ulex europaeus agglutinin (UEA I)	*	*	1.0ml	Sigma
Arachis hypogaea agglutinin (PNA)	*	*	2.0ml	Sigma
Helix pomatia agglutinin (HPA)	*	*	2.5ml	Sigma
Pisum sativum agglutinin (PSA)	*		1.0ml	Sigma
Vicia villosa agglutinin Isolectin B4 (VVA B4)	*	*	2.5ml	Sigma
Lens culinaris agglutinin (LCA)	*		8.0ml	Pharmacia
Ricinus communis agglutinin (RCA-I)	*	*	2.5ml	Sigma
Griffonia simplifocia agglutinin II (GSA II)	*	*	1.0ml	Sigma
Wheatgerm agglutinin (WGA)	*	*	6.0ml	Pharmacia
Concanavalin-A (Con-A)	*	*	9.0 x 1.0cm	Pharmacia
Superose-12 (FPLC)	*	*	30.0 x 1.5cm	Pharmacia

[a] * indicates use of column as part of the MonEst or ComEst fractionation procedures

Table 4.2a: Summary of column performance for MonEst fractionation [a]

Column/Fraction	Protein (mg)	Acetate Esterase (U/min x 10^{-3})	Butyrate Esterase (U/min x 10^{-3})
Pre-Q Sepharose [b]			
(Serum/cell extract):	26125 (100%)	3262 (100%)	3338 (100%)
Q-Sepharose			
Unbound:	5820 (22.3%)	118 (3.6%)	376 (11.3%)
pH6.3 eluate:	2274 (8.7%)	72 (2.2%)	75 (2.2%)
pH5.5 eluate:	4506 (17.2%)	1473 (45.2%)	1639 (49.0%)
1.0M NaCl eluate:	4437 (17.0%)	931 (28.5%)	614 (18.4%)
Phenyl-Sepharose			
Unbound:	1705 (6.5%)	410 (12.6%)	238 (7.1%)
0.8/0.6M eluates:	365 (1.4%)	28 (0.9%)	83 (2.5%)
0.4M eluate:	286 (1.1%)	108 (3.3%)	450 (13.5%)
0.2/0.0M eluates:	465 (1.8%)	303 (9.3%)	295 (8.8%)
Urea eluate:	254 (1.0%)	355 (10.8%)	325 (9.7%)
Benz/Arg/Blue Series			
Unbound:	437 (1.7%)	440 (13.5%)	426 (12.8%)
NaCl eluate:	204 (0.8%)	47 (1.4%)	156 (4.7%)
Urea eluate:	49 (0.2%)	10 (0.3%)	54 (0.3%)
Protein A/G			
Unbound:	180 (0.7%)	nt	nt
Eluate:	145 (0.6%)	nt	nt
Lectin Series			
Unbound:	135 (0.5%)	119 (3.6%)	109 (3.3%)
Eluate:	18 (0.1%)	4 (0.2%)	4 (0.1%)
Con-A			
Unbound:	77 (0.3%)	24 (0.7%)	154 (4.6%)
Eluate:	42 (0.2%)	67 (2.1%)	75 (2.2%)

[a] Protein and fluorimetric enzyme assays performed as described in Materials and Methods. Fluorescent units/min were calculated using the equation:

$$\frac{\textit{Fluorescent units/ml} \times \textit{volume (ml)}}{\textit{Assay time (mins)}}$$

[b] Indicates data for the total amount of extract protein and esterase activity processed (following ultrasonication, detergent extraction, dialysis and centrifugation of the original serum/cellular material)

Table 4.2b: Summary of column performance for ComEst fractionation [a]

Column/Fraction	Protein (mg)	Acetate Esterase (U/min x10^{-3})
Pre-Q Sepharose [b]		
(Cell extract)	8107 (100%)	5106 (100%)
Q-Sepharose		
Unbound:	2100 (25.9%)	2000 (39.2%)
1.0M NaCl Eluate:	3583 (44.2%)	1969 (38.6%)
S-Sepharose		
Unbound:	1610 (19.9%)	200 (3.92%)
0.25M NaCl Eluate:	239 (2.95%)	1000 (19.6%)
1.0M NaCl Eluate:	120 (1.48%)	40 (0.78%)
Benzamidine/Arginine Series		
Unbound:	219 (2.70%)	610 (11.9%)
Eluate:	93 (1.15%)	20 (0.39%)
Lectin Series		
Unbound:	104 (1.3%)	680 (13.3%)
Eluate:	54 (0.67%)	5 (0.10%)
Con-A		
Unbound:	136 (1.68%)	110 (2.15%)
Eluate:	9 (0.11%)	210 (4.11%)

[a] Protein and fluorimetric enzyme assays performed as described in Materials and Methods. Fluorescent units/min were calculated using the equation:

$$\frac{\textit{Fluorescent units/ml} \times \textit{volume (ml)}}{\textit{Assay time (mins)}}$$

[b] Indicates data for the total amount of extract protein and esterase activity processed (following ultrasonication, detergent extraction, dialysis and centrifugation of the original serum/cellular material)

Table 4.3 Substrate specificities of purified MonEst

Substrate [a]	Assay Procedure	Specificity	Hydrolysis by MonEst
Acetate-4MB	Fluorimetry	Esterase	Yes
Butyrate-4MB	Fluorimetry	Esterase	Yes
Alpha-naphthyl acetate	UV Spectrophotometry	Esterase	Yes
Alpha-naphthyl propionate	UV Spectrophotometry	Esterase	Yes
Alpha-naphthyl butyrate	UV Spectrophotometry	Esterase	Yes
Glu-NMec	Fluorimetry	Aminopeptidase-A	No
Ala-NMec	Fluorimetry	Aminopeptidase-N	No
Gly-Pro-HyPro-	HPLC	Aminopeptidase-P	No
Glu-Trp-	HPLC	Aminopeptidase-W	No
Hip-His-Leu-	HPLC	Angiotensin converting enzyme	No
Hip-Lys-	HPLC	Carboxypeptidase-M	No
Gly-Pro-NMec	Fluorimetry	Dipeptidylpeptidase IV	No
Tyr-D-Ala-Gly-Phe-Leu-	HPLC	Endopeptidase-24.11	No
Insulin chain	Radiometric	Endopeptidase-24.11	No
Gly-D-Phe-	HPLC	Microsomal dipeptidase	No
L-γ-glutamyl-p-nitroaniline	Spectrophotometric	γ-Glutamyltransferase	No

[a] Esterase fluorimetric substrates; 4MB, 7-hydroxy-4-methylcoumarin (4-methylumbelliferone): Peptidase substrates; NMec, 7-amido-4-methylcoumarin.

