

"I grow old learning something new every day"

SOLON, 638 - 559 B.C.

Valerius Maximus

Book VIII

Chapter 7 Sect. 14

A
STUDY OF
CATECHOLAMINE METABOLITES OCCURRING
IN
NORMAL
AND
PATHOLOGICAL URINES

Thesis submitted for the D. Phil. Degree

University of York

by

Alan William Stott

Department of Biochemistry, Pathology Laboratory,
Scarborough Hospital, Scarborough, North Yorkshire

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A B S T R A C T

This thesis describes an investigation into the principal metabolites of the catecholamines dopamine, noradrenaline and adrenaline, present in urine samples from normal subjects in various circumstances and from patients suffering from various pathological conditions.

Methods have been developed for the quantitation of the phenolic acids, 4-hydroxy-3-methoxymandelic acid (HMMA) and 4-hydroxy-3-methoxyphenylacetic acid (HVA) and the phenolic amines, normetadrenaline (NMA) and metadrenaline (MA).

Applying these techniques, normal ranges of the metabolites output over twenty-four hours were established for babies, young children and adults. In addition, the rate of output of metabolites for normal adults and for healthy students under mental stress (University examinations) was determined on urines collected overnight, and that for healthy adults during hard prolonged exercise, was determined on pre- and post-exercise urine collections. Metabolite patterns emerge which are characteristic of these normal groups.

For example, when expressed relative to urinary creatinine the metabolite levels of babies are higher than those of young children. The metabolites of sleeping students show no significant variation in the short-term during a period of expected increased mental activity; there is some evidence, however, that their output of MA is higher compared with an older age group. Physical exercise causes marked increase in the urinary levels of metabolites in healthy adults but the relative levels of metabolites do not vary significantly over the period of the exercise.

Analysis of catecholamine metabolites made on urines from patients with sub-arachnoid haemorrhage, hypertension and catecholamine secreting tumours, show marked variations of the metabolite patterns when compared with an appropriate normal control group. For example, sub-arachnoid haemorrhage is associated with high urinary levels of normetadrenaline such that high sustained levels correspond with the poorest prognosis. Our patients with essential hypertension appear in general to have elevated overnight urinary levels of 4-hydroxy-3-methoxymandelic acid together with normal levels of normetadrenaline, in contrast to the high

normetadrenaline levels and variable levels of 4-hydroxy-3-methoxy mandelic acid associated with catecholamine secreting tumours.

On the assumption that raised urinary metabolite levels correspond with actual high plasma levels of the appropriate catecholamine, attempts are made to relate the aberrant patterns observed in metabolites in the urine in pathological conditions with the pathogenesis of the actual disease.

ABBREVIATIONS USED IN THESIS

- PA - phenylalanine
- TY - tyrosine, (4-hydroxyphenylalanine)
- DOPA - 3,4-dihydroxyphenylalanine
- DA - dopamine, 2-(3,4-dihydroxyphenethylamine)
- NA - noradrenaline, 2-(3,4-dihydroxyphenyl)-2-hydroxyethylamine
- A - adrenaline, 2-(3,4-dihydroxyphenyl)-2-hydroxy-N-methylethylamine
- 3MT - 3-methoxytyramine, 2-(4-hydroxy-3-methoxyphenethylamine)
- NMA - normetadrenaline, 2-(4-hydroxy-3-methoxyphenyl)-2-hydroxyethylamine
- MA - metadrenaline, 2-(4-hydroxy-3-methoxyphenyl)-2-hydroxy-N-methyl-ethylamine
- HVA - homovanillic acid, 4-hydroxy-3-methoxy-phenylacetic acid
- HMA - vanilmandelic acid, 4-hydroxy-3-methoxy-mandelic acid
- DOMA - 3,4-dihydroxymandelic acid
- HMPG - 1-4(-hydroxy-3 methoxyphenyl)ethane-1,2-diol
- COMT - catechol-O-methyltransferase
- MAO - monoamine oxidase

CHAPTER 1

INTRODUCTION

1.1 Historical Background

In 1856 a substance which gave a green colour with iron(III) chloride was detected in the human adrenal gland by Vulpian,¹ but it was not until 1901 that two workers^{2,3} independently isolated this material and determined its structure. The compound, which was shown to be the 'active principle' of the adrenal gland from its physiological properties, was named adrenaline.³

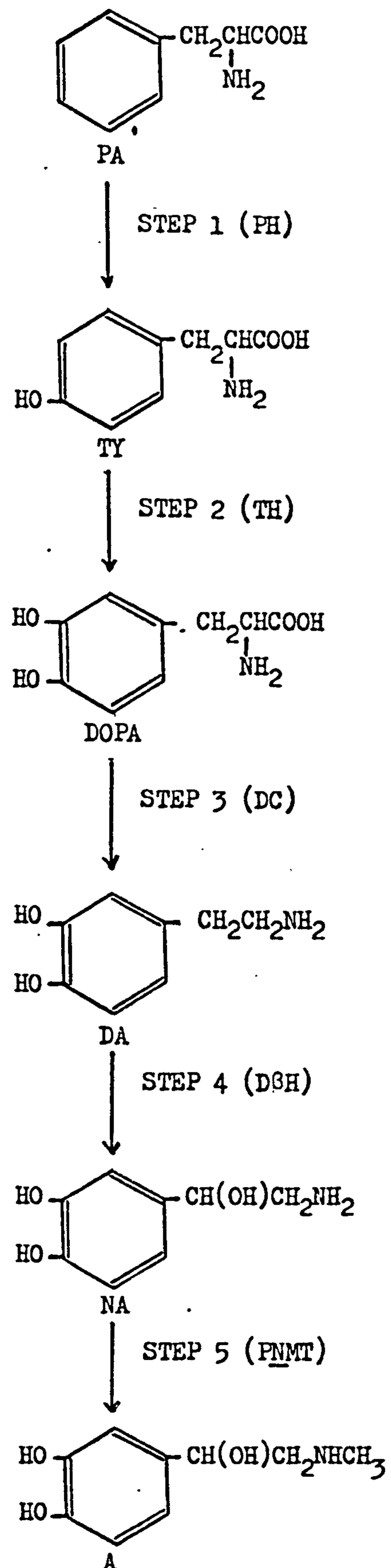
It had also been shown by Oliver⁴ that when an extract of the adrenal glands was injected into the arm of a child it produced a marked pressor effect and constricted the radial artery. Later, Oliver and Schafer⁵ found that the active substance is present in the adrenal medulla, but not in the cortex.

It was left to other workers^{6,7,8} to point out the similarity between the response to an injection of adrenal gland extract and the result of electrical stimulation of the sympathetic nerves to an organ and Elliott⁸ suggested that adrenaline is released at the ends of the sympathetic nerves when they are stimulated. However, this was such a radical idea that when Elliott published his work⁹ it was omitted from his discussion although he noted that 'adrenaline disappears in the tissues it excites'.

Noradrenaline which was first synthesised¹⁰ in 1904 was found to be a more powerful pressor agent¹¹ than adrenaline. Barger and Dale¹² reported that noradrenaline's physiological effects corresponded better with the action of the sympathetic nerves than did those of adrenaline, but they did not suggest it is noradrenaline and not adrenaline which is released at the sympathetic nerve endings, probably because at that time the existence of chemical transmitters in the autonomic system was unproven.^{8,9,13} It was not until 1921 that Loewi¹⁴ proved experimentally the existence of autonomic transmitters by stimulating the sympathetic nerves to a perfused frog's heart. He showed that the perfusate acquired the property of accelerating the rate of contraction of a second frog's heart, in the same way as an infusion of adrenaline.

Scheme 1 Synthesis of the Catecholamines

Dopamine, Noradrenaline and Adrenaline



Key - Scheme 1

	<u>Enzyme</u>
Step 1	Phenylalanine hydroxylase
2	Tyrosine hydroxylase
3	Dopa decarboxylase
4	Dopamine- β -hydroxylase
5	Phenylethanolamine <u>N</u> -methyltransferase

Similarly, results from the stimulation of the sympathetic nerves supplying the cat's liver liberated substances which had an 'adrenaline like' action when applied to other tissues, but the effects varied between different organs. This led Cannon and his co-workers to suggest that there might be two sympathetic transmitters operating, a Sympathin E¹⁵ producing excitation in the target organ receptors and Sympathin I¹⁶ which would inhibit the receptors.

The suggestion that Sympathin I could be adrenaline and Sympathin E could be noradrenaline¹⁷ was supported by further experimental work.¹⁸⁻²¹

In 1946 von Euler²² showed that extracts of adrenergic nerves and organs supplied by these nerves contained significant amounts of noradrenaline but only traces of adrenaline. Direct evidence that sympathetic nerves released noradrenaline was obtained by Peart²³ who found that after stimulation of the splanchnic nerves to the cat's liver, that the plasma separated from the blood obtained from the hepatic veins when applied to various pharmacological preparations, such as the isolated rat uterus and colon, caused them to relax. Comparing the activity of the extracts with that of adrenaline and noradrenaline on these preparations indicated that the main substance active in this plasma was noradrenaline. This work showed finally that noradrenaline is the main sympathetic nerve transmitter.

1.2 Formation of Adrenaline and Noradrenaline

The isolation of dopamine (3,4-dihydroxyphenethylamine) from mammalian urine and its formation in vivo by decarboxylation of DOPA (3,4-dihydroxyphenylalanine)²⁴ led Blaschko²⁵ to suggest that this could be one step in a series of reactions leading to the formation of noradrenaline and adrenaline from DOPA. Later work has confirmed this suggestion. Final proof that DOPA is the main precursor of adrenaline was obtained by Gurin and Delluva,²⁶ who incubated ¹⁴C-phenylalanine with rats' adrenal glands and subsequently isolated from this tissue ¹⁴C-adrenaline plus the suggested intermediates²⁵ (Scheme 1) Step 1.

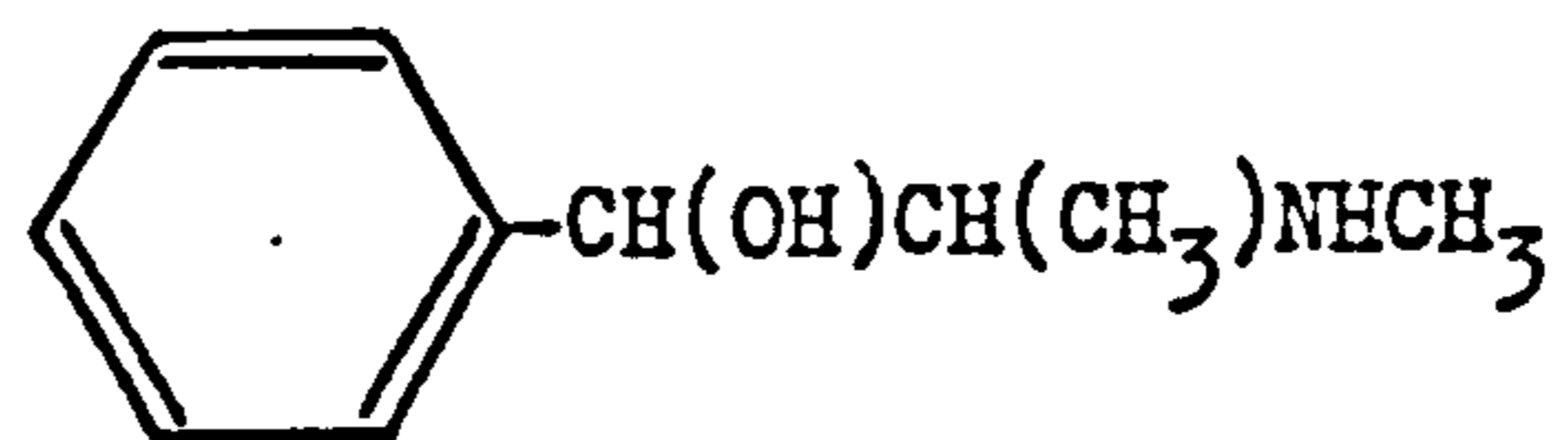
Radioactive tracers were further used to establish the particular steps involved at each stage in the biosynthesis of adrenaline and noradrenaline. Udenfriend and Wyngaarden²⁷ showed that ¹⁴C-tyrosine and ¹⁴C-DOPA are converted to ¹⁴C-adrenaline by the adrenal medulla and Demis et al,²⁸ showed that ¹⁴C-dopamine is converted to ¹⁴C-noradrenaline. The work of McGoodall and Kirshner^{29,30} also confirmed the in vivo pathway ¹⁴C-tyrosine - ¹⁴C-DOPA - ¹⁴C-dopamine - ¹⁴C-noradrenaline. Thus, the original suggestions of Blashko²⁵ in 1939 as to the biosyntheses of the catecholamines have been proved correct by the use of radio-isotopes.

However, the isolation of the enzymes responsible for each stage in the transformation was more difficult and has taken longer to achieve. Udenfriend and co-workers in 1954,³¹ showed that tyrosine could be converted to DOPA in a model system that required the presence of ascorbic acid, iron(II) and oxygen. Levitt et al³² and Udenfriend³³ finally showed that the enzyme responsible for converting tyrosine to DOPA was tyrosine hydroxylase (Scheme 1) Step 2. The rate of hydroxylation of L-tyrosine is slower than the subsequent enzymatic reactions and thus is the rate-limiting step in the formation of the catecholamines. The enzyme which requires the cofactor, 6-methyltetrahydropterin, for optimal activity is widely distributed in sympathetically innervated tissue and in the central nervous system (C.N.S.); also, it has been purified from the adrenal medulla.^{35,36,37}

The enzyme, DOPA-decarboxylase (Scheme 1) Step 3 was, as mentioned above, the first of the enzymes involved in catecholamine formation to be discovered and isolated. It decarboxylates only DOPA and 5-hydroxy-tryptophane³⁸ at significant rates and is widely distributed in mammalian tissues. It shows high activity in the kidney and has been purified from kidney and adrenal glands.^{39,40}

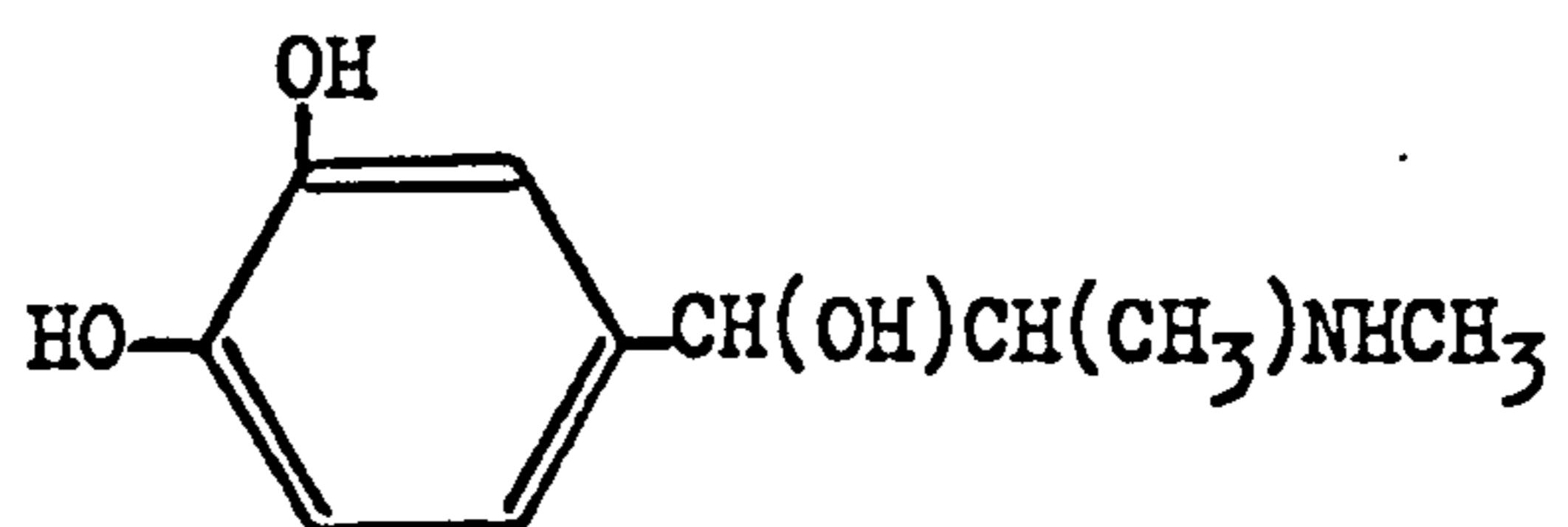
The fourth stage (Scheme 1) in the biosynthesis of the catecholamines, the β -hydroxylation⁴¹ of dopamine to produce noradrenaline, is catalysed by dopamine- β -hydroxylase, an enzyme that has a broad substrate specificity

Ephedrine



(I)

Corbasil (3,4-dihydroxyephedrine)



(II)

catalysing the hydroxylation of many phenethylamines to produce their respective β -phenylethanolamines. Dopamine- β -hydroxylase is present in high concentration in the chromaffin granules of the adrenal medulla,⁴²⁻⁴⁴ in the central nervous system, and most sympathetically innervated tissues. The enzyme contains copper^{45,46} and has been isolated in pure form from beef adrenal medulla.⁴⁶

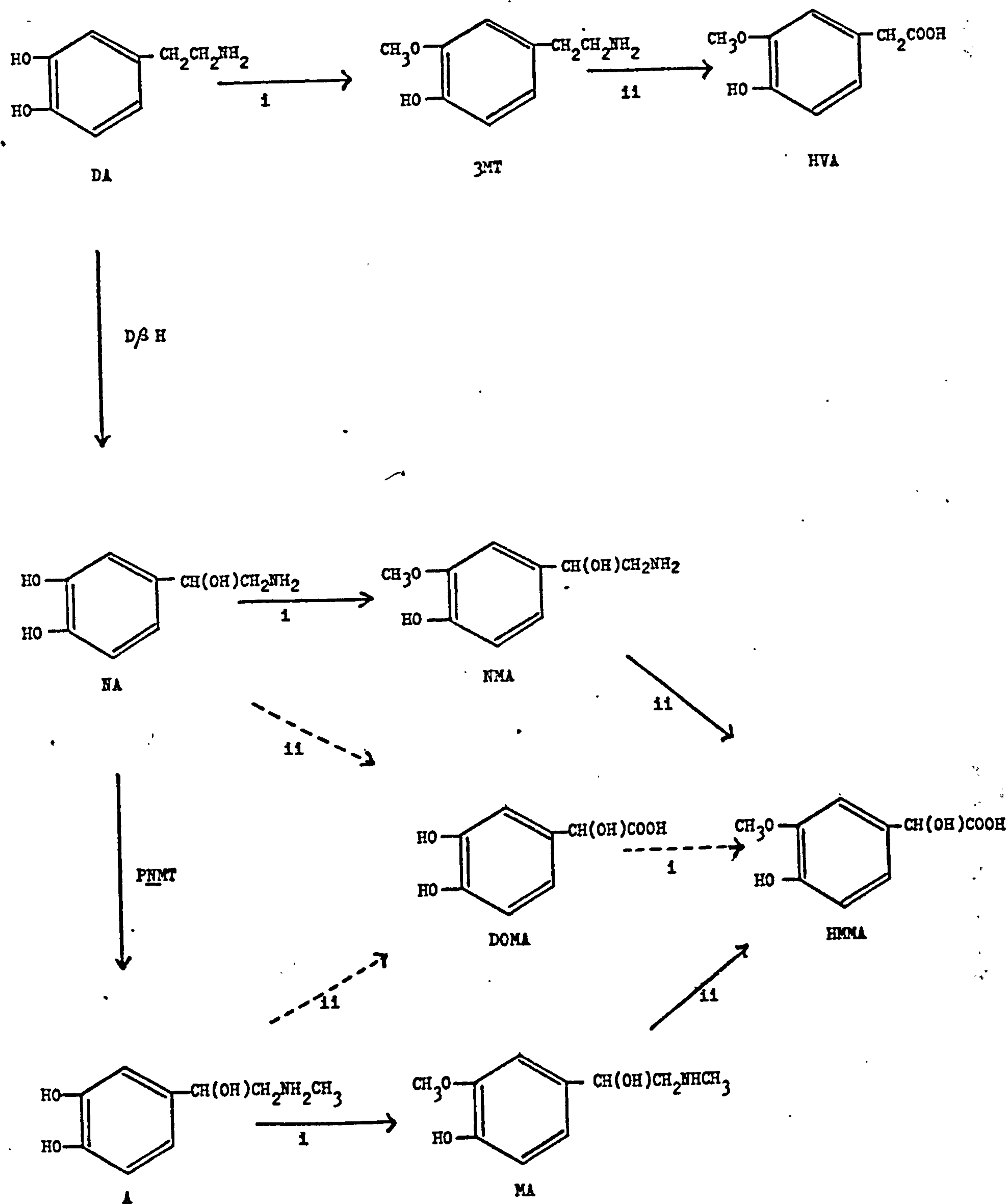
The final conversion in the biosynthetic chain (Scheme 1) Step 5, is brought about by the enzyme phenylethanolamine N-methyltransferase.^{47,48} This enzyme, which is soluble and specifically located in cells able to form adrenaline, methylates the amino function converting noradrenaline to adrenaline, principally in the adrenal medulla. It can also methylate adrenaline^{49,50} and various O-methyl derivatives (see later). The enzyme which requires S-adenosyl-L-methionine as methyl donor, but has no metal requirements, has been partially purified from beef adrenals.⁵¹

1.3 Metabolism of Adrenaline and Noradrenaline

In 1937, Blaschko and his colleagues⁵² showed that adrenaline could be oxidised and inactivated by an enzyme found in mammalian liver; this enzyme, monoamine oxidase (MAO), could also inactivate noradrenaline. Further data was obtained by Gaddum and Kwiatkowski in 1938⁵³ who noticed that ephedrine (I) sensitised various nerve preparations to NA. The effect was thought to arise from a blocking of the MAO in blood vessels and elsewhere by the ephedrine, resulting in the build-up of high levels of the neuro-hormones. However, when in 1955, von Euler and Zetterstrom⁵⁴ investigated the part played by MAO in inactivating the catecholamines in man, they gave intravenous injections of adrenaline, noradrenaline and corbasil (II) (3,4-dihydroxyephedrine) to normal subjects and found all three amines were inactivated to the same extent although corbasil is not subject to oxidation by MAO. From these results they suggested that circulating catecholamines were destroyed only to a minor degree by MAO and that some other enzyme system was largely responsible for their deactivation.

Scheme 2 Metabolism of Dopamine, Noradrenaline and

Adrenaline



Key - Scheme 2

i Catechol-O-methyltransferase

ii Monoamine oxidase

-----> Minor route

-----> Major route

Analysis of urine from a subject with pathologically high systemic levels of catecholamines (phaeochromocytoma) by Armstrong and his colleagues⁵⁵ in 1957, showed the presence of large amounts of 4-hydroxy-3-methoxy-mandelic acid (HMMA) and they suggested that HMMA could be a metabolite of the catecholamines. In the same year Axelrod,⁵⁶ after incubating rat liver slices with adrenaline in vitro showed that the 3-O-methyl derivative, metadrenaline, was formed and that incubating metadrenaline with an MAO system gave HMMA. Axelrod also showed that noradrenaline behaved in a similar manner. Thus, an alternative pathway to direct oxidation for catecholamines had been established for their metabolism (Scheme 2).

Kirshner et al⁵⁷ and La Brosse et al⁵⁸ showed that 80-90% of an injected dose of ³H-adrenaline can be recovered from urine as the sum of the metadrenalines and HMMA. Confirmation was provided by Kopin,⁵⁹ in 1960, who gave small doses of (+)-³H-adrenaline intravenously to male subjects and found that after collecting their urine for 54 hours, 95% of the original radioactivity was found in the urine, of which 97% was in the form of metabolites and only a tiny fraction was excreted unchanged. 3-O-Methylated compounds constituted 80% of the original dose, suggesting that 3-O-methylation is the principal pathway for deactivation (Scheme 2). When both 3-O-¹⁴C-metadrenaline and ³H-adrenaline were given together to student volunteers, 70% of the adrenaline was first O-methylated to the metadrenaline and 20% was deaminated by MAO to 3,4-dihydroxymandelic acid (DOMA). On the other hand 50% of the radioactive metadrenaline was excreted unchanged or as the sulphate conjugate and the other 50% was deaminated and then oxidised to give HMMA. Noradrenaline metabolism was observed to follow a similar pattern. Thus the major route in the inactivation of injected catecholamines is that of 3-O-methylation followed by oxidative deamination and not direct oxidation as was first proposed.

One of the enzymes responsible for the inactivation of the catecholamines, catechol-O-methyltransferase (COMT), catalyses the O-methylation of

a large variety of catechols⁶⁰ such as DOPA, dopamine, noradrenaline, adrenaline, and 3,4-dihydroxymandelic acid (DOMA). The enzyme shows very little stereospecificity for both (+) and (-) adrenaline are O-methylated at the same rate,⁶¹ but it shows regiospecificity in that 3-O-methylation predominates.⁶² The enzyme is usually soluble and found in most animal tissues,⁶¹ but a bound variant has been isolated from both brain⁶³ and liver.⁶⁴ The soluble enzyme from rat liver which requires S-adenosyl-L-methionine as the methyl donor and a divalent cation, probably magnesium, has been purified.^{60,61}

The enzyme which plays the minor part in the deactivation of the catecholamines has been shown to be a Group I MAO which is not inhibited by carbonyl reagents⁶⁵ and semicarbazide^{66,67} unlike Group II MAO which are inhibited by carbonyl reagents but are sensitive to semicarbazide.⁶⁵⁻⁶⁷

The oxidase was located in 1951 by Cotzias and Dole,⁶⁸ and Hawkins,⁶⁹ who independently showed that the enzyme was intimately bound in most organ tissues to the insoluble structures of mitochondrial membranes.

1.4 The Regulation of Catecholamine Biosynthesis and Metabolism

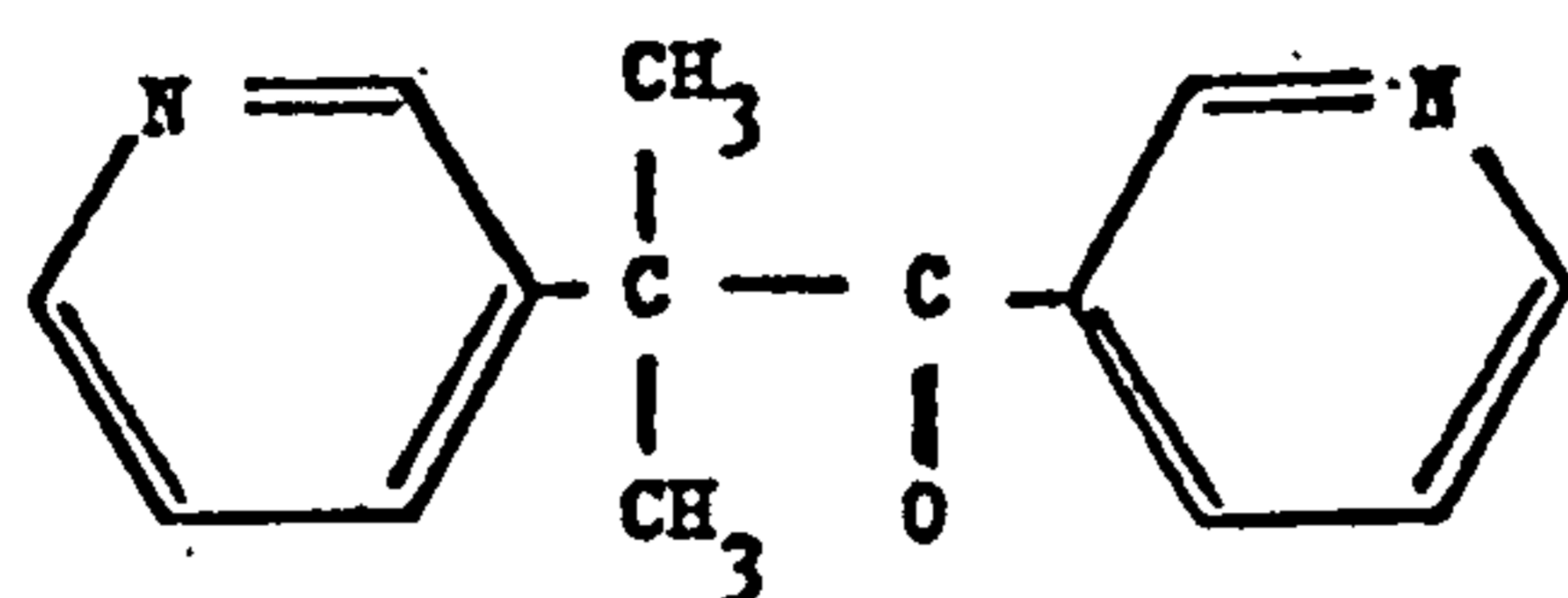
Catecholamines are normally released from storage organelles in amounts which depend on three factors; the storage capacity available, the rate of discharge and recharge of the stores, and the rate at which new organelles can be formed. The development of new storage organelles within the sympathetic system depends on the activity of the nerve growth factor,⁷⁰ its mode of action is, however, at present unknown.

The control of catecholamine synthesis appears to be mediated by inhibition of tyrosine hydroxylase by excess noradrenaline (i.e. end-product inhibition). Spector and his colleagues⁷¹ found that after blocking the breakdown of noradrenaline with a MAO inhibitor the levels of noradrenaline in the tissues of guinea pigs increased. If radioactive tyrosine was then given to the animals the formation of radioactive noradrenaline was reduced by comparison with a control group. However, when radioactive DOPA was given, the treated animals, by comparison with the control group, showed

neither an increase nor decrease in radioactive noradrenaline, suggesting that the excess noradrenaline produced as a result of the inhibition of the MAO inhibited the tyrosine hydroxylase. Subsequent work has supported this view⁷²; however, the site of action of the noradrenaline has not yet been elucidated. This inhibitory action of noradrenaline on the tyrosine hydroxylase appears to be the key factor in the short term regulation of catecholamine formation. Long term regulation is effected by control over the formation of new enzyme⁷³ an increase in the flow of nerve impulses acting on chromaffin or sympathetic tissue results in increased enzymatic activity.⁷⁴ This activity starts in the ganglionic cells and enzyme appears to travel along the axon of the nerve to the effector organ. Apart from the rate-limiting enzyme tyrosine hydroxylase, all the other enzymes involved in the biosynthesis of the catecholamines, both soluble and particle bound, appear to be increased and controlled in this way.⁷⁴ The mechanism by which neuronal activity in the pre-ganglionic sympathetic fibres induces, trans-synaptically, an increased enzymatic activity in the noradrenergic neurones is not clear.

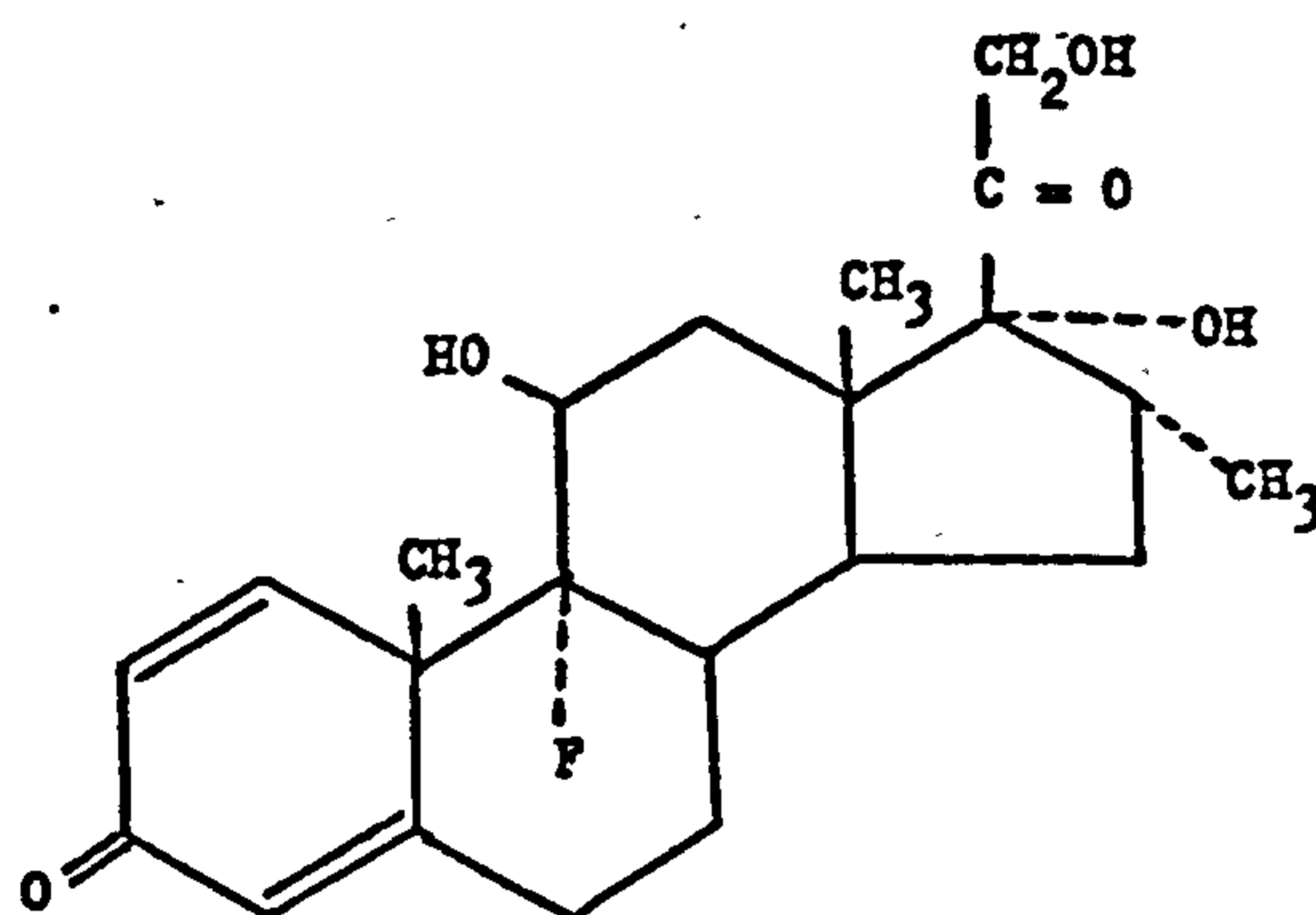
Coupland⁷⁵, in 1953, observed that chromaffin cells which were not in contact with adrenal cortical tissue contained only noradrenaline and he suggested that the development of phenylethanolamine N-methyltransferase for converting noradrenaline to adrenaline depended on the presence of cortical hormones. Later work by Wurtman and Axelrod⁷⁶ showed that hypophysectomy led to a severe reduction in the transferase level in rat adrenal medulla, which could be overcome by giving glucocorticosteroids.⁷⁷ Similarly the hypophysectomy reduced the level of dopamine- β -hydroxylase in adrenal medulla.⁷⁷ Axelrod and co-workers⁷³ had previously shown that after hypophysectomy tyrosine hydroxylase levels in the adrenal medulla fell; however, administration of corticotrophin ACTH prevented the fall. The reduction in activity of dopamine- β -hydroxylase and tyrosine hydroxylase following hypophysectomy could not be prevented by glucocorticosteroids.

Metirapone (Metapirone) 2-methyl-1,2-di(3 pyridyl)propan-1-one



(III)

Dexamethasone (9 α -fluoro-11 β , 17 α , 21 trihydroxy-16 α -methylpregna-1,4-diene-3,20-dione



(IV)

Thus a hormonal effect produced by the pituitary gland indicates a further control mechanism for catecholamine biosynthesis. The primary action of these pituitary hormones could be to produce adrenergic structures which would lead to an increase in either amine or enzyme levels. Support for this idea has come from the in vivo and in vitro work of Eränko and Eränko,^{78,79} who showed that giving hydrocortisone to newborn rats produces an increase in the number of small intensely fluorescing cells (S.I.F. cells) of sympathetic ganglia; the catecholamine content of the cells is also increased. S.I.F. cells appear to be intermediate between chromaffin cells and nerve cells and could form the chemical link between the pre- and post-ganglionic neurones.⁷⁸

The metabolism of the catecholamines by COMT and MAO has been investigated by Parvez and Parvez,⁸⁰ who observed that glucocorticosteroids inhibit MAO and COMT activities in vivo and in vitro. They gave adult rats Metapirone (III), a strong inhibitor of corticosteroid synthesis, and found that as the cortisol level fell both COMT and MAO activities increased in proportion. This led them to postulate that glucocorticosteroids act as a rate-limiting factor for catecholamine degradation. Other workers,⁷⁶ had given a synthetic glucocorticosteroid, dexamethasone (IV) and found that both MAO and COMT levels were lowered in rat adrenal tissue.

A study of the sub-cellular action of these steroid hormones by Litwack and Singer⁸¹ in relation to their molecular inhibition of the MAO and COMT indicated that the exact connection between receptor sites for glucocorticosteroids and their effect on the induction of new protein (enzyme) synthesis remains in doubt. It is possible that control is exerted at the genetic level within the cell.⁸²

In short, the complexity of the formation of the catecholamines and their subsequent breakdown involves the idea of a spatial separation of the enzymes from each other and an active movement of precursors from one site to another which is controlled by uptake mechanisms within the cells. A nerve growth factor,⁷⁰ appears to play an important part during the early

development of the adrenergic system. 'End-product' inhibition appears to govern short term regulation of catecholamine synthesis, coupled with this are factors, probably hormonal, which act upon storage particles and other structural elements in regulating the synthesis and metabolism of the catecholamines.

1.5 Release of Catecholamines from Sympathetic Nerves and Adrenal Medullary Tissue

The synaptic vesicles contained in the terminal varicosities of sympathetic nerves contain NA and it is commonly assumed that the NA released from the nerves originates from these vesicle stores. There are two principal ways in which the NA contained within the vesicles could reach the extracellular space. First, the NA could pass from the vesicle into the cell cytoplasm and from there pass through the cell membrane. However, in animals which have not been treated with a monoamine oxidase inhibitor, most of the NA passing from the vesicles into the cytoplasm will be oxidised by the MAO of the mitochondria in the nerve endings and will then leave the nerves as deaminated metabolites. This is found⁸³ to occur when animals are treated with reserpine, a drug which depletes the vesicles of their NA, and is in direct contrast to the action of a nerve impulse which evokes the release of NA at the nerve endings. The second method by which NA can be released is by discharging the vesicles directly into the extracellular space. It has now been established⁸⁴ that catecholamines are secreted from the adrenal medulla by exocytosis, by the fact that other soluble constituents (adenosine phosphate and proteins) of the chromaffin granules are also liberated stoichiometrically. A similar approach has been adopted in studying NA released from the sympathetic nerves. It has been found⁸⁵ that electrical stimulation of the splenic nerve evokes the release of two vesicle proteins, chromogranin and the enzyme dopamine- β -hydroxylase. Both are large protein molecules whose release from the nerve other than by a process of exocytosis, would be difficult to imagine. However, applying the model of the adrenal

medullary release of catecholamines to the sympathetic neurone presents difficulties, as any experiments with nerves must take into account the fact that NA after it has been released into the extracellular spaces can be taken up and bound both by nerve terminals and postsynaptic tissues.⁸⁶

The release of NA from the terminal varicosities of a sympathetic nerve is initiated by the arrival of 'action potentials' which originate within the cell-body. It has been suggested⁸⁴ that the link between depolarization of the nerve and release of NA is probably due to an influx of calcium ions into the nerve terminals. However, nothing is known regarding the role of calcium within the nerve endings. The vesicles probably reach the cell membrane by the Brownian movement within the cytoplasm⁸⁷ and once there the vesicle and cell membranes fuse, probably by means of the phospholipid molecules in the membranes. Lysolecithin has been shown to be present in quite large amounts both in the adrenal chromaffin granule membranes and in the purest preparations of noradrenergic vesicles so far isolated.⁸⁸ Once fusion has taken place a pathway is then open for the discharge of the NA into the extracellular spaces.

1.6 Catecholamine Uptake Processes

Burn in 1932⁸⁹ suggested that catecholamines might be taken up into tissue binding sites. However, it has only recently become clear that not one but several different processes exist for the uptake of the catecholamines into animal tissues. NA and related amines are known to be transported from the extracellular space across the axonal membranes of adrenergic nerves, a process usually termed 'Uptake₁'; a further mechanism exists to promote the free catecholamines from the cytoplasm into the nerve storage vesicles. Additionally catecholamines are transported across the membranes of smooth muscle and various other postsynaptic cells by another process termed 'Uptake₂'.

No equivalent of the enzyme acetylcholinesterase exists at adrenergic synaptic junctions, but inhibitors of the MAO and COMT enzyme systems do

not markedly prolong or potentiate the effects of NA after it has been released from sympathetic nerve terminals. The termination of the actions of released NA appears to be mediated not by enzymatic conversion but by a transfer of the amine back from its extracellular site of action into the nerve terminal. This recapture process is called 'Uptake₁'.

The NA after entering the nerves is rapidly accumulated into the intracellular storage vesicles both in the adrenal medulla and adrenergic nerves. The catecholamines are stored in an apparently stoichiometric complex with adenosine triphosphate (ATP).

Catecholamines are also taken up by the second transport system 'Uptake₂' into various extraneuronal peripheral tissues, such as vascular smooth muscle, cardiac muscle and certain glandular tissues. Arterial smooth muscle⁹⁰ in particular has a high affinity for concentrating and retaining NA. However, the actual mechanism is uncertain but a form of facilitated diffusion⁹¹ with some intracellular binding seems most likely. It has also been suggested⁹² that 'Uptake₂' like 'Uptake₁' may be a further method for inactivating physiologically active catecholamines. Gillespie⁹³ states that it is now generally accepted that extraneuronal uptake will inactivate some of the nerve liberated NA and also bind circulating adrenaline and that its importance may be much greater than the fraction of transmitter handled might indicate, since the transmitter which is being metabolised is that which has reached the effector cell membrane and is therefore remote from neuronal recapture.

In short, we can see that the inactivation of circulating noradrenaline or nerve liberated NA involves uptake both by nerves and by effector cells, including smooth muscle. The amine which is transported into nerves is largely protected by being incorporated into storage vesicles, whilst that transported into the smooth muscle is partly metabolised by the MAO and COMT enzyme and partly leaked back into the extracellular space where it may prolong the physiological response. If either 'Uptake₁' or 'Uptake₂'

is inhibited a compensatory increase occurs in the other mechanism, similarly inhibition of one of the two metabolic enzymes (MAO) (COMT) is accompanied by an increase in the other.

1.7 Plasma vs Urine Levels of Catecholamine Estimation

A frequent assumption regarding the measurement of catecholamines is that the urinary excretion rate of catecholamines reflects their plasma concentration; this assumption implies that the catecholamines are passively handled by the kidneys. Although the assumption is commonly made, there is no solid evidence available on this point in man. Observations on the fate of isotopically labelled NA injected into the circulating blood have shown⁵⁷⁻⁵⁹ that only a small fraction of the original dose appears unaltered in the urine, and an even smaller fraction of NA secreted from the nerve terminals escapes metabolism and appears unchanged in the urine.⁹⁴ Overy et al⁹⁵ however, demonstrated that in dogs which had been given trace quantities of radioactive NA, the free urinary NA was in fact derived from the total body production of the hormone rather than from local renal sympathetic activity. These workers suggested that their findings justified the measurement of urinary NA as a method of estimating the secretion of the sympathetic neurotransmitter produced from the whole body mass. They further showed that such measurements were not affected by urine pH, urine flow or by variation in the level of renal sympathetic activity. Similarly the urinary output of adrenaline might be expected to reflect that of the whole body mass which is essentially that of the adrenal medulla.

However, since 97% of these catecholamines are metabolised in vivo⁵⁹, and the small amount which escapes is extremely labile, measurement of neither NA nor adrenaline in the urine is likely to be a reliable guide to secretion rates. A better reflection of catecholamine secretion would be to measure plasma levels of these compounds. However, the levels are so low that special techniques which need both experienced personnel and sophisticated and expensive equipment are required to measure them. The

plasma catecholamines are also extremely labile, so that once a blood specimen has been obtained it must be centrifuged quickly and the plasma separated and, if not assayed immediately, it must be stored deep frozen at -20°C . Carruthers et al⁹⁶ have shown that within 30 min of collecting a blood sample approximately 40% of its catecholamine content is lost. As we have noted, because of the re-uptake and binding of active catecholamines within the body a random plasma analysis does not give a measure of the actual rates at which the amines are being secreted. Daggett and Carruthers⁹⁷ have recently shown that when blood is continuously sampled over 1 h periods, then the integrated catecholamine levels are higher than random estimations taken over the same period of time. Continuous blood sampling and measurement of catecholamines would probably give a much better picture of actual catecholamine secretion rates. However, this technique is not yet a routine procedure in the majority of hospitals.

It would seem that, rather than measuring the small amount of highly labile catecholamines which escape inactivation, a better reflection of amine secretion rates might be given by measuring the output of the urinary catecholamine metabolites over a particular time period. The end product of dopamine metabolism is HVA and of NA and adrenaline HMMA. These two phenolic acids are the most abundant catecholamine metabolites found in human urine. However, whilst the level of HVA could be taken to reflect dopamine secretion, the level of HMMA depends on the secretion rates of both NA and adrenaline. Therefore, to compare the relative activities of the adrenal medulla and the sympathetic nervous system, urinary levels of both NMA and MA have to be individually measured.

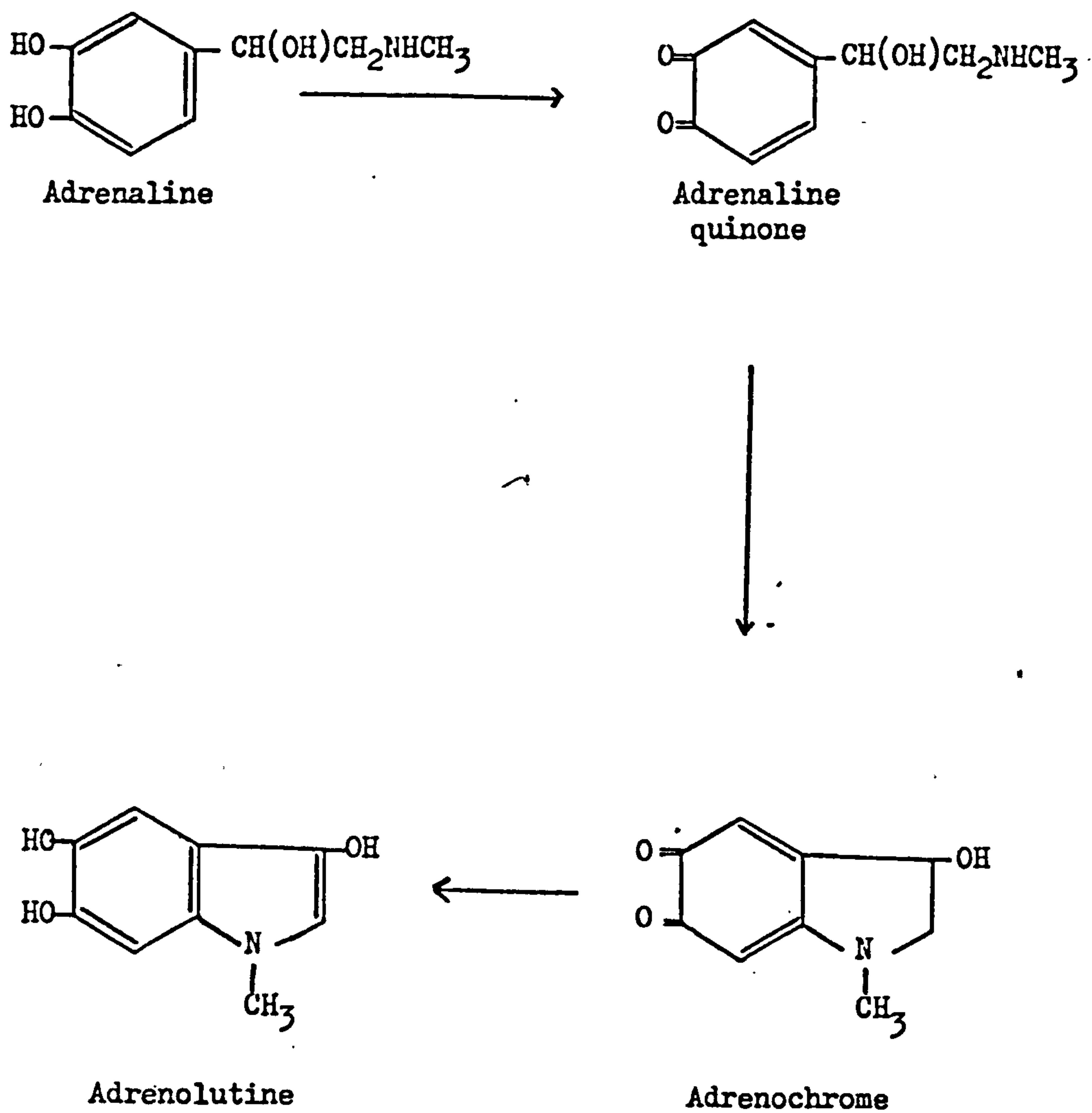
It seems reasonable to assume, although there is no direct evidence available to justify this, that the physiologically active catecholamines, after release, are rapidly metabolised and the metabolites produced are efficiently excreted by glomerular filtration through the kidneys regardless of their concentration in the blood, so that the rate of excretion of the

metabolites reflects the rate of their production. The work that follows has been done on this assumption to try to assess both the secretion of adrenaline and noradrenaline using simple techniques which can be performed in most clinical laboratories in both normal situations and those which are known to be associated with high secretion rates of the physiologically active catecholamines.

CHAPTER 2

THE MEASUREMENT OF PHENOLIC ACIDS AND
PHENOLIC AMINES FOUND IN URINE FROM
NORMAL SUBJECTS

Scheme 3 Formation of adrenolutine from adrenaline



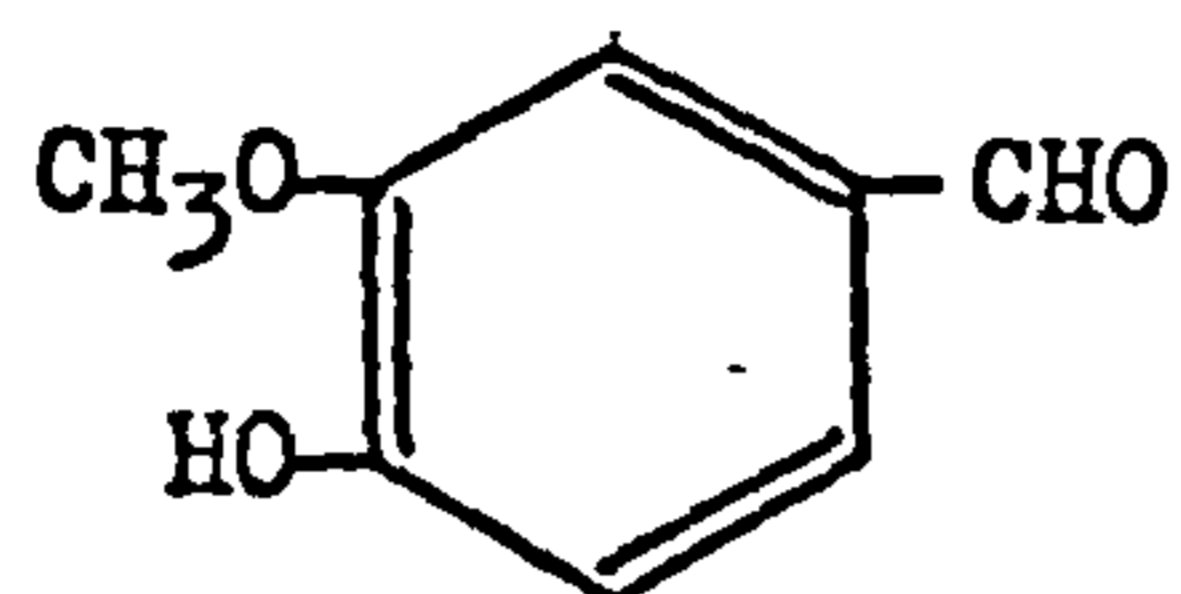
2.1 General Introduction

The elucidation of the biosynthesis and metabolism of the catecholamines in mammals and later studies on their storage, release and re-uptake (both in the sympathetic system and adrenal medulla) would not have been possible without sensitive and specific methods for measuring both the catecholamines and their major metabolites. Earlier methods employed biological techniques for measuring dopamine,⁹⁸ noradrenaline and adrenaline,^{99,100} in brain tissue, blood plasma and urine. However, such methods are time consuming and imprecise. More recently physico-chemical methods have been developed which are sensitive and more precise and can be used to carry out large numbers of determinations. In these methods the catecholamines are adsorbed on alumina,¹⁰¹ or ion-exchange resins,^{102,103} they are then eluted from the adsorbent and assayed by fluorimetric methods.

In general, two fluorimetric procedures have been developed for this purpose, namely the trihydroxyindole (THI)¹⁰⁴ and the ethylenediamine (EDA) condensation methods.¹⁰⁵ The THI method consists in oxidising adrenaline in alkaline solution to adrenochrome which then rearranges to form a fluorescent N-methyl-3,5,6-trihydroxyindole (adrenolutine) Scheme 3. Noradrenaline undergoes a similar reaction. Ascorbic acid is added as a reducing agent to prevent the oxidation of the relatively unstable adrenolutine. However, ascorbic acid itself can produce some fluorescence and lead to high blank values. To overcome this problem Weil-Malherbe¹⁰⁶ replaced ascorbic acid by mercaptoethanol to reduce fluorescence in the blanks.

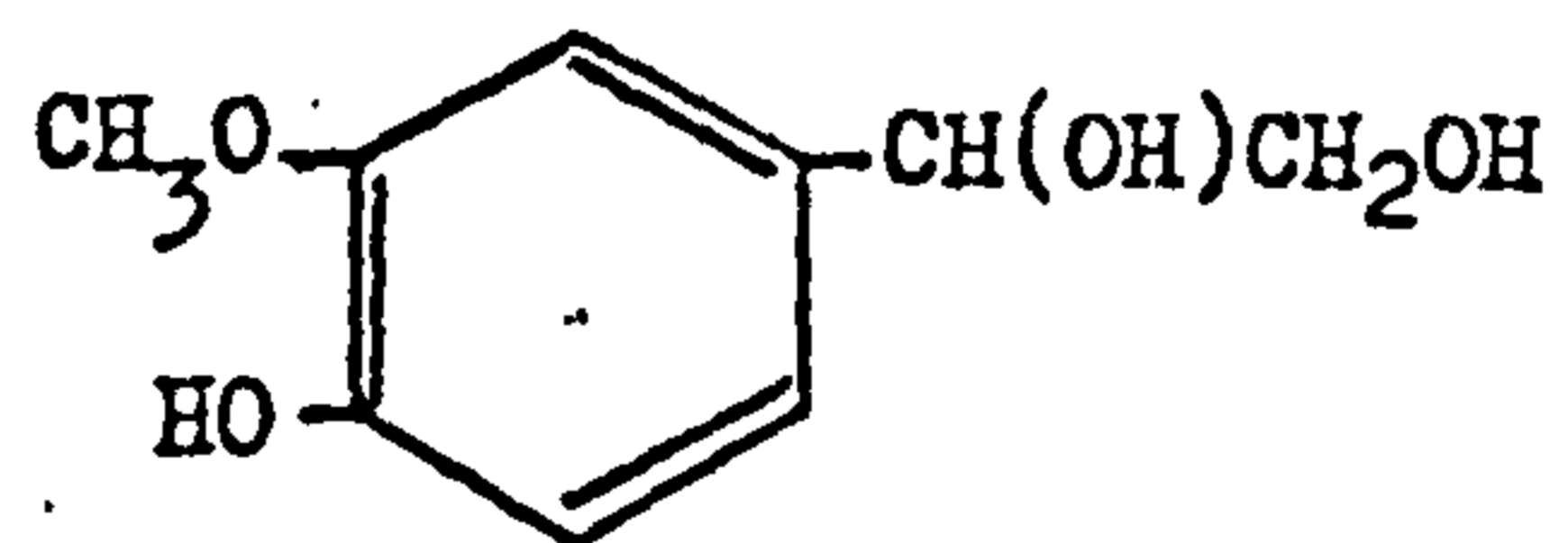
The need for expensive fluorimeters as well as the technical difficulties involved in measuring the small amounts of dopamine, NA and adrenaline found in normal urine and the interference from drugs, which are often present in urines obtained from hospital patients, suggested the need to develop simple techniques capable of separating and quantitating the individual urinary levels of the catecholamine metabolites, compounds which

Vanillin (4-hydroxy-3-methoxybenzaldehyde)



(V)

1-(4-hydroxy-3-methoxyphenyl)ethane-1,2-diol (HMPG)



(VI)

are relatively stable and certainly more abundant in urine than the free catecholamines.

2.2 Concurrent Measurement of 3-methoxy-4-hydroxy mandelic acid and Homovanillic acid in Urine

Introduction

Numerous methods have, in the past, been proposed for the determination of HMMA in urine. In earlier techniques,^{55,107,108} the phenolic acids were first extracted with an organic solvent from acidified urine, separated by paper or thin layer chromatography,^{109,110} and the spots, after development, were compared visually with known standards. Other workers measured HMMA either by a colorimetric technique based on a diazo-coupling reaction,¹¹¹⁻¹¹⁷ or by converting it to vanillin, (V) which was then estimated indirectly as a coloured complex¹¹⁸ or by direct spectrophotometric measurement at 360.nm.¹¹⁹⁻¹²¹

Other methods which require more complex instrumentation have been used to determine HMMA. These consist of low¹²²⁻¹²⁴ and high voltage¹²⁵ paper electrophoresis, radio isotope techniques,¹²⁶ column chromatography¹²⁷ and gas chromatography.¹²⁸

HVA has proved more difficult than HMMA to estimate quantitatively. The more specific techniques consist of repeated extraction of the HVA, followed by further purification by ion exchange chromatography, the final extract being converted to a derivative which is isolated and measured either colorimetrically,¹²⁹ by t.l.c.,¹³⁰ fluorimetrically¹³¹⁻¹³³ or by gas chromatography.^{134,135}

In the present work the method which has been developed estimates HMMA and HVA concurrently. The technique is simple and uses equipment which can be found in most hospital laboratories.

2.3 Isolation, Separation and Estimation of Phenolic Acids

a) Separation by high voltage electrophoresis

Preliminary work on the separation of urinary phenolic acids was

attempted using high voltage electrophoresis (HVE), a technique which appeared to combine ease of handling with rapid separation. Standard amounts of HMMA and HVA were extracted from aliquots of acidified distilled water (pH 3), using ethyl acetate. The organic solvent was removed and then evaporated to dryness after which the residue was redissolved in two drops of methanol and applied quantitatively to a sheet of chromatography paper which was then dipped in a pyridine-acetate buffer (pH 3.85) solution, blotted dry and placed on the HVE base plate. Each end of the paper was connected by soaked paper wicks to buffer-containing compartments and a voltage of 2,500 volts was applied across the chromatography paper for 30 min.

The phenolic acids were located by diazo coupling with 4-nitrobenzene-diazonium ion. This method gave a good separation of the phenolic acids. The coloured bands from the azo dyes were cut out, eluted using a mixture of aqueous sodium carbonate-methanol and the dyes were estimated spectrophotometrically. Unfortunately, after elution of the grey azo complex of HVA, levels of HVA below 10 μg could not be detected spectrophotometrically. The recovery of HMMA using this technique was not very good either, (60 - 65%). To try and overcome these problems the HMMA and HVA spots after their separation by HVE were scanned using a Chromoscan recording densitometer fitted with a red filter and integrator. This improved the limits of detection for HVA and resulted in recoveries of 73% and 79% for the HMMA and HVA standards respectively. However, when this technique was applied to the separation and estimation of the HMMA and HVA from normal urine, the spots from HMMA and HVA overlapped those from other phenolic acids, mainly dietary compounds, present in normal urine. In an attempt to overcome this problem the chromatograms after HVE were rechromatographed at right angles using propan-2-ol/ammonia/water as the solvent system. However, after development the phenolic acids were poorly separated and the chromatograms had a high degree of background colour.

As well as these technical problems, instrumental difficulties also arose which made electrophoresis unreliable for routine use. Furthermore, recently the D.H.S.S. has issued instructions to withdraw from service all HVE instruments of the make used because they have been found to be electrically dangerous to operate.

It was decided that a more reliable and safer method of separation was required which used simple equipment found in even the most poorly equipped hospital laboratory. As a result a method using organic extraction followed by two dimensional chromatography was developed.

b) Extraction and separation by two dimensional paper chromatography

The rapid extraction of phenolic acids both from aqueous solutions and urine samples at acid pH was found to be extremely efficient using the solvent ethyl acetate (see 2.4 a, b). After mixing and centrifuging the solutions, a clear demarcation line was present between the aqueous and organic phase which enabled a clean, uncontaminated aliquot of solvent to be easily removed and transferred to a small conical test tube prior to evaporation. This was carried out carefully under reduced pressure at room temperature. The residue containing the phenolic acids was re-dissolved in methanol and applied quantitatively as a small spot to one corner of a sheet of chromatography paper which was then subjected to two dimensional paper chromatography (Chapter 9,3-4). It was essential to adopt a two dimensional technique because of the large quantity and number (between 30 - 40) of phenolic acids present in normal urine. A basic solvent system was employed for the first chromatographic separation, followed by an acidic solvent system, which was run at right angles to the first solvent system, for the second separation. Using this technique maximum separation of the phenolic acids, HMMA and HVA, was achieved. After chromatography, the chromatogram was dipped in sodium carbonate, dried and re-dipped in a solution of diazotised 4-nitroaniline. Phenolic acids produce a wide variety of colours with this reagent, thus enabling the spots of HMMA (purple) and HVA (grey) to be easily located on the chromatogram (see Chapter 9.4).

After identifying the HMMA and HVA the spots were cut out from the paper and scanned using a Chromoscan densitometer, which recorded the integrated numbers as each spot was scanned. The amount of HMMA and HVA present on each chromatogram was then estimated by comparison with standard amounts treated in the same way as the tests and the results expressed as mg HMMA or HVA per 24 h (Chapter 9. 5-6).

2.4 Evaluation of Extraction, Separation and Estimation Procedures

a) Recovery of HMMA and HVA from distilled water

The recovery of HMMA, using the method above varied from 80 - 87% and of HVA from 80 - 85%. (Chapter 9.7a).

b) Recovery of HMMA and HVA from urine

Determination of the efficiency of recovery of HMMA and HVA from urine requires first the removal from the urine of all the HMMA and HVA initially present. This was done by oxidation with sodium periodate followed, after destruction of excess periodate, by the addition of known amounts of HMMA and HVA, after which the samples were treated as for normal urines. (9.7b)

The recovery of HMMA and HVA after this procedure ranged from 81 - 86% and 74 - 79% respectively, figures which are in close agreement with those for the recovery from distilled water and which were deemed to be acceptable.

c) Between batch reproducibility of method

Duplicate samples taken from 8 24 h urine collections were analysed on consecutive days for HMMA and HVA to assess precision. The mean excretion per minute of HMMA was 1.9 μg , the standard deviation was 0.06 μg with a coefficient of variation of 3.2%. Similarly the mean HVA concentration was shown to be 4.7 μg with a standard deviation of 0.3 μg and a coefficient of variation of 6.7%. (See Appendix)

2.5 Normal Urinary Levels of HMMA and HVA

The urinary outputs of HMMA and HVA in 24 h from 123 normal subjects, comprising 80 males (ages 18 - 78 years) and 43 females (ages 19 - 75 years) were as follows, where the quantities are expressed as mean outputs[±] 1 standard deviation. Results were not corrected for recovery which

Table 1

Comparison of 24 h mean outputs of HMMA and HVA in urine from 123 normal subjects, compared with published normal values

No. of Subjects	HMMA/mg	Range	HVA/mg	Range	Reference
68	3.7	(0.6-6.8)	-	-	118
<20	3.7 ± 1.1	(1.8-7.1)	-	-	120
6	3.3 ± 0.7	(-)	-	-	128
8	3.65 ± 0.9	(2.9-5.0)	-	-	126
11	11.0 ± 2.3	(-)	-	-	117
15	3.9 ± 0.7	(2.1-5.2)	-	-	119
9	13.5	(9 -17.9)	-	-	113
25	7.5	(2.0-13)	-	-	115
20	4.0 ± 1.5	(-)	-	-	116
14	5.4 ± 3.0	(-)	-	-	114
50	4.3	(0.5 -10)	-	-	109
14	4.3	(0.4-8.1)	-	-	137
8	-	-	4.45 ± 0.62	-	136
-	-	-	7.2 ± 2.1	-	130
-	-	-	6.0 ± 1.1	-	129
<12	3.6 ± 1.2	(1.7-6.3)	9.4 ± 2.5	(4.3-12.8)	127
20	5.7 ± 1.3	-	4.2 ± 1.5	-	134
25	3.4 ± 1.6	-	4.1 ± 1.8	-	135
7	4.8 ± 0.7	-	4.2 ± 0.8	-	133
123	3.3 ± 0.9	(1.6-7.5)	4.9 ± 1.3	(2.4-8.8)	*
80 males	3.3 ± 0.9	(1.6-7.5)	4.9 ± 1.3	(2.4-8.8)	*
43 females	3.2 ± 0.9	(2.3-6.4)	4.9 ± 1.3	(2.6-8.6)	*

* Present study

averaged 84% for HMMA and 77% for HVA.

	<u>HMMA/mg</u>	<u>HVA/mg</u>
All	3.3 ± 0.9	4.9 ± 1.3
Male	3.3 ± 0.9	4.9 ± 1.3
Females	3.2 ± 0.9	4.9 ± 1.3

Students' 't' test indicated no statistically significant difference between the male and female groups (Appendix)

The values for the mean and standard deviations of the catecholamine metabolites have been calculated based on the assumption that their outputs conformed to a normal Gaussian distribution pattern. Previous workers in this field (see Tables 1 and 9 for references) have all applied statistical treatments to their analytical results on the assumption of a normal distribution.

However, when the frequency distribution of the values obtained for HMMA, HVA, NMA and MA were plotted from our 123 normal group of adults the plots intimated a possible 'skew' distribution for the catecholamine metabolites. To confirm this possibility the data has been subjected to further computer analysis using specialised statistical tests to evaluate the 'degree of skewness' and 'transformation' of the data has been attempted to try and normalise any 'skew' distributions (For statistical detail, see Appendix, e)

The finding of a skew distribution means that statistical methods applicable to normal Gaussian curves cannot be applied to our results to determine means and standard deviations. Consequently empirical normal ranges have been put forward (Addendum, a) which are based on clinical interpretation of expected normality.

2.6 Discussion

Relatively few workers have measured HMMA and HVA concurrently employing a single technique. Roginsky and colleagues,¹³⁵ using a g.l.c. method for both analyses quoted a normal range of 3.4 ± 1.6 mg per 24 h for HMMA and 4.1 ± 1.8 mg per 24 h for HVA, obtained from 25 normal subjects.

The values for HMMA and HVA reported by other workers are tabulated in Table 1. From this table the value for HMMA output per 24 h obtained using the present method is in close agreement with those of Sunderman et al,¹¹⁸ O'Gorman¹²⁶ and Pisano et al,¹²⁰ who used colorimetric and spectrophotometric techniques and with those of Dekirmerjian and Maas,¹²⁸ Messiha et al¹²⁷ and Roginsky et al¹³⁵ who estimated HMMA using more sophisticated column-and gas-chromatographic methods. Similarly, the mean HVA value is comparable with those of Karoum et al¹³⁴ and Roginsky et al¹³⁵ who used g.l.c. analysis and Smith and Weil-Malherbe,¹³⁶ and Kahane and Vestergaard,¹³³ who used a modified fluorimetric technique, based on the original method of Sato,¹³² but is much lower than those reported by Messiha et al¹²⁷ who used the original method of Sato,¹³² and Sankoff and Sourkes,¹³⁰ who used a t.l.c. method.

In the past, estimation of HMMA in urine by direct colorimetric techniques^{113,115,117} without prior purification has tended to give high results for HMMA output per 24 h due to interference from drugs, such as salicylic acid, or from dietary phenols. For example, Mahler and Humoller¹¹⁴ using a direct colorimetric technique, obtained a mean value for HMMA of 5.4 ± 3.03 mg per 24 h. However, they stressed the need for strict dietary restrictions over a period of 48 - 72 h before the urine was collected.

Methods for HMMA estimation which use a preliminary extraction stage, followed by the oxidation of the HMMA to vanillin and subsequent spectrophotometric measurement^{119,120} appear to give lower results than colorimetric estimations. However, Sapira¹²¹ found that free HMPG 1-(4-hydroxy-3-methoxyphenyl)ethane-1,2-diol (VI), a natural constituent of

urine, which is extracted at acid pH is also oxidised to vanillin and can contribute between 4 - 56% of the total vanillin measured, which clearly would lead to falsely elevated results. More recent methods^{109,137} have used t.l.c. coupled with densitometric measurements of the spots to separate and estimate HMMA. These methods have similar mean values but tend to give a wider normal range than the present method. A criticism of the t.l.c. method is that great care must be taken to retain activation of the plate during the spotting procedure in order to obtain good separation of the phenolic acids. High humidity or the presence of pyridine, hydrochloric acid or ammonia vapours tends to deactivate the plates. Also the presence of drugs and dietary compounds in the urine extract can produce problems in a one-dimensional t.l.c. technique.

Values very similar to the levels found in the present study have been obtained by Dekirmerjian and Maas¹²⁸ who used sophisticated and expensive g.l.c. methods; their normal values, however, are based on a study of only six subjects. These were students who were living in a special metabolic ward and on a special diet which did not include oranges, bananas, tea, coffee, ice-cream or other substances which might conceivably elevate the urine levels of HMMA. It is impracticable, however, to have special dietary control in hospital prior to collecting 24 h urine samples for routine analysis of HMMA.

Two-dimensional chromatography produces good separation of the phenolic acids, thus allowing HMMA and HVA to be measured even when the subject is taking a normal diet and drugs such as aspirin. The only problem likely to be encountered using the proposed method is the appearance of a pink background on the chromatograms on development due to adventitious phenol in the chromatography area. The obvious remedy for this (exclusion of strong phenolic substances from the area in which the chromatography is performed) is a measure preferable to one which inconveniences patients or their treatment.

The method could also be used, by modifying the filters in the Chromoscan, to measure other phenolic acids present on the chromatograms. The significance of many of these is at present unknown.

2.7 Measurement of Normetadrenaline and Metadrenaline in Normal Adult Urine

Introduction

Metadrenalines are known to be about ten times more abundant in urine and to have a greater stability than their precursors, noradrenaline (NA) and adrenaline. Hence the estimations of normetadrenaline (NMA) and metadrenaline (MA) are easier to perform than those of NA and adrenaline and they may afford a better measure of the rates of turnover and secretion of NA and adrenaline.

Previous workers¹³⁸⁻¹⁴⁰ in this field, after a preliminary extraction of the metadrenalines from the urine used two-dimensional chromatography to separate them and they were either estimated semi-quantitatively^{138,139} or by elution followed by colorimetry¹⁴⁰ at 550 nm. Others^{141,142} used high voltage paper electrophoresis to separate the metadrenalines which were then coupled with diazotised 4-nitroaniline and the azo compounds were eluted and measured colorimetrically at 520 nm.

Pisano¹⁴³ in 1960, described a method for measuring the combined amounts of both metadrenalines which involves a preliminary extraction followed by oxidation of the metadrenalines with sodium periodate to vanillin which is extracted and measured spectrophotometrically at 360 nm.

Fluorimetric techniques¹⁴⁴⁻¹⁴⁷ have also been developed for the estimation of NMA and MA extracted from urine by ion exchange chromatography. A few g.l.c. procedures have been reported for the quantitation of urinary NMA and MA either as total metadrenalines by estimation of vanillin, from their oxidation by periodate, as its trimethylsilyl derivative¹⁴⁸ or individually as trifluoroacetyl derivatives.¹⁴⁹ However, no attempt has been made to determine normal urine values of NMA and MA by g.l.c. and few of the other sophisticated techniques¹⁵⁰⁻¹⁵² have been used for the routine analysis of NMA and MA in normal urine.

A method has been developed in this study using simple equipment found in most hospital laboratories which is quick, easy to perform by relatively unskilled personnel and gives reproducible and quantitative results for both NMA and MA.

2.8 Extraction of Metadrenalines from Standard Aqueous Solutions

a) Solvent extraction

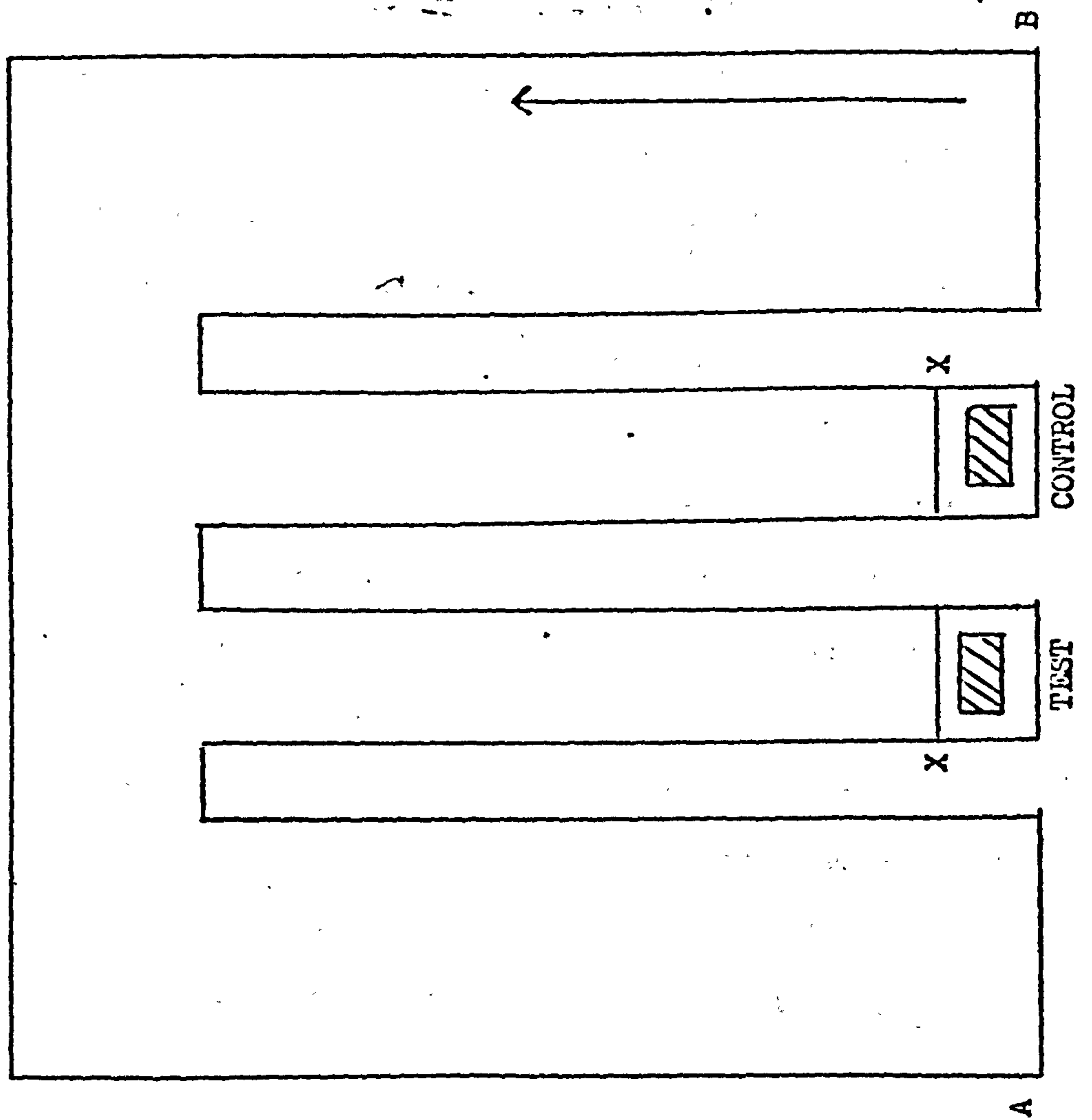
Preliminary extraction of the metadrenalines was first attempted using a procedure similar to that of Yoshinaga et al.,¹⁴² These workers hydrolysed urine samples for one hour at pH 1.0; after cooling, the samples were adjusted to pH 10 with sodium hydroxide, saturated with sodium chloride and extracted with 5 volumes of ethyl acetate. A modification of this procedure (Chapter 9. 8a) was tried in which acid hydrolysis was carried out for only 20 mins at pH 0.9 instead of pH 1.0. Although the difference in pH is relatively small previous workers¹⁴³ have noted losses were reduced when hydrolysis was carried out at pH 0.9. After hydrolysis the mixture was cooled, basified and saturated with sodium chloride prior to extraction using two separate aliquots of ethyl acetate, in an attempt to improve the yield. The organic extracts were pooled and evaporated to dryness and the residue re-dissolved in methanol and applied quantitatively to a sheet of chromatography paper which was then developed by two dimensional chromatography over a period of two nights. The NMA and MA spots were visualised using diazotised 4-nitro-aniline and the purple colour eluted from the paper using a sodium carbonate/methanol mixture (2:1) followed by colorimetric estimation, against known standards chromatographed and eluted in the same way. The percentage recovery of the metadrenalines ranged between 15 - 20%. An attempt was made to improve the recovery and to reduce the time factor. The extraction and evaporation procedures were repeated exactly as before, except that the residue dissolved in methanol was now applied to a sheet of chromatography paper cut to the dimensions illustrated in Fig.1. Test residues were applied to the hatched areas at the bottom of the paper strip labelled test, whilst standard amounts of NMA and MA were applied to a similar area on the corresponding strip, labelled control

Table 2

Results showing the variation with pH of the percentage of metadrenalines extracted from water or NaOH into ethyl acetate

pH of solution	Sample Number	NMA recovered/%	MA recovered/%	Mean/%	
				NMA	MA
7.0	1	20.6	20.6	17.2	20.7
	2	17.3	17.3		
	3	16.3	29.0		
	4	14.7	16.0		
8.0	5	16.2	22.7	18.0	22.5
	6	22.0	30.0		
	7	14.7	19.3		
	8	18.9	17.8		
9.0	9	21.2	24.2	22.8	17.6
	10	22.1	16.0		
	11	23.2	13.5		
	12	24.6	16.8		
10.0	13	18.3	16.0	20.0	17.0
	14	19.6	17.2		
	15	18.3	19.6		
	16	23.9	15.5		

Fig 1 Layout of chromatography paper for one-dimensional chromatography of NMA and MA



The chromatography paper was then developed overnight using a single solvent system (Chapter 9. 9b). The control strip was dipped in sodium carbonate, and after drying was dipped in a solution of diazotised 4-nitroaniline to locate the purple bands of NMA and MA. The areas on the test strip corresponding to the standards were cut out and eluted with alcoholic ammonia, converted with periodate to vanillin and measured spectrophotometrically (Chap 9. 9c). The reaction was quantitated using standard amounts of NMA and MA chromatographed in exactly the same way as the test. The mean NMA recovery for three extractions and estimations was 27% and for MA 33% which were an improvement on the previous figures but still unacceptable.

i) Effect of pH on the solvent extraction efficiency

It was felt that the poor recoveries of metadrenalines after ethyl acetate extraction could be due to their extraction at an incorrect pH. This factor was examined in more detail. Sixteen (20 cm³) of acidified (pH 0.9) distilled water containing known amounts of NMA (10 µg) and MA (10 µg) were, after being boiled and then cooled, adjusted to pH 7.0 (Samples 1 -4), pH 8.0 (Samples 5 - 8), pH 9.0 (Samples 9 - 12) and pH 10 (Samples 13 - 16) with sodium hydroxide, after which they were extracted with ethyl acetate as above, separated by one-dimensional paper chromatography and the NMA and MA content estimated spectrophotometrically (See Chapter 9 9 b - c).

The results (Table 2) indicate that organic extraction at pH 9.0 is the best, although the overall recovery of both NMA and MA is very poor over the whole pH range. This is in conflict with the results of Yoshinaga et al¹⁴² who quote an overall recovery of 80 - 90% using ethyl acetate at pH 10.

3-Methylbutan-1-ol extraction was also tried but difficulty was experienced due to the formation of emulsions. Organic extraction was finally abandoned and alternative extraction methods were examined.

b) Ion-exchange resins

Good recoveries of metadrenalines from ion-exchange resins have been reported by Bertoni et al¹⁵³ (90%) and Coward and Smith¹⁵⁴ (65 - 100%) who used the strong sulphonated polystyrene cation exchange

Fig 2 Standard Graph for estimation of NMA and MA after conversion to Vanillin

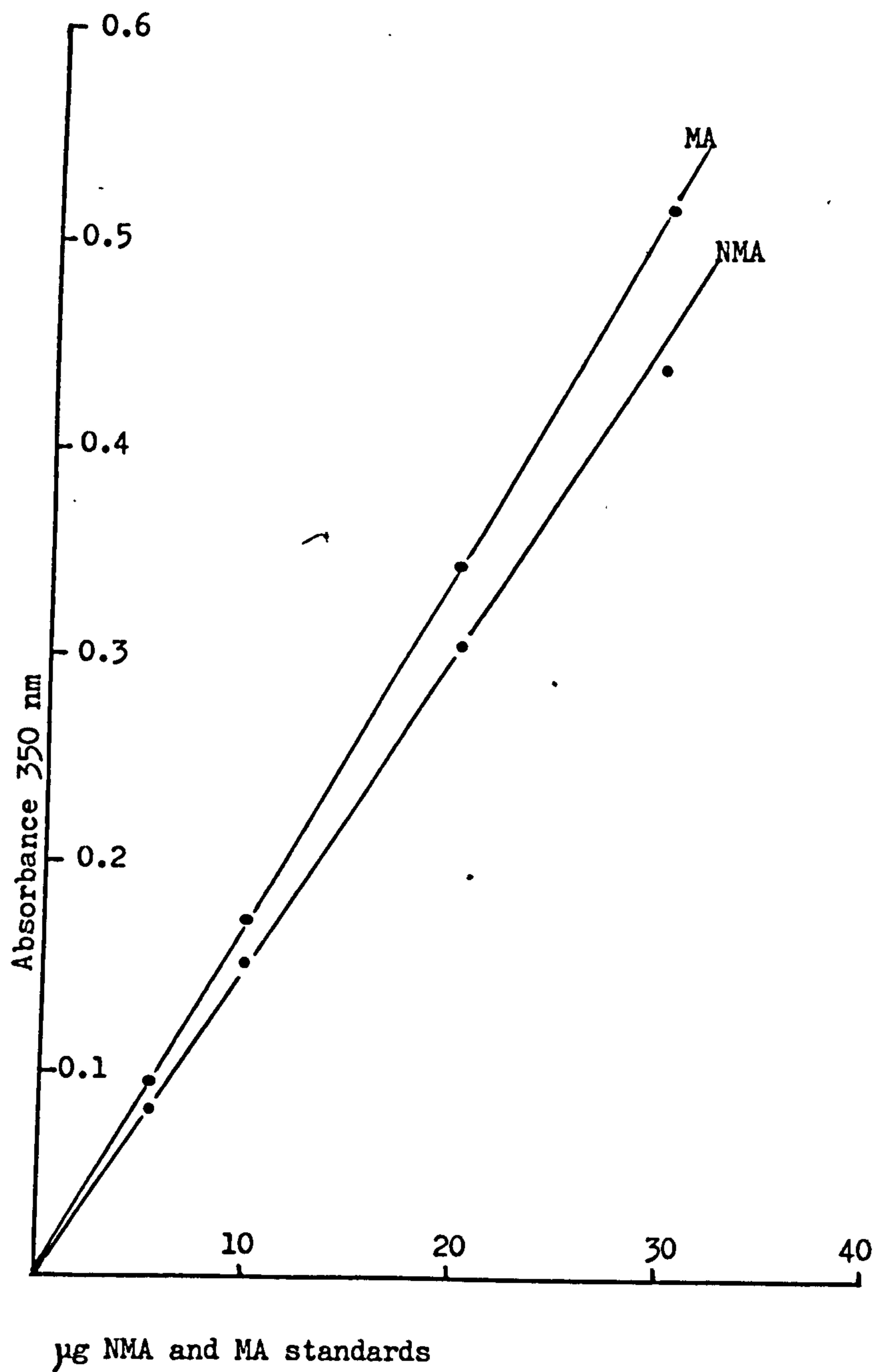


Table 3

Percentage recovery of NMA and MA extracted from
water by Dowex AG50-X8Na⁺ resin and eluted with alcoholic ammonia

Time used for elution/min	NMA recovered/%	MA recovered/%	Mean/%	
			NMA	MA
1	49	64	46	62
	44	55		
	45	67		
3	47	64	50	67
	51	69		
	52	68		
5	49	70	49	67
	52	62		
	46	66		
10	45	69	42	67
	38	69		
	43	63		
15	44	58	39	62
	36	56		
	37	72		

resins Dowex AG 50W - X2H⁺ and Dowex AG 50W-X8H⁺ respectively.

Sugiura¹⁵⁵ used a weaker cation exchange resin, namely Amberlite CG-50, but his recoveries (50%) were lower. It was decided to investigate the use of Dowex AG-50W-X8H⁺. An aliquot of a 24 h urine collection was first acidified and heated to liberate the free metadrenalines from their sulphate conjugates by hydrolysis. This solution was then cooled and brought to neutrality using sodium hydroxide.

An aqueous slurry of the Dowex cation exchange resin (prepared as in Chapter 9, 1a) was added and the mixture stirred for 15 mins to allow adsorption of the free metadrenalines to occur. The resin was allowed to settle and the supernatant decanted. The resin was then treated with successive washes of water (10 cm³), 50% (5 cm³) and 85% (10 cm³) ethanol to remove non-basic substances before it was finally stirred (5 mins) with 4M alcoholic ammonia to elute the phenolic amines. The mixture was filtered through a sintered glass filter funnel and the eluate measured and divided into equal portions labelled 'test' and 'control' both of which were evaporated under reduced pressure to dryness before being re-dissolved in methanol and applied respectively to the 'test' and 'control' strips of a one dimensional paper chromatogram (Chapter 9. 9b) which was developed overnight to separate the individual metadrenalines. Location and extraction was performed as in (Chapter 9.9c) and the results were quantitated using a standard calibration graph (Fig.2).

i) Recovery of metadrenalines from ion-exchange resin

The extraction of pure NMA and MA standards from Dowex cation exchange resin over timed periods ranging from 1 - 15 mins using 4M alcoholic ammonia (see Chapter 9.9a) suggests that a mixing time of 3 mins produced the best figures. The percentage recoveries were also reproducible with the recovery of MA being better than that of NMA (Table 3). However, the mean recovery level of 50 and 67% for NMA and MA respectively suggested that a high proportion of the standards was being lost during the extraction procedure. To track down the loss of metadrenalines each step of the extraction was checked. Thus a standard aqueous solution (10 cm³) of NMA (10 µg), after

Table 4

Effect of varying the concentration of alcoholic ammonia
on recovery of metadrenaline from Dowex AG50-X8Na⁺

Concentration of alcoholic % ammonia	NMA recovered/%	Mean/%	MA recovered/%	Mean/%
1M	46 44	45	53 57	55
2M	46 42	44	58 54	56
4M	37 45	41	60 62	61
8M	49 43	46	54 59	57

Table 5

Recovery of NMA and MA from distilled water using Dowex AG50-XSNa⁺
and elution with 2M aqueous ammonia

Standard	NMA recovered/%	Mean/%	MA recovered/%	Mean/%
1	86.7		83	
2	90	87.2	86	82.3
3	85.0		78	

Table 6

Separation of NMA and MA by paper chromatography and
recovery by elution with 4M alcoholic ammonia

Amount of NMA and MA added/ μ g	Amount Recovered NMA/ μ g	Mean Recovered/%	Amount Recovered MA/ μ g	Mean Recovered/%
1.5	1.75	110	1.55	102
	1.60			
	1.62			
2.5	2.66	105	2.57	102
	2.66			
	2.53			
5.0	4.85	97.8	4.76	96
	4.92			
	4.90			
7.5	6.55	88.3	7.20	95.5
	6.70			
	6.62			

Table 7

Reproducibility of estimation of NMA and MA in 24 h urine samples

Day	Metadrenaline levels in a normal urine /µg per 24 h		Metadrenaline levels in an abnormal urine /µg per 24 h	
	NMA	MA	NMA	MA
1	430	370	1,302	505
2	400	381	1,259	519
3	395	399	1,200	459
4	408	387	1,316	493
5	409	393	1,200	526
6	422	390	1,135	501
Mean	410	387	1,235	500
S.D.	±13	±10	±69	±24
Coeff. of variation/%	3.2	2.6	5.6	4.8

treatment with the ion-exchange resin, was examined for non-absorbed NMA by oxidation with periodate and estimation of vanillin. Similarly, each of the successive resin washes consisting of distilled water, 50% and 85% ethanol, after decantation, were made up to 10 cm^3 , divided, and after adding ten drops of 4M alcoholic ammonia and 0.1 cm^3 of periodate were scanned for the presence of vanillin. No vanillin was detected in any case. Vanillin, as expected, was found after oxidation of the NMA, eluted (3 min) by the 4M alcoholic ammonia (10 cm^3), but the recovery (31%) was lower than in previous findings. To try and overcome this problem the concentration of the alcoholic ammonia used to extract the metadrenalines was varied (Chapter 9, 8 b ii) and the supernatants removed and worked up as in (Chapter 9, 8 b i). The results (Table 4) show that all the solutions give comparable results with the mean recovery for NMA below 50% and below 60% for MA.

The possibility that the ethanolic ammonia in contact with the polystyrene resin beads causes the resin to distort, thus trapping some of the metadrenalines within the lattice framework was considered. To test this idea, the extraction procedure (Chapter 9, 8 b i) for NMA and MA was repeated, as described, but elution of the metadrenalines from the resin was carried out using 2M aqueous ammonia (10 cm^3) for 5 mins, the mixture was filtered and the eluate evaporated under reduced pressure to less than 10 cm^3 . The volume of each sample was readjusted to 10 cm^3 with distilled water before being divided into half. One half was oxidised and the other used as blank in the spectrophotometric estimation of vanillin. A standard solution of NMA ($10\text{ }\mu\text{g}$) in distilled water (20 cm^3) was evaporated, readjusted to 10 cm^3 , divided and treated as the tests. The results (Table 5) show a good overall recovery and reproducibility for the extraction of metadrenalines using aqueous ammonia. This method of elution was therefore used for the research reported in this Thesis.

2.9 Recovery of NMA and MA standards from one dimensional paper chromatograms, after elution and periodate oxidation

Table 8

Recovery of 10 µg amounts of NMA and MA added to 6 normal
urine samples

Sample Number	Recovery of metadrenaline/µg		Recovery/%	
	NMA	MA	NMA	MA
1	8.3	7.6	83	76
2	7.6	8.0	76	80
3	7.2	7.0	72	70
4	8.5	7.1	85	71
5	7.7	7.1	77	71
6	7.4	6.8	74	68
Mean	7.8	7.3	78	73

Table 9

Comparison of 24 h mean outputs of NMA and MA in urine from 123 normal subjects, compared with published normal values

Number of Subjects	NMA/ μ g	MA/ μ g	Total/ μ g	Reference
30			600 ⁺ 300	143
			less than 800	141
18			670 ⁺ 200	148
	600	150	750	140
6	176	205	380	142
19	160	80	240	144
47	288	137	425	146
	192	110	302	157
6	245 ⁺ 72	164 ⁺ 26	409	145
	420	88	508	156
123 males and females	394 ⁺ 95	352 ⁺ 94	746	*
80 males	404 ⁺ 95	369 ⁺ 89	773	*
43 females	377 ⁺ 94	320 ⁺ 95	697	*

* This study

Triplicate amounts of each standard (1.5, 2.5, 5.0 and 7.5 µg) were chromatographed, located, eluted, oxidised and quantitated spectrophotometrically using the adopted technique (Chapter 9, 9 a - c). The results (Table 6) show good recoveries for NMA and MA over the levels studied.

2.10 Reproducibility of the Adopted Method

A normal 24 h urine sample and a 24 h urine sample obtained from a patient suffering from a pheochromocytoma were analysed on six consecutive days to determine the day to day reproducibility of the method. The results (Table 7) show that the method has a good degree of reproducibility both at normal and high levels of metadrenaline excretion.

2.11 Recovery of NMA and MA from Normal Urine

Duplicate samples equivalent to a '30 min volume of urine' were taken from each of 6, 24 h samples. To one of each pair of duplicates was added 10 µg amounts of NMA and MA. The NMA and MA in each of the urine samples was extracted and estimated using the proven method (Chapter 9, 9 a - c). The recovery of the added NMA and MA was determined for each by subtracting the values for the urines without added amines from the value obtained for the corresponding duplicate to which amine had been added. The results (Table 8) indicate an overall recovery of 78% for NMA and 73% for MA, with a range of 72 - 85% and 68 - 80% respectively.

2.12 Normal Urinary Levels of NMA and MA

Twenty-four hour urine samples from 123 physically fit normal subjects were analysed for NMA and MA, comprising 80 males (ages 18 - 78 years) and 43 females (ages 19 - 75 years). Table 9 records the NMA and MA values (mean \pm 1 standard deviation) obtained in this study and those from other investigations (see Appendix appertaining to distribution studies).

A comparison of the results shows that output of NMA from the 80 males and 43 females are in close agreement; statistical analysis shows no significant difference of NMA output between males and females. However, the MA values of the males are higher than those of the females, and the difference is

significant at the 1% level. The values for NMA are similar to those obtained by Mattok et al¹⁵⁶ but higher than those of most other workers. The MA levels determined in this study are higher than have previously been reported, possibly due to a better recovery of MA using the extraction procedure described above. Most workers in the past have quoted normal ranges determined using only a few normal subjects and none have studied more than 50 subjects. This could account for some of the variability between the quoted normal ranges for NMA and MA.

2.13 Discussion

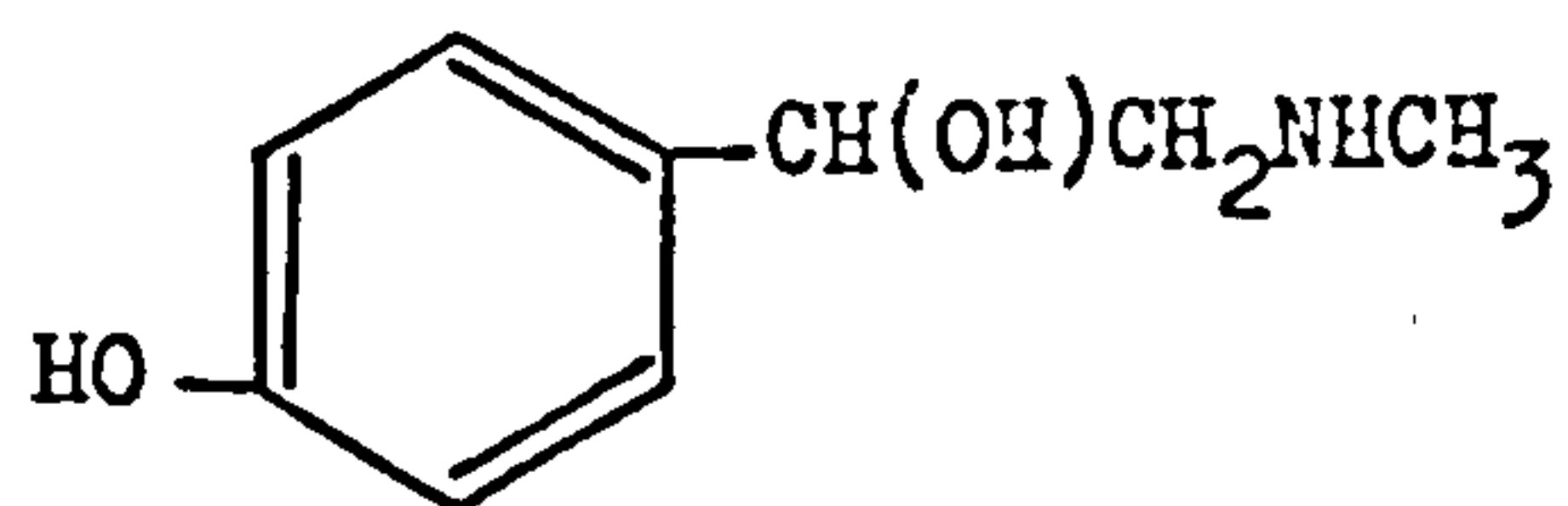
The amines NMA and MA are normally excreted in man as the sulphate conjugates, plus a small percentage as the more stable glucuronide.^{59, 158} Therefore, a preliminary hydrolysis stage usually with acid is required to liberate the free amines. Most workers in the past^{139, 142, 144} have carried out this hydrolysis at pH 1.0; however, Pisano¹⁴³ found that losses were reduced when the hydrolysis was done at pH 0.9 and this procedure has been adopted in this work.

Earlier workers^{138,140,142} extracted the metadrenalines from alkaline urine using organic solvents but this technique has been shown here to give very poor recoveries so this method was not used in this study.

The use of weak cation exchange resins such as Amberlite CG-50, to isolate the metadrenalines from urine has been reported.^{143,144} Smith and Weil-Malherbe¹⁴⁴ found that the isolation depended on a low urine salt concentration which required elaborate desalting techniques before adsorption of the metadrenalines could be carried out successfully. The observation¹⁴⁵ that free metadrenalines are quantitatively held by strong polysulphonated ion exchange resins and that 90% recovery of the metadrenalines from urine can be obtained using Dowex AG 50W - X2H⁺,¹⁵³ led to the trial of a Dowex polysulphonated resin in this study, namely Dowex AG-50W-X8H⁺ converted to the Na⁺ form. However, preliminary work using this strong cation resin was disappointing due to the low recoveries found after elution of the metadrenalines with 4M alcoholic ammonia, a result which was at variance with the findings of Coward and Smith¹⁵⁴ who obtained recoveries (65 - 100%) using 5M alcoholic ammonia. The good recoveries of NMA and MA found in the present study using 2M aqueous ammonia contradict the findings of other workers^{154,155} who found low recoveries when elution from Dowex 50-X8H⁺ was carried out using aqueous ammonia. The reason for the differences could be due to the form in which the resin is used. Alcoholic ammonia appears to be a better eluant when the Dowex resin is in the H⁺ form, whilst aqueous ammonia is a better eluant for Dowex resin in its Na⁺ form. The work of Coward and Smith¹⁵⁴ would support this idea. They obtained a recovery of 93% for NMA using Dowex 50-X2 (Na⁺ form) when eluted with 5M aqueous ammonia. To obtain consistent results with ion-exchange resins each batch was carefully prepared and standardised for recovery before being used for analysis.