Table 4.4 Kinetic studies to determine Vmax and Km values for myeloid esterase isoenzyme species [a]

Substrate	ComEst		MonEst	
	Vmax ($n\ mol.min^{-1}$)	Km (mM)	Vmax ($n\ mol.min^{-1}$)	Km (mM)
Alpha-naphthyl acetate	5.6	0.20	5.2	0.19
Alpha-naphthyl propionate	1.3	0.24	7.2	0.11
Alpha-naphthyl butyrate	*	*	10.0	0.05

[a] Kinetic studies of the two esterase isoenzyme species with each substrate were carried out as detailed in Materials and Methods. Maximum rates of substrate hydrolysis (Vmax) and Michaelis-Menten constants (Km) were calculated using Lineweaver-Burk and Eadie-Hofstee plots; the results are shown as the mean of these two plots and are expressed as n $mol.min^{-1}$ and mM respectively for Vmax and Km.

* indicates no significant substrate hydrolysis

Table 4.5 Inhibition studies of purified monocyte-specific (MonEst) and 'common' (ComEst) myeloid esterase species [a]

Inhibitor	Concentration (mM)	ComEst (pI 7.3 to 7.9)	MonEst (pI 5.5 to 6.2)
2-Nitro-4-carboxyphenyl N,N-diphenyl-carbamate (NCDC)	2.0 x 10-3	81	90
Phenylmethylsulphonyl fluoride (PMSF)	0.2	84	20
3-4, Dichloroisocoumarin (DCIC)	5.0 x 10-3	89	3
Neostigmine (methyl sulphate)	20	99	99
Sodium flouride (NaF)	1.0	52	5
Sodium dodecyl sulphate (SDS)	1.0 (%)	4	5
Sodium taurocholate	1.0	86	85
Acetylcholine iodide	0.1	91	99
Eserine	0.1	93	99
p-hydroxymercuribenzoic acid (HMBA)	1.0	96	100
Iodoacetamide	2.0	73	100
N-Tosyl-L-phenylalanine chloromethyl ketone (TPCK)	0.1	92	26
N-Alpha-p-tosyl-L-lysine chloromethyl ketone (TLCK)	0.1	100	84
Diethyl pyrocarbonate (DEPC)	1.0	16	11
1-10, Phenanthroline	0.1	94	113
Ethylenediaminetetraacetic acid (EDTA)	1.0	100	91
Urea	2.5 x 103	94	88

[a] Esterase activity was examined by UV spectrophotometric assay, using 0.5mM alpha-naphthyl acetate as substrate, in the presence and absence of inhibitors at the final concentrations stated. Esterase isoenzyme fractions were pre-incubated with each of the inhibitors for a constant period of five mins prior to assay. The results indicate the observed enzymic activity, where 100% represents the activity obtained in the absence of inhibitor.

Table 4.6 Mechanisms of esterase inhibition [a]

Inhibitor [b]	ComEst Species	MonEst Species	Analytical Methods
1% SDS	reversible	irreversible	Gel filtration
20mM NaF	reversible	reversible	Gel filtration and IEF
5.0mM PMSF	resistant	irreversible	IEF
0.05mM DCIC	resistant	irreversible	IEF
5.0mM DEPC	reversible	reversible	IEF
5.0mM TPCK	resistant	irreversible	IEF

[a] Purified monocyte-specific (MonEst) and 'common' myeloid (ComEst) esterase isoenzyme fractions incubated with various inhibitors and then analysed following gel filtration chromatography or isoelectric focusing (IEF) to assess whether inhibition was reversible or irreversible.

[b] Inhibitors: SDS, sodium dodecyl sulphate; NaF, sodium fluoride; PMSF, phenylmethylsulphonyl fluoride; DCIC, 3-4 dichloroisocoumarin; DEPC, diethyl pyrocarbonate; TPCK, N-tosyl-L-phenylalanine chloromethyl ketone.

Table 4.7 NaF, PMSF and DEPC inhibitor kinetic studies of 'common' myeloid (ComEst) and monocyte-specific (MonEst) esterase isoenzyme species [a]

Inhibitor	ComEst Species		MonEst Species	
	Inhibition type	K_i (mM)	Inhibition type	K_i/K_I (mM)
NaF: (0.0 to 1.0mM)	noncompetitive	1.28	noncompetitive	0.01
PMSF: (0.0 to 0.2mM)	no inhibition	-	mixed-inhibition [b]	
			(noncompetitive	0.01)
			(competitive	0.11)
DEPC: (0.0 to 0.1mM)	noncompetitive	0.05	noncompetitive	0.05

[a] Alpha-naphthyl acetate esterase activities were determined in the presence of varying concentrations of sodium fluoride (NaF), phenylmethylsulphonyl fluoride (PMSF) or diethyl pyrocarbonate (DEPC) by UV spectrophotometric assay. The type of inhibition was determined by Lineweaver-Burk plots and the K_i (or K_I for competitive inhibition) was estimated using secondary plots of 1/Vmax' against inhibitor concentration.

[b] At concentrations exceeding 0.5mM, PMSF was shown to cause irreversible inhibition of MonEst; K_i and K_I values for the noncompetitive and competitive components respectively are given separately.