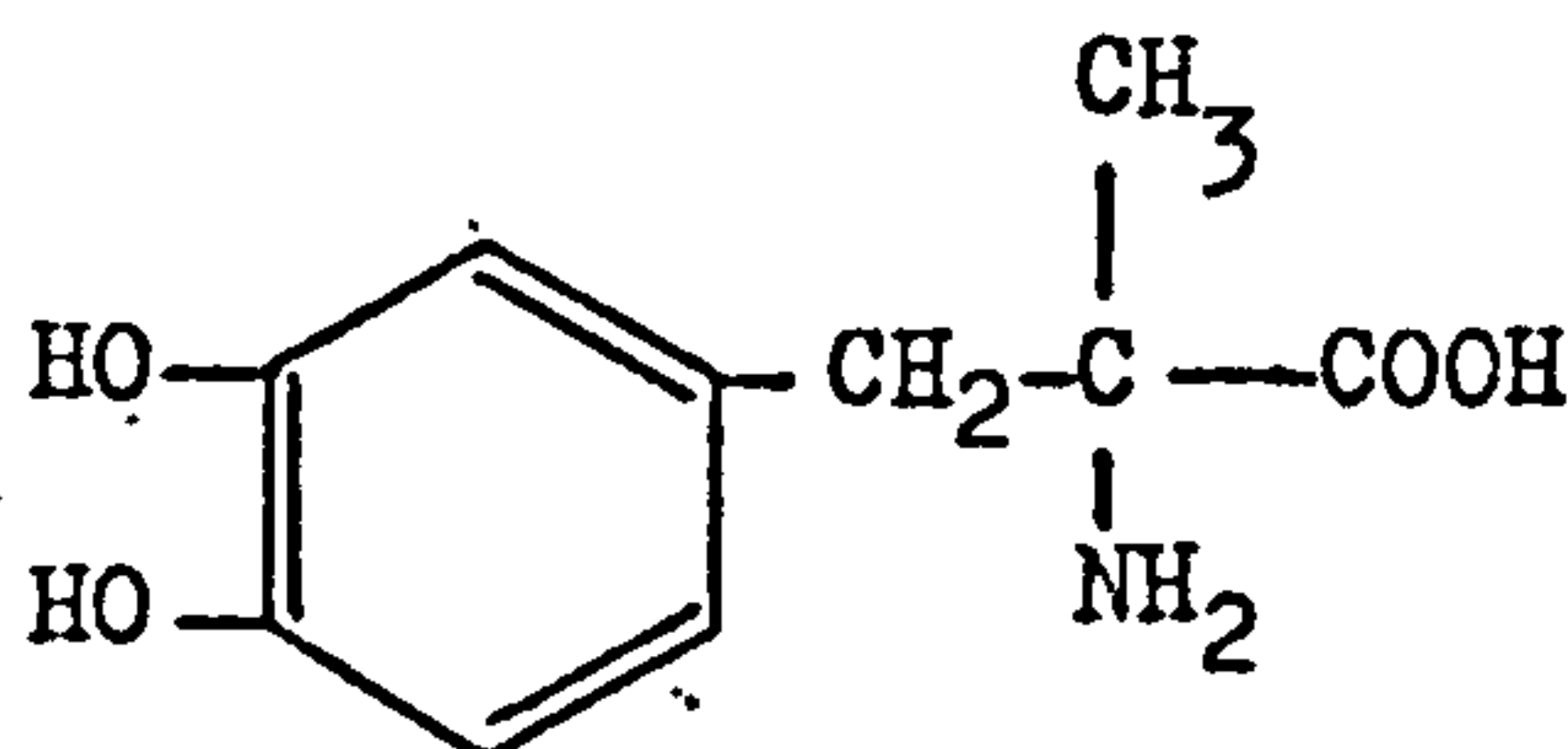
The elution time used to release the amines from the resin was fixed at 5 min, but the results suggest that a 3 min elution time would be

p-Sympatol (N-methyl-2-(4-hydroxyphenyl)ethanolamine)



(VII)

α -methylDOPA(2-amino-2-methyl-3-(3,4-dihydroxyphenyl) propanoic acid)



(VIII)

sufficient. The levels of NMA and MA recovered fell with an increase in extraction time possibly due to some re-adsorption onto the resin, emphasising the importance of keeping the elution time constant for reproducible results.

Many of the problems involving column techniques are due to the differences between column flow rates, producing poor reproducibility and in some instances low recoveries, due to marked variation in elution pattern, which correspond to marked changes in room temperature or resin quality. Wilk et al¹⁴⁷ overcame such problems by using tritiated NMA to determine the exact point at which the metadrenalines were eluted from the Amberlite CG-50 resin. Their technique, however, is too time consuming for the routine analysis of the metadrenalines. By stirring the resin with the metadrenaline solution at a constant speed and eluting for a fixed period of time most of these problems have been overcome in the present study. The other aspects of the extraction and estimation (chromatography, elution from the paper chromatogram and quantitation using periodate oxidation and spectrophotometry) were found to be almost quantitative for both NMA and MA.

In the original method of Pisano¹⁴³ both NMA and MA were extracted and converted to vanillin which was then estimated spectrophotometrically at a wavelength of 360 nm rather than at the max for vanillin (347 nm). The longer wavelength was used to minimise interference from other compounds which were also oxidised by the periodate, notably *p*-sympatol (VII) which is oxidised to 4-hydroxybenzaldehyde which adsorbs between 330 - 350 nm. Similarly certain drugs and their metabolites [such as α -methyl DOPA (VIII)] which can be found in the urine, are oxidised and will also give a falsely elevated value for total metadrenalines.^{159,160} The method which has been used here has overcome these problems, since the chromatography stage enables the individual metadrenalines to be located, separately extracted and quantitated, after oxidation, as vanillin at 350 nm without any interference from extraneous materials. The method has been shown to be very

reproducible, even at high metadrenaline levels, and gives good recoveries for MMA and MA. An interesting point is the finding of a significant difference in MA output between the male and female groups.

The value of screening hypertensive subjects, whose hypertension may be due to the presence of a phaeochromocytoma tumour, by estimating total metadrenalines, has been emphasised by Gitlow et al¹⁶¹ and by Sandler¹⁶² who state that urinary metadrenaline estimation is the best single test for the diagnosis of a phaeochromocytoma. Total metadrenaline estimation, however, suffers from the faults previously mentioned, in that it gives no indication, especially in the case of a phaeochromocytoma, as to which of the catecholamines, noradrenaline or adrenaline, is being secreted by the tumour, information which it is useful to have prior to any surgical procedure. The method reported here, however, provides this information. It can also be used to monitor levels of catecholamine metabolites, even when the subject is being treated with drugs such as α -methyl DOPA.

CHAPTER 3

THE ESTIMATION OF URINARY HMMA, HVA

NMA, AND MA LEVELS IN BABIES

AND YOUNG CHILDREN

3.1 Introduction

The discovery⁵⁵ of a raised level of HMMA in the urine of a subject with a catecholamine-secreting tumour, led to the development of many methods for estimating HMMA as an aid in the diagnosis of this type of tumour (phaeochromocytoma) and the related neural crest tumour (neuroblastoma) which occurs in babies and young children. In addition, the observation by McMillan¹⁶³ in 1956 that a malignant chromaffin tumour contained a high percentage of dopamine, was followed by the finding^{164 - 166} of an increased level of dopamine in the urine of children suffering from neuroblastomas.

The estimation of urinary dopamine is technically difficult. However, the output of its final metabolite HVA which would be expected to reflect any marked increase in secretion rates, has been carried out.

Gitlow et al¹⁶⁷ in a study of normal children suggested that the total output of metadrenalines (NMA + MA) varied less in children below the age of one than did their HMMA and HVA levels. As total metadrenaline output gives no guide to the individual secretion rates of NA and adrenaline, in the present work separate measurements of NMA and MA have been carried out. These might help to distinguish between normal children under conditions of stress sufficient to elevate their adrenaline secretion and thus increase their total metadrenaline output, from children with neuroblastomas.

The study first of all required the collection of accurate 24 h urine samples. These were collected from a group of eleven normal babies aged 1 - 12 months designated (Group A) who were admitted for minor surgical procedures (repair of squint or cleft palate defect) to the metabolic ward of the Birmingham Children's Hospital. The urines were collected under trained nursing supervision using the method described in Chapter 9, 2 . The second group, termed Group B, consisted of fourteen fit and healthy children, aged 2 - 11 years who collected 24 h urine samples under close supervision of adults.

The output of HMMA, HVA, NMA and MA were then measured in an attempt to establish the normal daily output and ranges for each group. The results have been compared with various parameters such as age, urine volume per 24 h

Table 10

Output of catecholamine metabolites per 24 h, urine volume per 24 h, mg creatinine per 24 h and body surface area in 12 babies aged 0-12 months (Group A) and 14 children aged 2-11 years (Group B)

	HMMA/ mg per 24 h	HVA/ mg per 24 h	NMA/ µg per 24 h	MA/ µg per 24 h	Urine Volume per 24 h/cm ²	Creatinine per 24 h/mg	Body Surface area/m ²
Group A (Babies)							
Mean ± 1 S.D.	0.8 [±] 0.4	1.2 [±] 0.4	74 [±] 16	49 [±] 16	162 [±] 85	47 [±] 20	0.35 [±] 0.09
Range	0.4-1.9	0.9-2.1	51-110	35-90	73-365	28-84	0.28-0.55
Group B (Children)							
Mean ± 1 S.D.	2.6 [±] 0.9	3.6 [±] 1.3	256 [±] 76	356 [±] 80	489 [±] 152	394 [±] 185	0.85 [±] 0.12
Range	0.6-4.0	0.6-5.5	72-367	90-364	280-870	35-806	0.71-1.10
't' value	-5.8	-5.8	-7.7	-7.6	-6.3	-5.9	-11.4
'P'	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Adults					1,522 [±] 235 ^a	1,565 ^b	
Range					700-3,170	1,360-1,770	

a Mean of 123 adults (see Ch. 2)

b Ref. 189

Fig 3 Output of urinary metabolites per 24 h compared with
age in years

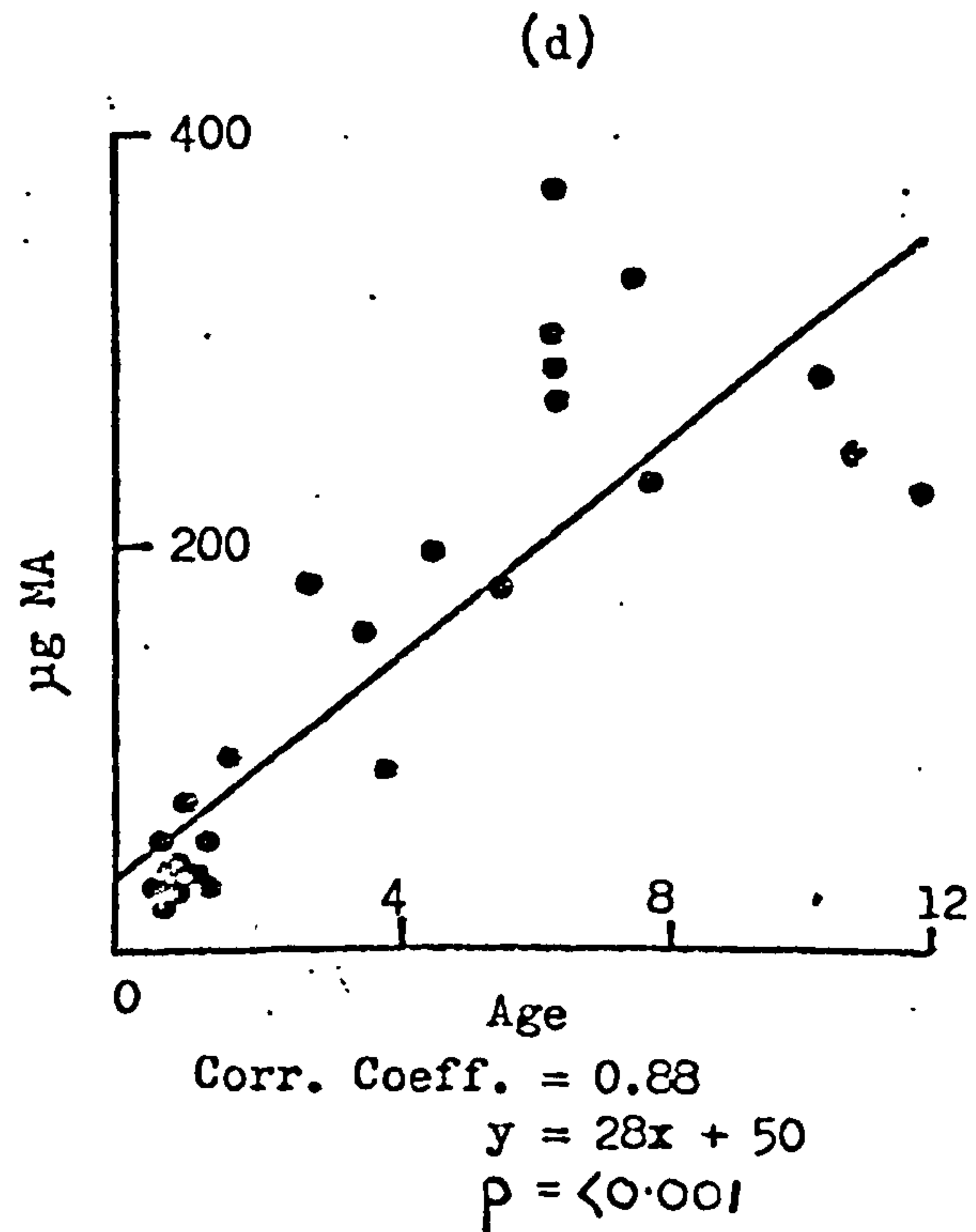
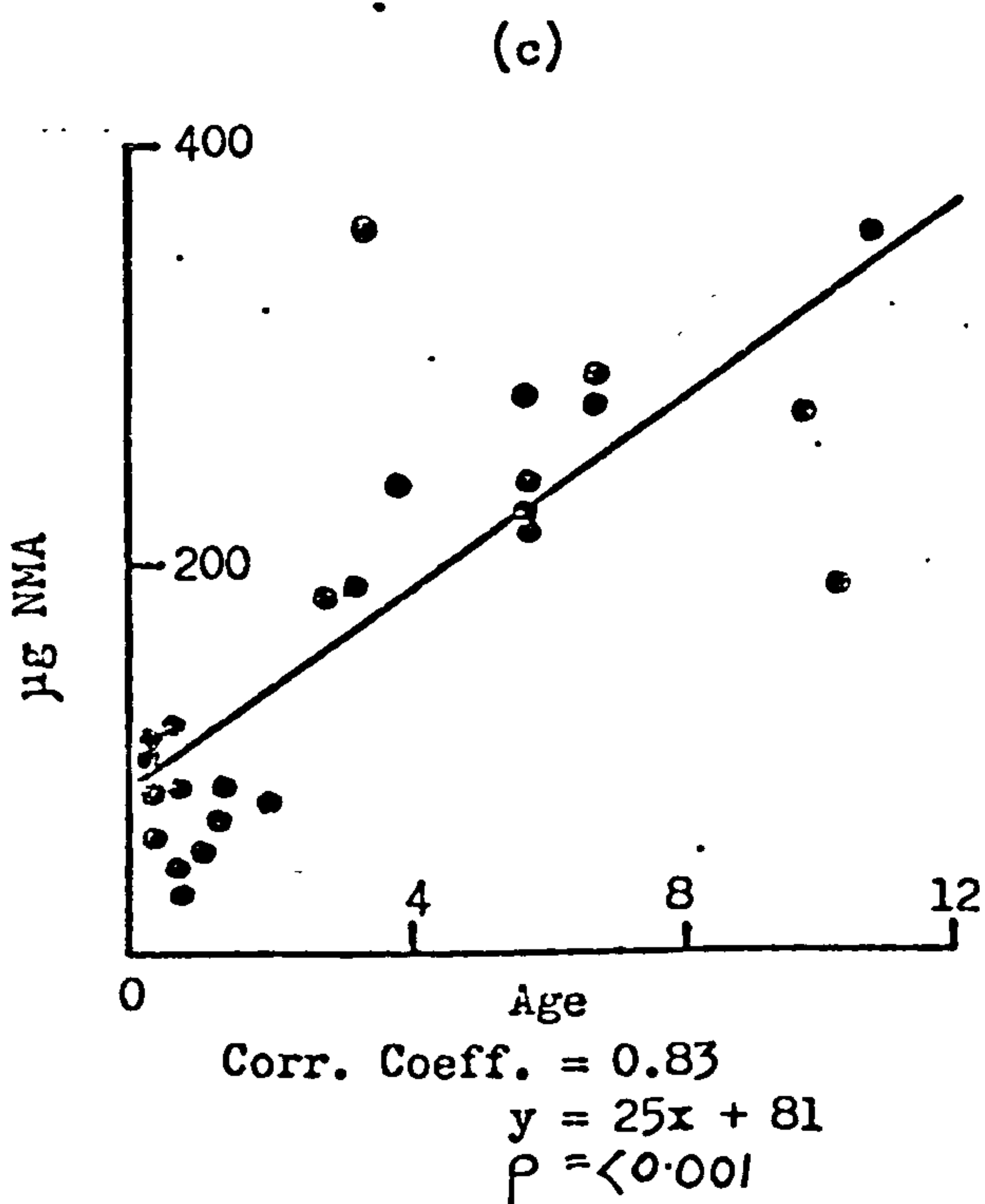
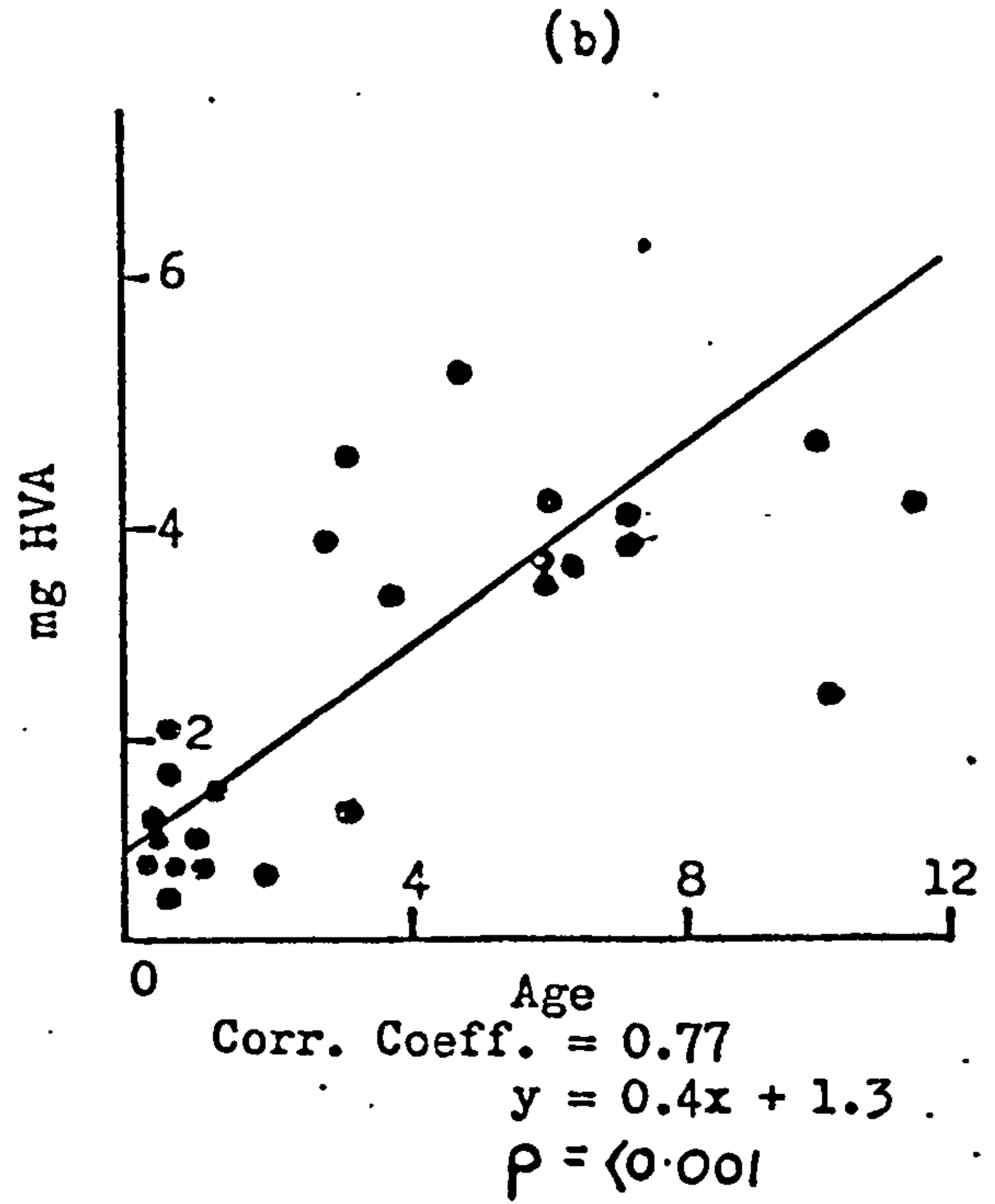
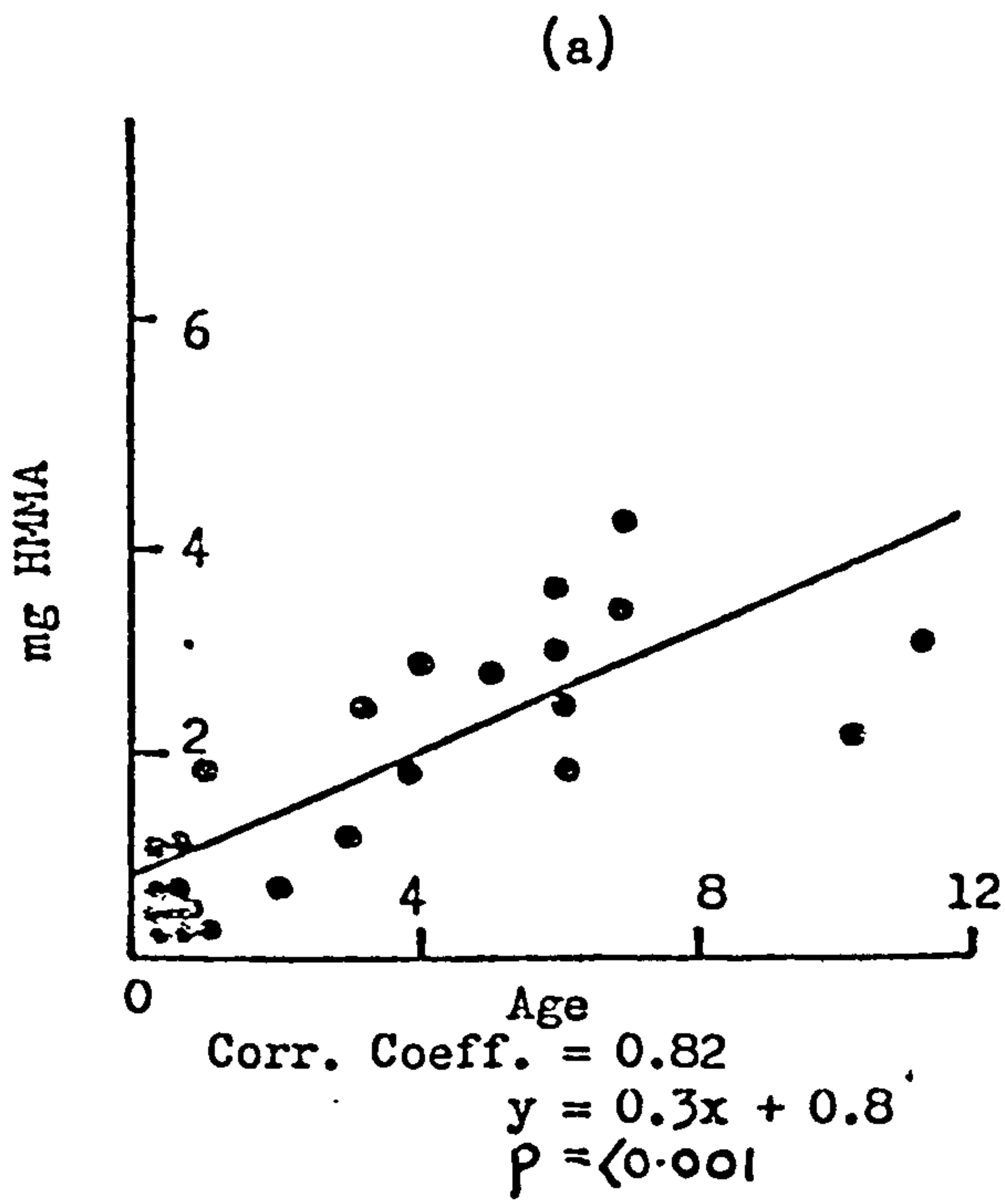


Fig 4 24 h output against urine volume

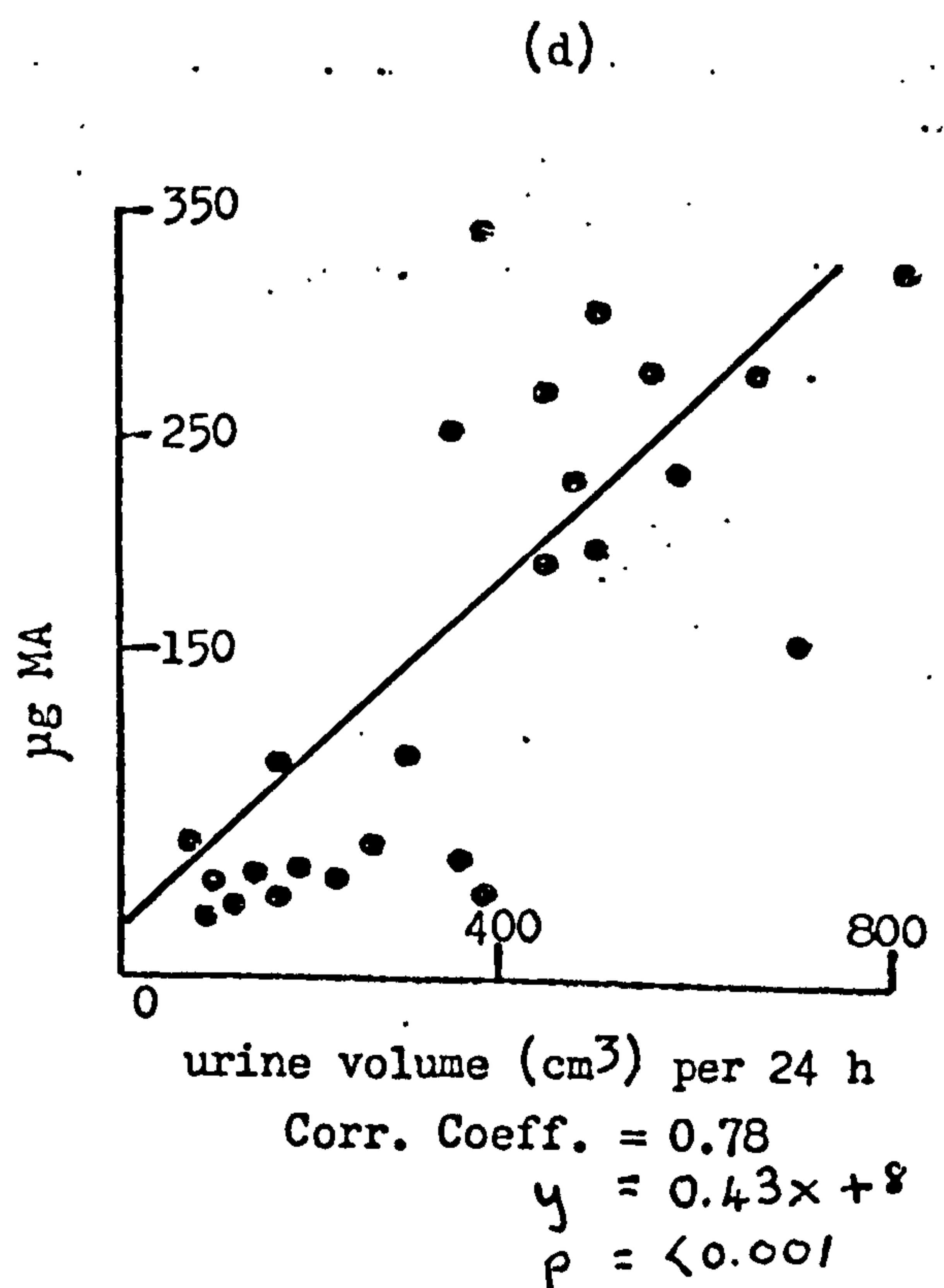
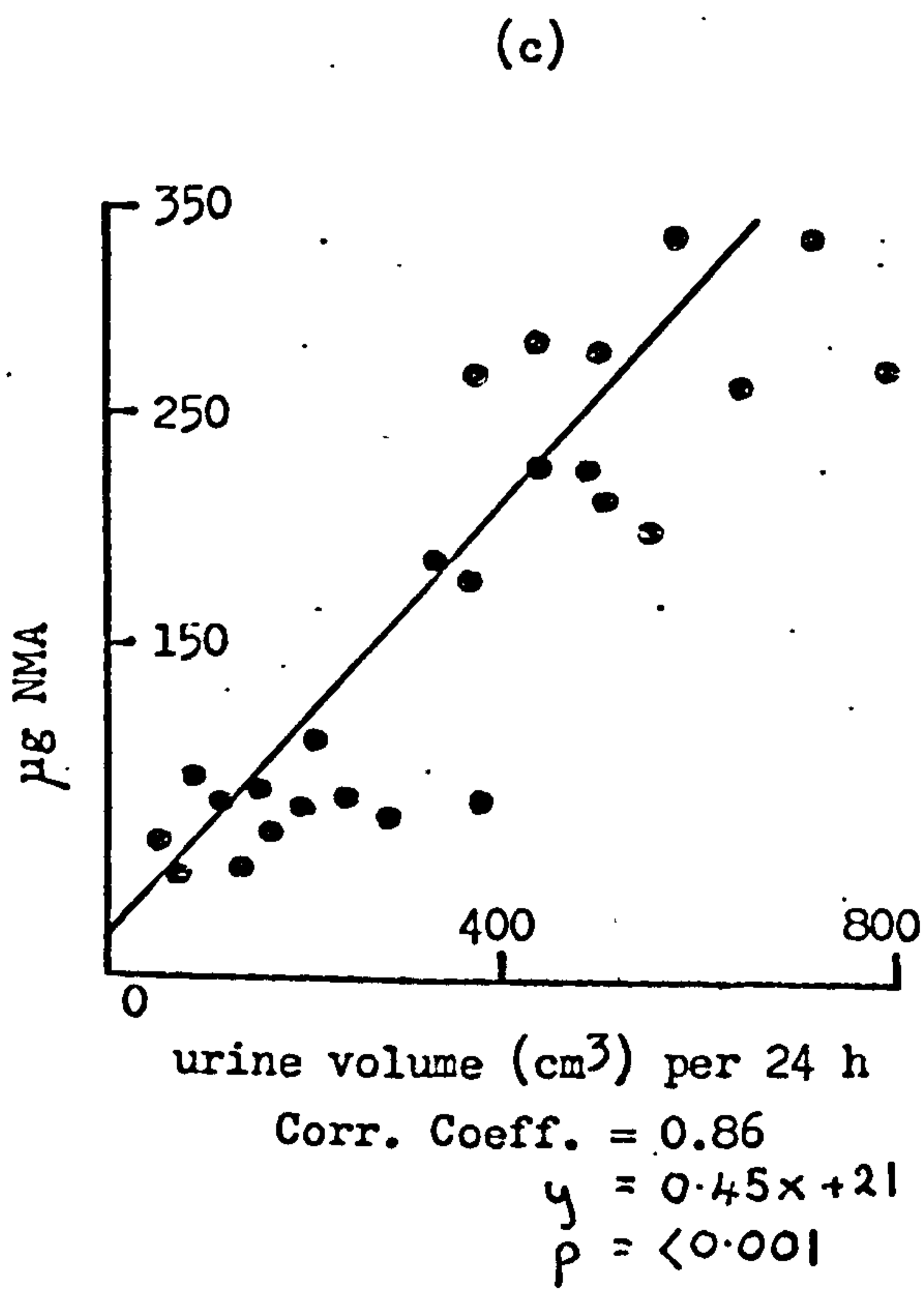
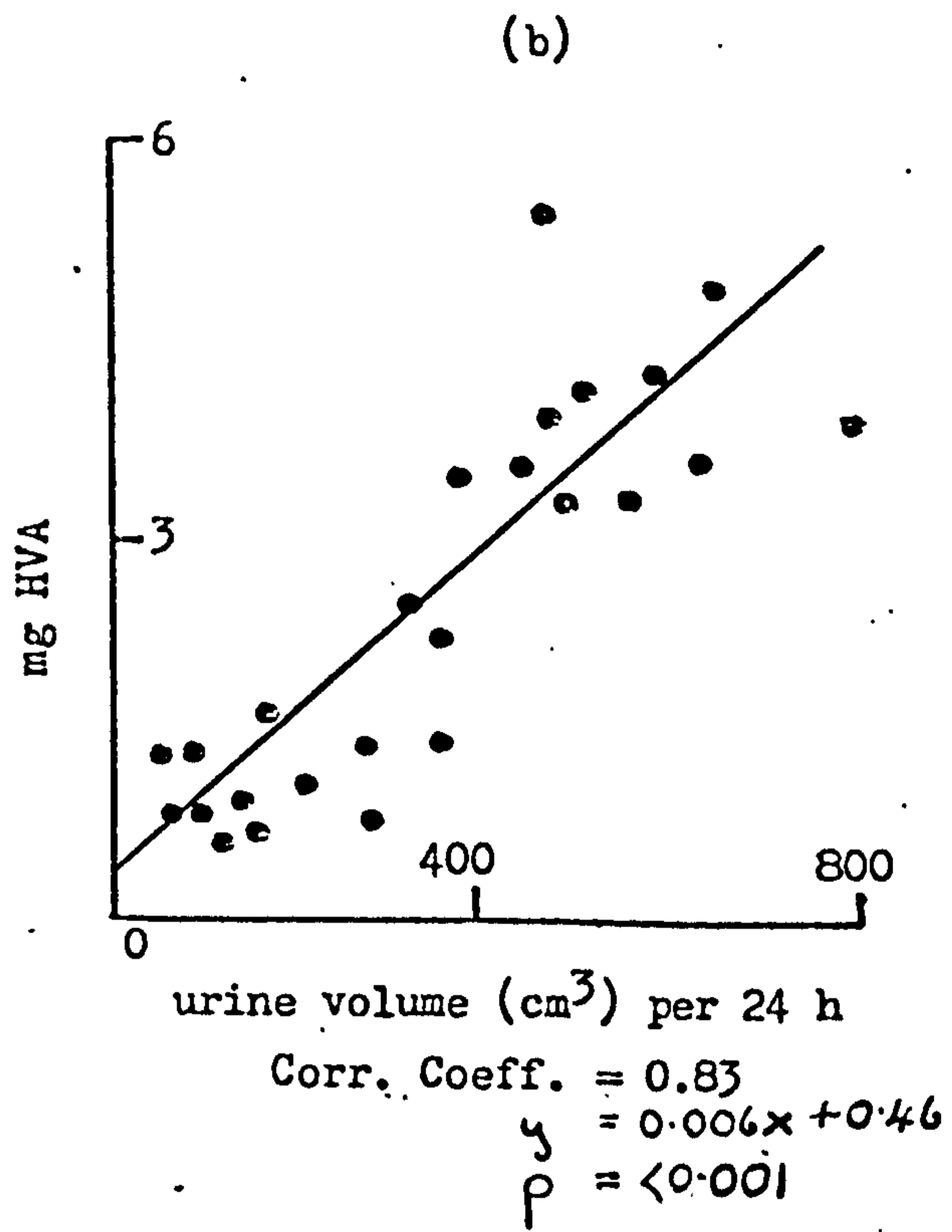
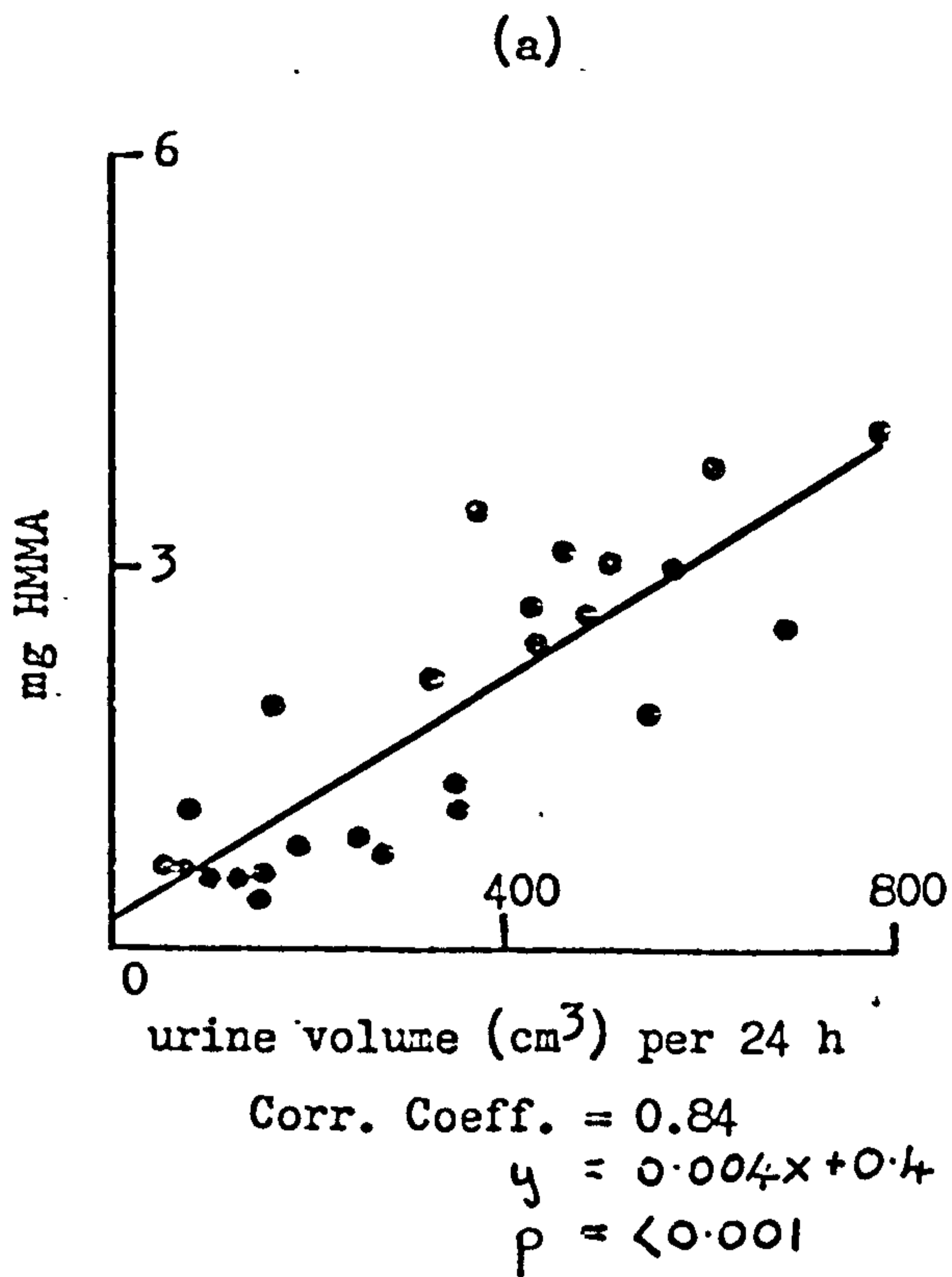
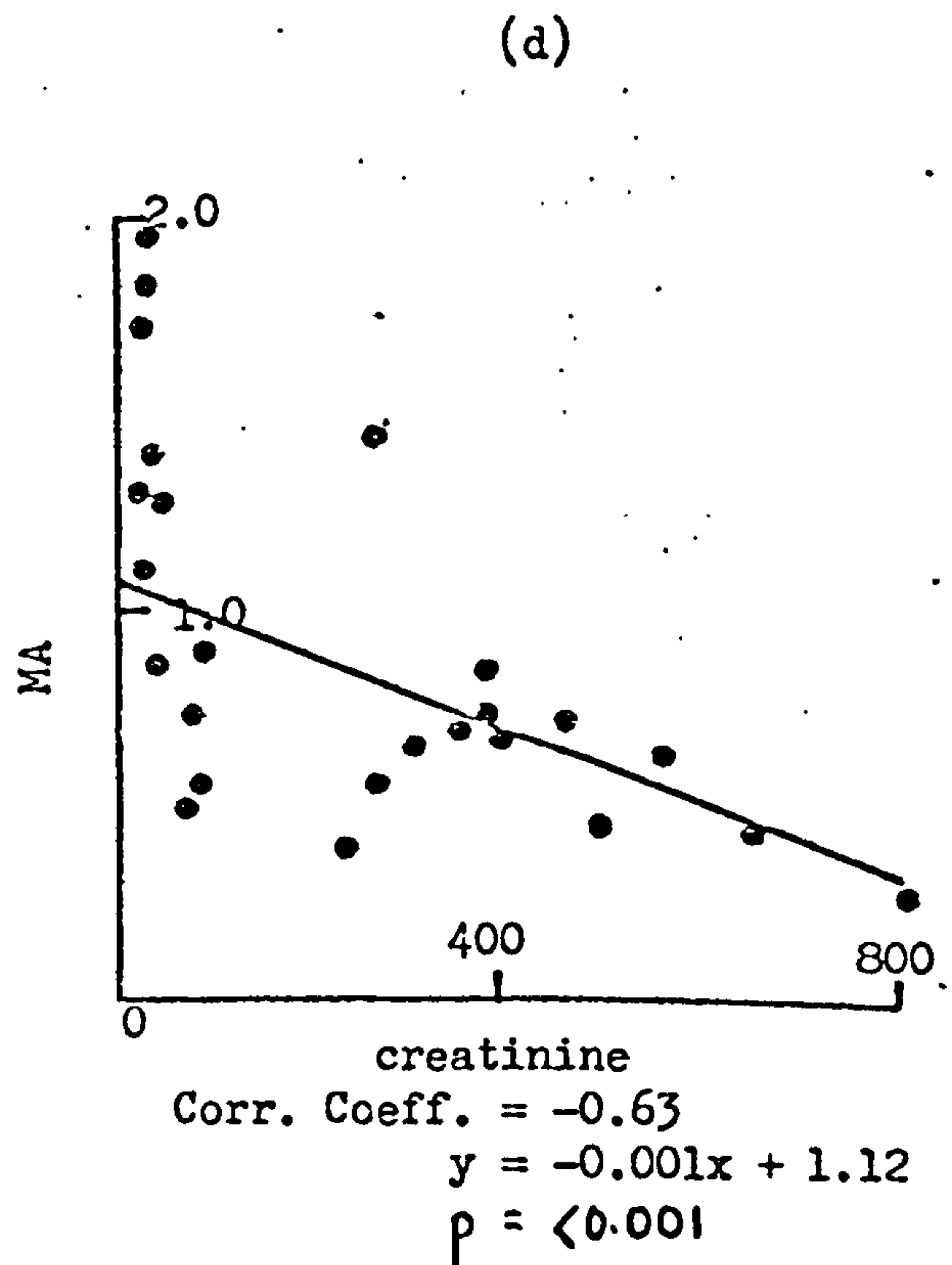
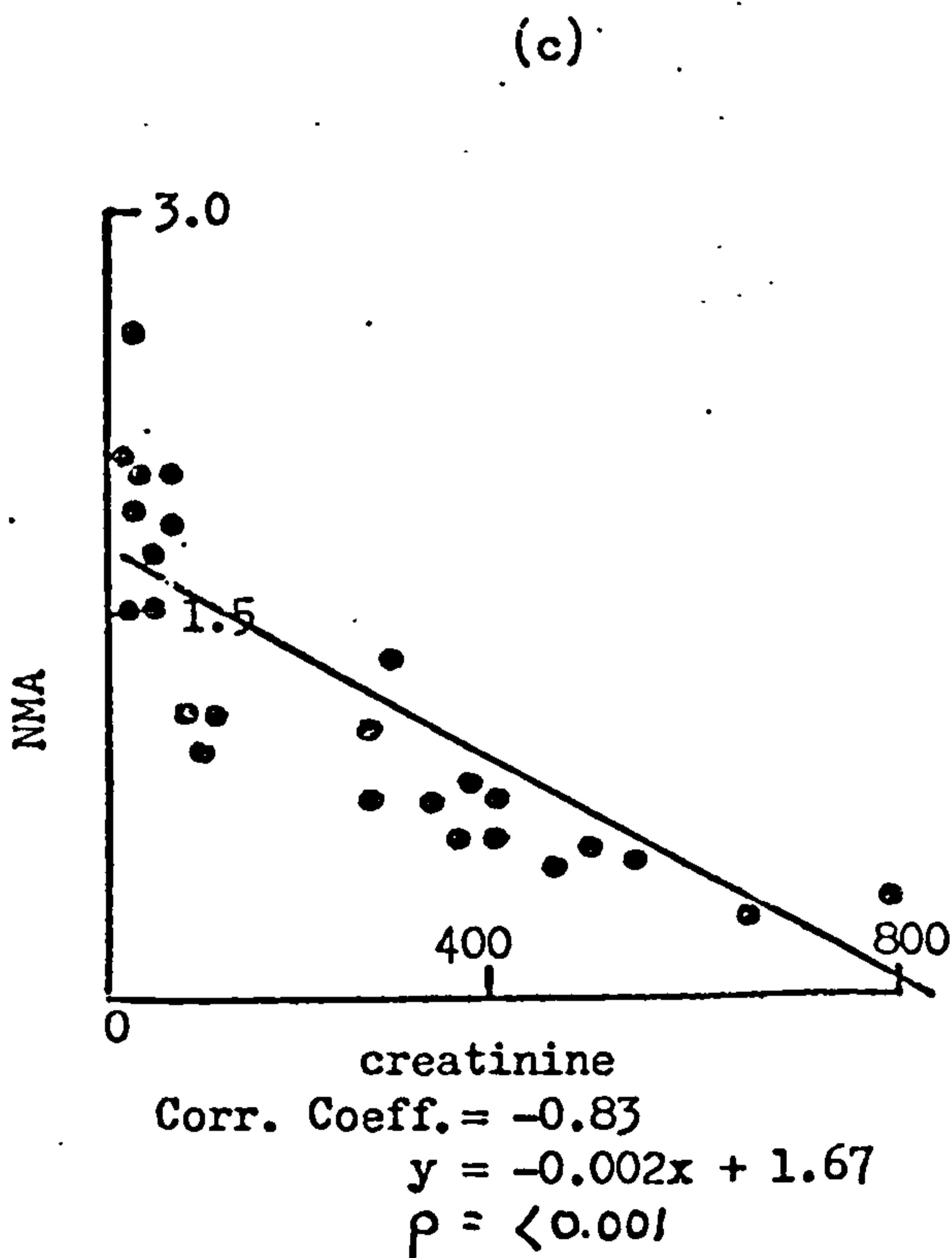
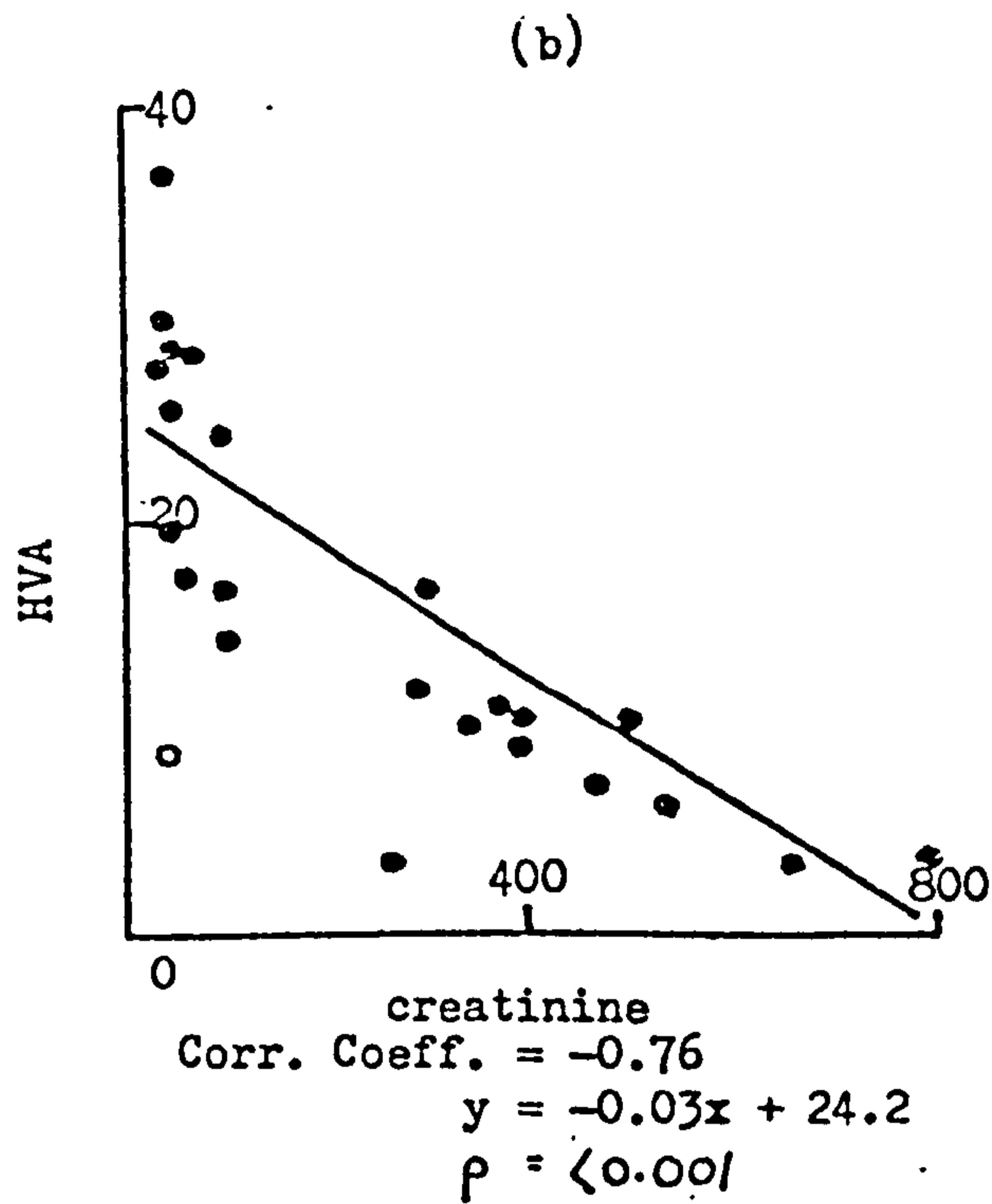
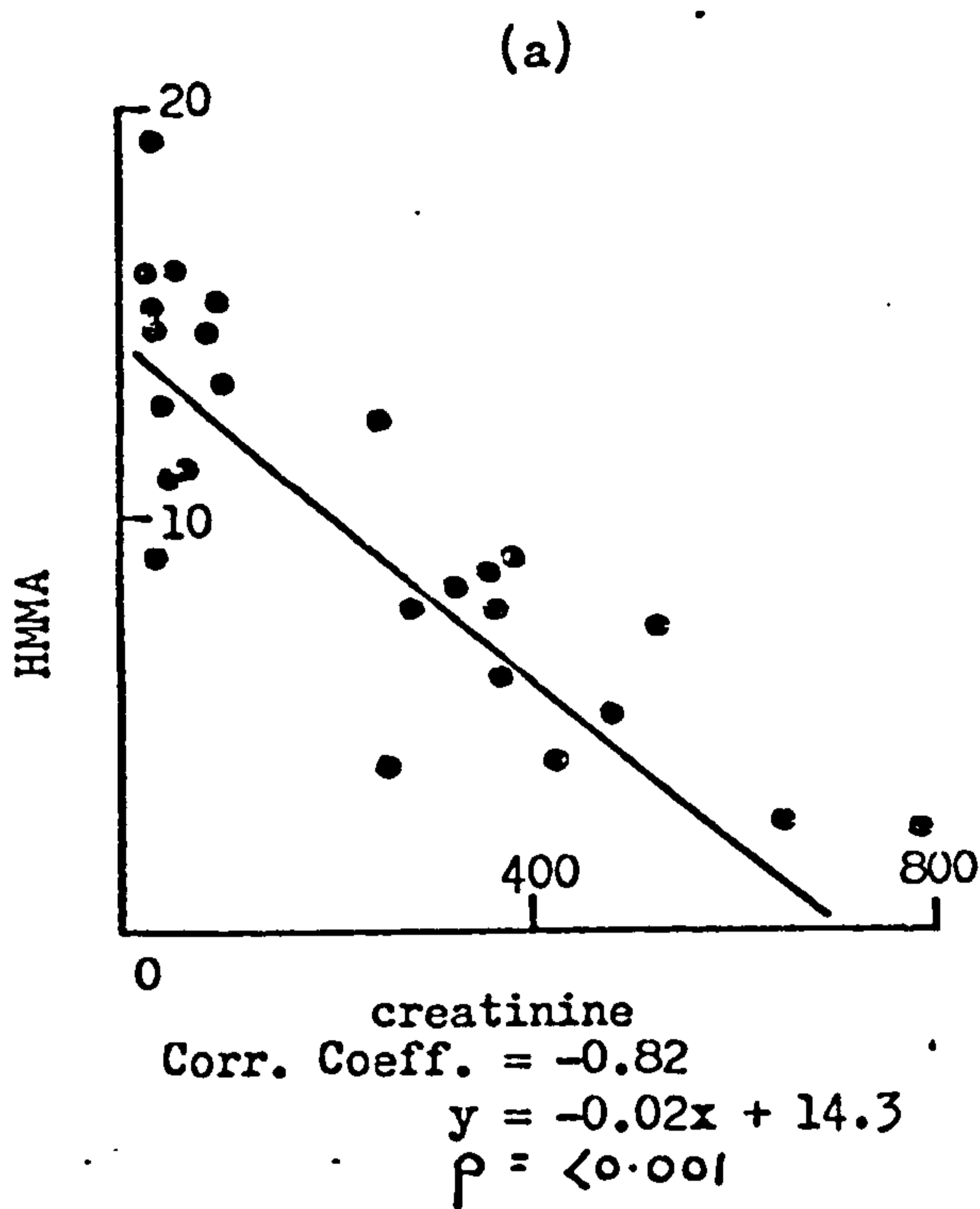


Fig 5 Output of metabolite in ug per mg creatinine compared with creatinine output mg per 24 h



and body surface area, in order to find the most reliable diagnostic index for assessing babies and young children who are thought to be suffering from a catecholamine secreting tumour.

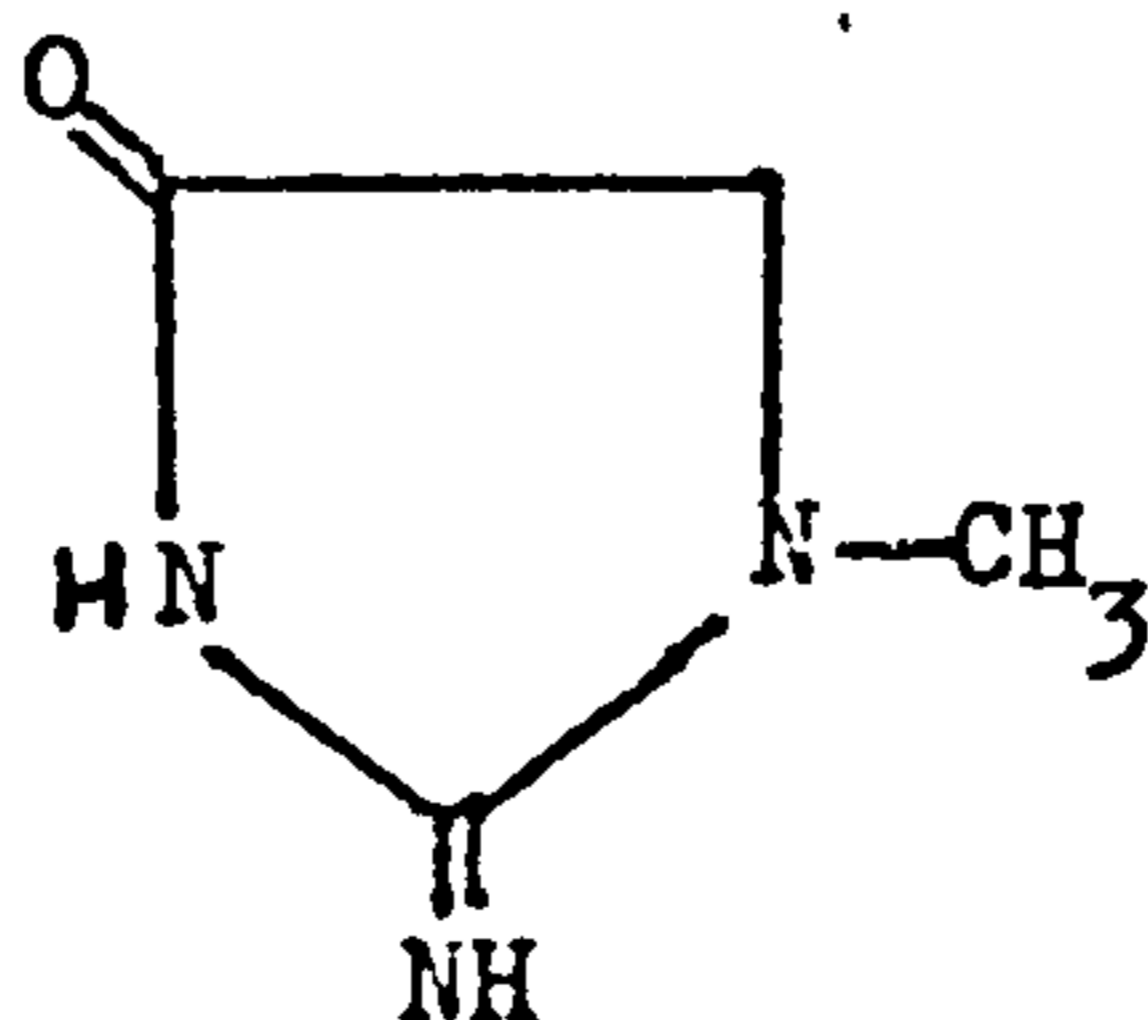
A further aim of this work has been to seek any relationships which might exist between the various pairs of metabolites and to establish whether the ratio of their levels is of diagnostic value (e.g. in neuroblastoma).

3.2. Results

The results in Table 10 indicate that the differences in the mean daily outputs of HMMA, HVA, NMA and MA from the young babies (Group A) and the children (Group B) are statistically significant ($p < 0.001$). Similarly, the differences in the mean outputs of urine per 24 h between the babies (Group A) and young children (Group B) are statistically significant ($p < 0.001$). Likewise the differences in the mean body surface area and the differences in the mean outputs of creatinine per 24 h were also shown to have 'p' values less than 0.001.

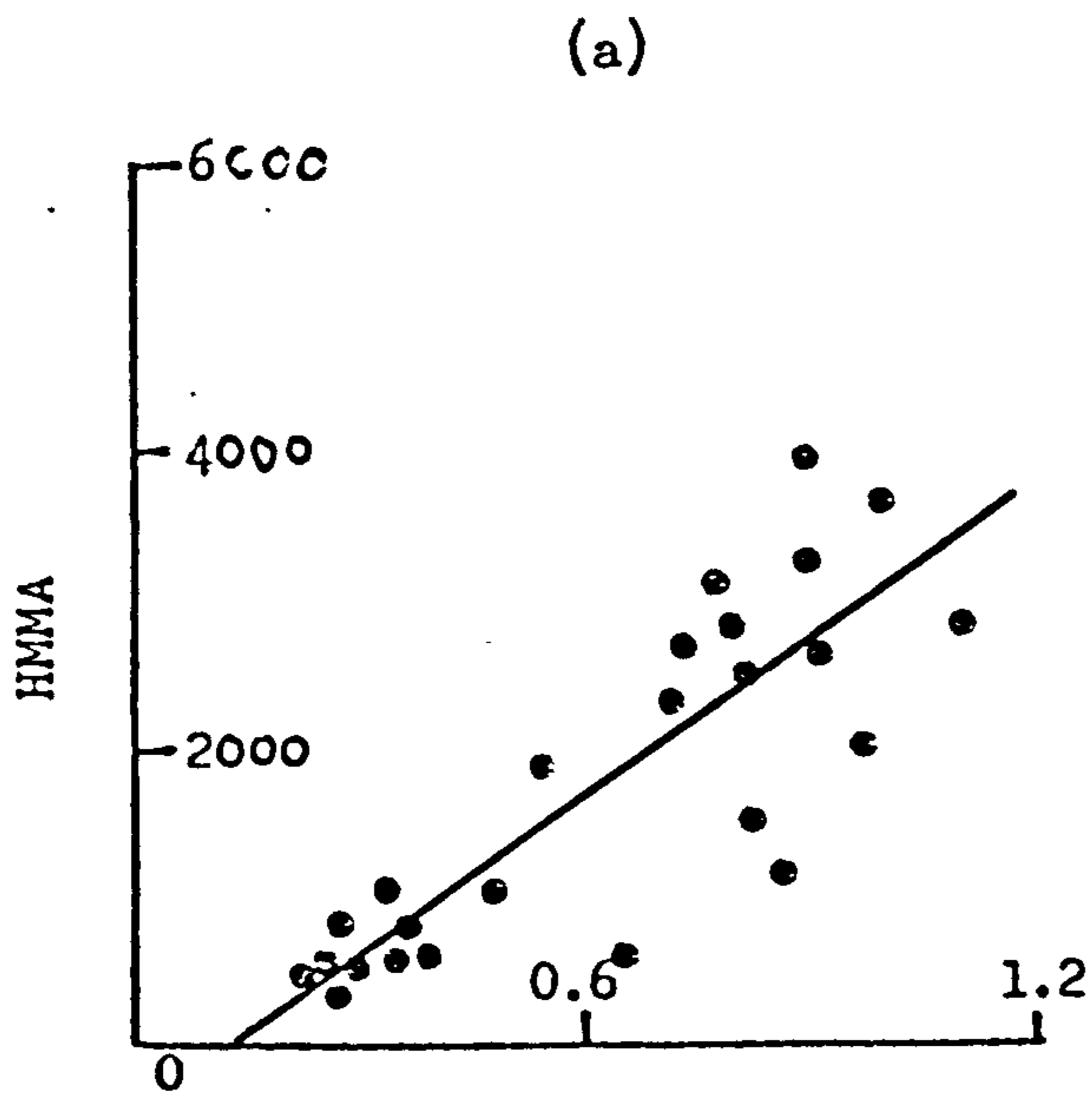
When the values for the daily outputs of HMMA, HVA, NMA and MA are plotted against age (Fig. 3 a - d) we see that each metabolite shows a good correlation. The results (Fig. 4, a - d) show that there is also a good correlation between the output of all four metabolites when compared with the urine volume per 24 h.

Because of the difficulties inherent in collecting 24 h urines accurately from young babies and children, creatinine (IX) levels were determined on all the urine samples and the results expressed as μg metabolite per mg of creatinine.

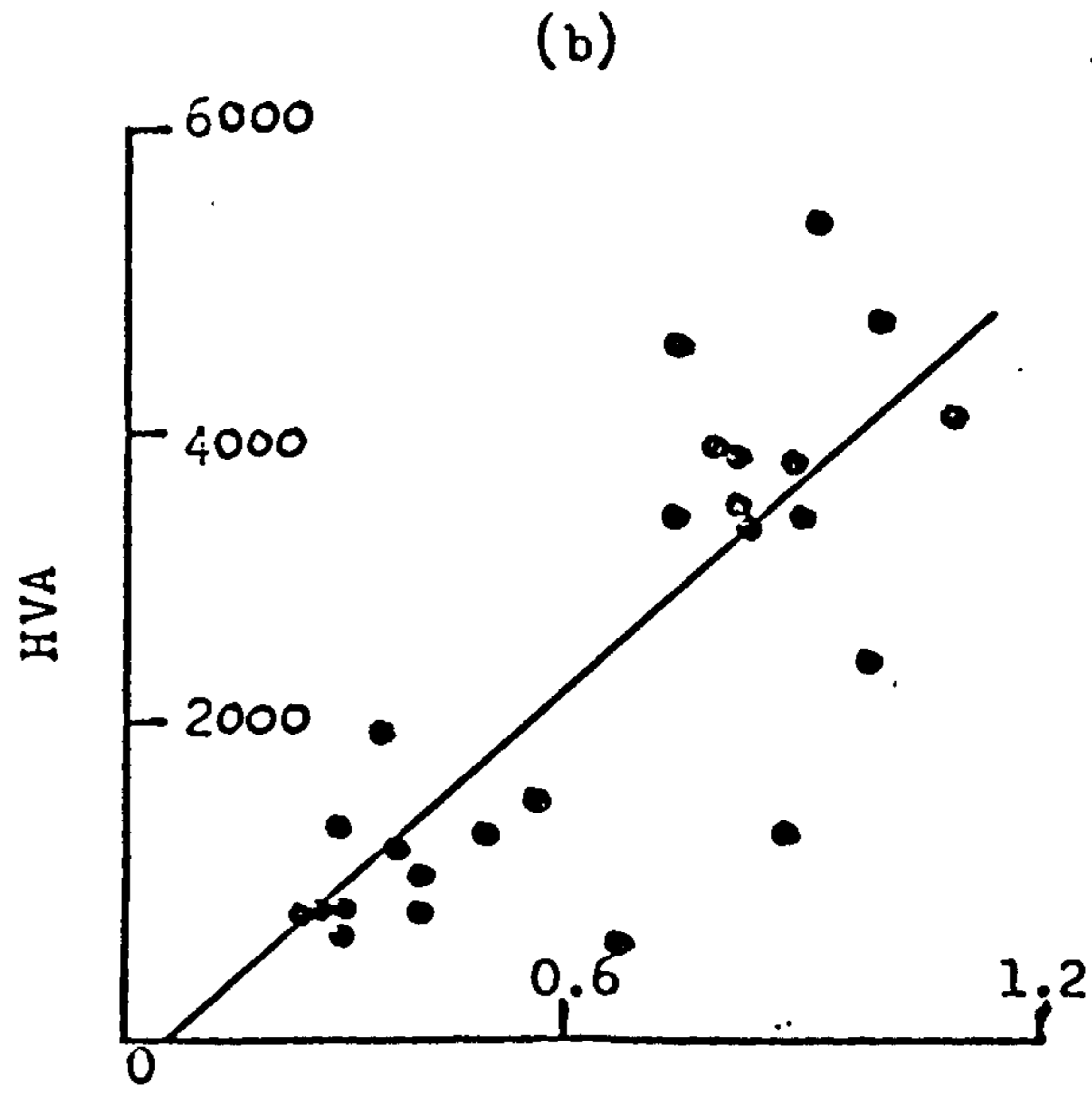


(IX)

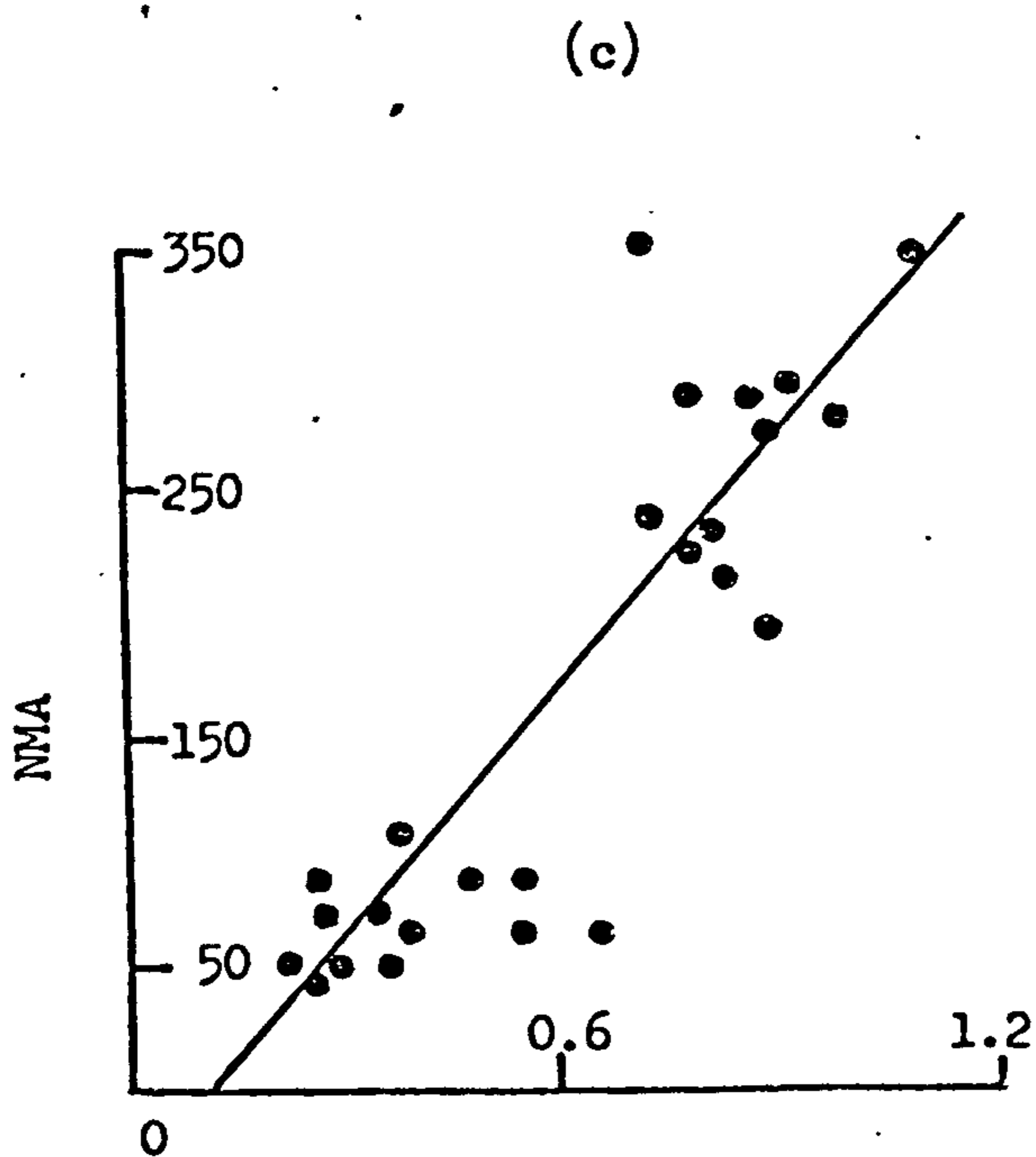
Fig 6 Output of metabolites μg per 24 h compared with body surface area (m^2)



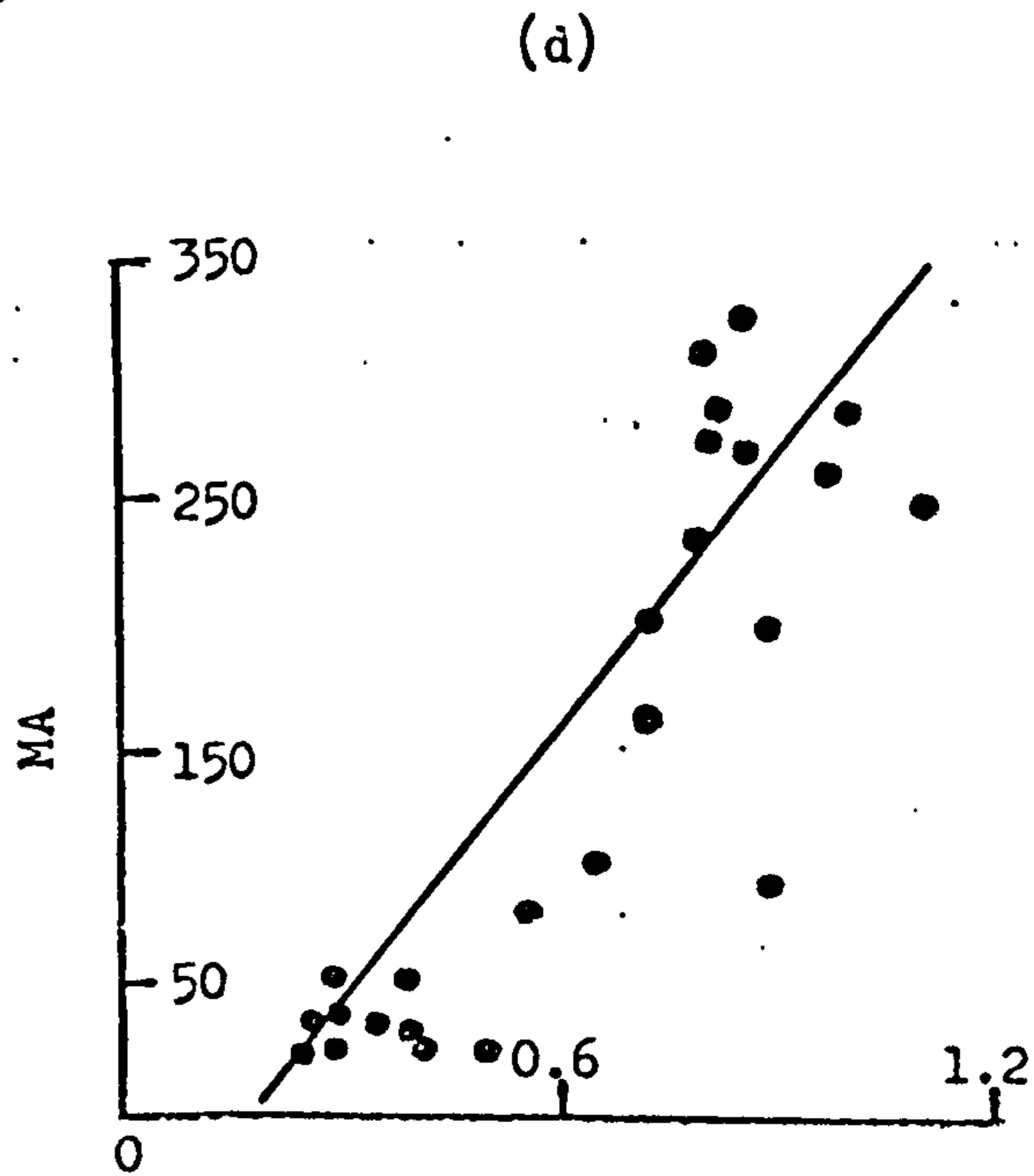
Body surface area
 Corr. Coeff. = 0.84
 $y = 3.6x - 0.5$
 $p < 0.001$



Body surface area
 Corr. Coeff. = 0.79
 $y = 4.5x - 0.3$
 $p < 0.001$



Body surface area
 Corr. Coeff. = 0.86
 $y = 338x - 38$
 $p < 0.001$



Body surface area
 Corr. Coeff. = 0.85
 $y = 349x - 67$
 $p < 0.001$

Table 11

Output of catecholamine metabolites expressed as (1) μg metabolite per cm^3 urine,

(2) μg metabolite per mg creatinine, (3) μg metabolite per m^2 body surface area

obtained from Group A (babies) compared with those from Group B (children)

	(1) μg metabolite per 24 h urine volume per 24 h (cm^3)				(2) μg metabolite per 24 h creatinine per 24 h				(3) μg metabolite per 24 h surface area (m^2)			
	HVMA	HVA	NMA	MA	HMMA	HVA	NMA	MA	HMMA	HVA	NMA	MA
Group A												
0-12 months	5.7 \pm 3.5	8.5 \pm 3.7	0.54 \pm 0.23	0.36 \pm 0.18	14.7 \pm 2.5	25.1 \pm 7.1	1.67 \pm 0.49	1.68 \pm 0.46	2,164 \pm 715	3,491 \pm 1,143	219 \pm 53	142 \pm 38
Range	2.8-12.6	5.3-15.8	0.22-0.98	0.12-0.78	11.3-19.4	14.5-37.1	0.94-2.62	0.34-1.90	1,385-3,454	2,461-6,165	131-303	74-215
Group B												
2-11 years	5.3 \pm 1.8	7.4 \pm 2.6	0.53 \pm 0.13	0.50 \pm 0.19	7.1 \pm 2.7	9.7 \pm 3.8	0.81 \pm 0.45	0.72 \pm 0.43	2,976 \pm 1,031	4,343 \pm 1,519	300 \pm 92	276 \pm 87
Range	2.1-8.9	2.3-12.8	0.26-0.75	0.24-0.98	3.3-12.5	3.7-17.1	0.3-2.1	0.3-1.9	892-4,539	969-6,662	111-511	103-404
t'	0.38	0.90	0.15	-1.81	7.2	6.9	4.6	1.99	-2.2	-1.6	-2.6	-4.7
	N/S	N/S	N/S	0.1>P>0.05	<0.001	<0.001	<0.001	0.1>P>0.05	0.05>P>0.02	N/S	0.02>P>0.01	<0.001

Fig 7 Output of urinary metabolites μg per cm^3 over a 24 h collection period compared with age in years

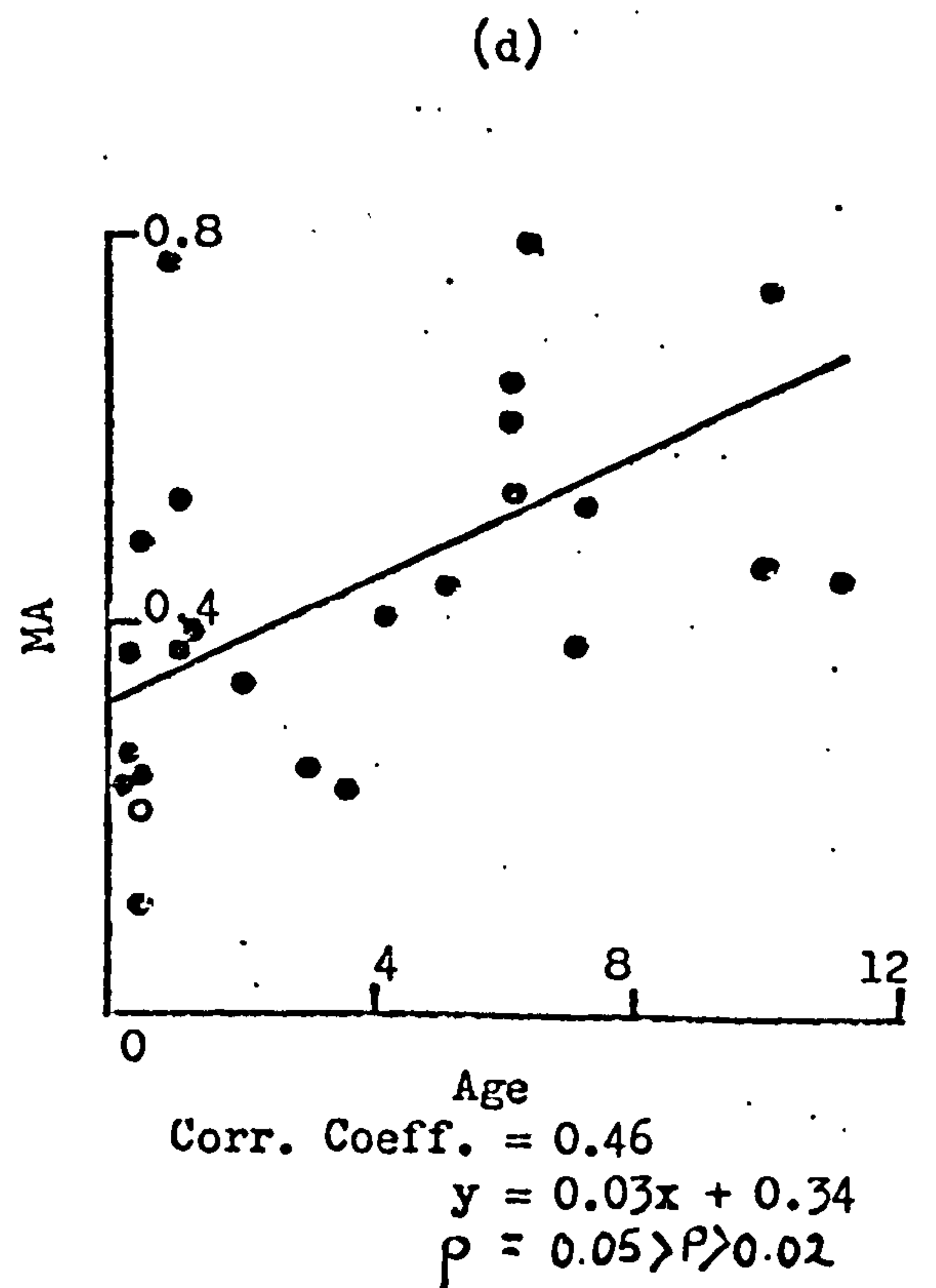
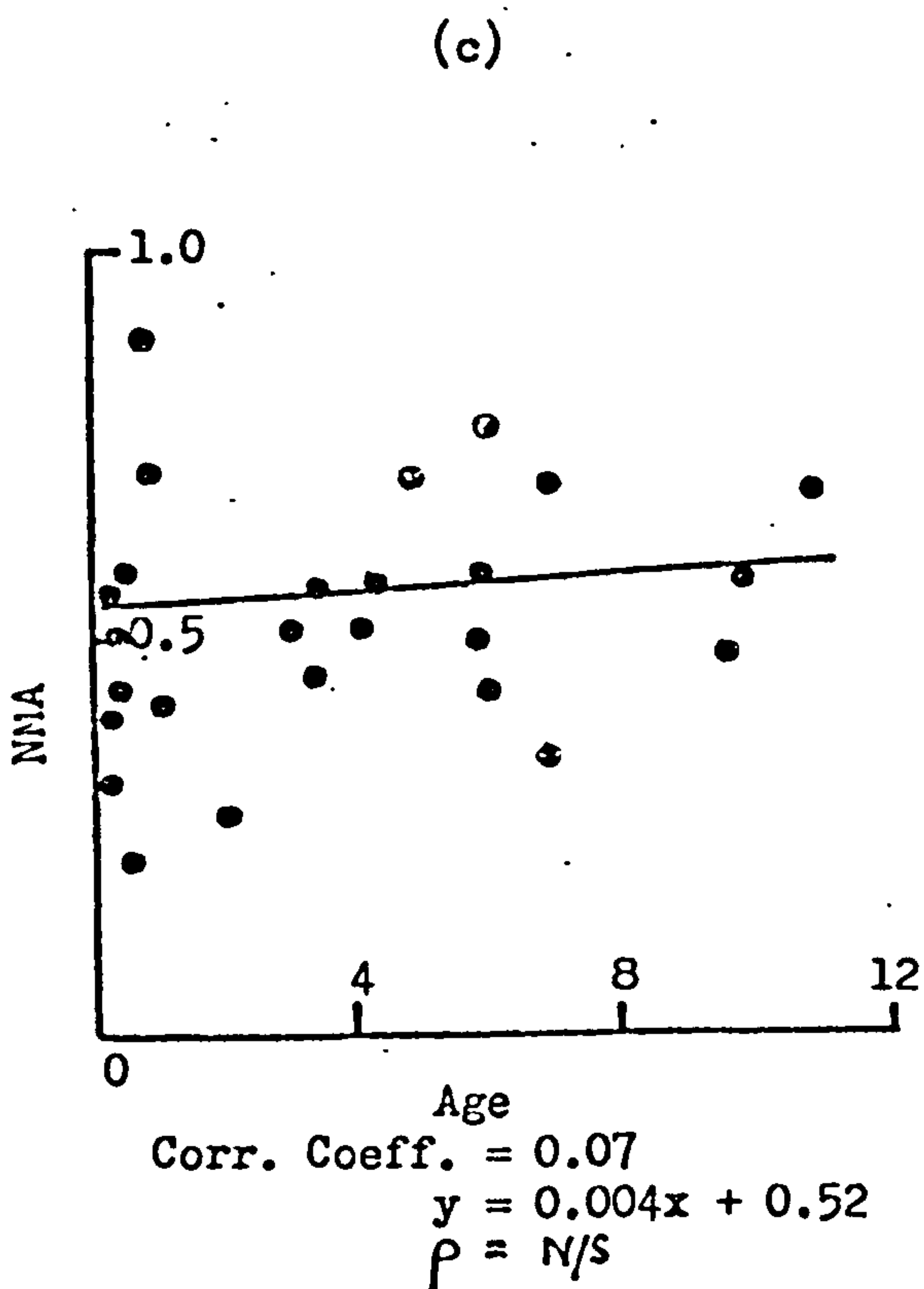
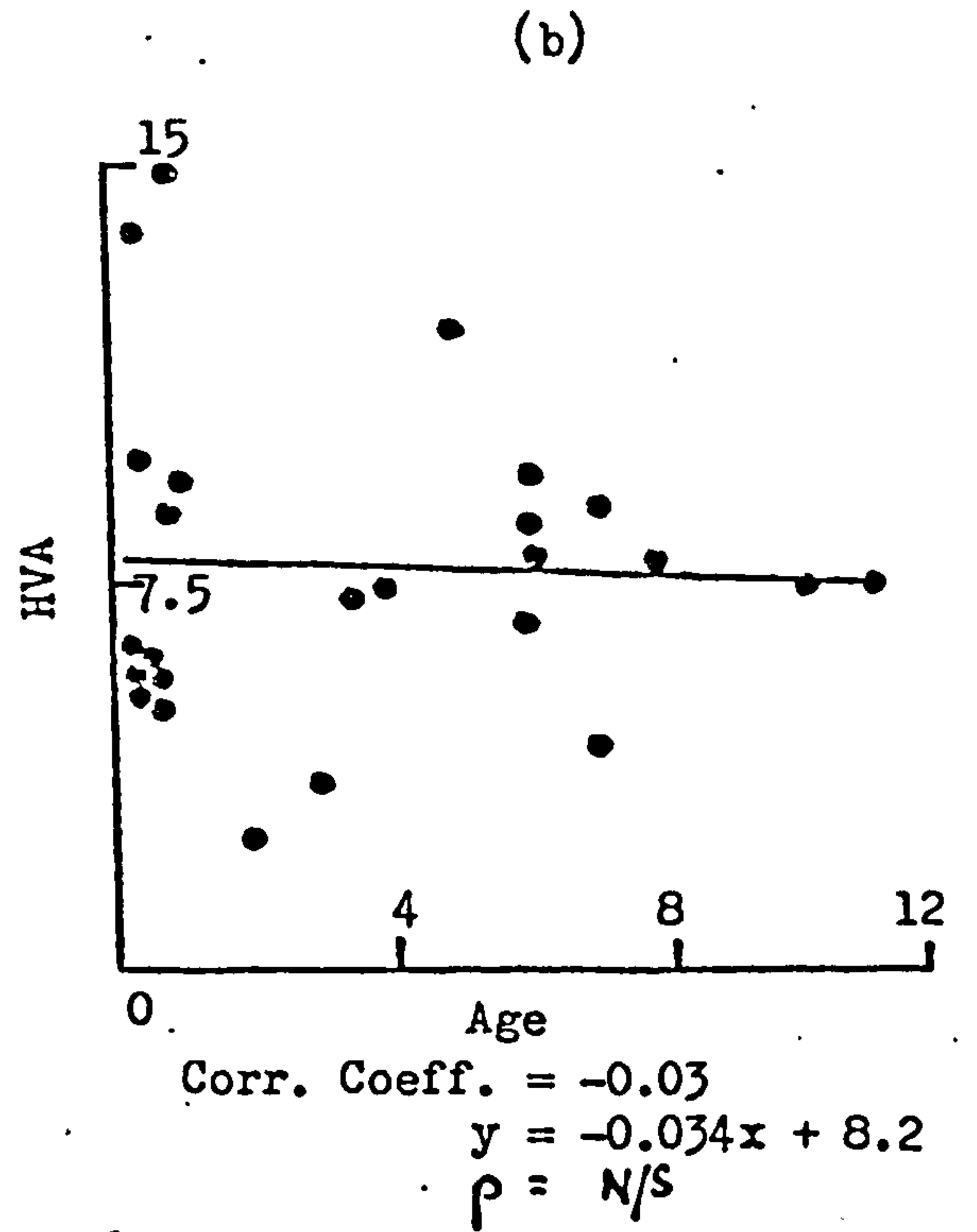
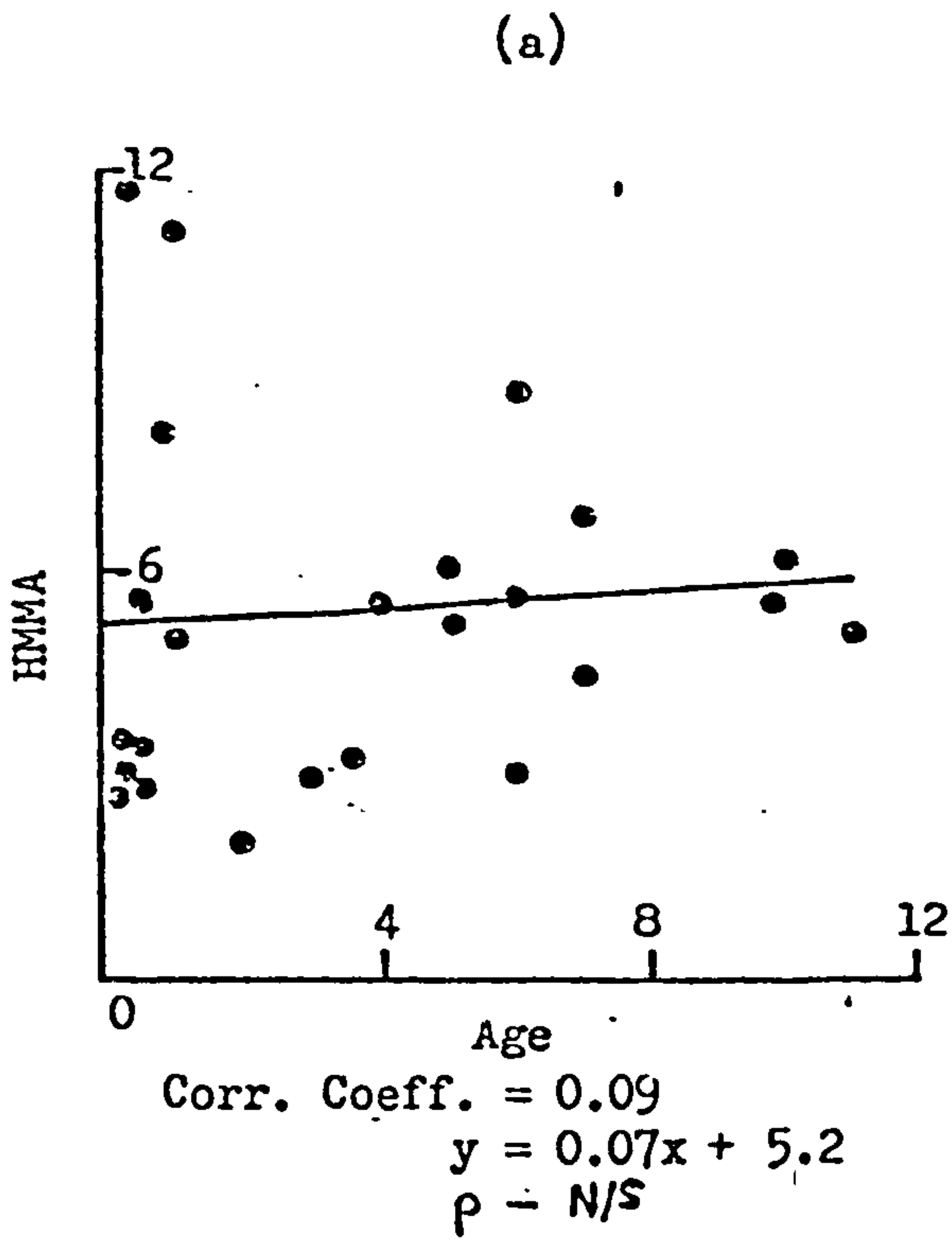
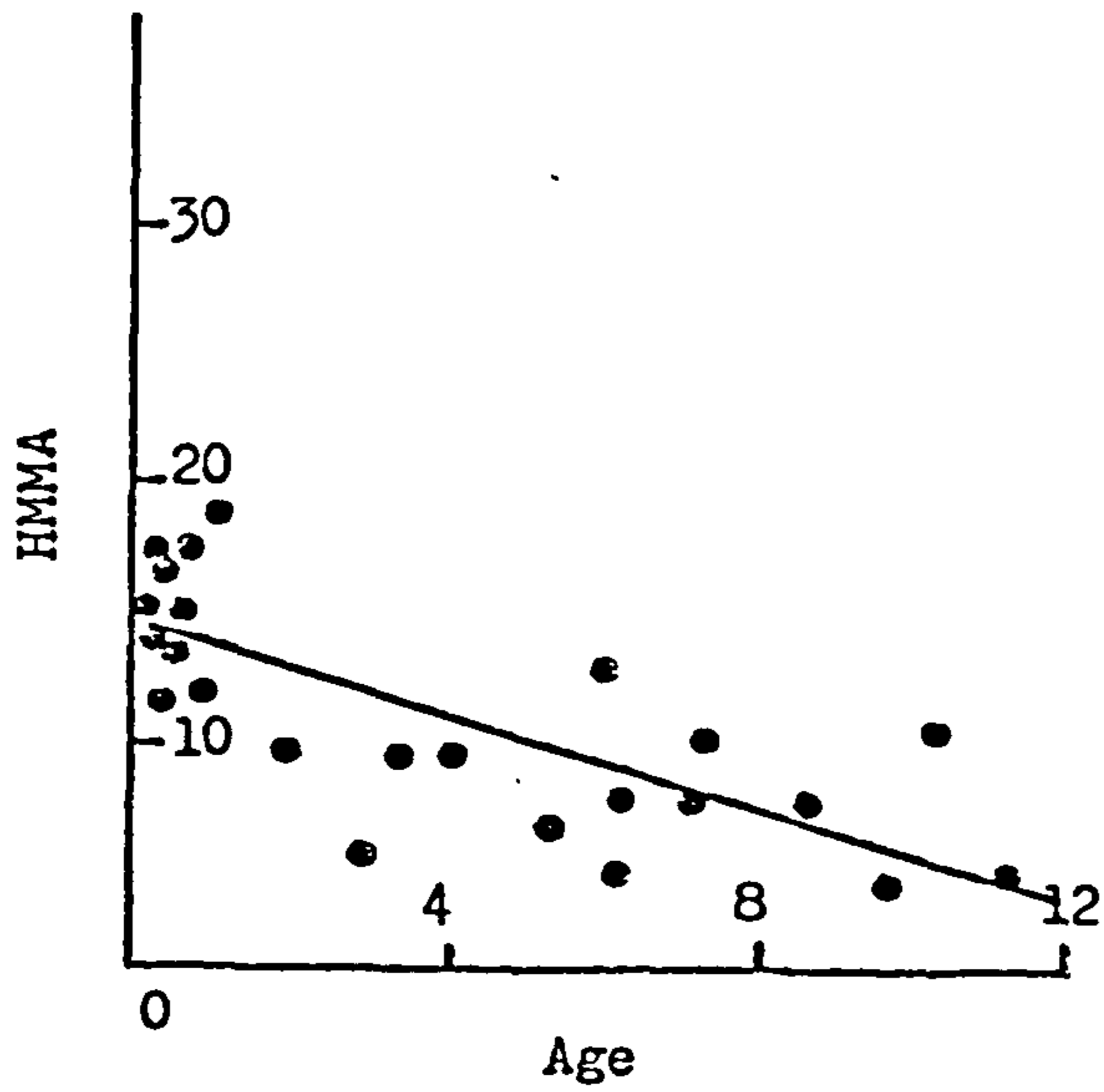


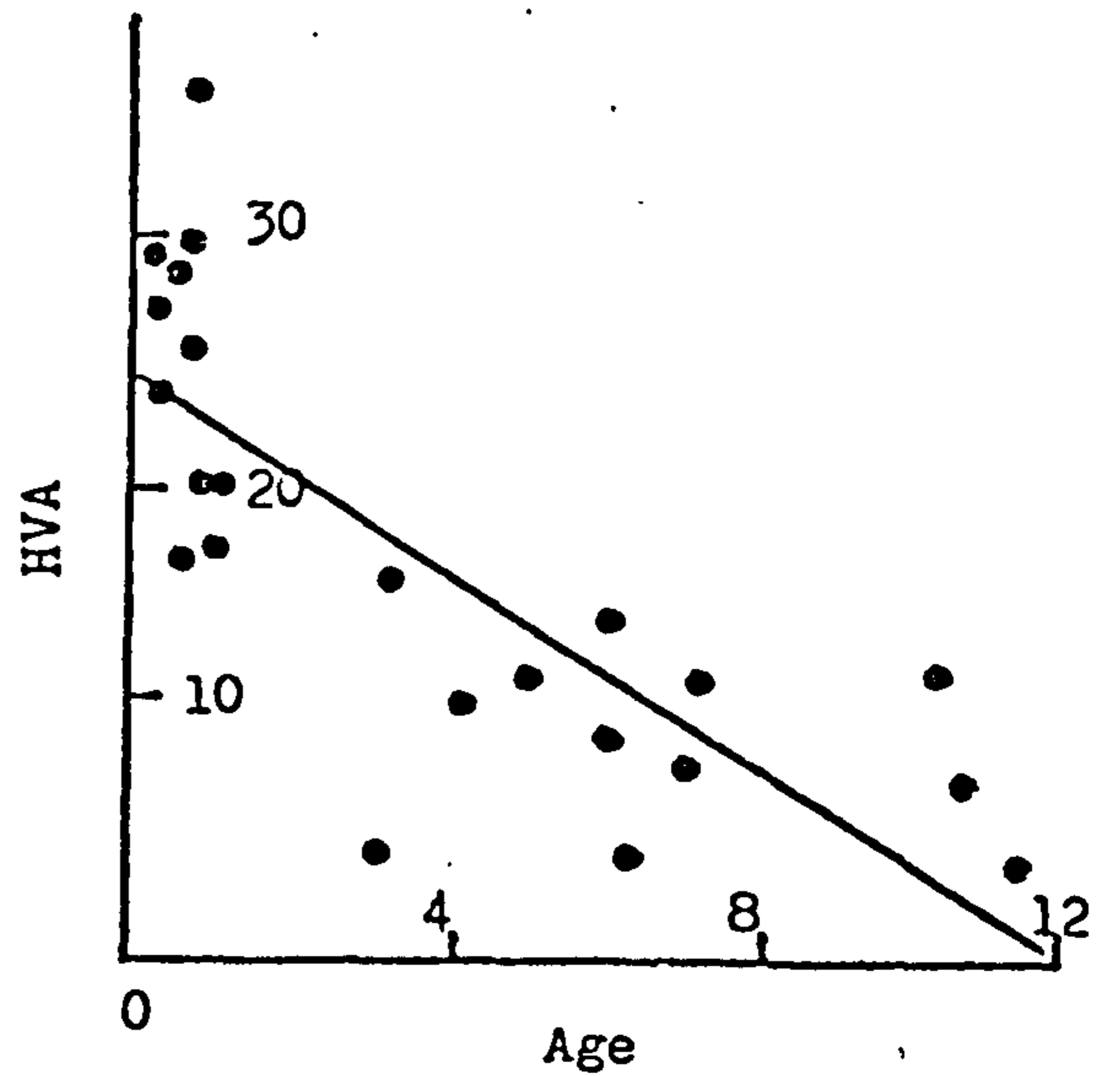
Fig 8 Output of metabolite in μg per mg of creatinine compared with age in years

(a)