Table 4.8 N-terminus amino acid sequence of purified human monocyte-specific esterase (MonEst) and comparisons with sequences obtained for carboxylesterases from other human and animal sources [a]

Human MonEst:	Residues 1-15	Gly His Pro Ser Ser Pro Pro Val Val Asp Thr Thr His Gly Lys
Human alveolar macrophage esterase:	Residues 1-15	Gly His Pro Ser Ser Pro Pro Val Val Asp Thr Val His Gly Lys
Rat liver carboxylesterase precursor E1:	Residues 19-32	His Pro Ser Ser Pro Pro Val Val Asp Thr Thr Lys Gly Lys
Rabbit liver microsomal esterase type I:	Residues 1-14	His Pro Ser Ala Pro Pro Val Val Asp Thr Val Lys Gly Lys
Human MonEst:	Residues 16-30	Val Leu Gly Lys Phe Val Ser Leu Glu Gly Phe Ala Gln Pro Val
Human alveolar macrophage esterase:	Residues 16-30	Val Leu Gly Lys Phe Val Ser Leu Glu Gly Phe Ala Gln Pro Val
Rat liver carboxylesterase precursor E1:	Residues 33-47	Val Leu Gly Lys Tyr Val Ser Leu Glu Gly Phe Thr Gln Pro Val
Rabbit liver microsomal esterase type I:	Residues 15-29	Val Leu Gly Lys Phe Val Ser Leu Glu Gly Phe Ala Gln Pro Val
Human MonEst:	Residues 31-39	Ala Ile Phe Leu xxx Ile Pro Phe Ala
Human alveolar macrophage esterase:	Residues 31-39	Ala Ile Phe Leu Gly Ile Pro Phe Ala
Rat liver carboxylesterase precursor E1:	Residues 48-56	Ala Val Phe Leu Gly Val Pro Phe Ala
Rabbit liver microsomal esterase type I:	Residues 30-38	Ala Val Phe Leu Gly Val Pro Phe Ala

[a] Amino acids underlined indicate substitutions compared to human MonEst.

Table 4.9 Active site amino acid sequence of an ox liver carboxylesterase and comparison with sequences from a number of other mammalian esterase and proteinase enzymes [a]

Enzyme	Amino acid sequence of the active site
Chymotrypsin A (EC 3.4.21.1)	Cys.Met.Gly.Asp.**Ser**.Gly.Gly.Pro.Leu.Val
Trypsin (EC 3.4.21.4)	Cys.Gln.Gly.Asp.**Ser**.Gly.Gly.Pro.Val.Val
Elastase (EC 3.4.21.11)	Cys.Gln.Gly.Asp.**Ser**.Gly.Gly.Pro.Leu.His
Thrombin (EC 3.4.21.5)	Cys.Gln.Gly.Asp.**Ser**.Gly.Gly.Pro.Phe.Val
Acetylcholinesterase (EC 3.1.1.7)	Glu. **Ser**.Ala
Cholinesterase (EC 3.1.1.8)	Phe.Gly.Glu. **Ser**.Ala.Gly.Ala.Ala.Ser
Carboxylesterase (ox liver; EC 3.1.1.1)	Gly.Glu. **Ser**.Ala.Gly.Ala.Glu.Ser

[a] Sequences quoted from Dayhoff et al., [1972].

CHAPTER FIVE

CONCLUDING DISCUSSION

Cytochemical studies are routinely used to supplement traditional morphological evaluations for the accurate identification and classification of leukaemic cells. In this context, the application of alpha-naphthyl acetate esterase (ANAE) cytochemistry is regarded as being particularly valuable for the delineation of monocytic/histiocytic cells and the subtyping of acute myeloid leukaemias (AML) [Scott, 1989; Li et al., 1989; Drexler et al., 1991a]. However, whilst the interpretation of ANAE staining patterns of normal haemopoietic elements is usually straightforward, atypical cytochemical reactivity of leukaemic cells is often difficult to interpret and may conflict with morphological and/or immunophenotypic impressions. Consequently, conventional ANAE cytochemistry in such cases is of limited value and a requirement for further discriminative analysis of ANAE isoenzymes is necessary. Early zymogram (PAGE and IEF) studies have shown that haemopoietic cells synthesise and express several ANAE isoenzyme components. With particular reference to myeloid cells, two distinct groups of ANAE isoenzymes have been documented; one which is commonly expressed by all myeloid cells (ComEst) and the other which is restricted to haemopoietic cells of monocytic lineage (MonEst) [reviewed in Scott & Drexler, 1989 and Drexler et al., 1991a, b]. The aims of this current study were initially to confirm and substantiate the previously reported relationships between normal and leukaemic myeloid cell cytochemical ANAE reactivity and the distribution of ANAE isoenzymes as defined by IEF with the second part of these investigations being designed to extend these 'baseline' studies by preparing purified ComEst and MonEst isoenzyme fractions in order to compare their molecular and biochemical characteristics. These latter studies were specifically undertaken to establish whether the two main myeloid esterase species represented variant forms of the same parent enzyme or, alternatively, were unrelated enzymes with a shared ability to hydrolyse simple non-physiological ester substrates.

To achieve the initial aims, esterase cytochemistries of a representative number of normal and leukaemic myeloid cells were examined and these were correlated with the qualitative expression of ANAE isoenzymes. For this, ANAE cytochemical reactions for a total of

309 acute myeloid leukaemias and 20 normal granulocyte/monocyte fractions were assessed. These investigations confirmed previous impressions [Flandrin & Daniel 1973; Li et al., 1973; Kulenkampff et al., 1977; Gordon & Hubbard 1978; Hayhoe & Quaglino 1980; Flandrin & Daniel 1981; Scott et al., 1987] that normal mature granulocytes and the blast cells of non-monocytic AML subtypes are typically ANAE negative (or show weak diffuse/granular staining) and that moderate to strong diffuse (M-type) ANAE reactions are restricted to normal and leukaemic cells developing along the monocyte/macrophage differentiation axis. Furthermore, zymogram (IEF) analyses of normal granulocyte and leukaemic AML-M1/M2 cell extracts consistently revealed the presence of ANAE isoforms (ComEst) within the pI range of 6.3 to 7.9. As the cellular concentration of ComEst components in these particular cell extracts was generally weak, it was apparent that the absence of detectable ANAE cytochemical staining of these cell types was a quantitative rather than qualitative phenomenon. This distinction is of some importance as a minor, but significant, proportion of acute myeloid leukaemias (AML-M1/2), as well as the abnormal promyelocytes of most hypergranular promyelocytic leukaemias, show relatively intense granular or focal ANAE reaction products. Although the presence of ANAE staining in these cases has been interpreted as being suggestive of 'mixed' or 'bilineal' granulocytic/monocytic differentiation [Tomonaga et al., 1985; Lemez, 1988], an analysis of ANAE isoenzyme distributions unequivocally showed that all such cases expressed ComEst isoforms only. Consequently, these findings indicate that the atypical cytochemical patterns of these particular leukaemias is due to a leukaemia-associated 'over-expression' of ComEst isoforms rather than resulting from the additional presence of MonEst or other ANAE isoenzyme species.

In contrast, normal monocyte fractions consistently showed moderate to strong diffuse cytochemical staining which correlated with the presence of high concentrations of MonEst ANAE isoforms within a narrow pI range of 5.5 to 6.2, in addition to the occurrence of myeloid cell ComEst components. However, additional analyses revealed that up to 5% of morphologically and immunophenotypically ($CD14^+$) defined normal

monocytes lacked significant esterase activity. For comparison, high proportions of blasts in 62% of the acute myelomonocytic (AMML-M4) and 35% of the acute monocytic (AMoL-M5) leukaemias examined also showed no apparent ANAE staining. When these cytochemical impressions were related to IEF zymograms, it was found that all cytochemically unreactive AMML-M4 and AMoL-M5 cases were characterised by a complete absence of MonEst components even though expression of ComEst isoforms in these cases was still observed.

Having confirmed the lineage association of the two main myeloid esterase isoenzyme species, and prior to the analysis of purified ComEst/MonEst forms, this study also undertook preliminary analyses (using representative unfractionated cell extracts) of their substrate specificities and inhibitor sensitivities. Histochemical staining of esterase isoenzymes following IEF separation, using substrates of increasing acyl chain length, provided further definitive insights into the relative substrate affinities of the two myeloid cell esterase species. These investigations showed that MonEst isoforms were capable of hydrolysing acetate, propionate and butyrate esters whereas ComEst components showed a decreasing substrate affinity with increasing acyl chain length (with no significant hydrolysis of alpha-naphthyl butyrate). These qualitative substrate studies therefore, confirmed the above interpellation that atypical ANAE cytochemistry of hypergranular APL-M3 cases resulted as a consequence of ComEst over-expression, in that the leukaemic promyelocytes of such cases were unreactive or only weakly reactive with butyrate esters.

Further analyses of unfractionated cell extracts for the inhibitory effects of various inorganic and organic compounds on MonEst and ComEst activities revealed that two compounds, sodium fluoride (NaF) and phenylmethylsulphonyl fluoride (PMSF), significantly inhibited MonEst but not ComEst. In agreement with previous studies [Scott et al., 1984a; Drexler et al., 1991a], it was additionally found that the inhibition of MonEst by NaF was a relative rather than specific phenomenon, as higher (>1.0mg/ml)

NaF concentrations also inhibited ComEst. However in contrast to previous observations [Cohn et al., 1987], PMSF at various concentrations specifically inactivated MonEst but had little effect on ComEst activity. Three other inhibitors examined (Neostigmine, eserine and p-hydroxymercuribenzoic acid; HMBA) showed no significant inhibition of either MonEst or ComEst, suggesting that these myeloid cell esterases were not arylesterases (HMBA-sensitive), acetylcholinesterases or cholinesterases (neostigmine and/or eserine-sensitive). From these preliminary substrate and inhibitor studies, it was concluded that MonEst and ComEst were likely to be unrelated esterolytic enzymes; an interpretation which provided sufficient impetus for their purification and further characterisation.

Using representative leukaemic cell extracts, the next part of the study evaluated the chromatographic characteristics of ComEst and MonEst components with a wide range of gel types in order to establish practical schemes for a scaled-up purification of ComEst and MonEst components. One of the most important observations from these initial analyses was that the cellular extracts contained, in addition to the histochemically demonstrable ComEst and MonEst species, a large number of esterolytic enzymes (including trypsin and chymotrypsin-like enzymes), which were capable of hydrolysing simple fluorimetric and naphtholic esters. Although further studies of these particular enzymes were not undertaken, as they were not the subject of these present studies, the recognition of their presence effectively meant that (a) the column procedures designed for the final chromatographic purification of the ComEst and MonEst species had to ensure their complete removal, (b) that the monitoring of column efficiencies and their ability to successfully resolve ComEst and MonEst could only be reliably achieved by histochemical zymogram analysis, and (c) that the conventional expression of enzyme purification and column yields (i.e. reporting enzyme activities relative to protein concentration) would be meaningless. This latter point is emphasised in particular by the investigations which examined correlations between total cell extract esterase activities (as defined by standardised fluorimetric assays) and esterase isoenzyme patterns. These

studies clearly indicated that there was no relationship between the quantitative level of esterase activity and the concentrations of ComEst/MonEst isoenzymes defined histochemically, therefore strongly suggesting the existence of additional esterolytic species.