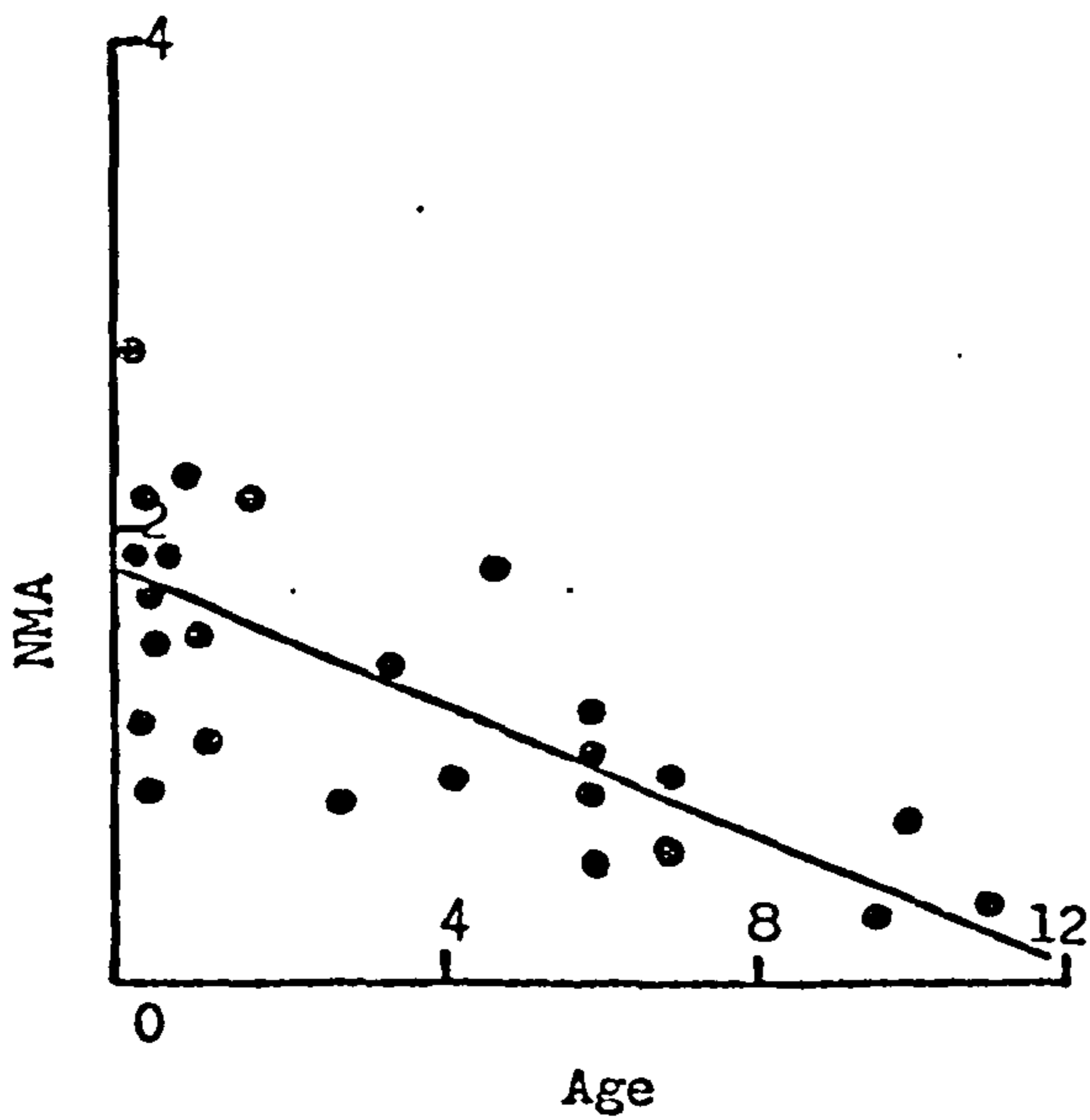
Corr. Coeff. = -0.73
 $y = -1.0x + 14$
 $\rho = <0.001$

(b)



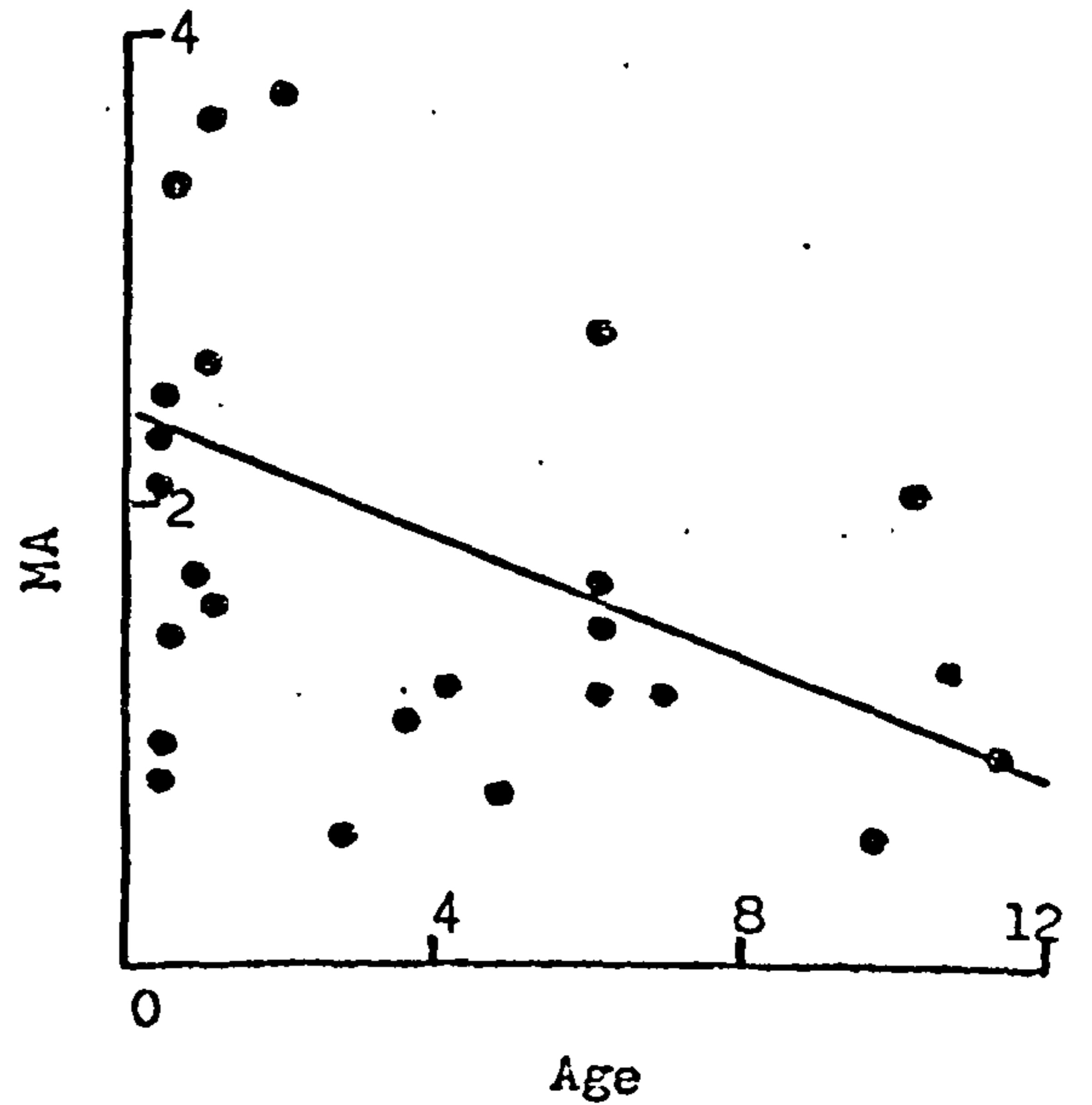
Corr. Coeff. = -0.75
 $y = -2.1x + 24$
 $\rho = <0.001$

(c)



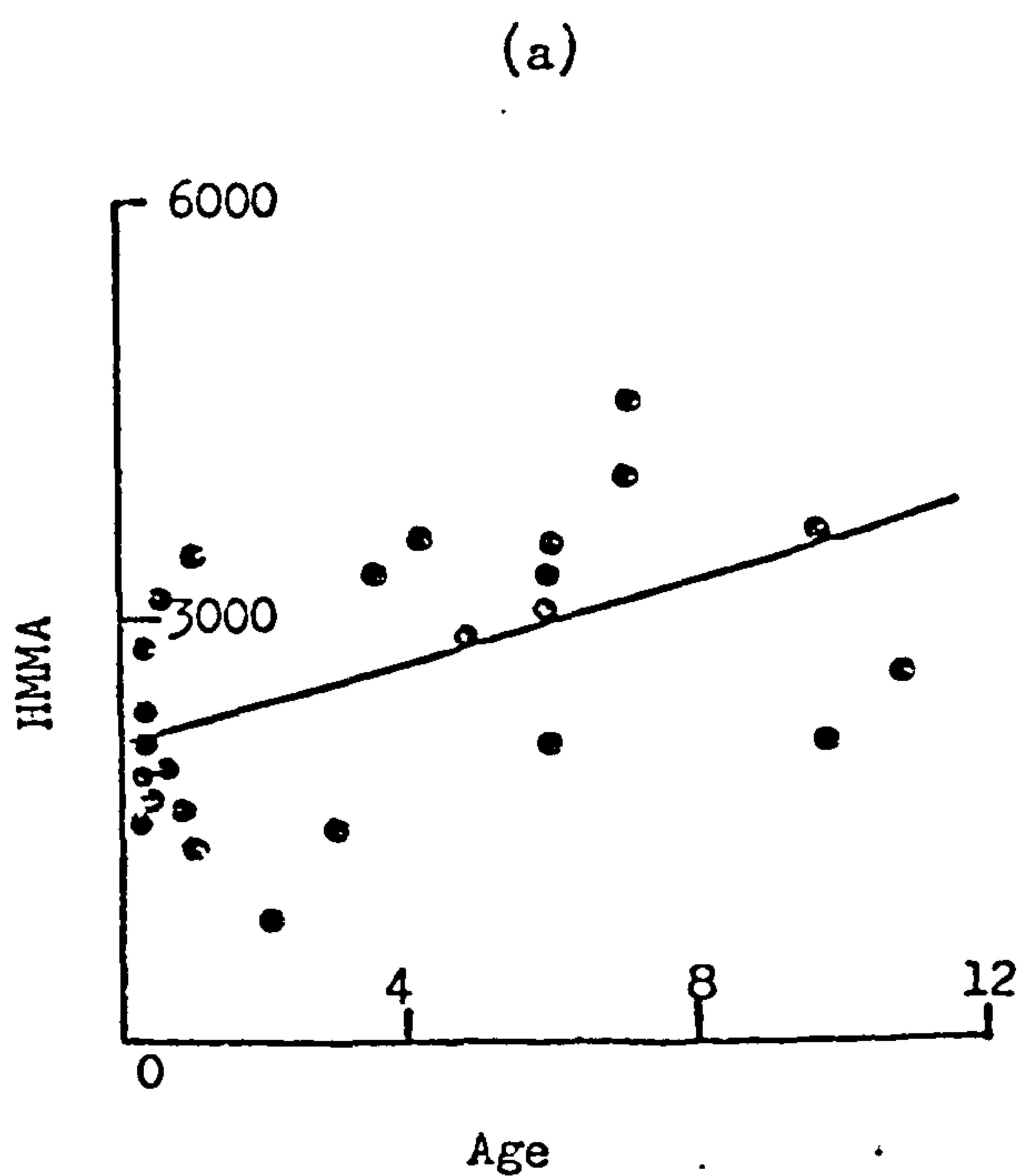
Corr. Coeff. = -0.76
 $y = -0.14x + 1.7$
 $\rho = <0.001$

(d)

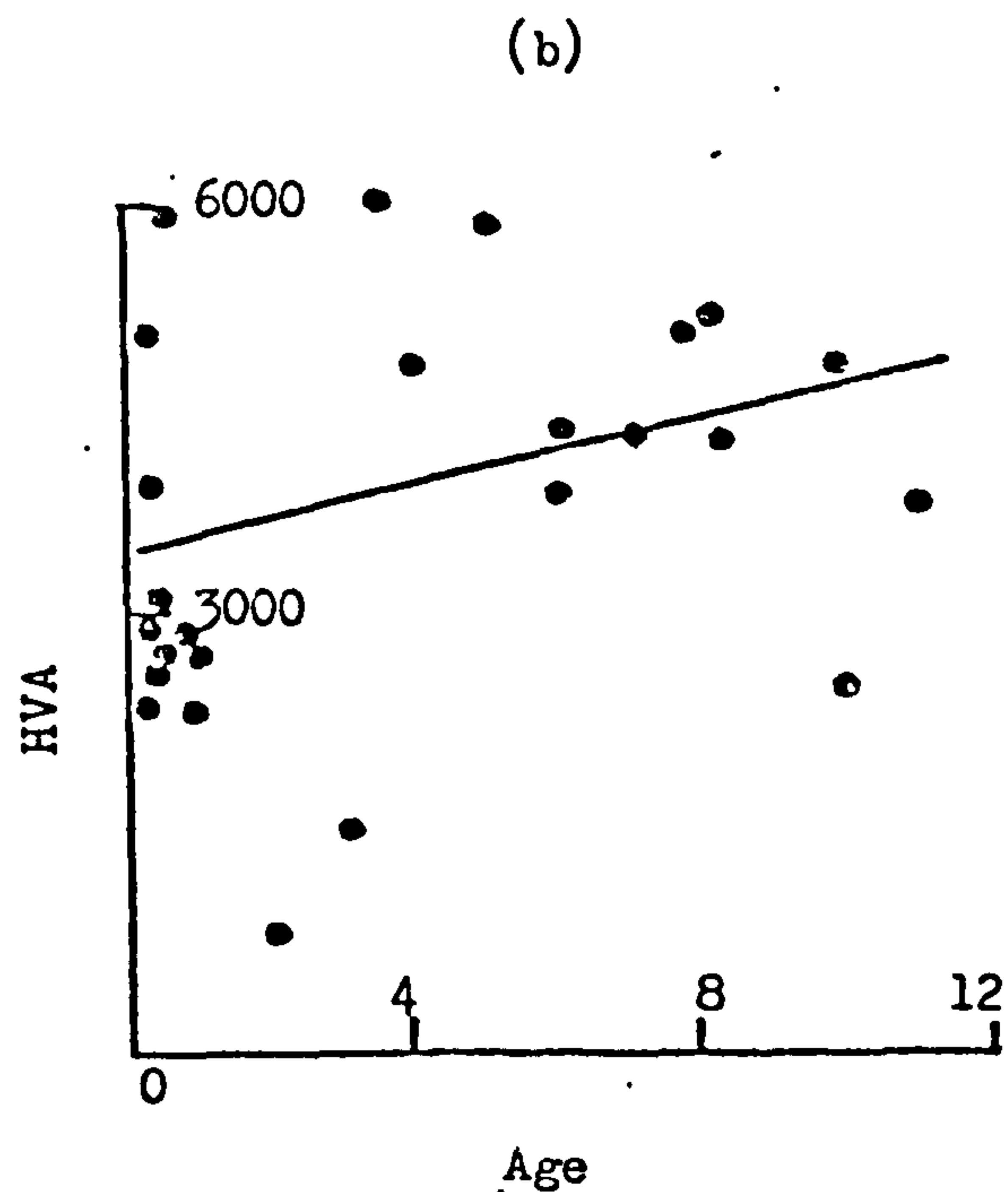


Corr. Coeff. = -0.46
 $y = -0.06x + 2.1$
 $\rho = 0.05 > P > 0.02$

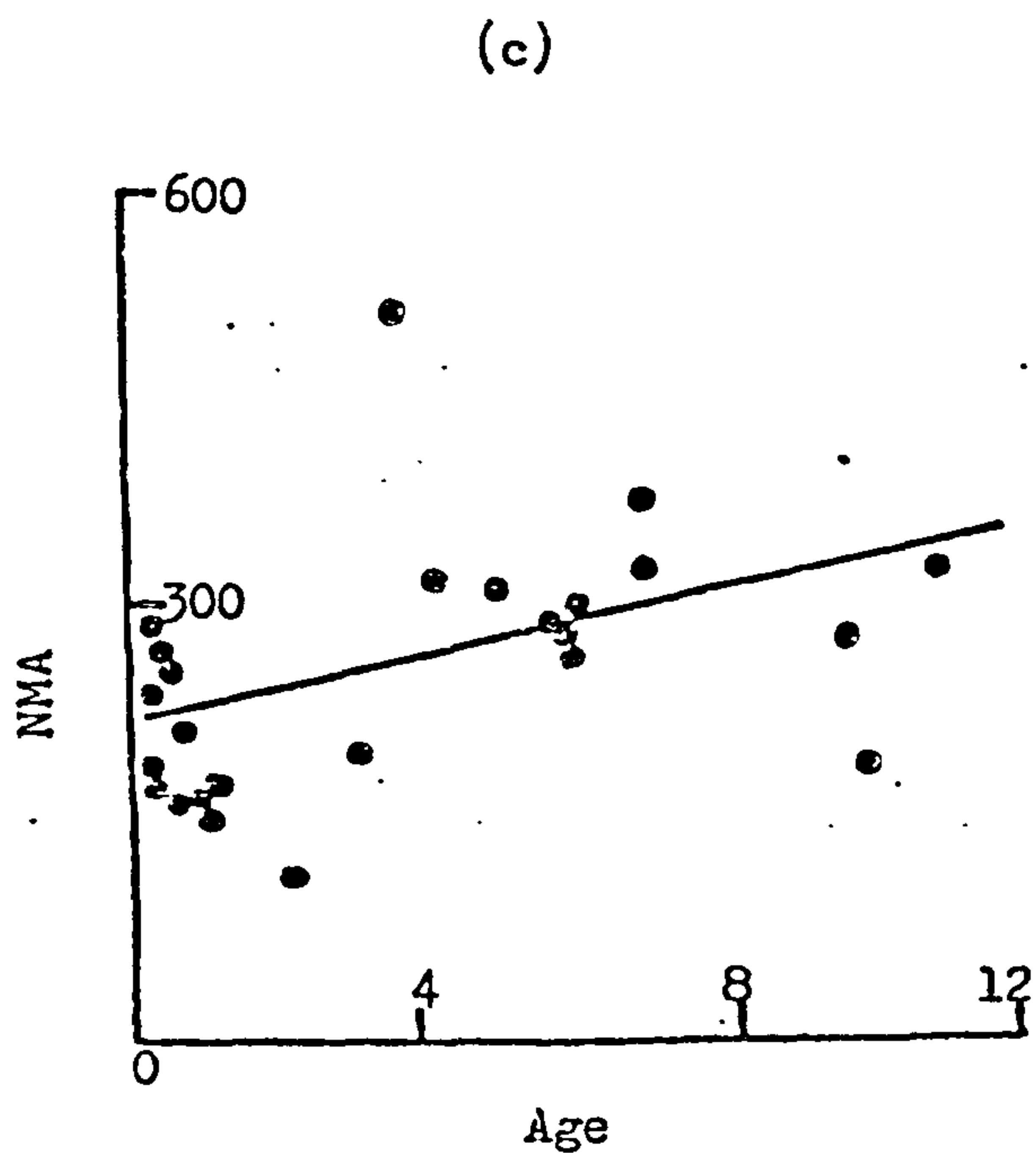
Fig 9 Output of metabolite in μg per m^2 of body surface area compared with age in years



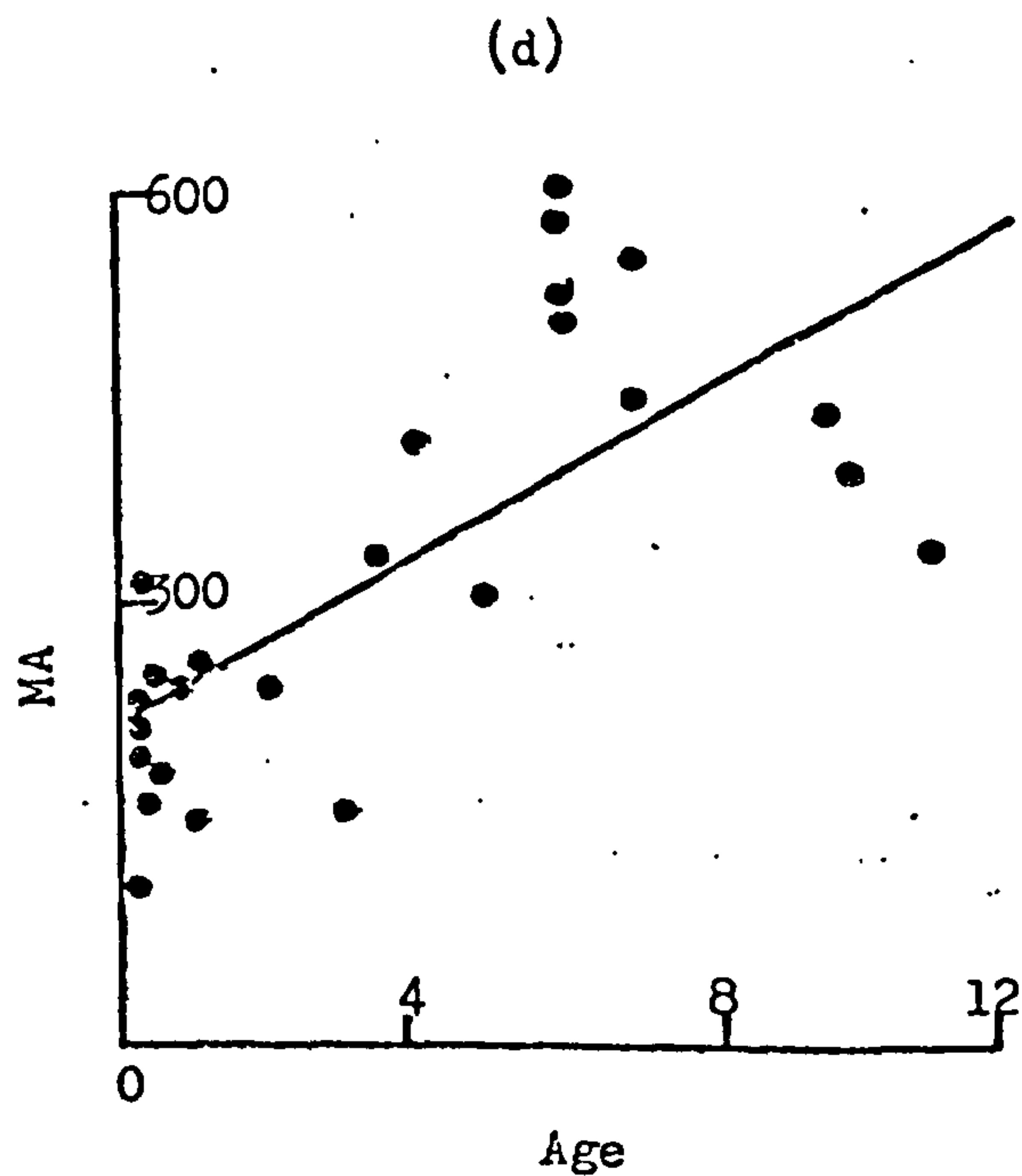
Corr. Coeff. = 0.49
 $y = 138x + 2113$
 $p = 0.02 > p > 0.01$



Corr. Coeff. = 0.26
 $y = 106x + 3501$
 $p = \text{N/S}$



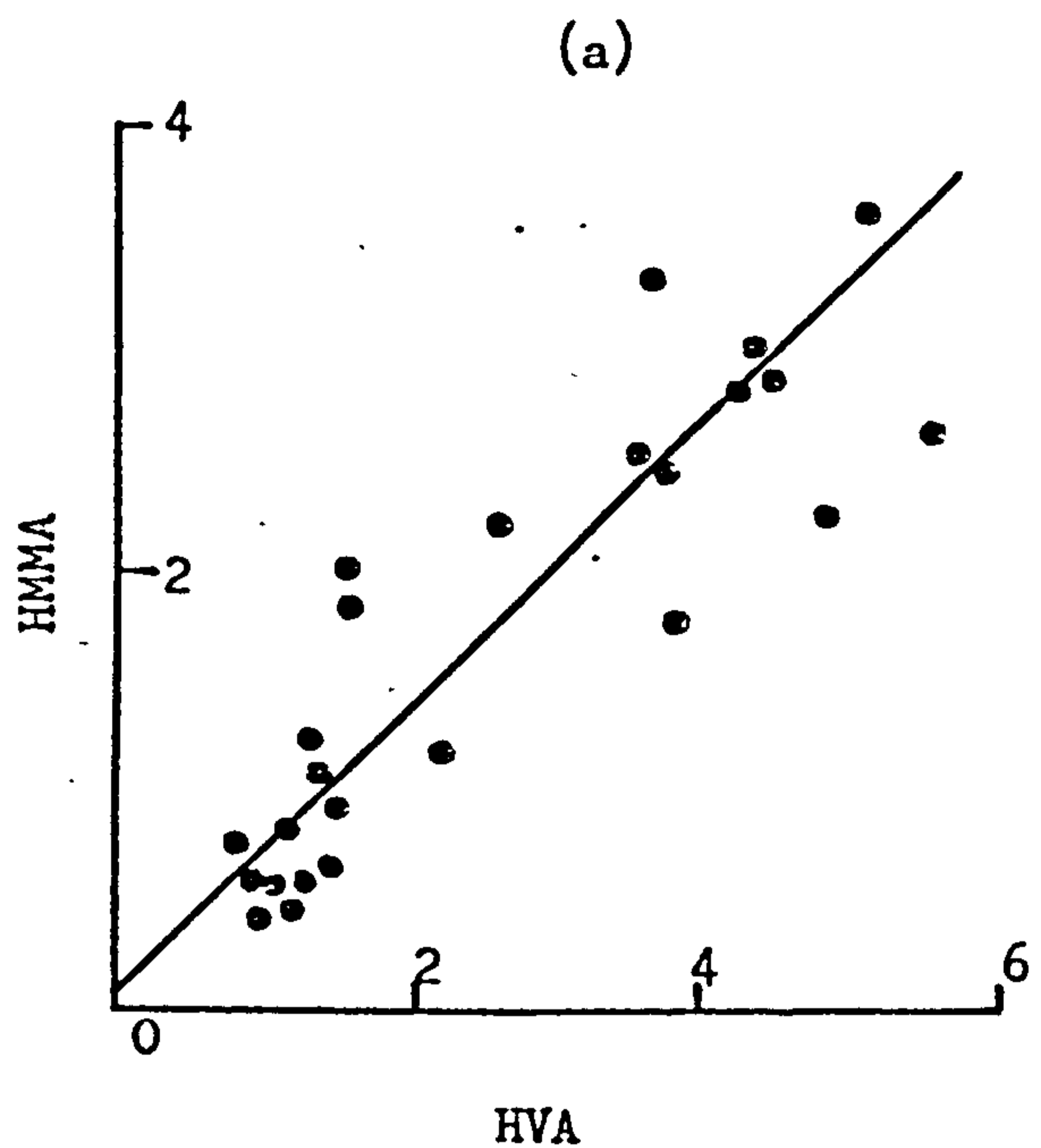
Corr. Coeff. = 0.40
 $y = 10x + 228$
 $p = 0.05$



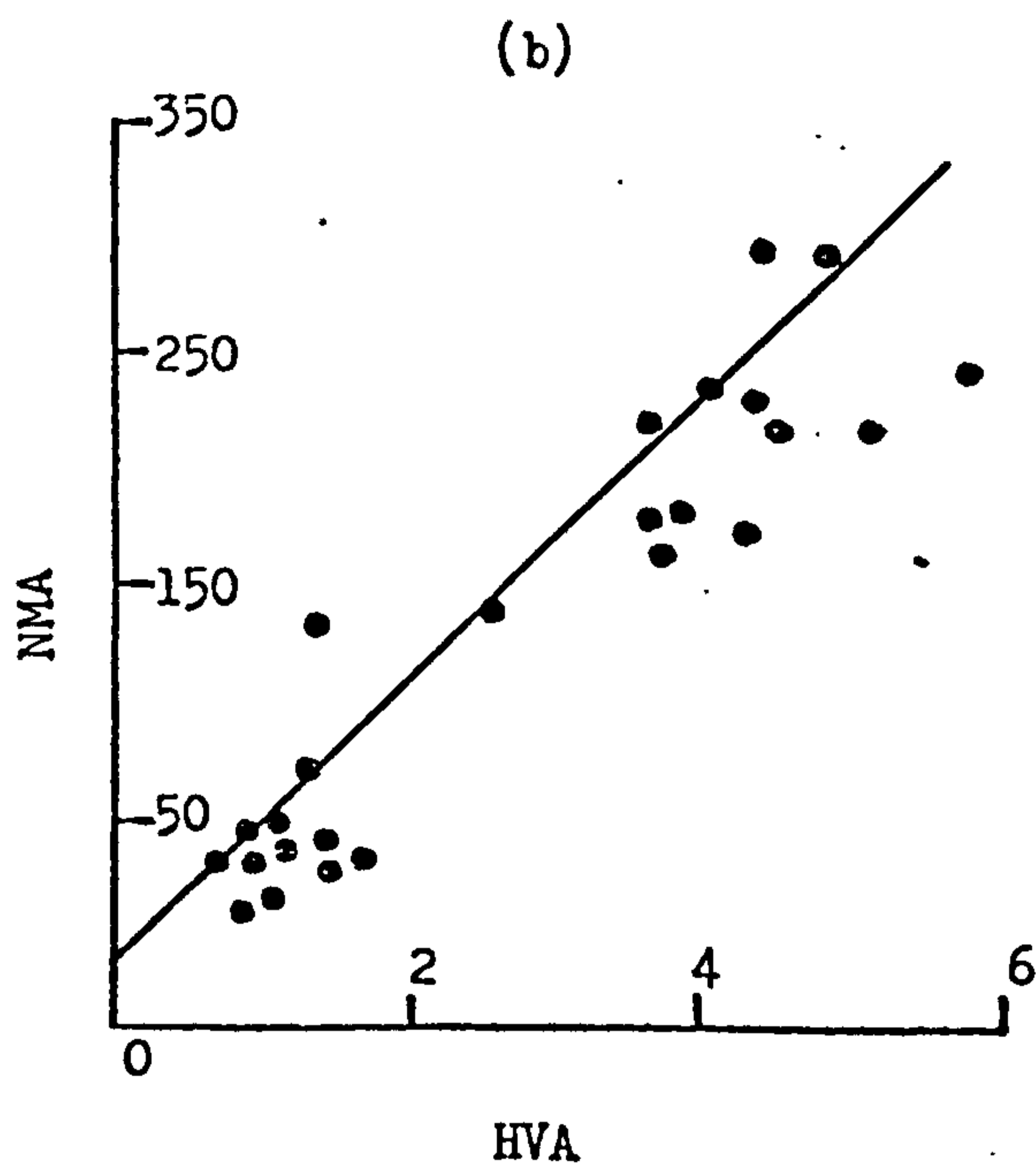
Corr. Coeff. = 0.70
 $y = 19x + 246$
 $p = < 0.001$

Fig 10 (a) Correlation mg per 24 h for HMMA compared with HVA mg per 24 h

(b) Correlation μ g per 24 h for NMA compared with HVA mg per 24 h



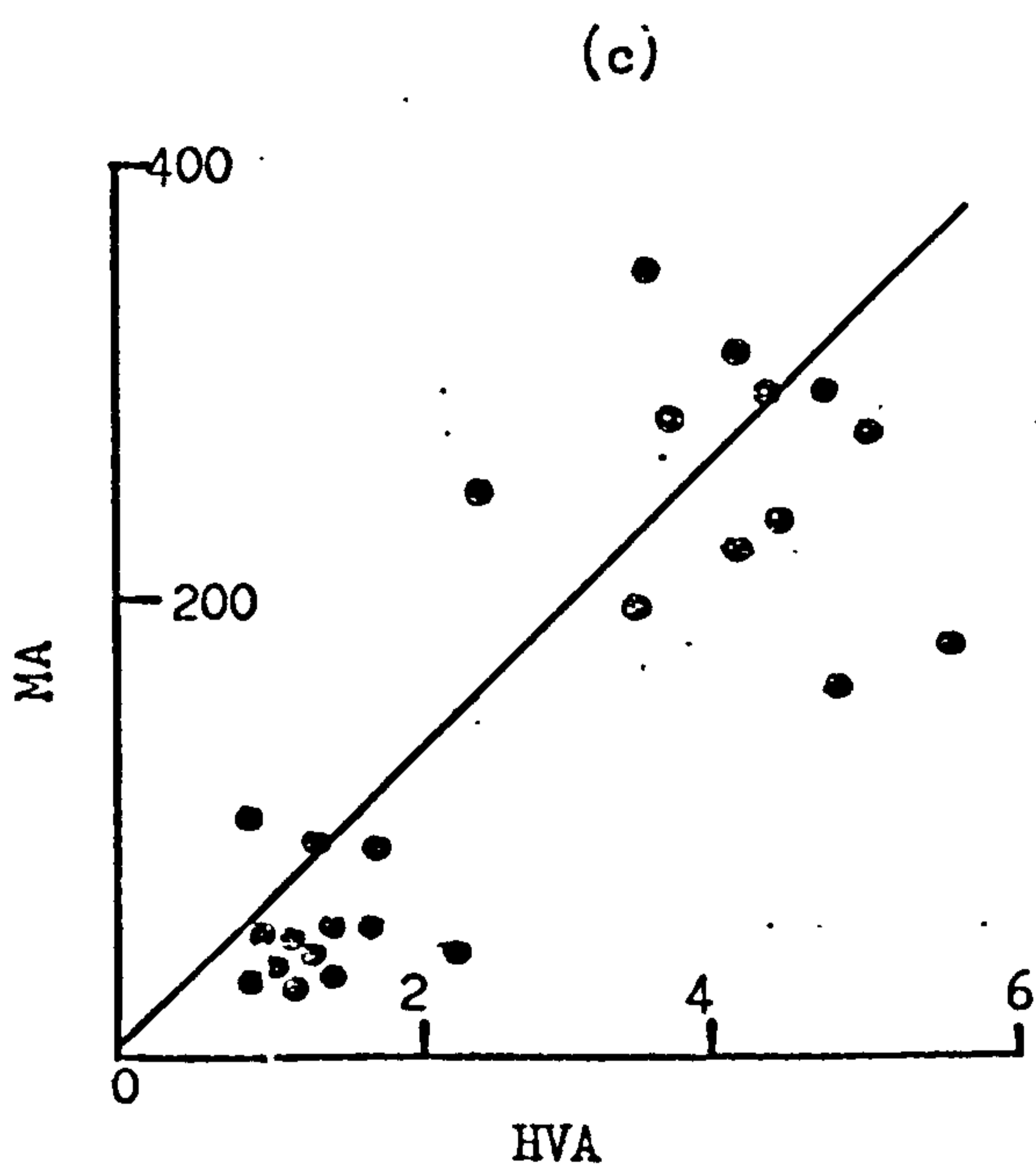
Corr. Coeff. = 0.89
 $y = 0.66x + 0.11$
 $p < 0.001$



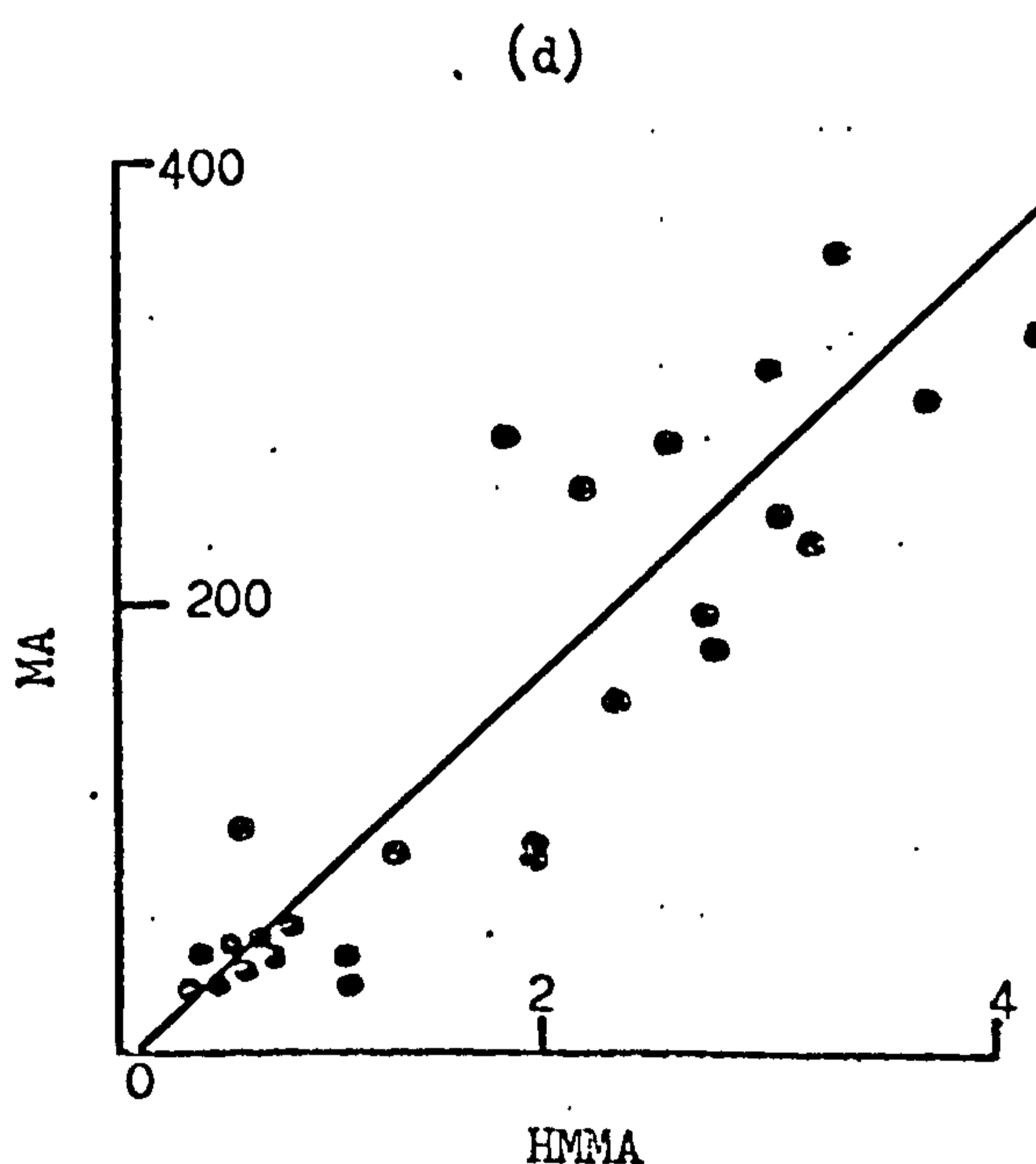
Corr. Coeff. = 0.90
 $y = .40x + 37$
 $p < 0.001$

(c) Correlation between MA μ g per 24 h compared with HVA mg per 24 h

(d) Correlation between MA μ g per 24 h compared with HMMA mg per 24 h



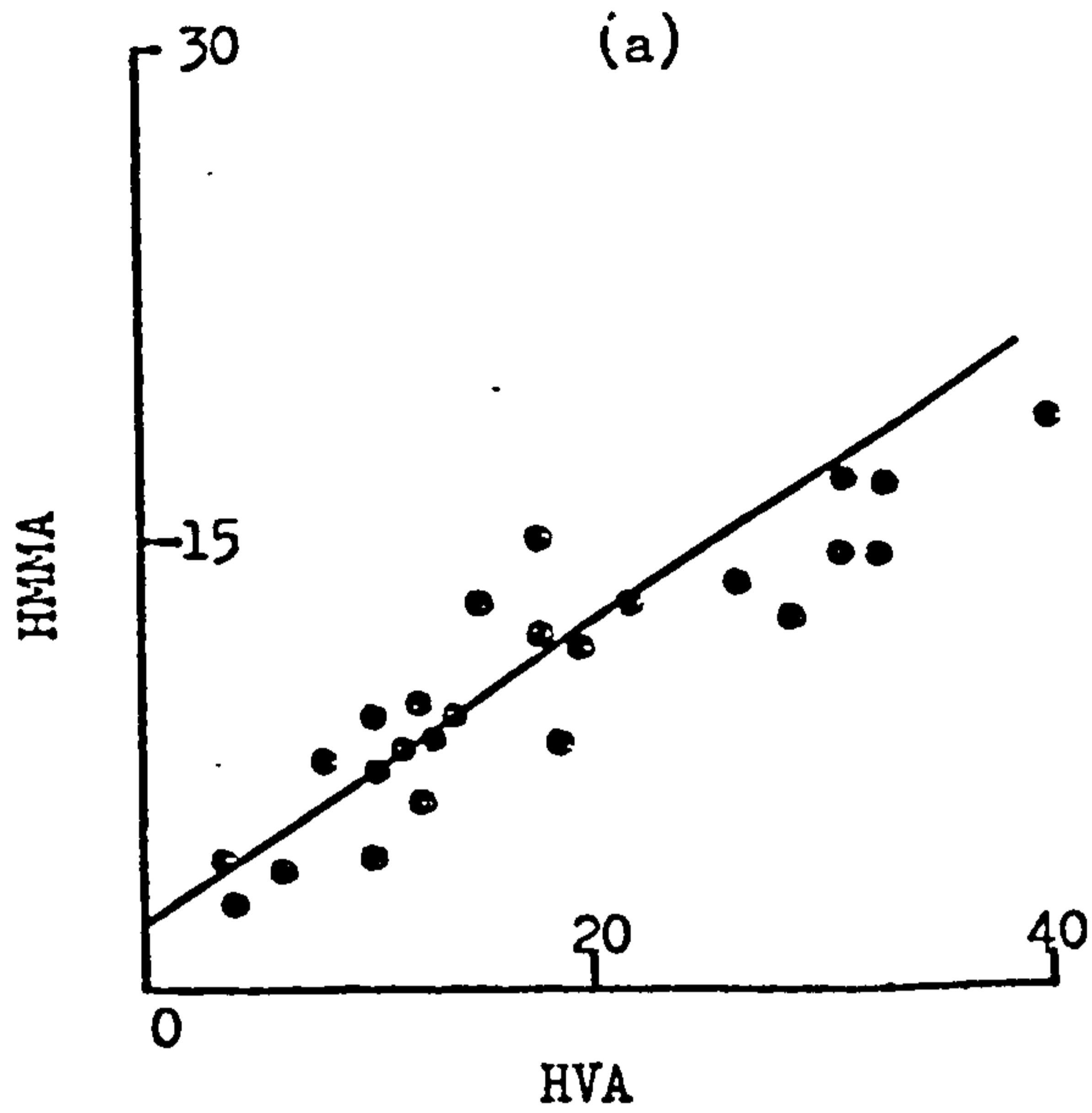
Corr. Coeff. = 0.80
 $y = 57x + 8$
 $p < 0.001$



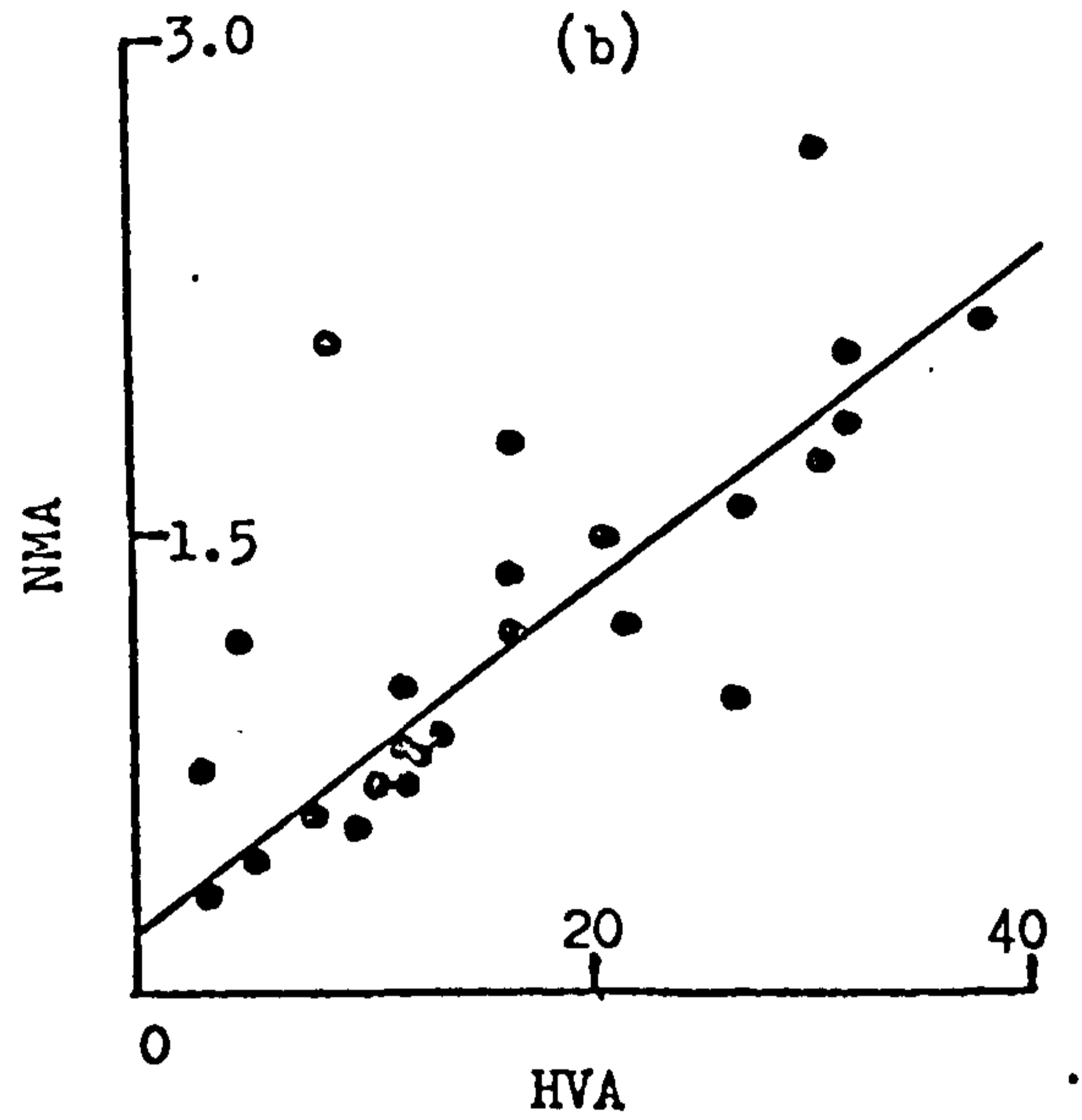
Corr. Coeff. = 0.90
 $y = 87x - 0.9$
 $p < 0.001$

Fig 11 (a) Correlation between HMMA μg per mg creatinine and HVA μg per mg creatinine

(b) Correlation between NMA μg per mg creatinine and HVA μg per mg creatinine



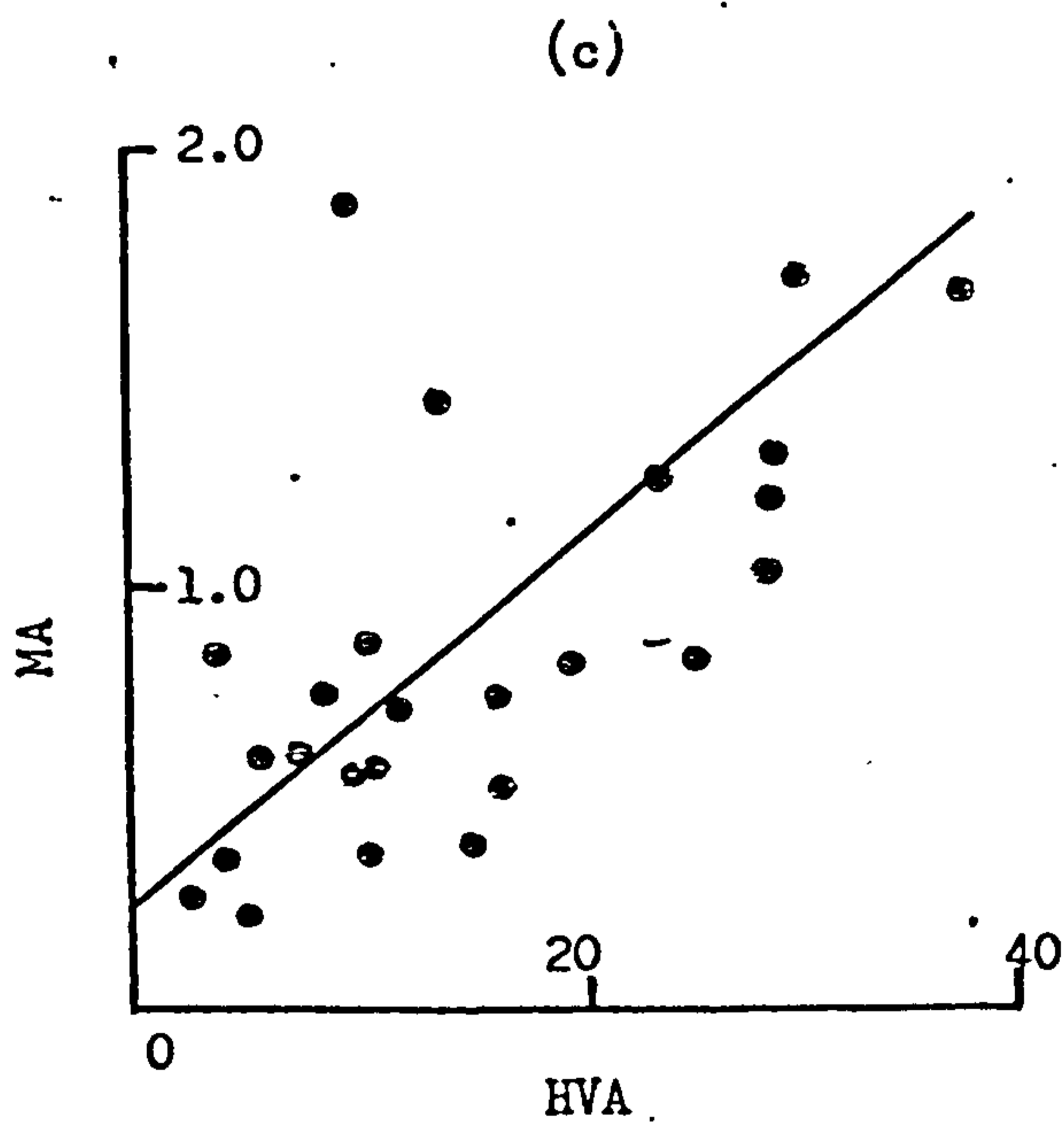
Corr. Coeff. = 0.92
 $y = 0.44x + 2.9$
 $p = < 0.001$



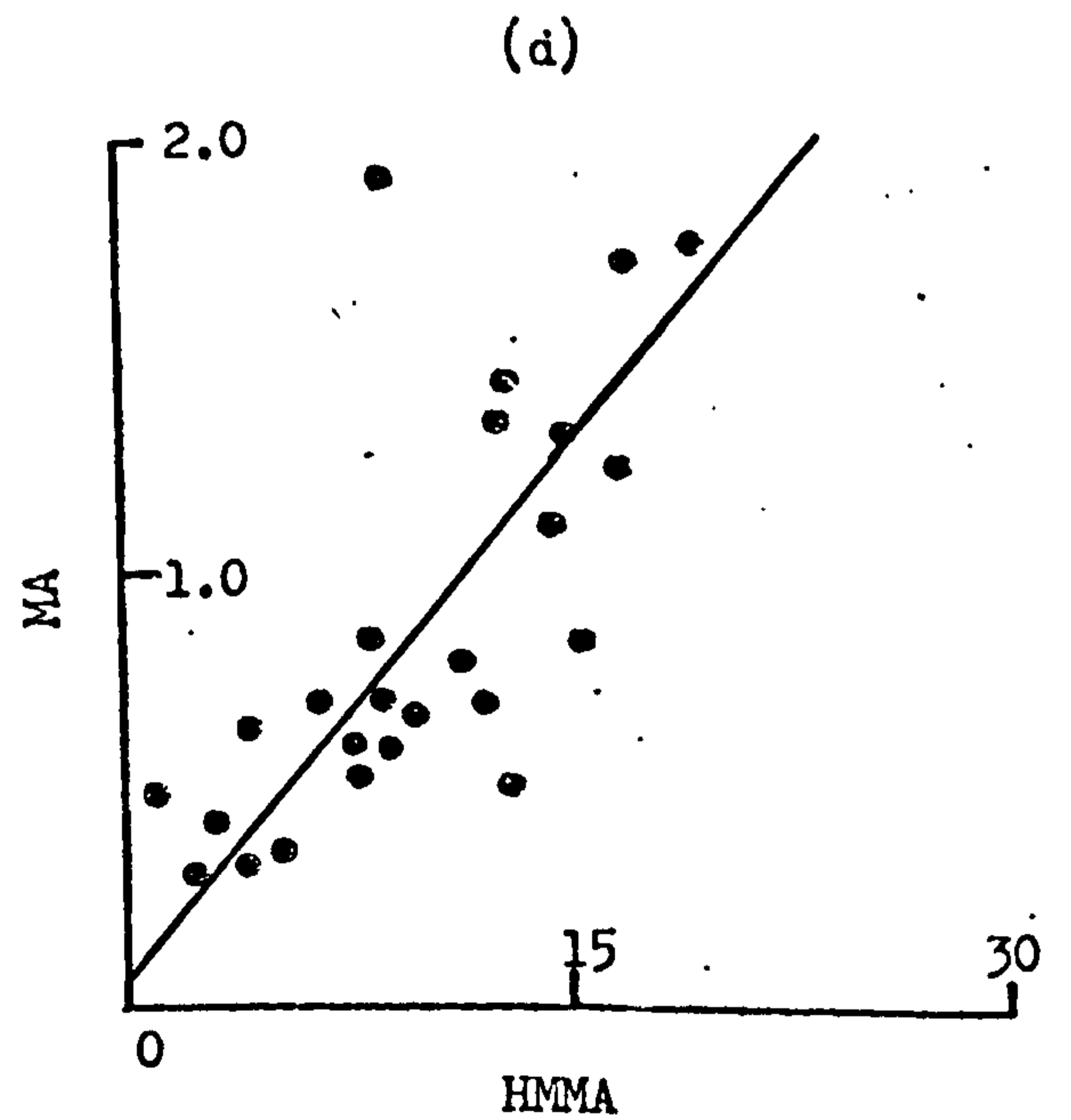
Corr. Coeff. = 0.89
 $y = 0.06x + 0.13$
 $p = < 0.001$

(c) Correlation between MA μg per mg creatinine and HVA μg per mg creatinine

(d) Correlation between MA μg per mg creatinine and HMMA μg per mg creatinine



Corr. Coeff. = 0.78
 $y = 0.04x + 0.25$
 $p = < 0.001$



Corr. Coeff. = 0.79
 $y = 0.08x + 0.07$
 $p = < 0.001$

Fig. 5, a - d indicates the output of HMMA, HVA, NMA and MA expressed as μg metabolite per mg creatinine compared with output of creatinine mg per 24 h. The finding of relatively high negative correlation coefficients for HMMA (-0.82), HVA (-0.76), NMA (-0.83), with a poorer degree of correlation for MA (-0.63) suggests that as the output of creatinine per 24 h increases the output of the catecholamine metabolites expressed as μg metabolite per mg creatinine falls in proportion.