Chromatographic evaluations additionally provided evidence of qualitative differences in the molecular characteristics of the ComEst and MonEst species. For example, the major ComEst components did not bind to anion exchangers with quaternary amino-ethyl groups whereas MonEst did, suggesting a possible conformational difference between the native structures of these two myeloid esterase types. In addition, preliminary lectin binding studies showed that whereas MonEst bound to *Concanavalin A* (Con-A), with no apparent affinity for any of the other lectins examined, ComEst showed partial affinities for *Pisum sativum agglutinin* (PSA) and *Lens culinaris agglutinin* (LCA) in addition to strong Con-A binding. When interpreted in the context of the differential sugar specificities of these lectins, these observations indicate significant differences in the carbohydrate structure of the two myeloid esterases. The pattern of lectin binding for MonEst was considered compatible with the presence of N-glycosidically linked oligomannosidic-type glycan(s) whereas, in contrast, ComEst showed a lectin binding pattern that was interpreted as being consistent with a mixture of (80-90%) biantennary N-acetyllactosamine-type (complex-) asparagine-linked glycan(s) and (10-20%) α-1,6-fucosylated biantennary N-acetyllactosamine-type glycan(s). In addition to revealing significant molecular differences between ComEst and MonEst, these investigations also provided a basis for constructing schemes for their chromatographic purification. Indeed, it was further apparent that because of the diversity of co-existing esterolytic enzymes in cell extracts, a wide range of chromatographic procedures would be required. Accordingly, the final chromatographic sequence was designed to maximise the removal of unwanted proteins and esterolytic enzymes and to minimise the loss of either the ComEst or MonEst species.

The final part of the study successfully achieved the purification of MonEst to homogeneity and ComEst to a highly enriched state using chromatographic procedures devised on the basis of results obtained in the preliminary evaluations. Although these procedures were extensive (due to the highly complex nature of the cell extracts used), compared to the relatively simple fractionation steps previously described for the purification of monocyte-associated esterases [Lam et al., 1978; Yourno, 1986; Saboori & Newcombe, 1990], the success of the purification schemes was, to some extent, evidenced by the isolation of approximately 40mg of homogeneous MonEst and 1.5mg of enriched ComEst. Isoelectric focusing of purified MonEst and partially purified ComEst revealed that both enzymes had been successfully resolved and showed no cross-contamination. Furthermore, the isoenzyme distributions and pI values of these purified esterases were identical to those of the unprocessed material, thus confirming that modifications in their charge characteristics had not occurred as a result of the purification procedures. Accepting the difficulty of estimating non-specific losses, the results of these studies also suggested that, when related to the original total esterase activities of the serum/cell extract used for the purification of MonEst, only 2.1% of the initial total acetate esterase and 2.2% of the butyrate esterase activities corresponded to the recovered purified MonEst enzyme. For comparison, the purified ComEst fraction constituted 4.1% of the total original cell extract acetate esterase activity. While there is little doubt that the extensive purification schemes used in this study led to some non-specific loss of the enzymes of interest, the results nevertheless indicate that a high proportion of the original esterolytic activity present in the starting materials was not of ComEst or MonEst type.

The subsequent investigations of purified material considerably clarified the molecular and biochemical characteristics of the two myeloid esterase species. Molecular weight estimations of purified MonEst indicated a relative molecular weight (Mr) in its native state of 157 kDa which, following SDS treatment, dissociated to an enzymatically inactive 63 kDa molecule. In contrast, ComEst comprised an active monomeric species

which had an apparent molecular weight of 53 kDa when determined by gel filtration chromatography and an Mr of 68 kDa by SDS-PAGE. Accepting the limitations of gel filtration chromatography for the accurate assessment of Mr, these findings suggest a possible monomeric and trimeric configuration for MonEst, an interpretation which is in accordance with previously reported observations [Lam et al., 1978; Yourno, 1986; Yourno et al., 1986; Cohn et al., 1987; Saboori & Newcombe, 1990], while the ComEst species showed no apparent subunit association as adjudged by reduced SDS-PAGE. In this context, it is important to emphasise that a trimeric configuration for MonEst is now widely accepted [reviewed in Drexler et al., 1991a] even though a recent communication [Zschunke et al., 1992] concluded from computer modelling studies that native MonEst is a dimeric membrane bound ectoenzyme. These authors essentially based their contention on the observation that the amino acid sequence of MonEst protein contains five cysteine residues, four of which are proposed to form internal stabilising disulphide bonds and the other forming an intramolecular disulphide bond (i.e. constituting the dimeric configuration). However, the probable existence of disulphide interactions is not supported by this current study, which consistently showed that MonEst is completely dissociated by SDS under non-reducing conditions and that under reducing conditions, there is no further change in Mr. Furthermore, investigations to ascertain the nature of MonEst subunit association, strongly suggested that individual subunits are associated through electrostatic interactions rather than covalent linkages.

Studies of chromatographically purified esterase species further confirmed the preliminary conclusions that the pattern of lectin binding for MonEst was compatible with the presence of N-glycosidically linked oligomannosidic-type glycan(s), whilst ComEst contained fucosylated and non-fucosylated biantennary N-acetyllactosamine-type (complex-type) asparagine-linked glycan(s). This was achieved by treating purified MonEst with endoglycosidase H, which acts on oligosaccharide units of the high-mannose type, and abrogating its binding to Con-A. Furthermore, although deglycosylation of MonEst decreased the Mr from 63 kDa to 60.1 kDa, this treatment had

no significant effect on the isoelectric points (pI) of its constituent isoenzymes. This latter observation is particularly important as it excludes the possibility that the carbohydrate components of this enzyme contribute to the pI and confirms previous studies [Scott et al., 1984c] that treatment of MonEst with neuraminidase also has no discernible effect on pI (i.e. consistent with the lack of terminal N-acetyl neuraminic acid groups). In a similar way, the predicted bianttenary glycan stricture of ComEst was further substantiated when treatment with endo H failed to affect its binding to Con-A.