Similarly, the outputs of HMMA, HVA, NMA and MA per 24 h volume all correlate well with body surface area (Fig. 6, a - d) with positive correlation coefficients ranging from (0.79 - 0.86). The results in Table II indicate that the difference between the mean daily outputs of HMMA, HVA, NMA and MA from the babies (Group A) and the young children (Group B), when expressed as μg metabolite per cm^3 urine is small except for MA which shows some significant degree of difference exists between the two groups. However, when the difference between the mean outputs for the four metabolites, HMMA, HVA, NMA and MA, calculated as μg metabolite per mg creatinine, is compared between babies (Group A) and young children (Group B), the results show very significant statistical differences ($p < 0.001$) between the two groups. The difference between the mean output of MA for the two groups, however, suggests a lower degree of statistical significance ($0.1 > p > 0.05$). Similarly, comparing the differences between the mean outputs of HMMA, HVA, NMA and MA, expressed as μg metabolite per m^2 of body surface area between babies (Group A) and young children (Group B), we find some degree of significance for HMMA, but no statistically significant difference for HVA between the two groups. The differences between the metadrenaline mean outputs for the two groups do show quite significant differences ($0.02 > p > 0.01$) for NMA and a much higher degree for MA ($p = < 0.001$).

The outputs of HMMA, HVA, NMA and MA per cm^3 of urine, when plotted against age (Fig. 7 a - d) show no significant degree of correlation for HMMA, HVA and NMA, although for MA there is some correlation (0.46). On the other hand, the outputs of these metabolites when plotted as μg per mg of creatinine against age (Fig 8 a - d), give a good negative correlation.

Further when the output of these metabolites expressed as μg per m^2 of body surface area are plotted against age (Fig 9 a - d) there is a slight correlation for the output of HMMA, HVA and NMA (correlation coefficient 0.49, 0.26 and 0.40 respectively), but a good degree of correlation (0.70) for MA output.

The relationships between the output of different pairs of metabolites expressed as mg per 24 h urine volume are shown (Fig 10 a - d). The correlation coefficients between HMMA/HVA, NMA/HVA, MA/HVA, and MA/HMMA are 0.89, 0.90, 0.80 and 0.90, suggesting good correlation between the catecholamine pairs.

A similar study (Fig 11 a - d) comparing the same metabolite pairs, but expressed on the basis of μg metabolite per mg of creatinine gives correlation coefficients ranging from 0.92 - 0.79.

3.4 Discussion

The relative merits of expressing the urinary output of catecholamine metabolites either as μg per mg creatinine or per 24 h urine volume, especially in young babies and children, has been an unresolved problem for a number of years. Because of the difficulty in obtaining accurate collections of urine from babies and young children, relatively few workers have studied the 24 h urinary excretion of catecholamine metabolites in normal children.

McKendrick and Edwards¹⁶⁸ measured the output of urinary HMMA in a group of 132 normal children and obtained a mean value, for ten children below the age of one, of 0.47 mg per 24 h, which is lower than that found in the present study; however, their range of 1.2 - 3.2 mg per 24 h for HMMA output in a group of children aged 1 - 12 years is more in line with our findings for children of a similar age.

The increase in the mean values of urinary HMMA, HVA, NMA and MA found for the older children over the babies shows that the output of these metabolites increases with age, an observation which is supported by the plots of metabolite output against age (Fig 3 a - d). Karki, in 1956,¹⁶⁹ showed that the output of urinary NA and adrenaline in a group of normal

children whose ages ranged from 1.5 - 16 years, increased with age.

His work was confirmed by Voorhess¹⁷⁰ who measured NA, adrenaline, dopamine and HMMA output per 24 h urine volume in normal children of various ages.

Our findings indicate that the output of the metabolites of dopamine, NA and adrenaline also increase with age.

There is a relatively steady increase in the mean urine output per 24 h in babies and young children with age which is due presumably to increasing body size and with it a concomittant increase in fluid intake and urine output. This results in the outputs of all the metabolites per 24 h when plotted against urine volume showing good correlations.

The validity of expressing results for children based on the output of creatinine especially in young infants and babies has been questioned by Applegarth and Ross,¹⁷¹ who found that the output of α -amino nitrogen expressed relative to creatinine suggested that the creatinine output in the first few years of life is much lower than the creatinine output in later life, a finding which makes the normal value of excretion of a metabolite expressed relative to creatinine extraordinarily and falsely age dependant.

Weetman et al¹⁷² found a significant diurnal variation in the outputs of HMMA and total metadrenalines expressed as μg metabolite per mg of creatinine which confirmed the finding of others^{168, 172} but was at variance with those of other groups.^{167, 174}

A possible explanation for this difference between the two groups of workers could be that the urine specimens in the first study¹⁷² were collected as timed overnight and daytime collections, whilst other groups^{167, 174} collected mid-morning samples where the creatinine levels would not be expected to vary as widely compared to the overnight/daytime creatinine levels.

In the present study, it is also apparent that there is a much larger increase in the mean output of creatinine per 24 h between the Group A babies and Group B children, due probably to the rapidly increasing muscle mass and increased activity of the children compared to the babies. When the output of the catecholamine metabolites are plotted as μg per mg of creatinine

against creatinine output per 24 h, quite good negative correlations are found for HMMA, HVA and NMA. MA output, however, does not correlate well with creatinine output. A possible reason for the good negative correlations for HMMA, HVA and NMA output per mg of creatinine against creatinine output (Fig. 5 a - d) is that the output of the sympathetic nervous system increases less with age than does the muscle mass and consequently as creatinine output increases so the relative outputs of HMMA, HVA and NMA to creatinine decrease. The secretion of adrenaline from the adrenal medulla and the subsequent urinary output of MA would not be expected to be closely related to body muscle mass as the secretion of adrenaline is dependant to a large extent on external stimuli acting on the organism. This could explain the poorer degree of correlation found for the output of MA when compared with creatinine output.

An interesting point, however, was the good correlation obtained between the outputs of all four catecholamine metabolites, expressed as output per 24 h compared with body surface area, a finding which suggests that an increasing body surface area results in an increase in urine volume; the output of MA also appears to be closely correlated with body size and urine volume, unlike the previous finding, when relating MA to creatinine output.

However, whilst there is an apparent increase in catecholamine metabolite output with age (Fig.3), an interesting point emerges when the average concentration of metabolites per cm^3 of urine over 24 h are compared between the two groups. Here we find no significant difference exists between the mean outputs of HMMA, HVA and NMA per cm^3 . There is, however, a significant difference between the two groups when the mean outputs of MA per cm^3 are compared. These findings become more apparent when all the catecholamine metabolite results, expressed as concentration per cm^3 of urine are plotted against age; only the output of MA increases with age,

This could suggest that the output per cm^3 of urine for HMMA, HVA and NMA is independent of age and that the production of NA and dopamine is closely related to the increasing size of the sympathetic nervous system. The increase per cm^3 of urine found for MA output with increasing age might

be a measure of the actual stress effects which would be expected to vary and probably show a positive increase with age, or it could be that in babies and young children the production of adrenaline is very low due to a delay in the maturation of the enzyme system which converts NA to adrenaline in the adrenal gland. Previous workers¹⁷⁵ have shown that the total catecholamine content of foetal adrenal glands is relatively low, reflecting the limited amount of adrenal chromaffin tissue, and that NA is the predominant amine in chromaffin tissue in foetal life, being present in greater absolute and relative amounts in extramedullary rather than medullary tissue.

Various groups of workers have measured urinary HMMA and HVA output as μg per mg creatinine in young babies and children. Von Studnitz found ranges of 1.2 - 9.5 μg HMMA and 3.9 - 39.9 μg HVA per mg of creatinine in twelve normal children aged 0.5 - 12 years using an electrophoretic technique. Gjessing¹⁷⁷ and Gitlow et al¹⁶⁷ used paper chromatography to separate HMMA and HVA which were estimated by visually comparing the spots with known amounts of standards. They reported ranges of 6 - 12 $\mu\text{g}/\text{mg}$ creatinine HMMA, 10 - 14 $\mu\text{g}/\text{mg}$ creatinine HVA and 1.4 - 15 $\mu\text{g}/\text{mg}$ creatinine HMMA and 12 - 35 $\mu\text{g}/\text{mg}$ creatinine HVA respectively, for children below the age of one year. Our ranges tend to be higher for HMMA, due perhaps to our more accurate method of scanning the developed spots which could detect amounts which would be missed by visual technique. Our HVA results show a closer agreement. Similarly, the outputs of HMMA and HVA in the children, aged 2 - 12 years, in Group B have higher ranges when compared to other workers' findings for HMMA, but are similar with regard to HVA output. Individual outputs of NMA and MA μg per mg creatinine have not been determined by other workers, to our knowledge. The linear relationships obtained when the output of the metabolites HMMA, HVA and NMA per mg creatinine were plotted against age were similar to those previously obtained by Gitlow et al¹⁶⁷ for HMMA, HVA and total metadrenalines, who stressed the extreme variability of HMMA and HVA excretion below the age of one. They also pointed out that the output of total metadrenalines against age, however, appeared to maintain linearity

below the age of one and suggested that the future assay of these amines might serve as a useful tool in differentiating normal children from those with neural crest lesions.

When the 24 h excretion of the four metabolites by the subjects in this study was related to body surface area the mean output of HMMA expressed as μg metabolite per m^2 for the older children (Group B) was found to show a slightly significant increase when compared with the mean value found in the babies (Group A) but no difference between the mean HVA output was observed between the two groups. The difference between the mean outputs μg metabolite per m^2 of NMA and MA for the babies (Group A) and the older children (Group B), however, showed a statistically significant increase for Group B over Group A.

However, when these outputs were plotted against age for all the children we found a poor degree of correlation for the outputs of HMMA, HVA and NMA, but the output of MA μg per m^2 of body surface area compared to age gave a good correlation. These findings are at variance with those found by Voorhess¹⁷⁰ who found little difference in adrenaline and HMMA output per m^2 of body surface area between her groups of children consisting of nineteen babies (birth to 1 year) and twenty-eight children (aged 1 - 15 years). Dopamine and noradrenaline outputs per m^2 were found, however, to be greater during the first year of life, a finding which she attributed to the rapid growth and maturation of the sympathetic nervous system with its increased production of NA. Our results for the outputs of HMMA, HVA and NMA do not support these findings, but tend to suggest that the output of catecholamines with age is directly proportional to increasing body size and urine volume. The catecholamine which one would expect to show the largest rise because of the maturation of the enzyme system plus the increase in external stress factors with age, would be adrenaline. However, Voorhess¹⁷⁰ found no difference in adrenaline output between her groups, unlike the findings in the present study which showed a significant increase in MA output per m^2

of body surface area when compared with age.

Scheme 2, Chapter 1, shows the metabolic pathways for the catecholamines. If we assume under normal conditions that the sympathetic nervous system produces most of the dopamine and NA and the adrenal medulla secretes adrenaline we might expect to find good correlations between selected pairs of metabolites. The good correlation between HMMA and HVA outputs per 24 h (Fig 10a) indicates a relationship between these metabolites as does the good correlation between NMA and HVA outputs (Fig 10b), suggesting that the rates of dopamine and NA production are closely related. The output of MA compared to HVA, whilst showing some degree of correlation (Fig 10c), had the lowest degree of correlation. The correlation between HMMA and MA, its precursor (Fig 10d) also had a high degree of correlation. When the same pairs of metabolites are expressed as μg metabolite per mg of creatinine, there is a good correlation between HMMA and HVA and NMA and HVA, (Fig 11a and b) but a lower degree of correlation between MA output and HVA or HMMA (Fig 11c and d). A likely explanation for this is that the outputs of HMMA, HVA and NMA appear to be closely related to muscle mass with attendant creatinine production, whilst the output of MA does not appear to be as closely linked to body muscle mass and hence creatinine output.

From these studies, it would appear that measuring and relating outputs of HMMA, HVA, NMA and MA per 24 h urine volume could enable us to assess the sympathetic and medullary activity of the body.

When the outputs of HMMA, HVA and NMA are expressed as μg per mg of creatinine they appear to be directly related to muscle mass. The values should therefore reflect any increase in sympathetic nerve activity resulting in the production of dopamine and NA. However, because the output of the stress hormone, adrenaline, from the adrenal medulla is closely related to external stimuli, the output of urinary MA is linked to a lesser degree with muscle mass and subsequent creatinine output than the other three metabolites.

The finding of correlation coefficients which range from (0.77 - 0.88) when the outputs of HMMA, HVA, NMA and MA per 24 h are compared to age and

urine volume and a lower range (-0.46 to -0.76) when the output of the four metabolites expressed as μg per mg of creatinine are compared against age and creatinine output, might suggest, especially regarding MA output, that in babies and young children the output of catecholamine metabolites expressed as μg or mg per day might be a better guide than μg metabolite per mg creatinine.

Our findings would tend to support the recent work of Weetman et al¹⁷² who suggested that urine samples used for screening for neuroblastomas in children below the age of five should be obtained from a 24 h collection of urine rather than from a random sample, and also support the work of Applegarth and Ross¹⁷¹ with regard to the variability of creatinine output in young children.

CHAPTER 4

A STUDY OF THE OVERNIGHT URINE OUTPUTS FOR HMA, HVA, NMA AND
MA FOUND IN A GROUP OF SECOND YEAR STUDENTS AT THE UNIVERSITY
OF YORK MEASURED AT VARIOUS TIMES BEFORE AND AFTER AN EXAMINATION

Table 12

Mean overnight urine output of HMMA, HVA, NMA and MA expressed as μg per min for the same twenty students in Groups 1, 2, 3 and 4 determined 8 weeks, 4 weeks and 2 days before and 5 weeks after an examination, showing a statistical comparison of Group I (Control) with

Groups 2, 3 and 4

	HMMA	HVA	NMA	MA
Group 1 Control	1.99 \pm 0.52	2.70 \pm 0.97	0.27 \pm 0.10	0.27 \pm 0.09
Group 2	2.27 \pm 0.49	3.35 \pm 1.05	0.29 \pm 0.06	0.33 \pm 0.07
Significance Group 1 - 2	N/S	0.17P>0.05	N/S	0.02>P>0.01
Group 3	2.32 \pm 0.69	3.00 \pm 0.67	0.29 \pm 0.06	0.33 \pm 0.06
Significance Group 1 - 3	N/S	N/S	N/S	0.02>P>0.01
Group 4	2.21 \pm 0.48	2.76 \pm 0.71	0.30 \pm 0.06	0.34 \pm 0.09
Significance Group 1 - 4	N/S	N/S	N/S	0.05>P>0.02

4.1 Introduction

A chance observation that the overnight output of NMA and MA in urine, expressed as μg metabolite per min, increased in a normal subject from a basal level to a much higher level in advance of a known stress situation, namely an hour's lecture, presented to a large audience, led to the suggestion that normal people faced with a future stress situation might respond by increasing their catecholamine secretion.

To test this suggestion a group of twenty normal healthy students at the University of York, consisting of fourteen males and six females, volunteered to collect a total of four overnight urine specimens starting eight weeks before an important examination. These urines were designated Group I (Control). Other overnight urine collections were made respectively at four weeks, Group 2, and two days, Group 3, before the examination. The final urine samples, Group 4, were collected five weeks after the examination at the start of a new term. The urines were accurately timed collections and were collected overnight in order to try to eliminate the effect of individual variations in physical activity and day-time 'stress factors' in the students in the hope that any increase in their overnight output of catecholamine metabolites might reflect 'on-going' stress factors.

4.2 Results

Table 12 shows the mean overnight outputs for HMMA, HVA, NMA and MA obtained from the twenty students who participated at each stage of the investigation. When the mean outputs of the catecholamine metabolites for Groups 2, 3 and 4 are compared against the Control Group 1, we find only a slight increase in the HMMA, HVA and NMA mean outputs. The output of MA, however, shows a steady increase in mean values from Group 1 through to Group 4. A statistical comparison between the Groups indicates that no significant difference exists between the Control Group and the other three Groups with regard to the outputs of HMMA and NMA. A slight degree of significance relating to the output of HVA was indicated between Group 2 and the Control Group, but not between

Groups 3 and 4 and the Control Group. Comparing the mean MA outputs of Groups 2, 3 and 4 against the Control Group indicated that a statistically significant increase in the output of MA had occurred over the period of the trial. However, there were no dramatic increases in catecholamine metabolite outputs as the examination approached as had been expected.

4.3 Discussion

Early this century Cannon¹⁷⁸ pioneered the study of the adreno-medullary response to stress. He attributed the physiological responses he obtained from experimental animals treated with a crude extract of adrenal gland tissue to a substance he called 'adrenin'. The adrenal medulla contains two active hormones, adrenaline and NA. Adrenaline is synthesised and stored in the chromaffin cells and is released into the circulation to act on distant organs when the body is subjected to a sudden physiological emergency, such as cold, fear, fatigue, shock, etc. In this sense, it mobilises what he termed the 'fight and flight' mechanism, a co-operative effort consisting of the adrenal medulla and the sympathetic nervous system.

One of the easiest emotions to elicit is that of anxiety, for example, in situations such as parachute jumping,¹⁷⁹ climbing wet and slippery rocks¹⁸⁰ or even when visiting the dentist.¹⁸¹ Stress of this nature tends, on the whole, to be quick and short-lived, although the response as measured by adrenaline output is often quite dramatic. In general, the body after a sudden short-lived stress soon returns to a normal state. Since stress situations as described above produce a demonstrable increase in adrenaline output previous work has in the main tended to concentrate on these short-lived stress situations, plasma or urine levels of catecholamines being measured immediately before and after the stress. Long-term responses to stress which may take place over weeks, months or years are obviously more difficult to measure. However, long-term stress factors, particularly environmental, must exist, as without them animals and man would not have adapted and attained their present evolutionary states. Certain groups of

people, such as pilots, because of their occupations, might be expected to be subjected to a greater degree of stress than other more sedentary workers, and, in fact, it has been shown¹⁸² that catecholamine levels are often double resting catecholamine values, during the time a pilot is at the controls. Carruthers¹⁸³ has suggested that airline pilots, because of the stress factor associated with their jobs should be biochemically screened for the early detection of heart disease. It has also been shown¹⁸⁴ that air traffic controllers at the busy O'Hare Airport, Chicago, who were studied daily, during two five-day work periods, using a battery of chemical tests including urinary NA and adrenaline, were shown to have greater stress levels than those obtained from pilots subjected to long or difficult flying operations - a 10-hour test in a flight-simulator, or prolonged decompression tests. It was also shown that these flight controllers, although asleep during the afternoon period of the study, excreted more adrenaline than was excreted at this same time of day by other groups of men who were awake and active and performing work which would not be regarded as stressful. It could be that long-continued or intermittent contact with a known stressor tends to raise thresholds and perhaps individuals may then become unresponsive to that particular stress and in fact adapt.

Lucas (1976)¹⁸⁵ points out that some students entering college for the first time and having to meet the challenge and stimulus of a new environment can develop stress symptoms, often in relation to study or examinations or both. Others presumably can adapt and cope reasonably well with their new environment. Krishnan¹⁸⁶ has shown that students at Leicester University who attended a Health Centre with symptoms referable to forthcoming examinations, could be helped by being given a drug (Oxprenolol) which relieves anxiety and tension by interfering with the action of the catecholamines. As a result they became more confident and performed better in their examinations than anticipated by their tutors, findings which

support the work of Brewer,¹⁸⁷ who studied a group of psychology students sitting an examination. The students were classed as having 'high test anxiety' or 'low test anxiety'. These students were given propranolol, a β -adrenergic blocker or a placebo, before the examination. The results showed that propranolol does not impair examination performance, and in fact it did improve performance in those students who had severe anxiety symptoms.

Our study group consisted of students who had already attended the University for one year and were starting their second year of study. Our findings of only a slightly significant statistical difference between the outputs of MA, the metabolite of adrenaline, at various stages before and after their Part 1 Examination and no difference in the outputs of HMA, HVA and NMA could suggest that the second year students have already adapted quite well to the University life with its attendant pressures and therefore did not respond significantly to the 'thoughts' of a forthcoming examination. However, when the students' overnight values are compared with the overnight values found in a normal, but much older (average age 43 years) group of adults (Chapter 6, Table 15) there appears to be quite marked differences between the two groups regarding the mean outputs of HMA, HVA and NMA, in particular the mean overall output of MA found for the students was over 70% higher than that of the adult group.

An explanation of these findings could be that for the young, active, intellectual University students our results are in fact 'normal' for this group, suggesting that perhaps overnight production of catecholamines falls with increasing age, due to a decrease in physical and mental activity. Alternatively, these findings could imply that young adults after entering a University are being subjected daily to hard, mentally stimulating experiences which could, over an extended period of time, lead to an increased production of catecholamines. This idea, of an ongoing stress might account for the enhanced level of MA found in Group 4 at the start of a new term.

The concept of students functioning at a higher level of catecholamine activity would also explain why the prospect and actual sitting of an important examination did not produce the biochemical stress response that was anticipated. The students appear to be already working under a high degree of applied pressure and were able to cope quite readily with the extra stress imposed by an examination without the need for a further increase in catecholamine production.

It would be interesting to repeat the trial with groups of 1st, 2nd and 3rd year students, or the same group of 1st year students followed throughout their University life, compared to a normal group of non-University subjects matched for age and sex. In this way it might be possible to detect changes in catecholamine metabolite output patterns over a longer period of time to determine if the methods are in fact sensitive enough to provide a chemical means of measuring the physiological adaptive responses to an ongoing stress.

CHAPTER 5

A STUDY OF THE INFLUENCE OF A 54-MILE RACE ON URINARY
CATECHOLAMINE METABOLITE LEVELS

5.1 Introduction

The North Yorkshire Moors' Search and Rescue Team annually organise a race called the Five Crosses Walk. It starts and finishes at Goathland, a village at the centre of the North Yorkshire Moors. The competitors cover a distance of 54 miles non-stop. This race is open to anyone, male or female, over the age of 16 years and the winner in 1975 for the second year in succession was a Sheffield man, who covered the distance in 9.5 h.

It was felt that a comparison of the competitors' urinary catecholamine levels before and after the walk might yield information on the effect, in healthy subjects, of the acute physical and possible mental stress associated with the exercise.

Every walker who volunteered to participate in this study was given a typewritten sheet of instructions which set out in detail the procedure to follow for the accurate collection of the urine samples.

A sample of urine was obtained from 23 volunteers, aged between 20 - 70 years, immediately prior to the start of the race at noon, on Saturday, 12th July, 1975.

The volunteers, after producing their first sample, were each given a numbered adhesive label which corresponded to their pre-race sample number and asked to produce a urine sample at the completion of the race and to attach their number to the bottle. A box of empty plastic bottles was left at the finishing point for this purpose. As the majority of competitors took between 16 - 20 hours to complete the race, these post-race urine bottles were collected from the finishing point at mid-day, Sunday, 13th July. Fourteen post-race urine samples were obtained from the original 23 volunteers - these urines were returned to the laboratory and treated as (in Chapter 9.2) As the walkers were competing in a race for which prizes were being awarded, it was felt that it would be unfair to the volunteers to ask them to collect every urine sample which they produced during the race, as it would have meant them carrying extra bottles and other equipment. Therefore, only pre- and post-race samples were collected from the walkers.

Table 13

Mean level of creatinine expressed as μg per 100 cm^3 and urine levels of HMA, HVA, NMA and MA expressed as μg per mg creatinine from 14 walkers before and after a 54-mile race

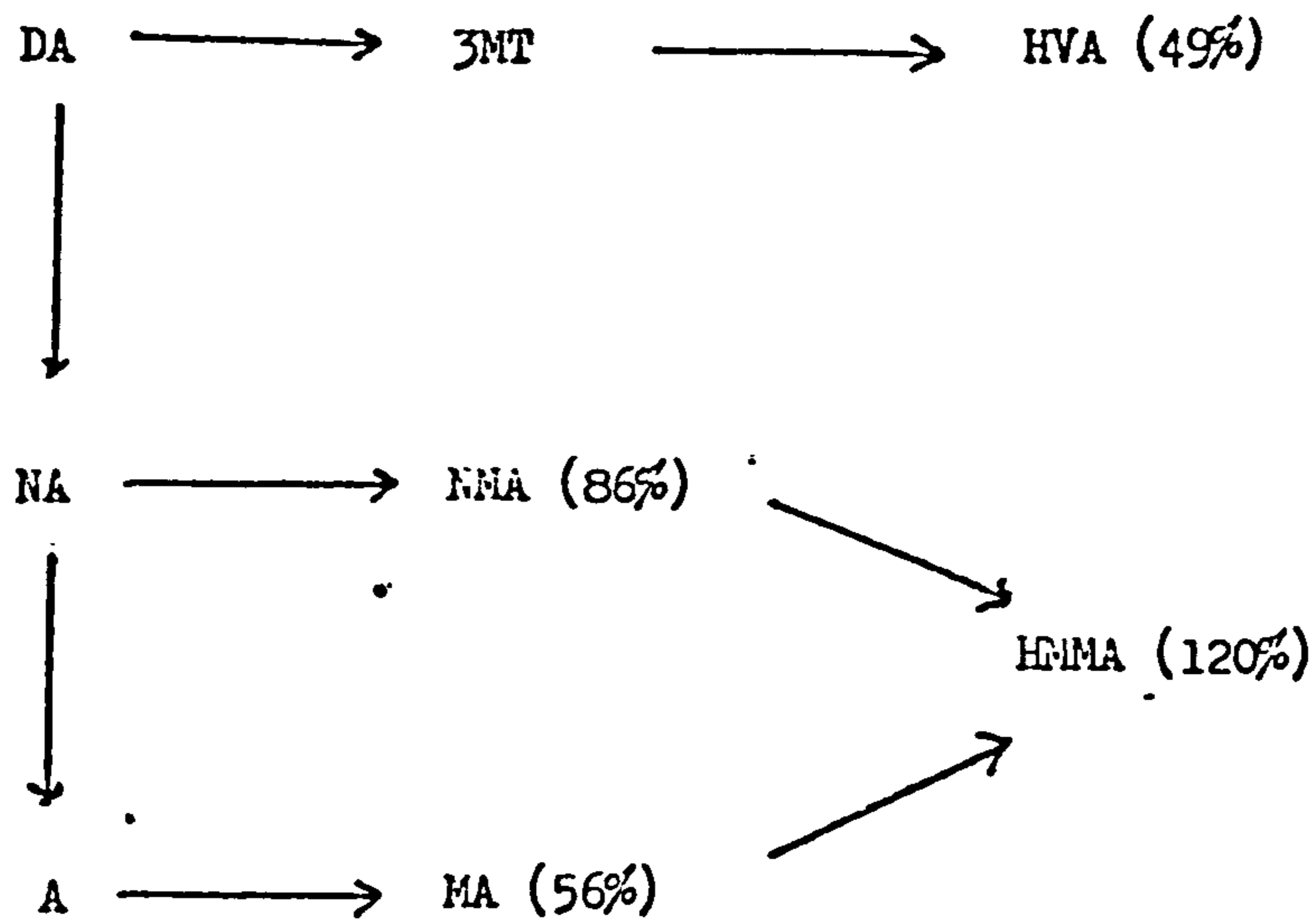
	Before Race	After Race	't'	'P'
Creatinine $\mu\text{g}/100\text{ cm}^3$	102 ± 35	163 ± 46	-3.8	<0.001
HMA	2.5 ± 0.9	5.5 ± 2.5	-4.2	<0.001
HVA	3.5 ± 0.8	5.2 ± 2.4	-2.6	$0.02 > P > 0.01$
NMA	0.27 ± 0.06	0.49 ± 0.14	-5.4	<0.001
MA	0.25 ± 0.08	0.39 ± 0.10	-3.9	<0.001
Ratio $\frac{\text{NMA}}{\text{MA}}$	1.23 ± 0.65	1.31 ± 0.50	-0.37	N/S
Ratio $\frac{\text{HVA}}{\text{NMA}}$	13.2 ± 3.2	11.3 ± 5.1	1.21	N/S
Ratio $\frac{\text{HMA}}{\text{HVA}}$	0.72 ± 0.23	1.2 ± 0.6	-2.7	$0.02 > P > 0.01$

Table 14

Mean levels of HMMA, HVA, NMA and MA expressed as $\mu\text{g per cm}^3$
urine volume from 14 walkers before and after a 54-mile race

	Before Race	After Race	't'	'P'
HMMA	2.6 ± 1.3	9.5 ± 5.0	-4.8	<0.001
HVA	3.6 ± 1.6	8.8 ± 6.9	-2.6	0.02 > P > 0.01
NMA	0.28 ± 0.12	0.87 ± 0.38	-5.4	<0.001
MA	0.25 ± 0.11	0.65 ± 0.23	-5.7	<0.001
Ratio $\frac{\text{NMA}}{\text{MA}}$	1.2 ± 0.7	1.4 ± 0.6	-0.5	N/S
Ratio $\frac{\text{HVA}}{\text{NMA}}$	13.3 ± 3.1	10.8 ± 5.4	1.4	N/S
Ratio $\frac{\text{HMMA}}{\text{HVA}}$	0.7 ± 0.24	1.3 ± 0.6	-2.9	0.01 > P > 0.002

Scheme 4 Outline of catecholamine metabolism showing mean values for catecholamine metabolite outputs based on creatinine and expressed as the percentage increase of pre- to post-walk



5.2 Results

The mean output of creatinine expressed as μg per 100 cm^3 urine (Table 13) was found to be significantly increased between the pre- and post-walk specimens.

Tables 13 and 14 show the mean level of HMMA, HVA, NMA and MA, pre- and post-walk, expressed as μg metabolite per mg creatinine and also as μg metabolite per cm^3 urine volume for the fourteen volunteers who successfully completed the race; also included is a comparison of the pre- and post-walk ratios, NMA/MA, HVA/NMA and HMMA/HVA. These results suggest that a statistically significant difference exists between the pre- and post-walk values for HMMA, HVA, NMA and MA and similarly between the pre- and post-walk HMMA/HVA ratios, in both sets of data, but no significant difference is apparent between the NMA/MA and HVA/NMA pre- and post-walk ratios.

Scheme 4 illustrates the mean percentage increase, based on creatinine output, for each of the four metabolites found after the race. This indicates that the mean output of HVA increased by 49% compared to the 120% for HMMA. Similarly NMA and MA increased by 86 and 56% respectively.

5.3 Discussion

The possibility that our results might be influenced by the effect of dehydration was considered. The competitors were all physically fit and experienced long distance runners over this type of terrain and were familiar with the problems of dehydration. The mid-day start to the race meant that the walkers were subjected to the hot afternoon sunshine and had urine samples been collected throughout the race, instead of just prior to the race and at the completion, it may have been possible to detect changes due to dehydration which may have occurred over this period. Unfortunately, it was not possible to collect urine specimens throughout the entire race. Most competitors completed the race in 16 - 20 h. As a result the latter part of the race was completed during the night and early morning. The weather during this period was very wet, misty and cold and the competitors made ample use of the hot soup and drinks available at the check points situated at 8-mile intervals along the route. The final urine specimens, taken on completion of the race, were probably produced with the individuals in a reasonable state of water balance.

When the amount of each metabolite per cm^3 of urine before and after the race were calculated and compared, the results indicated a significant increase had occurred for HMA, HVA, NMA and MA output. However, the relative amounts as measured by the ratios NMA/MA and HVA/NMA remained relatively constant, only the HMA/HVA ratio showing a significant change. These results are very similar to those obtained by Sarviharju¹⁸⁸ who found the ratio of NA/A levels before and after exercise to be similar.

The increase in urinary creatinine levels found between the pre- and post-walk samples is almost certainly due to the breakdown in muscle creatine¹⁸⁹ as a result of the hard physical exercise involved in racing 54 miles. The increase in the urinary levels of the catecholamine metabolites expressed as μg metabolite per mg creatinine found after the race, suggests that the rates of secretion of the catecholamines, dopamine, NA and adrenaline, have all increased due to the hard physical work involved and its accompanying stress factors.

It is now generally accepted¹⁹⁰ that the adrenal gland plays a key role in regulating the response of an animal to 'stressors'. Taggart and co-workers¹⁹¹ measured plasma levels of NA and adrenaline in eleven subjects before and after a seven minute period of vigorous exercise on a bicycle ergometer, and found both NA and adrenaline levels to be increased, results which are consistent with the finding of the increased urinary output of NMA and MA in the present study. The increase in NA secretion during muscular exercise is due to the increased sympathetic nerve activity. However, why there is a concomitant increase in adrenaline output during exercise is not clear. It has been suggested¹⁹² that there is a consistent relationship between the amount of adrenaline released on the one hand and the degree of mental stress or unpleasantness on the other and that the increase in adrenaline found after hard physical exercise is associated partly with the quality of the emotional^{193,194} response to the physical stress. However, it is well known¹⁹⁵ that adrenaline released from the adrenal medulla has the effect of increasing the conversion of glycogen to glucose in liver and muscle cells, thus enabling the body to cope with strenuous physical exercise by providing the necessary fuel (glucose) from the glycogen store. Similarly, in times of stress, the release of adrenaline is an important means whereby fatty acids are made available for metabolism.¹⁹⁶

The increased urinary outputs of the catecholamine metabolites found after the race could be due to an increase in sympathetic activity as a result of increased muscle activity coupled with an increased adrenal medullary output of adrenaline, whose effect would be to increase the overall metabolic rate of the body tissues thus enabling the body to cope with the increased physical and mental stresses. Unfortunately no blood sugar samples were obtained from our volunteers. However, what proportion of the adrenaline secretion is due to physical stress and how much is produced by mental stress factors is unknown. Conrad et al¹⁹⁷ showed that the plasma levels of catecholamines increased after physical work in a group of subjects even

though they were maintained in a hyperglycemic state by means of a glucose infusion. Sarviharju¹⁸⁸ also found that plasma glucose levels fell significantly after quite vigorous exercise, possibly due to glucose uptake into the muscle cells, whilst the urine NA and adrenaline levels over the same period rose significantly.

It would appear from our urinary levels of MMA and MA that the secretion of both NA and adrenaline increased during the race in proportion to their pre-race levels. Other workers¹⁹⁸ who studied a group of racing car drivers, found that the drivers' plasma level of NA and adrenaline were very high 3 min after the race had finished compared to the values obtained 3 min before the start. However, if one expresses their data as the ratio NA/A, values of 6.6 and 6.4 are found for pre- and post-race levels respectively, findings which are comparable with the observations in this study of the ratios of MMA/MA levels when expressed either as $\mu\text{g per cm}^3$ or $\mu\text{g metabolite per mg creatinine}$. The reason for the significant difference between the MMA/HVA ratio pre- and post-race appears to be that the output of HVA/ $\mu\text{g per mg creatinine}$ does not show the same relative increase as the output of MMA/ $\mu\text{g per mg creatinine}$, possibly due to a rapid increase in the synthesis of NA from its precursor dopamine, as a result of the increasing physical stress. The secretion of dopamine, as well as NA and adrenaline, does not appear to have been studied under conditions of physical stress. In the normal state the sympathetic nerves liberate amounts of dopamine and NA in the same proportion as that in which they are stored in the nerves.¹⁹⁹ This suggests that an increase in the output of NA after sympathetic nerve stimulation, should be accompanied by a similar proportionate increase in dopamine output. Provided that the increase in NA observed after physical exercise is due only to increased sympathetic nerve activity, then the urine output of MMA and HVA, the products of NA and dopamine metabolism respectively, might be expected to show some degree of correlation and reflect overall sympathetic nerve activity. In the

present study the lack of any statistical difference between the mean before and after walk HVA/NMA ratios could suggest that the excess NA produced as a result of hard physical exercise, is in fact derived from an increase in sympathetic nerve activity rather than from the adrenal medulla.

It would appear therefore that after any hard, strenuous physical activity the secretion of the catecholamines, NA and adrenaline, increases probably in proportion to the severity of the stress, which is reflected by the finding of a relatively constant relationship between the urine and plasma ratios of NA to adrenaline as well as the urine levels of NMA/MA before and after physical exercise. The increase in plasma adrenaline and urine MA levels found after physical exercise is probably due in the main to an increase in body metabolism, but a small percentage may also be produced due to mental stress factors. Our findings also suggest that the urine levels of the catecholamine metabolites are a fair reflection of the plasma catecholamine values, as was hypothesised at the outset of this research.

CHAPTER 6

A STUDY OF THE OVERNIGHT URINE CATECHOLAMINE METABOLITE
LEVELS FOUND IN NORMAL AND HYPERTENSIVE SUBJECTS

6.1 Introduction

It has been shown²⁰⁰ that a consistently raised blood pressure is potentially dangerous because of the attendant risks of subsequently developing cerebrovascular, coronary or renal disease.

Hypertensive subjects, in the main, can be divided into two major groups, (i) those whose raised blood pressure is due to a variety of pathological conditions and is therefore a secondary condition, and (ii) those who have a consistently high blood pressure, even when resting, and for which no pathological cause can be found. The latter are said to have 'essential hypertension'.

It is known^{201, 202} that the sympathetic nervous system plays an important part in controlling blood pressure and that certain drugs which interfere with the normal sympathetic control of blood vessels, induce a much larger fall in blood pressure in hypertensive subjects than in normotensives. It is possible that the elevated blood pressure of patients suffering from essential hypertension is associated with excessive sympathetic activity and that these patients will have a corresponding increase in the plasma and urine levels of the sympathetic hormone, N.A.. Studies with hypertensive patients have produced conflicting results. Holtz et al²⁰³ in 1947, were the first to observe an increase in pressor amines in the urine of a hypertensive subject, but no data were given. Later von Euler²⁰⁴ studied a group of 500 hypertensive subjects and observed that the majority of these patients excreted normal amounts of urinary NA when compared to a control group, but 16% had levels which were significantly above normal. Some research²⁰⁵⁻²⁰⁸ has confirmed von Euler's findings, whilst other work^{209,210} has found no apparent differences in urinary NA levels between normal and hypertensive subjects.

Strong evidence for an abnormality in the function of the sympathetic nervous system in hypertension was obtained with sophisticated plasma assay

Table 15

A comparison between the mean overnight and daytime outputs for HMA
HVA, NMA and MA expressed as μg per minute for a group of seventeen
normotensive subjects

	<u>Mean</u>	<u>'t'</u>	<u>'P'</u>
HMA overnight	1.6 [±] 0.5		
		-3.03	0.01 > P > 0.001
HMA daytime	2.4 [±] 0.8		
HVA overnight	2.2 [±] 0.8		
		-4.2	< 0.001
HVA daytime	3.4 [±] 0.8		
NMA overnight	0.23 [±] 0.06		
		-1.8	0.1 > P > 0.05
NMA daytime	0.26 [±] 0.06		
MA overnight	0.18 [±] 0.06		
		-2.2	0.05 > P > 0.02
MA daytime	0.22 [±] 0.06		

Table 16

Overnight output of catecholamine metabolites (μg per minute) from
twenty hypertensive patients compared with 17 normal subjects

	<u>Mean</u>	<u>'t'</u>	<u>'P'</u>
Hypertensives HMMA	$2.4^{\pm}0.7$		
		3.7	P<0.001
Normals HMMA	$1.6^{\pm}0.5$		
Hypertensives HVA	$2.2^{\pm}0.5$		
		0.1	N/S
Normals HVA	$2.2^{\pm}0.8$		
Hypertensives NMA	$0.22^{\pm}0.06$		
		-0.41	N/S
Normals NMA	$0.23^{\pm}0.06$		
Hypertensives MA	$0.19^{\pm}0.07$		
		0.75	N/S
Normals MA	$0.18^{\pm}0.06$		

techniques involving gas-chromatography²¹¹ and double-isotope labelling studies.²¹²⁻²¹⁴ These studies showed the presence of increased circulatory NA levels in patients with essential hypertension and that the levels were positively correlated with the diastolic blood pressure.^{212,213} Nevertheless, it would be premature to suggest that a direct causal relationship exists between the circulating NA level and the increased blood pressure. It might be expected, however, that both hypertensive and normotensive subjects would, during a state of rest such as occurs during sleep, have lower plasma levels of NA than during the daytime active period. Recently, Brézinová and Carruthers²¹⁵ found for four sleeping hypertensive patients raised plasma levels of NA which were higher than the daytime plasma NA levels determined in a group of resting normotensive individuals,⁹⁶ showing that hypertensive subjects, even when asleep, have increased amounts of NA in their blood.

Using our simple techniques for the estimation of urinary HMMA, HVA, NMA and MA, we have compared the output of these catecholamine metabolites in timed overnight urine collections obtained from a group of seventeen normal subjects, ten males and seven females, whose ages ranged from 32 - 55 years and who were neither taking medicants nor were suffering from any disease which could have interfered with the tests, with those obtained from a group of twenty hypertensive patients. This group was comprised of fourteen males and six females, all completely untreated and previously undiagnosed, whose ages ranged between 45 - 65 years and whose blood pressure ranged from 175/110 to 250/160.

6.2 Results

Table 15 gives the mean outputs per minute obtained from the overnight and daytime urine collections in the normal subjects, for each of the catecholamine metabolites, HMMA, HVA, NMA and MA. These results indicate that significant statistical differences exist between the mean night-time and day-time values, particularly with regard to the output of HMMA and HVA.

A comparison between the overnight results for the hypertensive group and the normals (Table 16) expressed as μg per minute output, shows that there is a very significant difference between the mean output of HMMA in the hypertensive group compared to the normal group, but no significant differences are apparent between the two groups regarding the overnight

mean outputs of HVA, NMA and MA, which in the hypertensive patients tend to be lower than those obtained in the normal subjects.

6.3 Discussion

There are three probable reasons for the conflicting results regarding the association of raised blood pressure with raised blood and/or urine levels of NA in hypertensive subjects.²⁰⁶⁻²¹⁵

First, the estimations have either been carried out on random urine samples¹⁴⁶ or 24 h urine collections¹⁴² or have been related to output per hour²¹⁶ and consequently the values from different research groups cannot easily be compared.

Secondly, the patients and the normal controls have varied. Thus some hypertensives have been compared against hospital in-patients²¹⁶ whilst others have been compared against hospital personnel.¹⁴⁶ Often the patients are resting or lying down during the collection period, whilst the control subjects are active and being subjected to everyday stresses. Thirdly, many of the hypertensive subjects have been treated with anti-hypertensive drugs prior to the urine collection, this treatment being stopped for varying periods of time before the start of the test period. As a result the patients are no longer 'untreated' hypertensives.

Prior to this study, no-one has used overnight urine outputs of catecholamine metabolites to compare untreated hypertensive patients with normotensive subjects. Further, in this study, both the control subjects and the patients were under the same conditions of posture, lying resting or asleep, and during the urine collection period both groups were in their own familiar surroundings, thereby eliminating any mental stress factors which could arise from the subjects being placed in unfamiliar surroundings, such as a hospital.

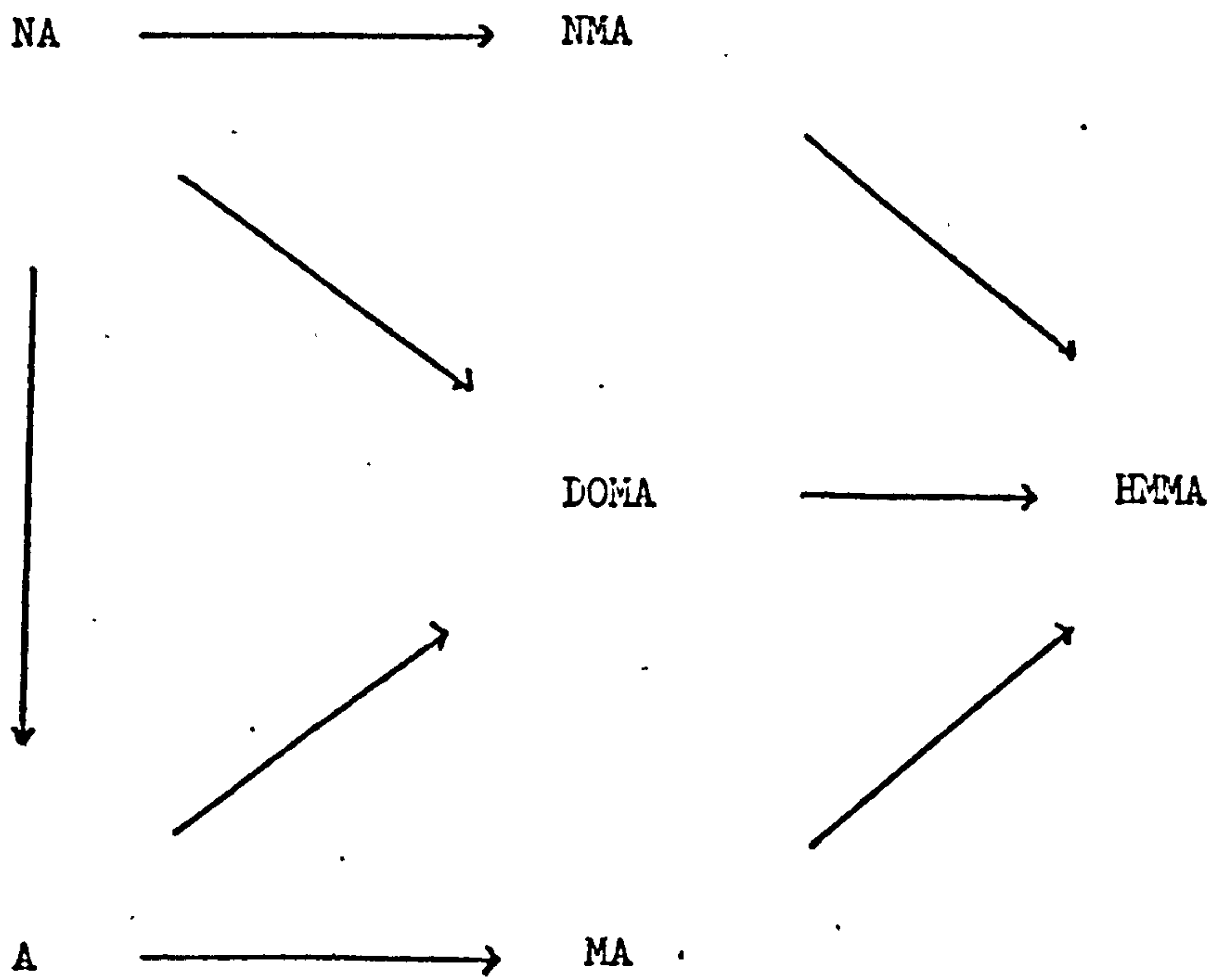
The results obtained from the normotensive subjects clearly illustrate the marked increase in catecholamine metabolite outputs, when expressed as μg per minute, during the daytime compared with the night-time. This day-

time increase is expected due to the increase in physical and mental activity which occurs during the daytime compared to the night-time sleeping period.