In addition to their molecular differences, kinetic studies revealed distinct substrate reactivity patterns for ComEst and MonEst. K_m and V_{max} estimations showed that the ComEst species had higher affinities and turnover rates for the shorter acyl chain esters (acetate > propionate), with negligible turnover of the butyrate ester. In contrast, the MonEst species showed considerably higher affinities and turnover rates for the longer acyl chain esters (butyrate > propionate > acetate). Purified MonEst and ComEst preparations were also extensively investigated to determine their inhibitor sensitivities with respect to types of inhibition (competitive, uncompetitive, non-competitive, reversible or irreversible). For this, representative carboxyl ester hydrolase and protease inhibitors were used, firstly to elucidate differences between the MonEst and ComEst enzymes, and secondly to provide further insights into the nature of their active sites. Of the 17 inhibitors examined, only two (SDS; and diethyl pyrocarbonate, DEPC) induced significant (>50%) inhibition of the ComEst species, with a further two compounds (NaF; and iodoacetamide) exerting partial (20-50%) inhibition. For comparison, a total of six inhibitors (NaF; PMSF; DEPC; SDS; 3-4 dichloroisocoumarin, DCIC; and N-tosyl-L-phenylalanine chloromethyl ketone, TPCK) caused significant (>50%) inhibition of the monocyte-specific MonEst species. NaF, SDS and DEPC were therefore the only compounds to induce inhibition of both esterase species. Inhibition of both species by NaF was shown (for the first time) to be noncompetitive, with MonEst having a 130 fold increased sensitivity to this compound compared to ComEst. Another significant difference between ComEst and MonEst revealed by these studies, was that MonEst was

irreversibly inhibited by SDS whilst the inhibition of ComEst activity was found to be reversible. Additionally, whereas the requirement of serine and histidine residues was implicated for MonEst activity (i.e. because of irreversible inhibition by PMSF/DCIC and TPCK), their requirement was not evident for ComEst. Together, the substrate and inhibitor studies provide sufficient evidence to conclude that MonEst shows characteristics consistent with its classification as a carboxylesterase (EC 3.1.1.1) while ComEst displays characteristics more in keeping with its classification as an acetylesterase (EC 3.1.1.6). The molecular and biochemical characteristics of MonEst and ComEst are summarised in Table 5.1. Collectively, these observations indicate that these two myeloid esterase species are unrelated enzymes which share a common ability to hydrolyse simple non-physiological ester substrates.

Because the MonEst species had been purified to homogeneity, this study was also able to ascertain its N-terminal amino acid sequence. As the complete amino acid sequences of monocyte/macrophage-associated carboxylesterase species have recently been reported by two independent groups, it was possible to make comparisons with the results obtained for purified MonEst. The first of these (in which the initial 47 amino acids at the N-terminus were not determined [Zschunke et al., 1991]) was obtained by analysing a cDNA sequence of purified carboxylesterases from the U937 cell line; the second was obtained from limited amino acid sequencing of alveolar macrophage esterase and subsequent screening of the corresponding cDNA library [Munger et al., 1991]. These comparisons revealed a high degree of homology between MonEst and carboxylesterase from human alveolar macrophages [Munger et al., 1991] and the U937 cell line [Zschunke et al., 1991]. Furthermore, extensive literature and data base searches also indicated significant sequence homology with human liver esterase [Ozols, 1989; Riddles et al., 1991; Long et al., 1991], and carboxylesterases from at least two other non-human sources [Korza & Ozols, 1988; Long et al., 1988; Takagi et al., 1988; Robbi et al., 1990]. Consequently, the similarities in esterase cytochemistries and their undoubted ontogenetic relationships, suggest that the purified MonEst analysed in this current study

is very similar if not identical (not withstanding possible minor amino acid substitutions, which may reflect molecular polymorphism) to alveolar macrophage and U937 carboxylesterases.

Amino acid sequence data is frequently valuable in determining the targeting of newly synthesised proteins. This is a particularly useful strategy when assessing whether synthesised protein is retained within the cytoplasm, transported to specific subcellular organelles (e.g. mitochondria, endoplasmic reticulum, Golgi apparatus, lysosomes, plasma membrane or other cellular membranes), or secreted from the cell. In this respect, the sequence data presented by Munger and colleagues [1991] is informative in that it represents the complete gene product sequence prior to co- or post-translational protein modification, whereas the N-terminus sequence derived in this current study effectively represents that of the post-translationally modified and functionally active protein. The presence of a transient 16-17 residue N-terminus signal peptide [Munger et al., 1991], together with the observation that MonEst is N-glycosylated, strongly suggests that its biosynthesis occurs within the endoplasmic reticulum (ER). Furthermore, the presence of His-Ile-Glu-Leu (HIEL) COOH-terminal tetrapeptide [Munger et al., 1991; Zschunke et al., 1991] on MonEst protein, which is now widely accepted as being analogous to the Lys-Asp-Glu-Leu (KDEL) ER retention sequence [Andres et al., 1990; Munger et al., 1991; Haugejorden et al., 1991; Robbi & Beaufay, 1991] and, to a lesser extent, the observation that its glycosidic structure remains as a high mannose type and is not further modified, indicates that MonEst could indeed be retained in the ER. This view is further supported by the findings of Robbi and Beaufay [1991], who reviewed the COOH terminal sequences of several wild-type and mutant liver carboxylesterase species and concluded that the presence of a KDEL-like carboxyl terminal sequence (including HIEL) is strongly indicative of protein retention in the ER. However, Zschunke and colleagues [1991, 1992] postulate from hydropathy calculations and computer modelling of their amino acid sequence that monocyte esterase is an integral membrane protein. This observation is partially substantiated by Munger and

colleagues [1991], who report that macrophage carboxylesterase is insoluble in the absence of solubilising agents (a view not shared by either this current study or that of Zschunke et al., [1991]). However, these authors also suggest that macrophage carboxylesterase may be a secretory protein, but these conclusions appear to contradict what is currently understood about proteins expressing KDEL-like carboxyl terminal tetrapeptides, which are generally soluble and rarely secreted [Andres et al., 1990; Haugejorden et al., 1991; Robbi & Beaufay, 1991]. It is however important to note that the degree of protein retention within the ER, whilst requiring the presence of a retention signal, is also influenced by the remaining sequence. Consequently, although considered unlikely it is nevertheless feasible that despite expressing the retention tetrapeptide, the remaining sequence of MonEst may not be conducive to its retention in the ER. Alternatively, as suggested in a recent report [Zschunke et al., 1992], MonEst may be localised within the ER in resting monocytes/macrophages but, following activation, it may be translocated to the plasma membrane. In this context, it is pertinent to note that these present studies found that both the ComEst and MonEst esterase species remained soluble in the absence of detergent throughout their purification, and that virtually all detectable ComEst and MonEst activity could be extracted from cell fractions without the inclusion of detergents in the extraction buffers. These observations, in conjunction with the information obtained from amino acid sequence analysis, indicate that MonEst is essentially soluble and not a peripheral or intrinsic membrane protein, thus contradicting the widely held belief that MonEst is a plasma membrane ectoenzyme [Bozdech & Bainton, 1977, 1981; Monahan et al., 1981; Zschunke et al., 1991, 1992]. However, although this matter remains unresolved, it is nevertheless clear that determination of the subcellular localisation of MonEst will have fundamental implications with regards to elucidating its physiological function(s).