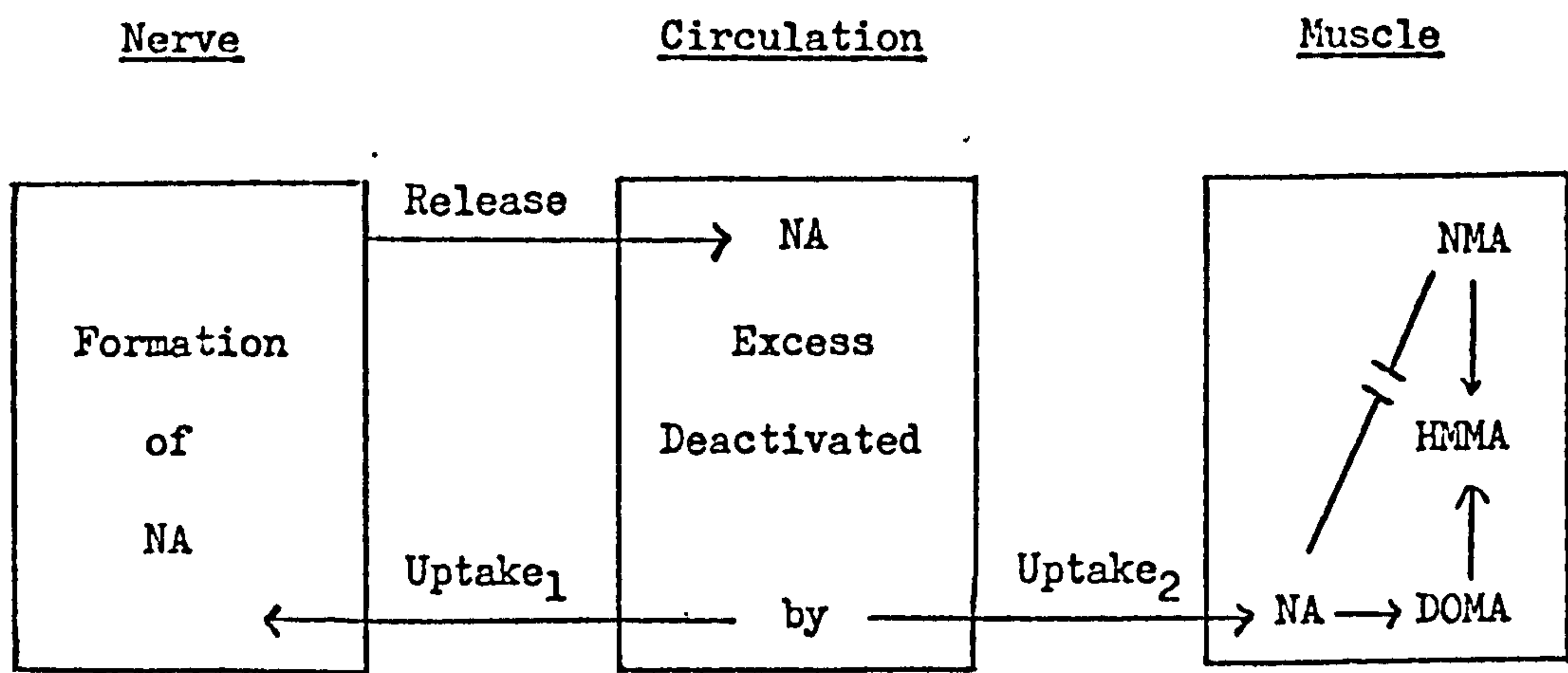
Evidence which suggests a possible abnormality in the functioning of the sympathetic nervous system in hypertension has recently been inferred from assays of the plasma levels of catecholamines²¹¹⁻²¹⁴ which have shown an increase in circulating levels of NA in hypertensive patients. From our study of normal and hypertensive overnight urine samples we found a significantly higher mean overnight output of HMMA, expressed as μg per minute, in our hypertensive group when compared with the normal group. (Interestingly, the overnight output of HMMA by hypertensives is very similar to the normal daytime output for normotensives, a finding which closely parallels the work of others.^{96,215}) The increase in the overnight output per minute of HMMA found in our hypertensive patients implies that even when sleeping, untreated hypertensive patients have a higher plasma level of catecholamines or a more active sympathetic nervous system than normal sleeping subjects. These catecholamines in turn are metabolised (see Chapter 1, Scheme 2) to give increased urine levels of HMMA. However, our finding of similar overnight outputs for HVA, NMA and MA, expressed as μg per minute, for the two groups, is at first sight, puzzling.

One reason for the finding of an increased plasma level of NA in hypertensive patients could conceivably be due to an increased secretion of NA from the ends of the sympathetic nerves. It has been shown,^{217,218} that discharge of NA, by exocytosis, is closely linked with a corresponding discharge of the enzyme dopamine- β -hydroxylase from the nerve vesicles. Louis and co-workers²¹⁹ found a proportionate increase in both NA and dopamine- β -hydroxylase levels in hypertensive subjects which appeared to correlate well with their elevations in resting diastolic blood pressure. Other workers^{211,220} have also found increased plasma levels of dopamine- -hydroxylase in hypertensive patients. In contrast, Åberg et al²²¹ found no

Scheme 5 General outline of catecholamine metabolism



Scheme 6 An alternative method whereby the excess plasma noradrenaline levels found in hypertensive subjects might be metabolised



significant difference in dopamine- β -hydroxylase levels between his hypertensive and normal groups. If the finding of an increased plasma level of NA and dopamine β -hydroxylase in hypertensive patients is valid, then under normal conditions one would expect that the increased secretion of NA would be rapidly deactivated by a corresponding increase in the COMT and MAO enzyme systems, to produce increased metabolite levels in the urine (Scheme 5). On the other hand, if the normal metabolic mechanisms were impaired this could account for enhanced plasma levels of NA and a distorted urinary metabolite pattern, as we observe. Support for the suggestion that the enzymes responsible for metabolism of NA are perturbed in hypertension comes from recent work²²² which has shown that untreated hypertensive patients have significantly higher blood cadmium levels than normal subjects. It might be that replacement of the natural divalent Mg^{++} co-factor^{60, 61} for the COMT enzyme system by cadmium, itself a divalent metal, leads to a less active or inactive enzyme system.

Alternative mechanisms which could account for the increased plasma level of NA found in hypertensives, are failure of the normal re-uptake process²²³ due either to nerve degeneration or to an abnormality in the binding mechanism. It has been shown²²⁴, in cats, that severance of sympathetic nerves leads to a marked inability of the degenerating nerves to bind and store active catecholamines. However, there is no evidence to suggest that hypertensive patients have any degeneration of their sympathetic nervous system, unlike people who suffer from an inherited disease, known as familial dysautonomia, in which degeneration of part of their autonomic system is a clinical feature. Furthermore, the finding²²⁵ of low urinary levels of HMMA in this condition is contrary to our findings in hypertensive patients of a normal urinary output of NMA with raised levels of HMMA and suggests that actual degeneration of nerve fibres leads to a reduced rather than an

increased output of catecholamines. In the second mechanism inability of the nerves to re-bind secreted NA, results in an increased plasma level, then given the normal mechanisms of metabolism we would again expect to observe a general increase in urinary metabolite levels.

Our finding of a normal urinary level of NMA in hypertensives lends support to the idea (Scheme 6) of a partial metabolic block occurring in the normal breakdown of NA in hypertensive patients. The effect would be a decrease in the rate of inactivation of released NA, some of which could perhaps be re-bound by the sympathetic nerves. However, it is known²²⁶ that if neuronal uptake is interfered with then extra-neuronal uptake (uptake₂) increases and that any excess NA is removed from the circulation by being loosely bound to vascular smooth muscle and also cardiac muscle.²²⁷ The uptake of catecholamines by this method is rapidly followed by their intracellular catabolism by MAO and COMT enzymes, and constitutes a mechanism whereby extra-cellular catecholamines are destroyed. However, if the extra neuronal COMT enzyme system is partially defective in hypertensive subjects, then any excess NA would tend to be deactivated by the usually minor pathway via the MAO system, to produce 3, 4-dihydroxymandelic acid (DOMA).

Thus one might postulate that the increased plasma NA levels which have been found in hypertensive patients could, in part, be caused by a defect in the extra neuronal COMT enzyme, due either to the presence of an abnormal metal (Cd^{++}) which may compete with the normal metal co-factor, or the binding of cadmium to an abnormal iso-enzyme, the presence of which has recently been suggested by Glauser *et al.*²²² If the latter idea is accepted then the excess DOMA resulting from the presence of an abnormal COMT iso-enzyme in the extra neuronal uptake processes will be converted by the normal liver COMT enzyme, to produce ultimately increased urine levels of HMMA without any significant elevation of NMA output, results which are consistent with our findings. The excess NA attached to the vascular smooth muscle could perhaps be responsible for the increased vascular constriction which would produce the increase in blood pressure which is symptomatic of essential hypertension.

To confirm the above proposal, it would be of value to measure urine levels of DOMA and compare the specific rates of reaction for the MAO and COMT enzyme systems using radioisotope labelled substrates in normal and hypertensive subjects, as well as a study of 'trace metals' in this disease.

CHAPTER 7

URINARY CATECHOLAMINE METABOLITE LEVELS FOUND IN PATIENTS

AFTER SUBARACHNOID HAEMORRHAGE

7.1 Introduction

It is now well established²²⁸ that the cerebrovascular tree is innervated by sympathetic nerve fibres. Histochemical studies²²⁹ have demonstrated noradrenergic nerve endings in the walls of the intracerebral vessels and electron microscopy²³⁰ has confirmed the presence of typical autonomic nerves with dense localised swellings which are thought to contain NA.

The precise physiological role of these nerves, however, is unknown. Recent work^{229, 231} has suggested that the autoregulatory mechanism of cerebral blood flow is controlled by the sympathetic nervous system. Harper et al²³² studied the effects of stimulating the cervical sympathetic nerve trunk and the intracarotid infusion of noradrenaline on anaesthetized baboons maintained at varying levels of arterial carbon dioxide pressure. They concluded that the extraparenchymal vessels are influenced by the sympathetic nervous system, whilst the intraparenchymal vessels are under local intrinsic metabolic regulation, the pial vessels being influenced by both systems. However, although sympathetic discharge may constrict the extraparenchymal and pial vessels, normal autoregulatory processes affecting the intraparenchymal vessels will tend to maintain a normal flow. Following a subarachnoid haemorrhage (SAH) radiological evidence²³³ often indicates the presence of spasm in the cerebral vessels. Similarly, the observations^{234 - 235} of high urine catecholamine levels in patients who have suffered a SAH and what role, if any, catecholamines might play in the production and maintenance of cerebral spasm is uncertain.

The aim of this investigation was to study the variation in the excretion of catecholamine metabolites of patients who had suffered a SAH and to relate these levels to the clinical state of the subject, in order to assess the usefulness of serial measurement of catecholamines as a possible aid to prognosis in the SAH patient.

Table 17

Overall mean urinary outputs of the catecholamine metabolites obtained from twelve relatively fit patients (Group I) after SAH, compared with results from ten patients (Group II) who were drowsy or disorientated after SAH

	HMA mg per 24 h	HVA mg per 24 h	NMA µg per 24 h	MA µg per 24 h
Group I	3.4 ± 1.3	3.5 ± 1.5	459 ± 225	355 ± 171
Group II	4.54 ± 1.8	3.8 ± 1.2	692 ± 347	511 ± 172
't'	-3.2	-0.8	-3.4	- 3.8
P	0.01 > P > 0.001	N/S	0.01 > P > 0.001	< 0.001

Table 18

Comparison between the overall mean urinary outputs of the catecholamine metabolites pre- and post-operation obtained from the ten SAH patients in

Group III

	HMMA mg per 24 h	HVA mg per 24 h	NMA µg per 24 h	MA µg per 24 h
Group III				
a) - before operation	3.8 ± 2.0	3.5 ± 1.5	514 ± 243	542 ± 201
b) - after operation	4.1 ± 1.6	3.6 ± 1.2	507 ± 291	412 ± 180
't'	0.6	-0.4	-0.1	-2.5
P	N/S	N/S	N/S	0.02 > P > 0.01

Table 19

Comparison between the overall mean urinary outputs of the catecholamine metabolites pre- and post-operation (Group III) with those of the relatively fit patients (Group I)

	HMMA mg. per 24 h	HVA mg per 24 h	NMA µg per 24 h	MA µg per 24 h
Group I	3.4 ± 1.3	3.5 ± 1.5	459 ± 225	355 ± 171
Group III (a) Before operation	3.8 ± 2.0	3.5 ± 1.5	514 ± 243	542 ± 201
't'	-1.1	-0.02	-0.9	4.1
P	N/S	N/S	N/S	<0.001
Group III (b) After operation	4.1 ± 1.6	3.6 ± 1.2	507 ± 291	412 ± 180
't'	-2.1	0.3	-0.8	-1.3
P	0.05 > P > 0.02	N/S	N/S	N/S

Thirty-eight patients admitted consecutively to a neurological centre were studied. Their ages ranged from 11 - 65 years. The patients were studied over the period of their stay in hospital, an average of two weeks. On day 1, 2 and 3, after admission and then every 2 - 3 days afterwards, a 24 h urine sample was collected for the estimation of HMMA, HVA, NMA and MA. On the basis of their clinical condition, the patients were classified into four groups, by the neurosurgeon in charge of the unit, as follows:

Group I - consisted of 12 patients who were classed on examination as being relatively fit and alert after a SAH (No. of observations - 39)

Group II - consisted of 10 patients who were drowsy, disorientated with or without neurological signs (No. of observations - 34) after a SAH.

Group III - consisted of 10 patients who had an operation within 2 - 3 days to relieve their symptoms (No. of observations - 55, one of whom subsequently died after operation) - defined as pre-and post-operation SAH patients.

Group IV - consisted of 7 patients (one post-operation) who subsequently died within 3 - 7 days after their SAH (No. of observations - 24)

7.2 Results

Comparison of the catecholamine levels between the different clinical groups revealed several interesting points. For example, comparing Group II with Group I patients (Table 17) showed significantly higher urinary mean outputs of HMMA, NMA and MA, but not of HVA in the Group II patients. There was apparently therefore some degree of correlation between an impaired level of consciousness and high urinary catecholamine metabolite levels.

A study of the Group III patients (Table 18) before and after operation, indicated that no significant increase or decrease after the operation took place in the urinary levels of HMMA, HVA and NMA, but a decrease in the mean output of MA was observed after the operation, a finding which might suggest that the operation relieved some 'stress' factor which was present before the operation.

Table 20

Comparison between the overall mean urinary outputs of the catecholamine metabolites found in Group I, with seven patients, Group IV, who died after a SAH

	HMMA mg per 24 h	HVA mg per 24 h	NMA µg per 24 h	MA µg per 24 h
Group I	3.4 ± 1.3	3.5 ± 1.5	459 ± 225	355 ± 171
Group IV	5.2 ± 1.6	4.6 ± 1.3	866 ± 531	606 ± 266
't'	4.4	2.6	4.1	4.2
P	<0.001	0.02 > P > 0.01	<0.001	<0.001

Fig. 12 Daily mean output of HMMA mg per 24 h for
Groups I, II, III and IV after SAH

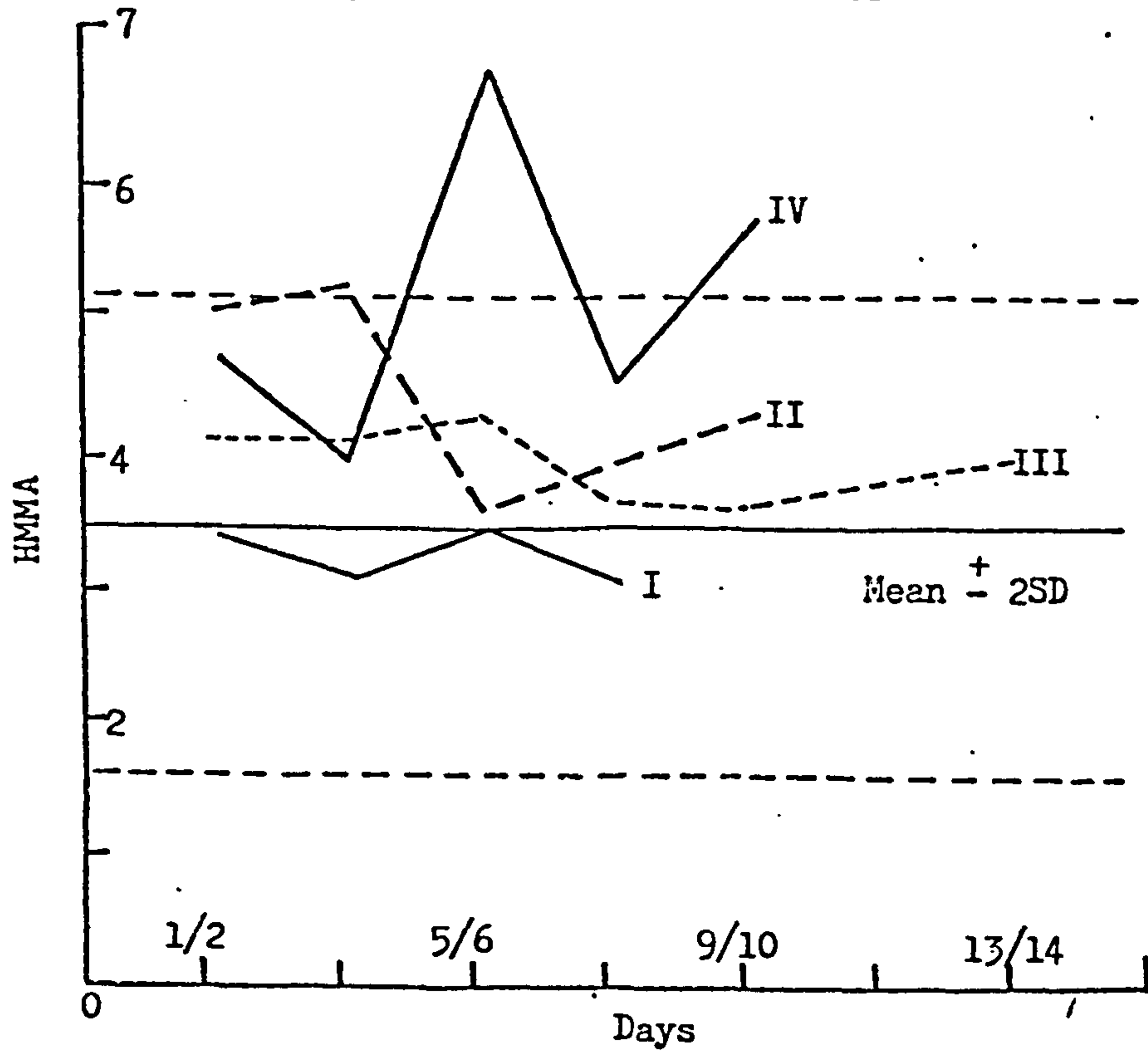


Fig. 13 Daily mean output of HVA mg per 24 h for
Groups I, II, III and IV after SAH

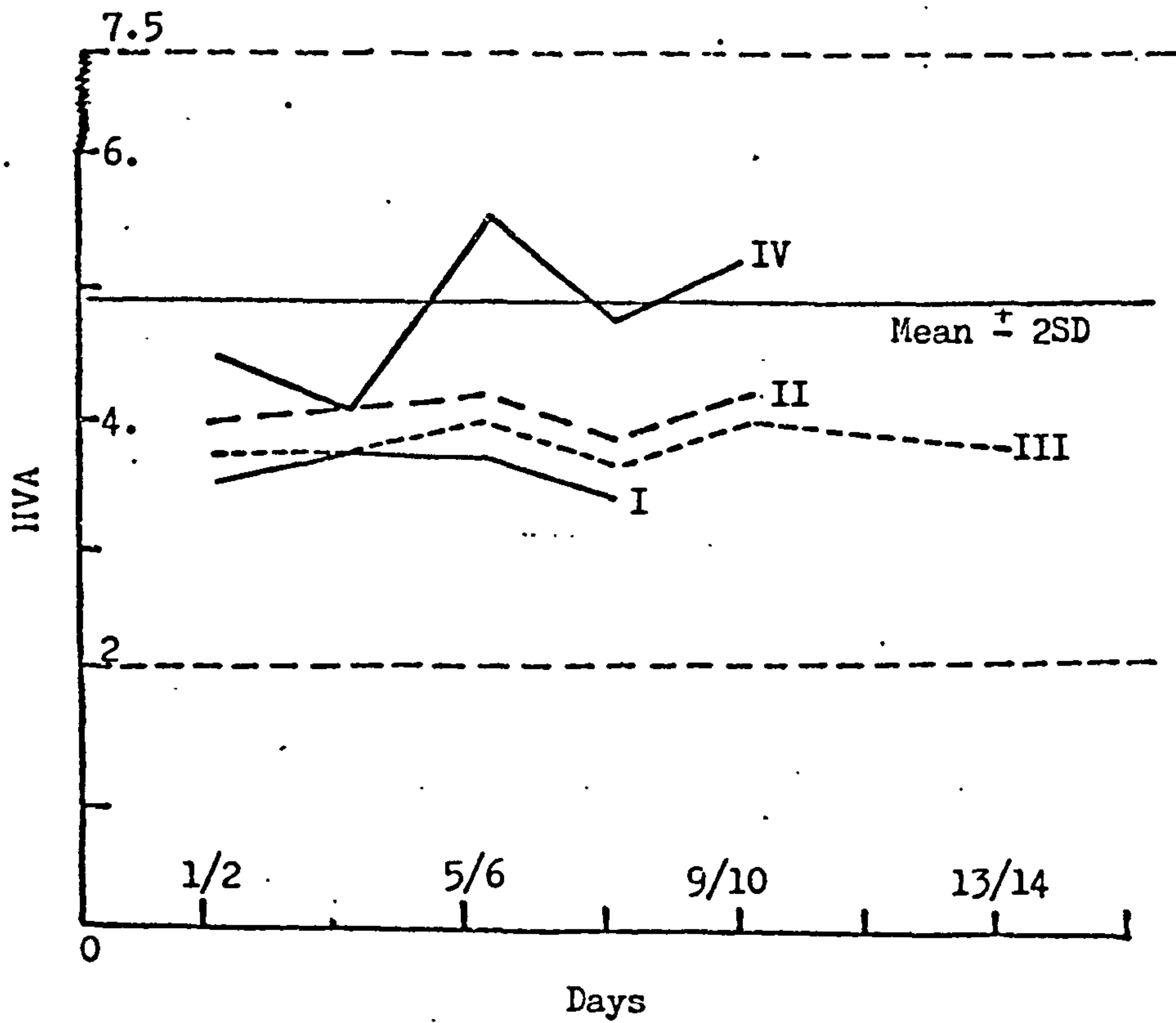


Fig 14 Daily mean output of NMA μg per 24 h for Groups I, II, III and IV after SAH

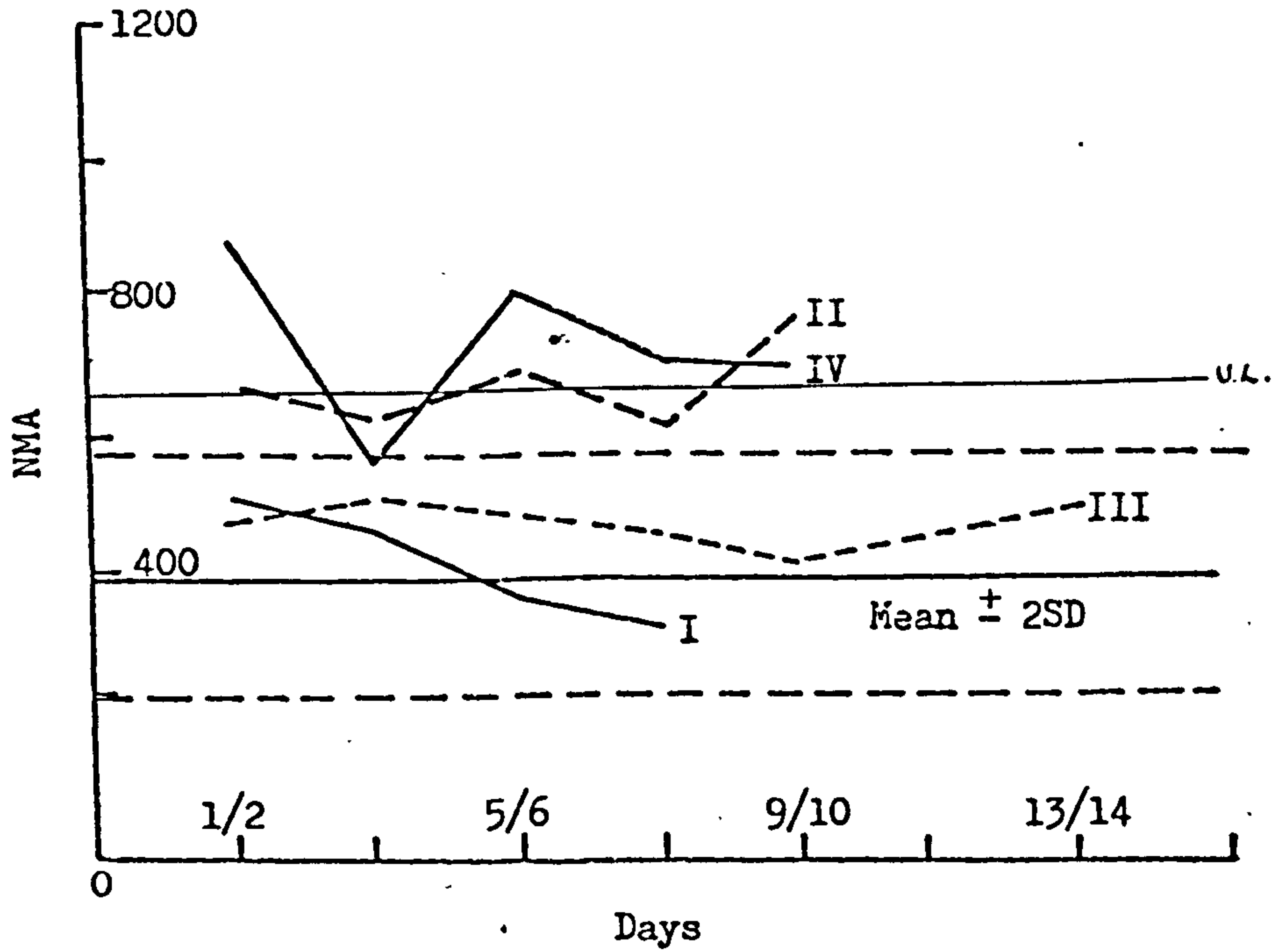
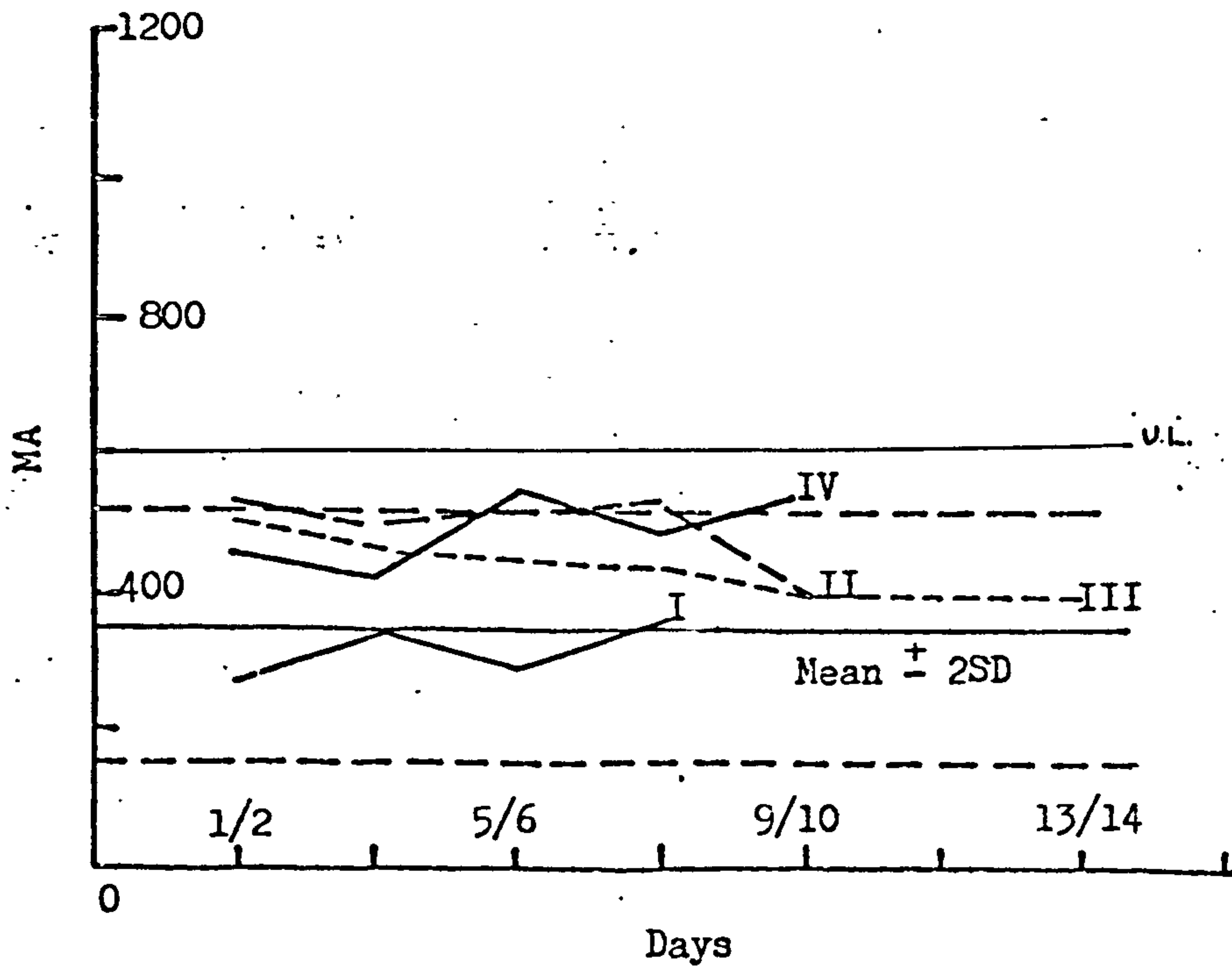


Fig 15 Daily mean output of MA μg per 24 h for Groups I, II, III and IV after SAH



When a comparison was made of the alert Group I patients, with the Group III pre- and post-operation patients (Table 19), no significant increase between the Group IIIa (pre-operation) and the Group I values for HMMA, HVA and NMA was apparent, but there was however, a significant difference between the Group I and Group IIIa patients with regard to the high MA values in the pre-operative patients. A comparison between Group I and Group IIIb (after operation) indicated a slight increase in HMMA, but no significant differences between the HVA, NMA and MA - the operation apparently reducing the output of MA closer to the Group I value. A comparison between Group I and Group IV - the patients who subsequently died after a SAH (Table 20) showed that all four urinary mean levels of catecholamine metabolites were significantly increased in the Group IV patients, some of the values for NMA output reaching levels comparable with those encountered in phaeochromocytoma.

A study of the daily mean values for HMMA (Fig 12) shows that the Group I patients who recovered spontaneously from a SAH showed no apparent increase in HMMA output. The Group II subjects have elevated HMMA values for the first four days after a SAH, but the level then falls. The patients, Group III, who underwent operation, have HMMA levels well within the range established in Chapter 2. The patients who died, Group IV, had the widest fluctuations in HMMA output with a peak level much greater than those found in the other three groups by the sixth day.

Similarly, a study of the HVA output in each Group (Fig 13) appeared to suggest that for Groups I, II and III the mean values were similar and tended to lie nearer the lower end of the observed normal range (Chapter 2.5). Only in Group IV did the HVA show a peak, which tended to parallel the HMMA peak, but even this level was still well within the observed range for HVA. A study of the NMA output between the four groups (Fig 14) produced some interesting results. The mean level of NMA in the Group I subjects is higher on day 1 than subsequent days, but the results would be considered normal. The Group III patients show a rise in NMA the day after their

operation, but their NMA mean levels tend to remain within the observed range.

However, when we consider the mean NMA output in Groups II and IV the results, apart from those found in Group IV (day 3/4), would tend to fall above the mean \pm 2 S.D. limits of the method. We have shown (see Appendix e) that the distribution of the catecholamine metabolites from our normal group have a positive skew distribution. As a result empirical top normal limits have been imposed on our data (see Addendum). As a rough guide the upper level (UL) for NMA has been set at 650 $\mu\text{g}/24$ h. Using this empirical figure we find that the mean daily output for the Group II patients is much higher than those of Groups I and III and fluctuates either side of this arbitrary upper limit, but the mean level does not fall with time over the study period. The mean values for NMA in the patients who died, Group IV, tend to lie above the arbitrary level of 650 $\mu\text{g}/24$ h and in general would be classed as abnormal.

Similarly, setting our upper limit for MA at 600 $\mu\text{g}/24\text{h}$ (Fig 15) would result in all the mean daily values for Groups I, II, III and IV being classed as normal. A closer look at the data shows that the MA mean levels from Group I tend to rise as NMA falls. In Group III the mean level of MA falls steadily with time. The patients in Group II tend to have higher mean MA levels which tend to remain high and do not fall until 8 or 9 days after the SAH. The Group IV patients show wide fluctuations in mean MA output.

7.3 Discussion

High urinary levels of catecholamines have been found in the majority of patients with acute cerebral lesions.^{234, 235} The highest levels have been reported in SAH patients. However, these workers expressed their results not as output per 24 h but as μg catecholamine per mg creatinine for NA, adrenaline and total catecholamines. Some of their urine samples were collected days after admission, and in only eleven patients were more than three measurements carried out. Nine patients had total metadrenalines estimated at least once, but elevated levels were found only in one unconscious patient.

In the present study, the daily mean outputs of NMA and MA suggest that certain SAH patients in Group I and Group III appear to exhibit a normal stress reaction, which after an initial rise, was followed by a fall in urinary NMA and MA output. Groups II and IV however, had continuing high urine levels of NMA and MA which did not fall with time, suggesting that these patients have a high production or rapid turnover of NA and adrenaline. The mechanism which produces this prolonged effect is unknown.

Sesenbach et al²³⁷ in 1951, demonstrated in man that NA when administered parentally, produced a greater increase in cerebral vascular resistance (58%) than the increase in arterial blood pressure of 31% resulting in a reduced cerebral blood flow. Other workers²³⁸ reported in man that adrenaline produced an increase in mean cerebral arterial blood pressure and cerebral blood flow without a significant change in cerebrovascular resistance, while NA increased cerebrovascular resistance, producing a decrease in cerebral blood flow despite a substantial increase in mean cerebral arterial pressure. The effect of a high circulating level of catecholamines on the cerebral blood vessels has been reported²³⁹ in a patient who was found to be suffering from a catecholamine secreting tumour, who was shown angiographically to have diffuse vasoconstriction of the cerebral vessels. It has also been demonstrated²⁴⁰ that spastic vessels can themselves release NA. These findings could suggest that at a critical level of circulating catecholamines the cerebral vessels will start to constrict. These spasms may then cause NA to be released from the spastic vessels which may initiate further cerebral artery spasms which would have the effect of prolonging and maintaining the spasms due to the high level of NA. A suggestion which our finding of continued high levels of urinary NMA in many of our badly affected patients might lend support.

The part played by this increase in NA in maintaining cerebral artery spasm is not clear. It could be a natural attempt by the body to prevent blood flow from the ruptured vessels. Boulay²³³ who studied spasm in

cerebral arteries after SAH showed that spasm is more common on the same side as the aneurysm, but spasm can also be seen in arteries distant from the initial bleed, which have normal lengths of arteries separating them. Death occurred in half his cases who were shown to have spasm on angiography, most were in coma and all had a severe degree of arterial spasm. He also showed after a SAH that spasm of the arteries took between 1 - 4 weeks to relax. Why these spasms are produced in some patients and not in others is difficult to explain. One suggestion might be that the degree of constriction of the arteries depends on the severity of the initial bleed. This would result in a variable degree of physical stress occurring within the body with the concomitant production from the adrenal medulla of NA and adrenaline in amounts which would be directly related to the severity of the stress. It is known¹⁹¹ that NA and adrenaline production may vary with the type of stimulus. The increase in urine output of NMA and MA found in our four groups of patients might perhaps lend support to the suggestion of an increasing stress factor which could be related to the severity of the haemorrhage.

It would certainly appear that the appearance of spasm in the cerebral arteries can be related to high levels of circulating NA in a non-SAH patient.²³⁹ Comparable urine levels of NMA to those produced by patients with catecholamine-secreting tumours were found in the patients who died and many of the more severely affected patients in Group II.

The mechanism whereby spasm in the cerebral arteries after the initial bleed is maintained over long periods is difficult to explain. A possible suggestion could be that the hypothalamus has a 'direct effect' on the sympathetic nervous system. An increased hypothalamic response could perhaps be induced by spasm of the small arteries supplying the hypothalamus. The effect of this would be to increase sympathetic activity and lead to an increased production of catecholamines. Ischaemic lesions, due to anoxia, have been found at autopsy in the hypothalamus of patients who died following a SAH.²⁴¹ The increased sympathetic drive, resulting in increased plasma

levels of catecholamines, would in turn potentiate the spasms, thereby increasing the hypothalamic ischaemia, thus creating a vicious circle of events which contribute to the high morbidity and mortality found in this condition.

A clinical pointer which emerged from this study is the finding of mean urine levels of NMA which lie within the empirical normal limit in SAH patients who recover either spontaneously (Group I), or after operation (Group III), compared to the mean values for NMA found in the SAH patients who subsequently died (Group IV) or who had a marked degree of paralysis or were disorientated (Group II), whose mean NMA values tended to lie above the empirical range and in many cases were still above the empirical normal limits days after the SAH had occurred. It would appear that daily NMA estimations in SAH patients may be useful in predicting the possible outcome. A falling NMA or a value within the normal limits would be good, an increasing NMA or a value lying above the upper limit would be bad.

A further interesting point from this study is the quite low mean daily values for HVA found in the first three SAH groups compared to the calculated normal mean level for HVA (Chapter 2, 5), which might suggest that the production of dopamine may be reduced after a SAH. Even the patients who subsequently died did not increase their mean output of HVA more than one standard deviation higher than the calculated normal mean value. Recent work^{242 - 244} in relation to the condition known as hyperprolactinaemia has indicated that the secretion of prolactin by the pituitary is under direct tonic inhibition by the hypothalamus and that dopamine is thought to be the major prolactin inhibiting factor operating between the hypothalamus and the pituitary. It would be pure speculation to suggest that the prolonged excess of NA found in some cases of SAH could be due to failure of their hypothalamic-pituitary feed-back mechanism, due to a reduced dopamine level. It would be interesting to measure and compare blood levels of dopamine and noradrenaline in a group of SAH patients.

CHAPTER 8

STUDIES ON THE URINARY LEVEL OF CATECHOLAMINE
METABOLITES FOUND IN PATIENTS WITH EITHER A
PHAEOCHROMOCYTOMA OR A NEUROBLASTOMA TUMOUR
AND THE UTILITY OF THE METABOLITE ANALYSES
IN ACCURATE DIAGNOSIS

PART I	-	PHAEOCHROMOCYTOMA
PART II	-	NEUROBLASTOMA

8.1 Introduction

Phaeochromocytoma, although closely related histogenetically to the highly malignant neuroblastoma, is usually benign. Like the adrenal medulla, itself a major derivative of neural crest ectoderm, these tumours are examples of sympathetic tissue functioning as an endocrine gland; this results in the overproduction of a variety of catecholamines with their subsequent adverse effect on the host. One of the major clinical effects on the patient is either paroxysmal or, more frequently, persistent hypertension, due to the sudden or constant secretion of excess catecholamines from these tumours. The output of catecholamines, however, is variable and appears to be closely related, according to Crout and Sjoerdsma,²⁴⁵ to the size of the tumour. Small tumours (less than 50g) tend to have a rapid rate of output and secrete pure amine into the host producing severe symptoms, whilst larger tumours have a slower output of the physiologically active catecholamines and tend instead to put out a higher proportion of metabolites. This apparent relationship between tumour size and catecholamine excretion probably relates to the type of tumour rather than its actual size since a low secretion rate may not be detected at an early stage unless the individual is subjected to external stress either as a result of surgical procedures or perhaps after parturition. A phaeochromocytoma can therefore be considered as a potential danger.

Engel and von Euler²⁴⁶ in 1950 were the first to demonstrate that urine obtained from a patient with a phaeochromocytoma contained an excess of catecholamines and they suggested that estimation of the urinary free catecholamines might be used as a diagnostic test for phaeochromocytoma.

However, the measurement of free catecholamines is unlikely to be a sure indication of the relative rates of secretion of catecholamines by the tumour. The actual amount of free catecholamines found in the urine is only a very small fraction of the total catecholamines secreted. Various

Table 21

Output of HMMA, HVA, NMA and MA mg per 24 h urine volume
found in 19 cases of pheochromocytoma compared with outputs
determined for a group of 123 normal adults

<u>Case No.</u>		<u>Sex</u>	<u>HMMA</u>	<u>HVA</u>	<u>NMA</u>	<u>MA</u>
1	J.D.	M	18.6 * 3.6 6.4 5.1 6.1	6.3 2.8 5.8 4.3 4.2	2.46 * 2.20 * 1.16 * 1.02 * 0.81 *	0.33 0.36 0.27 0.25 0.49
2	K.M.	M	29.2 * 26.7 *	3.8 5.2	9.74 * 8.03 *	1.49 * 1.97 *
3	P.W.	M	6.2 7.3	12.3 * 13.7 *	1.68 * 1.80 *	1.28 * 1.55 *
4	D.M.	M	3.1	10.3 *	0.79 *	5.92 *
5	V.T.	F	21.6 *	17.5 *	4.77 *	0.40
6	B.S.	F	11.8 *	10.3 *	1.66 *	0.40
7	B.L.	F	12.2 *	17.9 *	1.69 *	3.59 *
8	O.B.	F	1.4 3.7	6.5 2.9	2.91 * 3.61 *	0.32 0.94 *
9	W.H.	M	8.9 *	3.8	2.95 *	5.48 *
10	A.J.	F	27.6 *	7.1	9.18 *	0.93 *
11	J.R.	F	4.2 4.5	2.8 4.7	1.11 * 1.57 *	1.10 * 1.83 *
12	I.M.	M	7.3	4.3	5.69 *	3.85 *
13	R.F.	M	5.6 3.2 4.6 6.1	2.6 1.4 4.1 5.5	1.95 * 2.73 * 0.85 * 1.32 *	8.57 * 3.86 * 2.88 * 4.54 *
14	I.C.	F	7.8 *	3.2	1.33 *	1.75 *
15	E.M.	M	7.0 45.8 * 10.7 * 16.6 * 15.8 * 17.6 * 46.6 *	4.7 5.2 3.6 7.9 7.4 7.2 6.0	1.49 * 1.62 * 4.21 * 6.29 * 3.78 * 6.36 * 6.84 *	0.91 * 0.96 * 0.37 0.40 0.49 1.08 * 0.54

cont/

Table 21 (cont)

<u>Case No.</u>		<u>Sex</u>	<u>HMMA</u>	<u>HVA</u>	<u>NMA</u>	<u>MA</u>
16	P.B.	F	19.5 *	7.6	5.26 *	3.88 *
			10.7 *	6.2	3.37 *	2.63 *
			10.9 *	5.7	4.54 *	3.23 *
			7.9 *	4.8	2.57 *	2.45 *
			13.6 *	6.5	4.21 *	3.33 *
			10.9 *	7.1	3.97 *	3.74 *
			7.2	8.2	3.30 *	3.19 *
17	B.C.	F	8.8 *	5.1	2.62 *	0.88 *
			7.5	5.0	2.98 *	1.10 *
18	H.P.	F	28.3 *	24.7 *	2.29 *	2.86 *
19	J.H.	F	10.6 *	5.9	2.62 *	2.96 *
Normal adults						
	Mean		3.3	4.9	0.39	0.35
	Ranges		(1.6-7.5)	(2.4-8.8)	(0.25-0.65)	(0.25-0.55)

* Values falling above the observed normal ranges

Table 22

The distribution of catecholamine metabolite levels within and above the normal range in 43 24 h urine samples taken from 19 subjects known to have a phaeochromocytoma

	<u>Above Normal Range</u>	<u>Within Normal Range</u>	<u>% Normal</u>
HMMA/mg per 24 h	24	19	44
HVA/mg per 24 h	7	36	84
NMA/mg per 24 h	43	0	0
MA/mg per 24 h	33	10	23

Table 23

Nineteen cases of pheochromocytoma classified as having 'elevated'
or 'normal' metabolite levels according to the metabolite analysis used

	<u>Elevated</u>	<u>Normal</u>	<u>% Missed</u>
HMMA/mg per 24 h, as sole test	13	6	32
HVA/mg per 24 h, as sole test	7	12	63
NMA/mg per 24 h, as sole test	19	0	0
MA/mg per 24 h, as sole test	16	3	16
Combined HMMA+HVA/mg per 24 h	15	4	21

workers,^{120,247} in this field have measured the urinary levels of HMMA but as we saw from (Chapter 1) Scheme 2, in man the catecholamines are first metabolised to NMA and MA before their conversion to HMMA. Because of this, measurement of HMMA alone gives no indication of the relative amounts of NA and adrenaline which have been secreted. The output of dopamine in connection with phaeochromocytoma was not considered important until 1956 when MacMillan¹⁶³ analysed tissue from an atypical phaeochromocytoma and found that it contained a high concentration of dopamine which she suggested might be characteristic of a malignant phaeochromocytoma. Robinson²⁴⁸ et al also found in a study of 48 phaeochromocytomas, that one was malignant and that both the primary and secondary growths produced dopamine which resulted in high urine levels of HVA.

The urinary catecholamine metabolites, HMMA, HVA, NMA and MA were measured in 19 proven cases of phaeochromocytoma and compared with the normal adult range for each metabolite which had been determined using the previously described methodology, to evaluate which, if any, of the four catecholamine metabolites might serve as a reliable index for the detection of an active phaeochromocytoma.

8.2 Results

Table 21 shows the extreme variability in the outputs of the four metabolites, HMMA, HVA, NMA and MA found in different patients and even in the same patient on different days.

Table 22 shows the distribution of the metabolite analyses for the known pathological urines within and above the normal range for each metabolite. The results indicate that using the measurement of HMMA and HVA (mg per 24 h), to detect a phaeochromocytoma would have produced false negative results in 44% and 84%, respectively, of the cases. The output of MA (mg per 24 h) would have been negative in 23% of the results. However, the output of NMA (mg per 24 h) would have been diagnostic for all 43 urine samples.