With regards to origin (genetic or post-translational) of esterase polymorphism, Peters [1982] described a total of 17 different structural genes which control the expression of murine esterase enzymes. Each locus was reported to contain a minimum of two

different alleles, and clusters of esterases encoded by these multiple gene loci were noted to display differences in tissue distribution and subcellular locations. In a further study, Coates & Cortner [1986] described an alpha-naphthyl butyrate esterase (designated isoform B3) of human mononuclear leucocytes which was expressed as two allelic variants with differing molecular and catalytic properties. These authors concluded that whilst the B3 isoform exists as an active trimer of identical subunits, its allelic variant (designated isoform $B3_2$) existed as an active monomer; therefore the primary phenotypic difference between the two forms appears to result from an inability of the $B3_2$ subunits to form trimers. Although the genetic mechanisms which regulate the synthesis and expression of the two myeloid esterase enzymes are unknown, one suggestion [Yourno et al., 1984] has been that monocyte-specific MonEst isoenzymes may be derived from pre-formed esterases with a higher pI (ComEst). This concept is based on the assumption that the ComEst and MonEst species are related at the genetic level and that the synthesis of MonEst results from post-translational processing of the ComEst protein, following specific commitment to monocytic differentiation. However, there has been no convincing scientific evidence to support this view and, indeed immunological studies, using rabbit polyclonal antisera with apparent specificity against MonEst, clearly indicate antigenic differences between MonEst and ComEst isoenzyme forms [Radzun et al., 1981; Cohn et al., 1987]. An alternative view [Yourno & Mastropaolo, 1981] is that there are distinct gene systems encoding ComEst and MonEst proteins. In this respect, it is considered that the expression of ComEst and MonEst may be dependant on the differential activation and suppression of distinct esterase encoding-genes (i.e. gene(s) coding for ComEst protein are activated during both granulocytic and monocytic differentiation whilst MonEst gene(s) are activated following specific monocytic commitment or, alternatively, are suppressed during granulocytic differentiation).

When considering the origin of the individual charge (pI) isoforms of ComEst and MonEst enzymes, evidence of a multiple gene origin of MonEst isoforms comes inadvertently from a recent report [Zschunke et al., 1991], which suggested that

hybridisation of digested genomic DNA with a 151-bp 5'-terminus cDNA fragment of monocyte-associated esterase resulted in a pattern of several bands on Northern blots. The significance of these data, which was surprisingly not considered by the authors, indicate that if MonEst isoforms were indeed encoded by a single gene, then the DNA sequence of the transcribed gene would comprise several periodic repeating bases with >80% homology for the 151-bp 5'-terminus fragment. As this is not readily apparent from examination of the cDNA sequence provided by these authors, an alternative view is that the observed hybridisation pattern represents recognition of several related genes from a multigene family. These findings therefore suggest that MonEst polymorphism may originate at a genetic level (i.e. true isoenzymes) through differences in constituent amino acid residues or their sequence. Primary modifications (e.g. substitution of basic amino acids with acidic residues) of this nature could also account for their minor charge differences, with no discernible effect on molecular size, and could also explain the apparent charge association of MonEst monomeric subunits to form active trimers (e.g. electrostatic attraction between amino acid residues with opposite polarities).

As would be expected from the diversity of esterase species, there appears to be a large degree of confusion regarding the physiological role of esterases. Some evidence has been presented [Junge & Krisch, 1975] suggesting that carboxylesterases may be associated with the hydrolysis and detoxification of xenobiotic substrates. In this context, it is perhaps relevant that the MonEst purified in this current study showed some N-terminus sequence homology with human cytochrome P450 and furthermore, as the cytochrome P450 cascade of enzymes is essentially localised in the smooth ER of hepatocytes, this lends some support to the possible ER localisation of MonEst in monocytes/macrophages. The biological role of esterases has also been associated with the hydrolysis of arylamine carcinogens [Lund-Pero et al., 1989], steroid esters (including sex steroid hormones) and glucocorticoids [Schottler & Krisch, 1974], although a recent report [Saboori & Newcombe, 1990] has shown that purified monocyte esterase itself does not hydrolyse these compounds. Other investigators have reported

hydrolysis of the xenobiotic compound triphenyl phosphate [Paxman et al., 1988] by monocyte-associated esterase. However, as these studies used whole cell preparations to investigate esterase function, it is quite possible that enzymes other than esterases could have catalysed the hydrolysis of these compounds, a view substantiated by Saboori & Newcombe [1990]. The results of this present study did however, additionally indicate that MonEst was incapable of hydrolysing aminopeptidase, angiotensin converting enzyme, carboxypeptidase, dipeptidylpeptidase, endopeptidase, microsomal dipeptidase, or glutamyltransferase substrates and therefore this particular enzyme is unlikely to be involved in primary protein modification processes. As with MonEst, little is known of the function of ComEst. This current study has shown that this esterase species is likely to be an acetylesterase, and ultrastructural cytochemistry [Monahan et al., 1981] further indicates that it is primarily localised within cytoplasmic lysosomes/granules. This conclusion is supported by conventional cytochemical observations that the increased ComEst activity of APL-M3 hypergranular promyelocytes parallels increased granulation in these cells, and the finding that focal staining in some AML blasts is associated with the presence of Auer rods which are known to be formed from neutrophlic azurophil granules. Primary functions associated with lysosomal enzymes include degradation of nucleic acids (acid RNase; acid DNase), carbohydrates (β-galactosidase; α-glucosidase; lysozyme), mucopolysaccharides (β-glucuronidase; lysozyme; hyaluronidase), and proteins (cathepsins; collagenase). Additional lysosomal enzymes include phosphatases, esterases and sulphatases. It is therefore considered quite likely that the function of ComEst is associated with the degradation of internalised (phagocytosed) esterified molecules or molecular structures of particle membranes (e.g. bacteria) following fusion of the lysosome with phagocytic vesicles. However, in the absence of known physiological substrate(s) the biological significance of ComEst remains elusive.

Compared to ComEst, there have been more intensive studies, largely because of its highly lineage-restricted nature, to investigate the functional role(s) of MonEst. Indeed, it has been speculated that MonEst protein may be involved in a diverse range of

physiological functions including pinocytosis [Cohn et al., 1987], facilitation of diapedesis and migration through tissues [Monahan et al., 1981], inflammation and growth regulation [Yourno et al., 1986], and detoxification and metabolic processing of drugs [Li et al., 1973; Oertel & Kastner 1984; Yourno 1986]. Involvement of MonEst in monocyte associated cytotoxicity has also been speculated [Oertel et al., 1985], although this contention was based on the rather tenuous assumption that reduced cytotoxicity results from specific inhibition of MonEst activity by bis-(4-nitrophenyl)-phosphate in an *in vitro* assay system measuring cytotoxic lysis of tumour target cells. However, this inhibitor is likely to have simultaneously inhibited other cellular enzymes, and therefore the direct effect of MonEst on tumour cell cytotoxicity remains unproven. Nevertheless, some circumstantial evidence that MonEst may play a role in tumour cell killing has been provided by a report [Markey et al., 1990] of a statistically significant correlation between hereditary MonEst deficiency and the occurrence of malignant neoplasia. These investigators have hypothesised that severe diminution in MonEst activity may, to some extent, predispose to certain malignancies or be associated with such a predisposition [Markey et al., 1987]. The major limitations to this conclusion however are (a) that the great majority of patients with haemopoietic and non-haemopoietic malignancies appear to have normal monocyte esterase activities; (b) there have been no subsequent reports of hereditary MonEst deficiency to confirm these impressions; and (c) monocyte esterase deficiency often occurs as a secondary (acquired) event in a wide range of haematological and non-haematological disorders (e.g. rheumatoid arthritis [Bell et al., 1992]). An alternative approach to this was recently provided by Newcombe [1992], who hypothesised that an absence or inhibition of MonEst (and perhaps esterases of other immune surveillance cells such as natural killer cells, lymphokine-activated killer cells, and cytotoxic T lymphocytes) may impair the immune and cytotoxic functions of such cells thereby allowing tumour development. This contention is primarily based on previous findings that the prevalence of lymphoproliferative disorders appears to be increased in workers exposed to organophosphorous compounds [Blair et al., 1985; La Vecchia et al., 1989; Alavanja et al., 1990; Hall & Rosenman, 1991] and the finding that

MonEst activity is irreversibly inhibited by a wide range of such compounds [Saboori et al., 1991]. However, Newcombe [1992] neglects to add the fact that many enzyme systems (including by definition all serine proteases) that are also present in immunocompetant cells, are almost certainly simultaneously inhibited when exposed to organophosphates. As such, these hypotheses remain highly speculative even though they clearly deserve further investigation.

In conclusion, the initial aims of this study were to undertake an extensive analysis of normal and leukaemic myeloid cell esterases in order to confirm the relationships between myeloid cell differentiation and esterase isoenzyme patterns, and to specifically analyse by chromatographic, molecular and kinetic techniques whether or not the esterase isoenzyme species that are expressed by granulocytic and monocytic cells represent different gene products or are variant post-translational forms derived from the same gene product. It is considered that the results of these investigations have comprehensively demonstrated the distinct nature of the ComEst and MonEst species and shown that these enzymes are almost certainly synthesised in granulocytic and monocytic myeloid cells as a result of differential gene activation following appropriate lineage commitment and differentiation.

CHAPTER 5

Table 5.1

Table 5.1 Summary of the molecular, biochemical, kinetic and inhibitor characteristics of the myeloid ComEst and MonEst esterase species

	ComEst	MonEst
Molecular Size (gel filtration):	53 kDa	157 kDa
Molecular Size (SDS-PAGE):	68 kDa	63 kDa 60 kDa (deglycosylated)
Molecular composition:	Monomer	Trimer
pI range of isoforms:	6.3-7.9	5.5-6.2
Glycan structure: [a]	Biantennary	High-mannose
Con-A binding following endo H: [b]	Yes	No
Substrate hydrolysis:		
alpha-naphthyl acetate	+++	+++
alpha-naphthyl propionate	+	+++
alpha-naphthyl butyrate	0	+++
Inhibitor studies: [c]		
20mM NaF	partial (reversible)	marked (reversible)
1% SDS	marked (reversible)	marked (irreversible)
5mM DEPC	marked (reversible)	marked (reversible)
5mM PMSF	resistant	marked (irreversible)
0.05mM DCIC	resistant	marked (irreversible)
5mM TPCK	resistant	marked (irreversible)

[a] Biantennary indicates a mixture of biantennary N-acetyllactosamine-type (80-90%) and α-1,6-fucosylated biantennary N-acetyllactosamine-type (10-20%) glycans; High-mannose indicates the presence of oligomannosidic-type glycans N-glycosidically linked to the protein.

[b] Indicates the effect of binding to Concanavalin-A following treatment with endoglycosidase H.

[c] NaF, sodium fluoride; SDS, sodium dodecyl sulphate; DEPC, diethyl pyrocarbonate; PMSF, phenylmethylsulphonyl fluoride; DCIC, 3-4, dichloroisocoumarin; TPCK, N-tosyl-L-phenylalanine chloromethyl ketone.

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