Table 23 expresses the same analytical results in a rather different way; it groups the 19 actual cases of phaeochromocytoma as having an 'elevated'

Fig 16 Relationship between NMA and HMMA mg per 24 h in urine
from patients with phaeochromocytoma

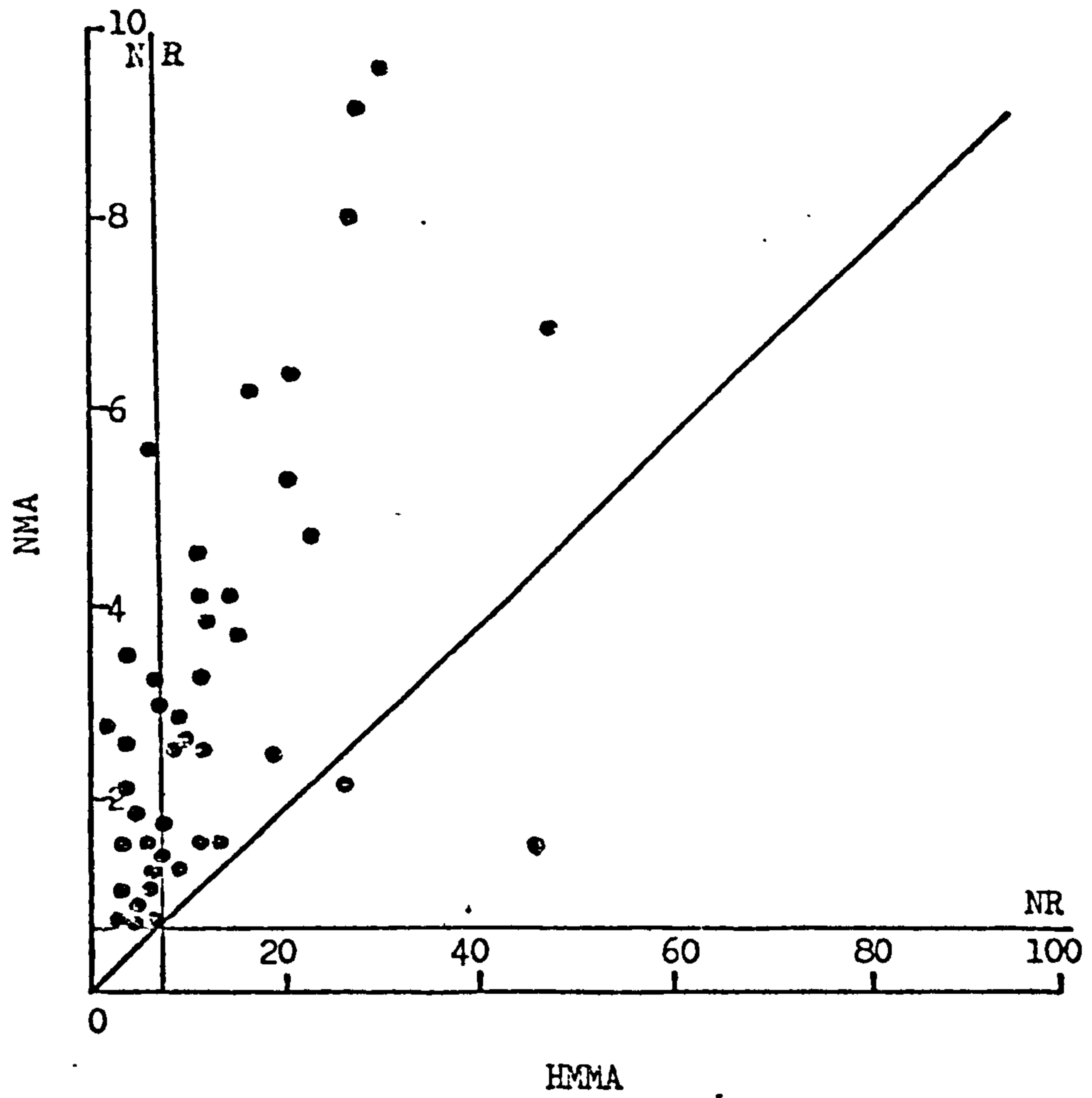


Fig 17 Relationship between HVA and HMMA mg per 24 h urine
from patients with phaeochromocytoma

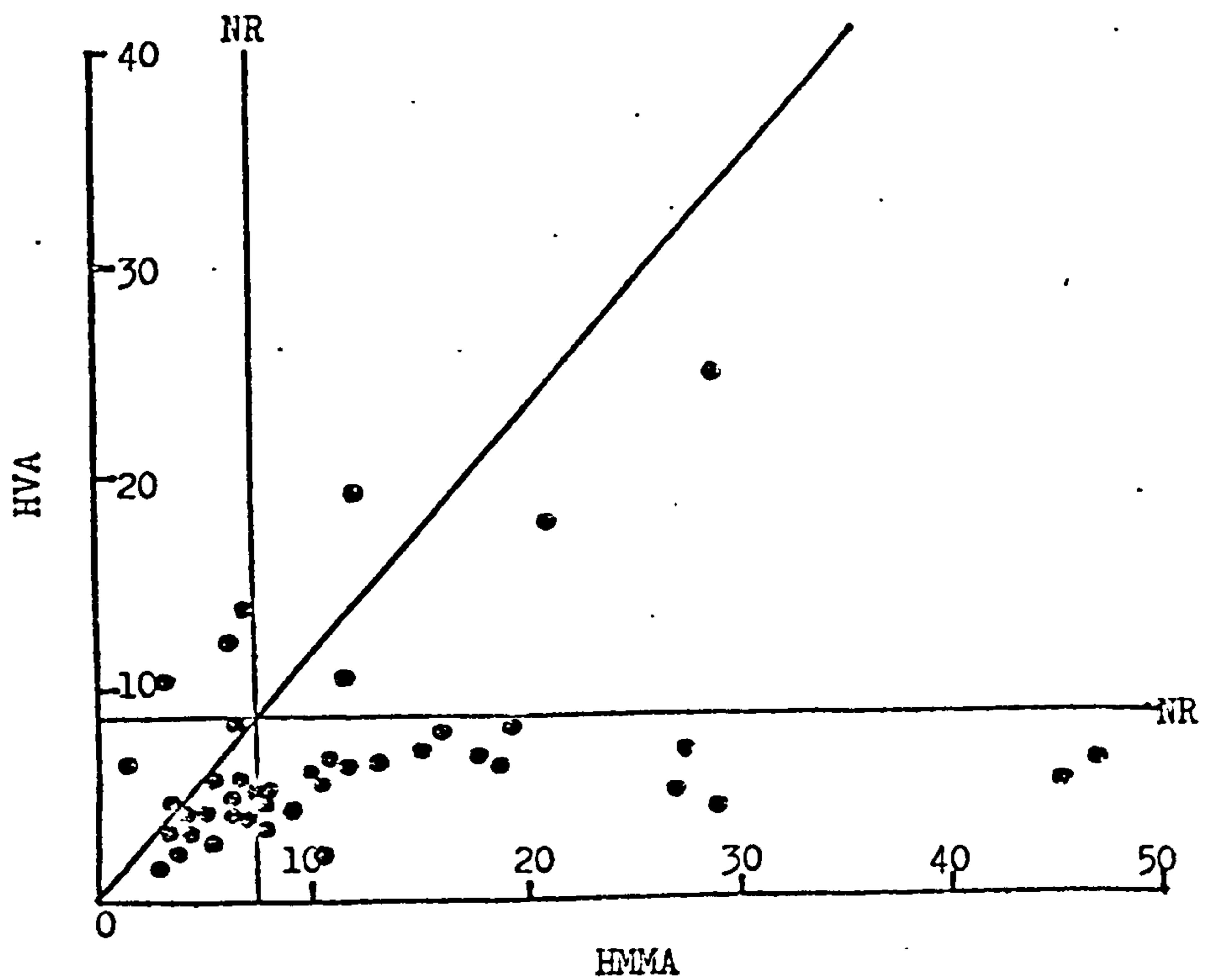


Table 24

Output of HFMA, HVA, NMA and MA/ug per mg creatinine found
in 17 cases of pheochromocytoma compared with normal ranges

<u>Case No.</u>		<u>Sex</u>	<u>HFMA</u>	<u>HVA</u>	<u>NMA</u>	<u>MA</u>
1	J.D.	M	6.7 *	2.3	0.9 *	0.12
			2.6	2.0	1.6 *	0.26
			3.5	3.2	0.6 *	0.15
			2.6	2.2	0.5 *	0.13
			3.4	2.4	0.5 *	0.28
2	K.M.	M	19.7 *	2.6	6.6 *	1.0 *
			23.4 *	4.6	7.0 *	1.7 *
3	P.W.	M	20.0 *	39.6 *	5.4 *	2.9 *
			34.8 *	65.2 *	8.6 *	7.4 *
4	D.M.	M	2.6	8.7 *	0.7 *	5.0 *
5	V.T.	F	15.9 *	12.9 *	3.5 *	0.3
6	B.S.	F	15.9 *	13.9 *	2.2 *	0.5 *
7	B.L.	F	8.2 *	12.1 *	1.1 *	2.4 *
8	O.B.	F	2.2	10.0 *	4.5 *	0.5 *
11	J.R.	F	3.0	2.0	1.7 *	1.7 *
			3.9	4.1	1.4 *	1.6 *
12	I.M.	M	5.6 *	3.3	4.4 *	3.0 *
13	R.F.	M	4.2	2.0	1.5 *	6.4 *
			4.3	1.9	3.7 *	5.2 *
			3.8	3.4	0.7 *	2.4 *
			4.1	3.7	0.9 *	3.0 *
14	I.C.	F	11.5 *	4.7	2.0 *	2.6 *
15	E.N.	M	10.4 *	7.0 *	2.2 *	1.4 *
			42.4 *	4.8	1.5 *	0.9 *
			14.3 *	4.8	5.8 *	0.5 *
			12.6 *	6.0 *	4.8 *	0.3
			16.1 *	7.6 *	3.9 *	0.5 *
			11.9 *	4.9	4.3 *	0.7 *
			56.8 *	7.3 *	8.3 *	0.7 *

/cont.

Table 24 (cont)

<u>Case No.</u>		<u>Sex</u>	<u>EMMA</u>	<u>HVA</u>	<u>NMA</u>	<u>MA</u>
16	P.B.	F	19.9 *	7.6 *	5.4 *	4.0 *
			16.0 *	9.3 *	5.0 *	3.9 *
			10.4 *	6.3	3.9 *	3.2 *
			18.1 *	8.7 *	5.6 *	4.4 *
			17.3 *	11.3 *	6.3 *	5.9 *
			13.8 *	15.2 *	6.1 *	5.9 *
17	B.C.	F	11.9 *	6.9 *	3.5 *	1.2 *
			10.0 *	6.7 *	4.0 *	1.5 *
18	H.P.	F	24.9 *	10.6 *	2.5 *	10.2 *
20	J.R. (Died)	F	13.6 *	3.0	4.8 *	11.2 *

Normal ranges

Mean	2.2	3.3	0.26	0.23
Range	(1.1-5.0)	(1.6-5.9)	(0.16-0.4)	(0.16-0.37)

* Results above the observed ranges.

Table 25

The distribution of catecholamine metabolite levels, compared with creatinine, above and outside the normal range in 39 urine samples taken from 17 subjects known to have pheochromocytoma

	<u>Above</u> <u>Normal Range</u>	<u>Within</u> <u>Normal Range</u>	<u>% Normal</u>
HMA/ μ g per mg creatinine	27	12	31
HVA/ μ g per mg creatinine	20	19	49
NMA/ μ g per mg creatinine	39	0	0
MA/ μ g per mg creatinine	34	7	18

Table 26

Seventeen cases of pheochromocytoma classified as having 'elevated'
or 'normal' metabolite levels according to the metabolite analysis used

	<u>Elevated</u>	<u>Normal</u>	<u>% Missed</u>
HMMA/ μ g per mg creatinine	13	4	24
HVA/ μ g per mg creatinine	10	7	41
NMA/ μ g per mg creatinine	17	0	0
MA/ μ g per mg creatinine	15	2	12
HMMA+HVA/ μ g per mg creatinine, combined test	16	1	6

or 'normal' level for each metabolite analysis of their urine samples

The data suggests that the best single test to use in detecting a suspected pheochromocytoma would be to measure the output of NMA expressed as mg per 24 h urine volume. The output of MA would have produced 3 normal tests, whilst the outputs of HMMA and HVA (mg per 24 h), if measured individually, would have given normal tests for 6 (32%) and 12 (63%) patients respectively. The concurrent estimation of HMMA and HVA, however, would have produced better figures with only 4 normal findings out of the 19 cases.

When we examine the relative amounts of NMA and HMMA expressed in mg per 24 h (Fig 16) in relation to the line defined by normal subjects (cf Chapter 2 Table 1) there is no doubt that the output of NMA relative to HMMA is in general, proportionally greater in the pathological urines. The figure clearly illustrates that although one can find normal levels of HMMA in these pheochromocytoma patients, their NMA levels were all elevated above the normal range. Fig 17 shows the relationship between HMMA and HVA. This suggests that in pheochromocytomas, unlike neuroblastomas (see later) the output of HMMA relative to HVA, in general is proportionally much greater in the pathological urines than in normal urine.

When the results are expressed as μg metabolite per mg of creatinine instead of as a 24 h output rather similar conclusions may be drawn. For 17 cases of pheochromocytoma, (Table 24) indicates, as before, marked variability in the outputs of the individual catecholamine metabolites from patient to patient. Similarly, when pathological urines are classified, on the basis of 39 results for each metabolite we find (Table 25) that whilst the output of NMA expressed now as μg per mg creatinine, was outside the normal range in all cases, the output of MA was normal in 18% of cases, and the output of HMMA and HVA were within the normal ranges in 31% and 49% of tests respectively.

The findings presented in Table 26 suggest that the best single

Fig 18 Relationship between NMA and HMMA/ μ g per mg creatinine in urine from patients with phaeochromocytoma

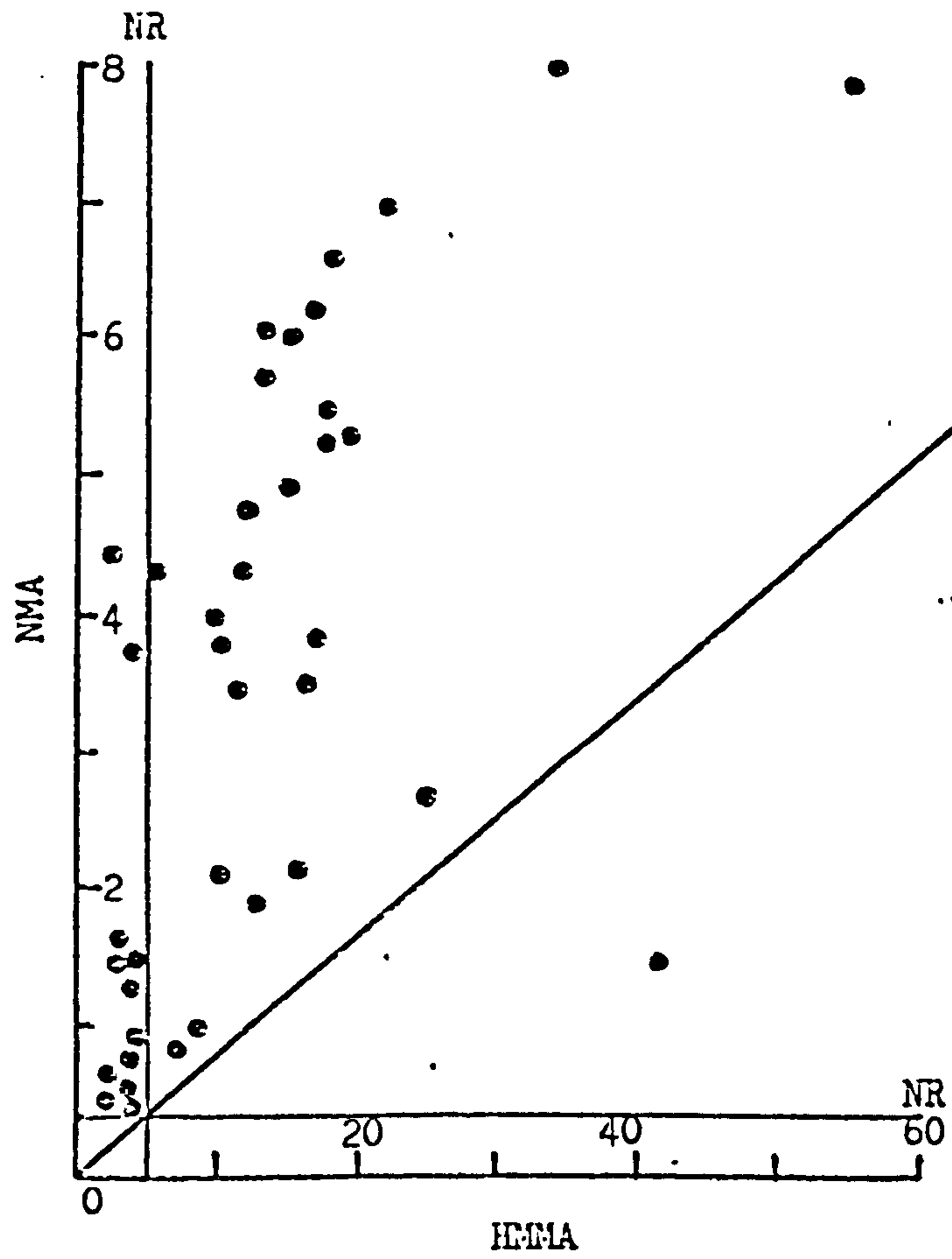
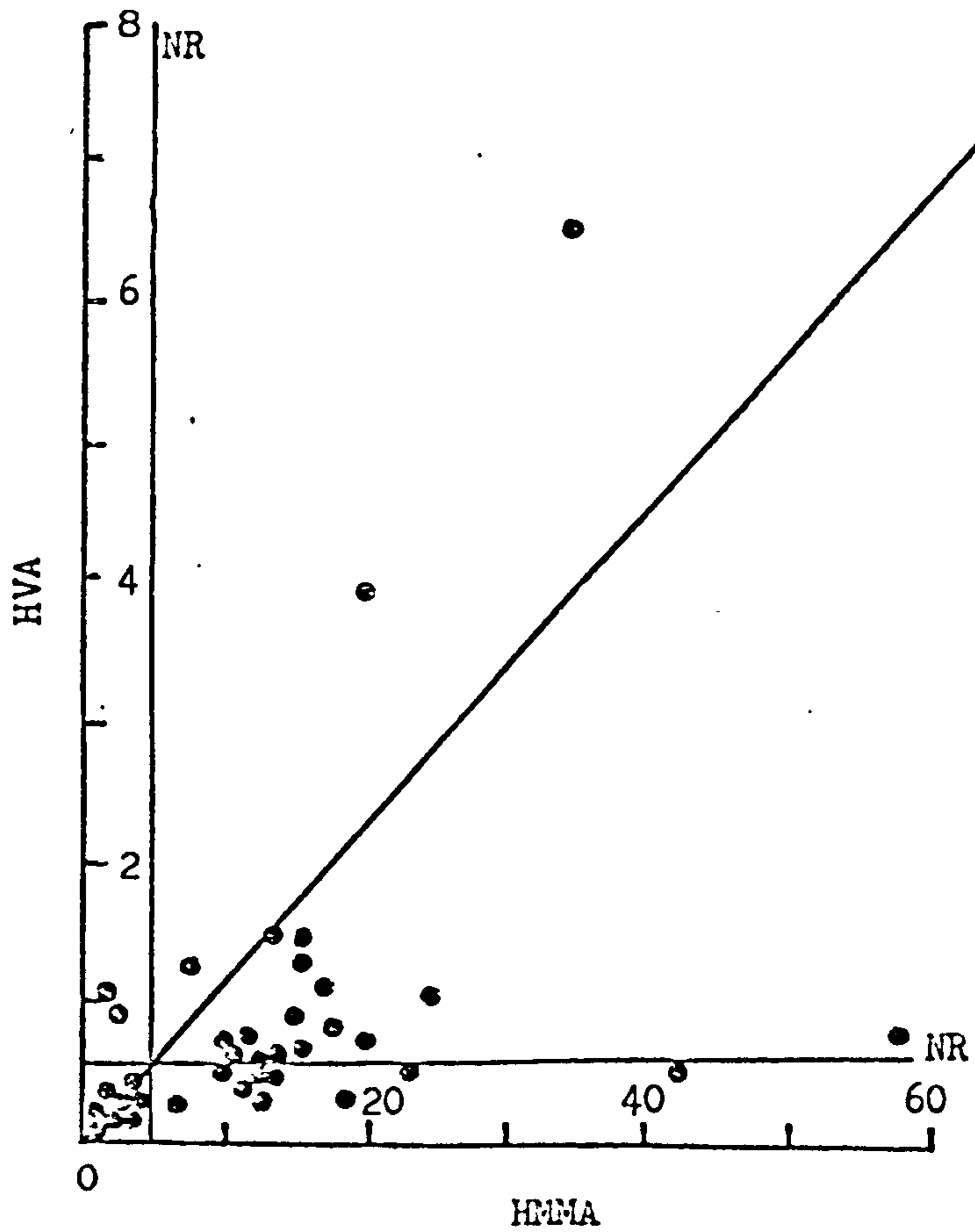


Fig 19 Relationship between HVA and HMMA/ μ g per mg creatinine in urine from patients with phaeochromocytoma



test of the metabolites for detecting a phaeochromocytoma, if a random sample of urine is used, would be the output of NMA expressed as μg NMA per mg creatinine whilst the output of MA would have produced two false normal results, out of

Interpretation of the outputs of HMMA and HVA separately would have produced 4 (24%) and 7 (41%) false normal findings out of the 17 cases; the combined test, however, produced only one normal result.

When we compare the relative amounts of NMA and HMMA, expressed as μg per mg of creatinine in relation to normal behaviour (Fig 18), the results are similar to those of (Fig 16) in that in the pathological urines the output of NMA is much greater in proportion to that of HMMA and, as before, the results for NMA lie above the normal range. More of the HMMA values tend to fall above the normal range when expressed as μg HMMA per mg creatinine rather than mg per 24 h.

Similarly, Fig 19 compares the relative outputs of HMMA and HVA in relation to the normal outputs and shows that in phaeochromocytoma HMMA levels tend to be increased more than HVA levels, relative to normal behaviour, although expressing the output of HVA as μg per mg creatinine tends to produce more abnormal results than when the output of HVA is expressed in mg per 24 h.

8.3 Discussion

In many hospital laboratories the standard procedure used to screen for the presence of a phaeochromocytoma is to measure the urinary output of HMMA per 24 h. This is done either chromatographically¹⁰⁸ or using a spectrophotometric technique.¹²⁰

One of the problems encountered in screening in this manner is the fact, as can be observed from our results, that the outputs of the individual metabolites can vary with time. Richards *et al*²⁴⁹ described two patients, one of whom had a urinary HMMA value of 7 mg per 24 h one day and thirteen days later the value was 46 mg per 24 h. The other patient on six separate

occasions had urine HMMA levels of 72 - 420 mg per 24 h, findings similar to those found in three of our patients (Nos. 1 J.D, 15 EM and 16 PB).

Normal levels of HMMA have been found^{250,251} in urine from patients with known pheochromocytomas. In a careful study of 39 pheochromocytoma patients Kelleher et al²⁵¹ found only one normal HMMA urine level in 70 tests. The finding of normal values for 44% of the HMMA analyses in the present study is a little disturbing; in part explanation perhaps is the fact that the 43 samples used came from a smaller number (19) of pheochromocytoma patients, and additionally, were not distributed evenly over the donors. The finding by Crout et al²⁵⁰ of 3 out of 23 cases of pheochromocytoma which had normal levels of HMMA (13%) is closer to our findings of 6 normals out of 19 cases (32%).

The finding of an increased urinary output of HVA in six of our cases might indicate that these pheochromocytomas have malignant characteristics if MacMillan's postulate is correct.¹⁶³ Robinson et al²⁴⁸ studied a patient who had a malignant tumour. This patient prior to the removal of his tumour was excreting large amounts of HMMA, HVA, NMA and MA in his urine. The tumour was removed and histologically appeared to be benign. The patient's excretion of catecholamines and catecholamine metabolites returned to normal for eleven months, after which his HMMA and NMA output started to increase until at the time of his death his secondary growths were producing excess catecholamines, the metabolites of which were being excreted in amounts similar to those obtained in the case of his primary tumour. This indicated that both primary and secondary growths secreted large amounts of DOPA and dopamine.

Louise et al²⁵² in a study of 11 pheochromocytoma patients found two whose urine contained large amounts of DOPA and dopamine, as well as raised levels of NA and adrenaline; both patients curiously were normotensive. Histological examination of their tumours confirmed that they were pheochromocytomas, but no information was given regarding follow up studies in these two

patients in order to detect the development of possible malignant secondary tumours. Hypersecretion of DOPA is a characteristic of non-chromaffin sympathetic tumours such as neuroblastomas,^{253,255} tumours which are normally highly malignant. Patients with phaeochromocytoma tumours who are found to have excess levels of HVA in their urine should be carefully followed up routinely, after the tumour has been removed in order to detect any signs of secondary growths becoming functionally active.

The concurrent measurement of HMMA and HVA expressed in mg per 24 h would have proved more successful in diagnosing phaeochromocytoma than separate analyses. Apparently the distortion of the catecholamine metabolism pattern caused by the tumour has variation from individual to individual for these two metabolites and thus the distortion is best discerned when the two parameters are measured. The metabolite whose analysis alone, expressed as output in 24 h, was found to be the best indication of phaeochromocytoma was NMA. All cases showed abnormal levels. This finding extends and confirms the work of others²⁵¹ who measured total metadrenalines using the Pisano technique¹⁴³ and also two-dimensional chromatography with quantitation by visual comparison of the spots obtained. They found abnormally high metadrenaline levels in all urine samples obtained from phaeochromocytoma patients. Crout et al²⁵⁰ considered that the levels of urinary NA and adrenaline were the most reliable diagnostic indices of a phaeochromocytoma, but there is much evidence^{251,255,256} to suggest that the total output of metadrenalines, or indeed the individual levels of NMA and MA are equally useful as guides in detecting phaeochromocytoma. Our results certainly support this contention. Furthermore, using the present method for analysis of NMA and MA, neither the specialised equipment nor the specialised techniques¹⁴⁸⁻¹⁵² previously required are necessary.

When we compare outputs in 24 h of HMMA and NMA we find our results closely parallel those of Kelleher et al²⁵¹ in that there is a much greater proportional increase in normetadrenaline than HMMA. A comparison between

HMMA and HVA suggests that in phaeochromocytomas, unlike neuroblastomas (see later) there is a relatively greater increase in HMMA. An increase in HVA compared to HMMA, might suggest the presence of a DOPA or dopamine secreting atypical or malignant phaeochromocytoma, or in a child a neuroblastoma (see later).

Because of the relative ease in collecting accurate 24 h urine specimens from adults most catecholamine metabolite analyses are quoted as output per 24 h. Very few figures have been produced which relate output of catecholamines to that of creatinine, the usual reference when random urine samples are analysed. Kelleher et al²⁵¹ quote results for the output of HMMA in two of their cases. One of them, when converted to μg HMMA per mg creatinine, would have been within the present method's normal range, the other would have been abnormal. However, both had NMA outputs eight times the normal level we have found for this metabolite when it is expressed in terms of μg per mg of creatinine.

Gupta et al²⁵⁷ produced normal urinary ranges for total metadrenalines based on a modified Pisano¹⁴³ technique for random urine specimens and expressed their results as mg total metadrenalines per g creatinine. Using their method they found no false positives in 150 hypertensive patients but five cases of neural crest tumours gave very high figures. These workers suggested using their modified technique as a supplement to urinary HMMA estimation.

The outputs of HMMA and HVA when expressed in terms of creatinine appeared to give fewer negative findings than when the results were expressed as mg per 24 h, a finding which is difficult to explain, although work carried out by Sunderman²⁴⁷ who studied the urinary output of HMMA per 24h suggested that with urine volumes from 400 - 800 cm^3 per 24 h the excretion of HMMA was significantly lower than when the urine volume lay between 800 - 3,000 cm^3 per 24 h. He did not give any reason for

this variation apart from suggesting that the urine volume of adults should exceed 800 cm^3 if measurement of HMA is to be carried out. In the present study, only 4 out of the 43 24 h urine collections had urine volumes within the $400 - 800 \text{ cm}^3$ range, and one of these was obtained from a young child.

It is apparent from the relationships between HMA and NMA in whichever mode they are plotted, that the estimation of NMA is the best of the metabolites for the detection of a phaeochromocytoma. It was the only test which would have indicated the presence of an active phaeochromocytoma for all our test samples.

An advantage in the separate assay of NMA and MA over the estimation of total metadrenalines is that the outputs of NMA and MA when measured individually reflects to a certain degree the secretion of NA and adrenaline from the tumour. From a surgical point of view the need to know whether a tumour is secreting a lot of adrenaline is important because of the possibility of cardiac dysrhythmias occurring during operation. This information cannot be derived from a total metadrenaline estimation. Tumours which are found to be extra-adrenal, particularly malignant secondaries, usually produce NA only, whilst the commoner adrenal tumours often secrete a mixture of NA and adrenaline. In these cases the differential assay of NMA and MA would be superior as a diagnostic tool.

On the basis of the results of this work it is suggested that the routine screening of hypertensives who may be suspected of harbouring a phaeochromocytoma²⁵⁸ should consist of a quantitative estimation of total urinary metadrenalines produced in 24 h. Any urines found to have raised levels could then be analysed for NMA and MA individually. Further, it is suggested that the practice of initial quick screening for excess HMA should be re-considered in view of our findings, but that if it is performed the results may be more reliably expressed as μg per mg creatinine rather than as mg per 24 h.

Additionally, all cases of phaeochromocytomas, where high levels of

NA secretion are indicated, should have HVA estimations carried out as an indication of whether they are malignant,¹⁶⁴ especially in the cases where a tumour has previously been detected and removed.

8.4 Introduction

A neuroblastoma is a highly malignant tumour which develops from the neural crest tissue of children. It is one of the commonest types of infantile tumour, yet it is frequently misdiagnosed in spite of the fact that neural crest tumours are unique in producing easily detectable amounts of catecholamines in up to 90% of patients.²⁵⁹ The reason is probably because the primary tumour may never become clinically obvious.

The first report²⁶⁰ of an increase in the excretion of pressor amines from a patient with a neuroblastoma occurred in 1957. This was followed by a further report²⁶¹ in which the excess catecholamines were measured using biological techniques. Later workers^{166,262,263} also found elevated levels of urinary NA in these patients.

The finding¹¹⁷ of an increased output of HMMA, led other groups^{264,265} to measure this compound as an aid to the detection of neuroblastomas. However, the most noteworthy discovery was the finding^{165,166} of high levels of dopamine in urine obtained from patients with neuroblastoma. This led to the demonstration that HVA, a metabolite of dopamine, was also present in large amounts.¹⁶⁶ Studies^{117,139,264} on the output of the O-methylated catecholamines are fewer in number. Sandler¹⁶² commented in 1964 that the metabolites which probably undergo the largest proportional increase in excretion are these amines, but little work has been done in measuring the individual levels of NMA and MA.

In this work a study was made, using the methods which have been developed, to evaluate which, if any, analyses of urinary metabolites might prove useful for the early detection of neuroblastoma.

8.5 Results

Table 27 gives the results for the output of HMMA, HVA, NMA and MA expressed as weight per 24 h urine volume found in nine children with proven neuroblastoma; normal ranges for children aged 2 - 11 years are given for

Table 27

Output of HMMA, HVA, NMA and MA mg per 24 h urine volume found in 9 cases, aged 3 - 8 years, of neuroblastoma compared with normal ranges for children aged 2 - 11 years

<u>Case No.</u>		<u>Sex</u>	<u>HMMA</u>	<u>HVA</u>	<u>NMA</u>	<u>MA</u>
1	N.S.	F	2.9	8.1 *	0.95 *	0.32
2	J.H.	F	15.0 *	12.2 *	2.05 *	0.45 *
3	C.S.	F	5.0 * 6.3 *	14.4 * 12.8 *	0.95 * 1.08 *	0.41 * 0.51 *
4	R.H.	M	2.4 1.2	4.8 3.2	0.60 * 0.46 *	0.33 0.29
5	C.C.	F	2.6	3.7	0.45 *	0.64 *
6	A.H.	M	10.1 *	33.9 *	2.2 *	0.23
7	R.C.	M	14.1 *	34.5 *	1.23 *	0.29
8	B.M.	M	21.6 * 29.8 * 32.6 * 17.7 * 14.2 * 8.0 *	24.3 * 28.9 * 32.7 * 23.2 * 17.1 * 20.1 *	3.22 * 7.32 * 8.00 * 1.38 * 1.16 * 0.72 *	0.37 * 0.58 * 0.71 * 0.50 * 0.34 0.30
9	C.L.	M	4.5 * 2.9	9.4 * 9.4 *	2.28 * 0.66 *	1.27 * 0.27

Normal children (age 2 - 11 years)

Mean	2.6	3.6	0.26	0.24
Range	(0.6-4.0)	(0.6-5.5)	(0.07-0.37)	(0.09-0.36)

* Results above ; the range defined by two standard deviations on either side of the normal mean

Table 28

The distribution of catecholamine metabolite levels within and above the normal range in 17 24 h urine samples from 9 subjects known to have neuroblastoma

	<u>Above Normal Range</u>	<u>Within Normal Range</u>	<u>% Normal</u>
HMMA/mg per 24 h	12	5	29
HVA/mg per 24 h	14	3	18
NMA/mg per 24 h	17	0	0
MA/mg per 24 h	9	8	47

Table 29

Nine cases of neuroblastoma classified as having 'elevated' or 'normal' metabolite levels according to the metabolite analysis used

	<u>Elevated</u>	<u>Normal</u>	<u>% Missed</u>
HMMA/mg per 24 h, as sole test	6	3	33
HVA/mg per 24 h, as sole test	7	2	22
NMA/mg per 24 h, as sole test	9	0	0
MA/mg per 24 h, as sole test	5	4	44
Combined HMMA+HVA/mg per 24 h	7	2	22

Fig 20 Relationship between NMA and HMMA/mg per 24 h in urine
from children with neuroblastoma

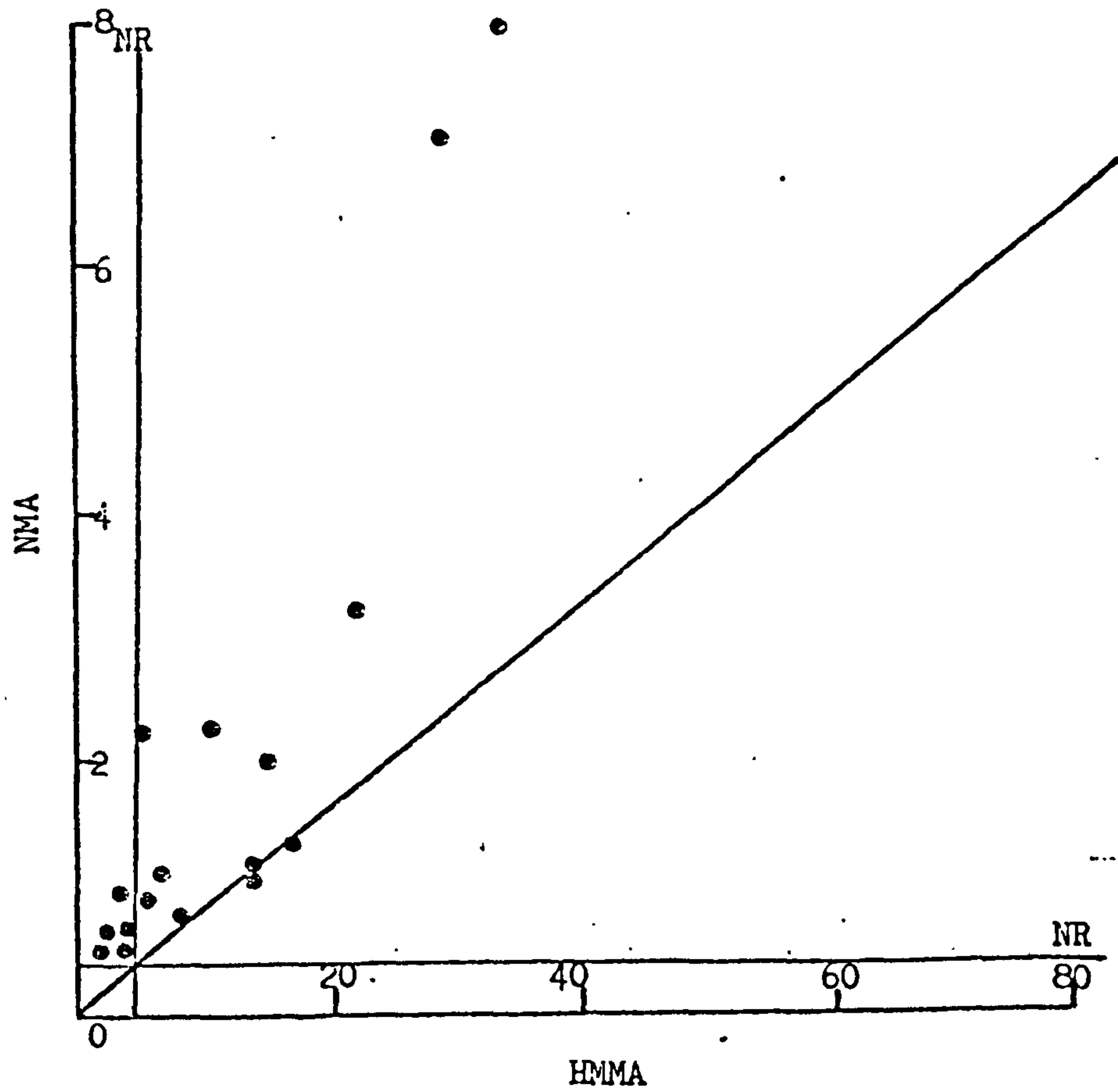


Fig 21 Relationship between HVA and HMMA/mg per 24 h urine
from children with neuroblastoma

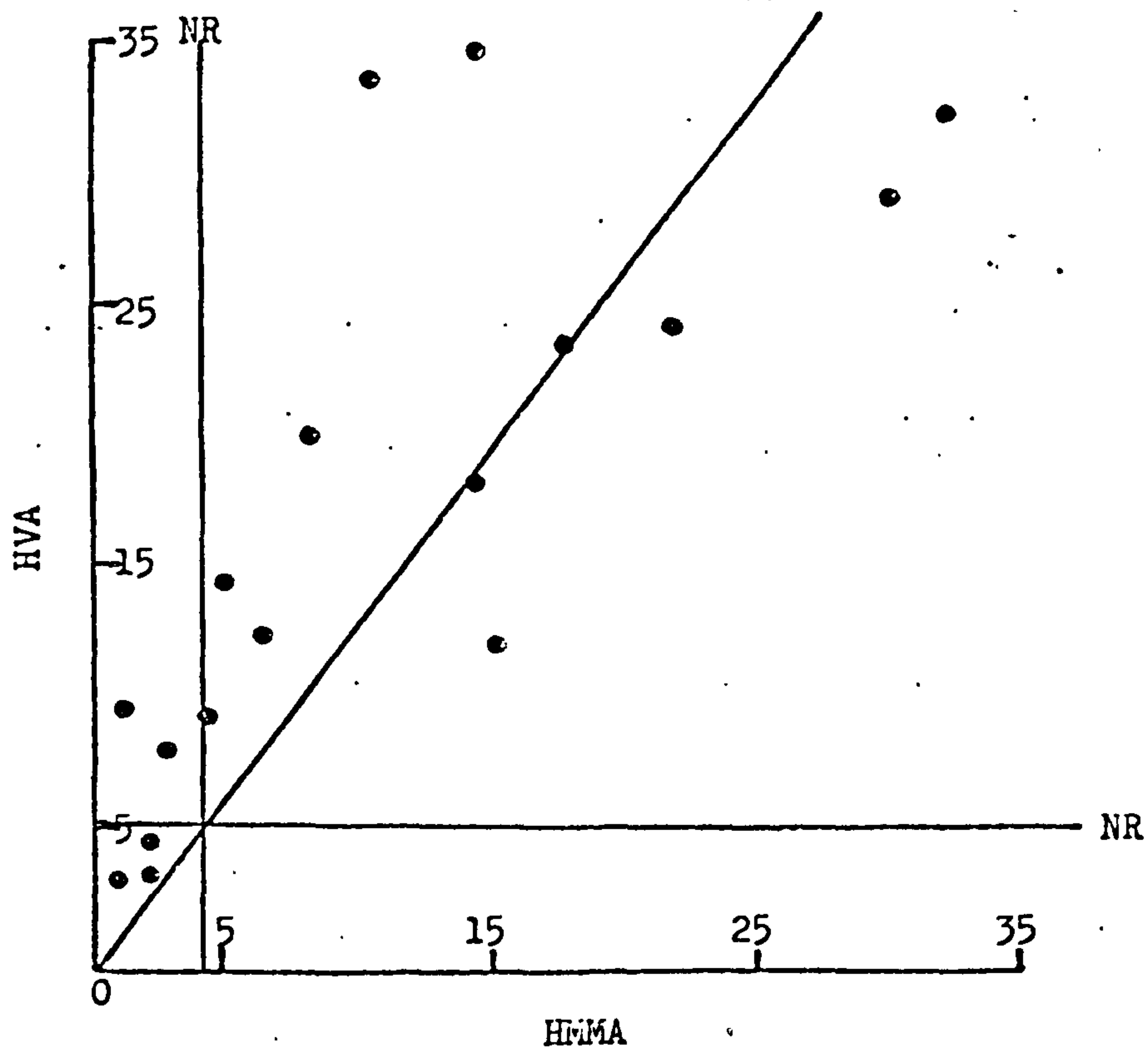


Table 30

Output of HMMA, HVA, NMA and MA μ g per mg of creatinine found in 10 cases of neuroblastoma, aged 3 - 8 years (original nine plus one) compared with normal ranges for children aged 2 - 11 years

<u>Case No.</u>		<u>Sex</u>	<u>HMMA</u>	<u>HVA</u>	<u>NMA</u>	<u>MA</u>
1	N.S.	F	35.0 *	98.0 *	3.7 *	0.15
2	J.H.	F	63.3 *	51.5 *	8.6 *	1.9
3	C.S.	F	14.2 * 22.8 *	41.0 * 46.4 *	3.0 * 3.9 *	1.3 1.8
4	R.H.	M	29.2 * 13.0 *	78.0 * 25.9 *	11.2 * 3.3 *	7.1 * 1.8
5	C.C.	F	5.5	7.9	0.9	1.4
6	A.H.	M	41.0 *	138.0 *	9.0 *	0.9
7	R.C.	M	31.0 *	98.0 *	2.7 *	0.6
8	B.M.	M	126.0 * 113.0 * 166.0 * 60.0 * 60.0 * 19.0 *	142.0 * 110.0 * 160.0 * 78.0 * 73.0 * 48.0 *	18.8 * 27.8 * 43.4 * 4.6 * 4.9 * 1.7	2.2 2.2 3.6 * 1.7 1.4 0.7
9	C.L.	M	12.7 * 9.0	26.6 * 29.0 *	6.4 * 2.0	3.6 * 0.8
10	J.M.	F	31.4 *	22.0 *	12.8 *	0.8

Normal children (age 2 - 11 years)

Mean	7.1	9.7	0.8	0.8
Range	(3.3-12.5)	(3.7-17.1)	(0.5-2.1)	(0.3-2.9)

*Results above the range defined by two standard deviations on either side of the normal mean

Table 31

The distribution of catecholamine metabolites, compared with creatinine, within and above the normal range in 18 urine samples taken from 10 subjects known to have neuroblastoma

	<u>Above Normal Range</u>	<u>Within Normal Range</u>	<u>% Normal</u>
HMMA/ μ g per mg creatinine	16	2	11
HVA/ μ g per mg creatinine	17	1	5.5
NMA/ μ g per mg creatinine	15	3	16
MA/ μ g per mg creatinine	3	15	83

Table 32

Ten cases of neuroblastoma classified as having 'elevated' or 'normal'
metabolite levels according to the metabolite analysis used

	<u>Elevated</u>	<u>Normal</u>	<u>% Missed</u>
HMMA/ μ g per mg creatinine, as sole test	9	1	10
HVA/ μ g per mg creatinine, as sole test	9	1	10
NMA/ μ g per mg creatinine, as sole test	7	3	30
MA/ μ g per mg creatinine, as sole test	3	7	70
Combined HMMA+HVA/ μ g per mg creatinine	9	1	10

Fig 22 Relationship between NMA and HMA/ μ g per mg creatinine in urine from children with neuroblastoma

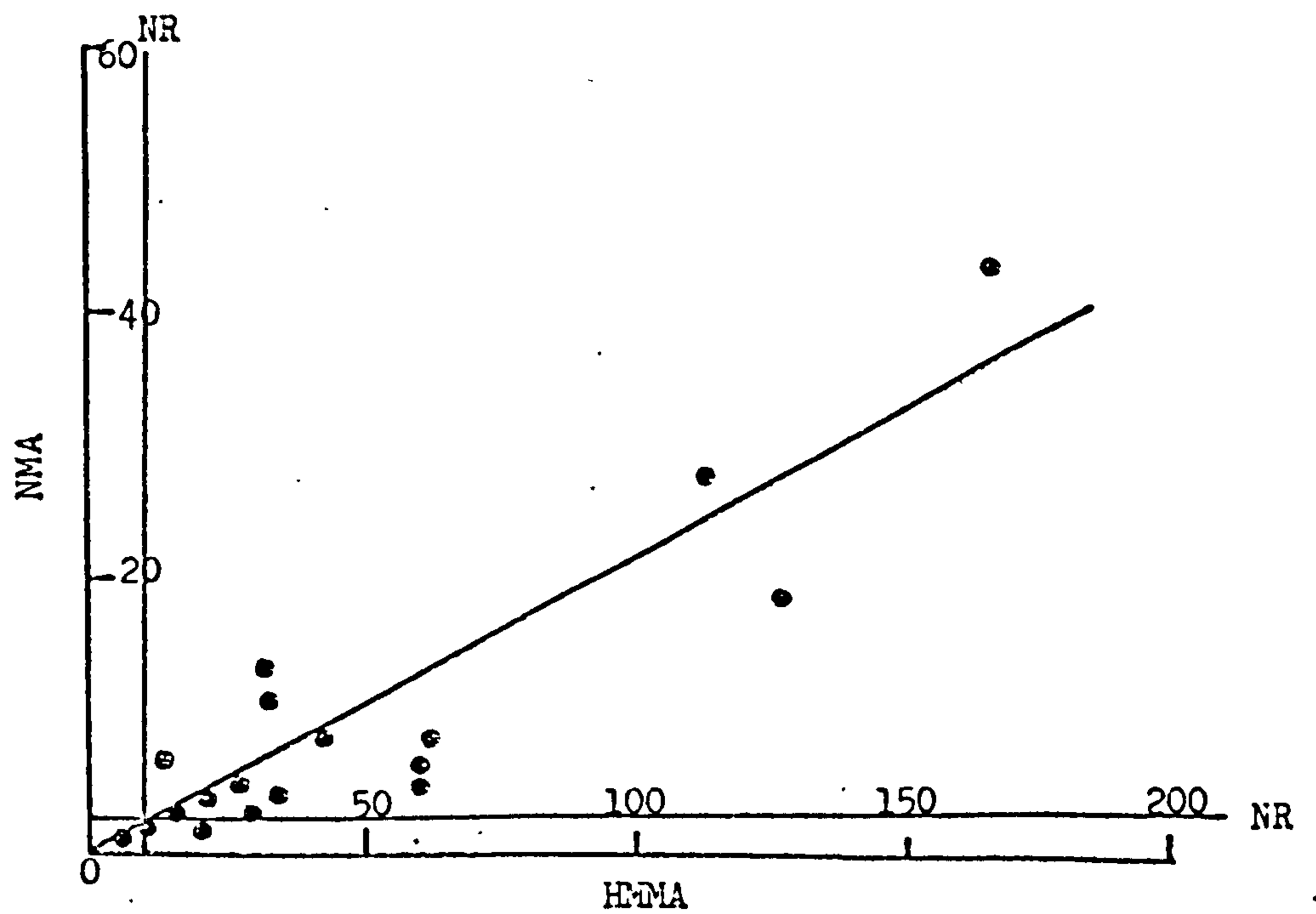
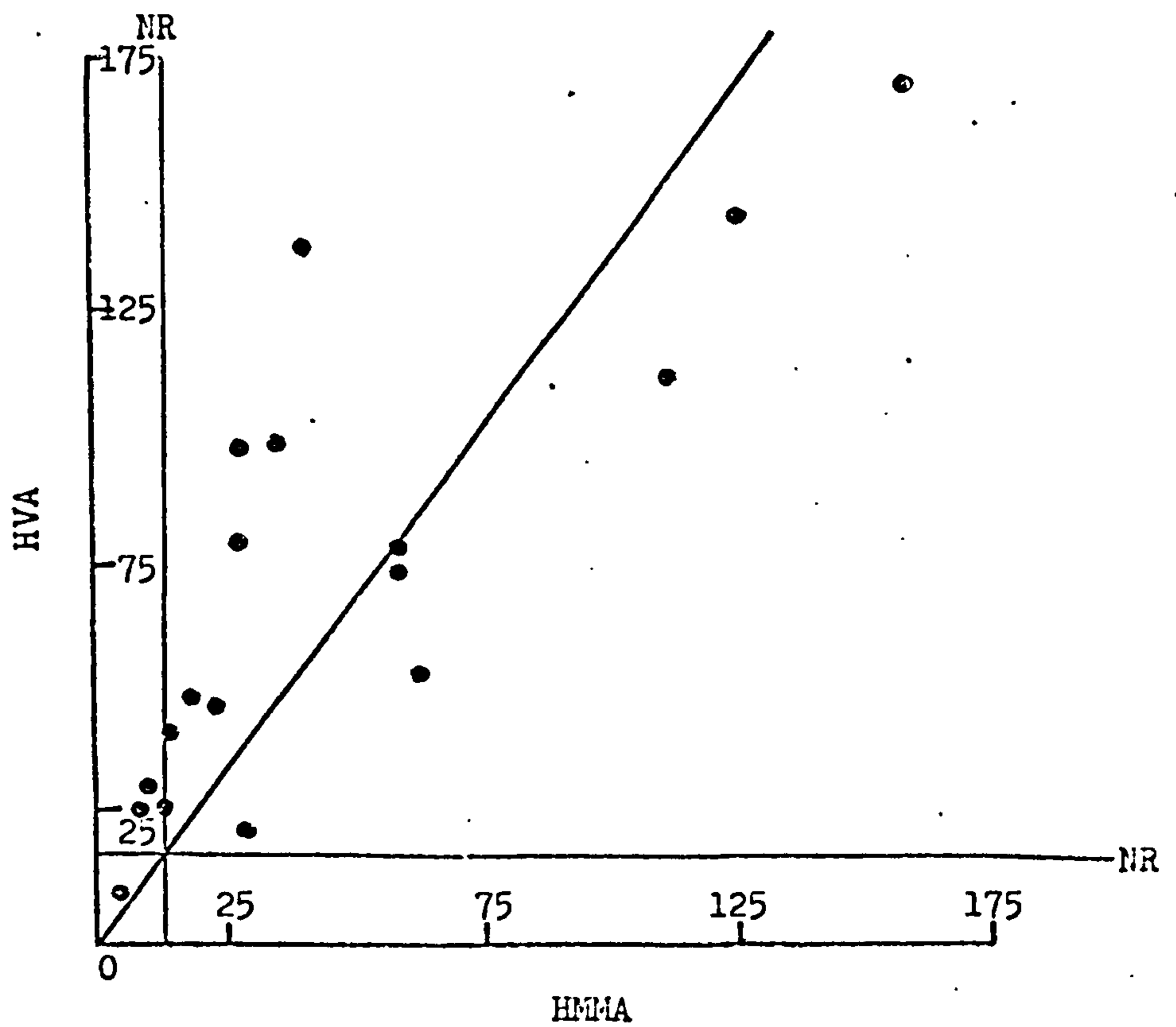


Fig 23 Relationship between HVA and HMA/ μ g per mg creatinine in urine from children with neuroblastoma



comparison. Values marked with an asterisk denote results which fall outside a range which covers two standard deviations on either side of the normal mean. Table 28 shows the distribution of the 17 analyses for the four metabolites from the 9 subjects within and above the normal range. The analyses for NMA alone were above the normal range in all cases. For the other metabolites varying proportions of the analyses were above the normal range.

When the results for each individual neuroblastoma patient are studied (Table 29) to decide which single analysis, if any, would have been 100% positive in detecting all 9 cases, we find that the best is that for NMA which was elevated in all cases. This is followed by HVA (78%) and HMMA (67%) respectively.

Fig 20 shows the relationship between the 24 h outputs of HMMA and NMA in relation to the normal proportions and illustrates the finding of a relatively greater increase in NMA compared to HMMA. Similarly, Fig 21 shows the relationship between output of HMMA and HVA. This indicates that neuroblastoma patients appear to have a greater output of HVA relative to HMMA by comparison with normal (contrast the pheochromocytoma patients Fig 17). Again we may alternatively express the levels of urinary metabolites in terms of creatinine output instead of on a time basis.

Table 30 shows the total results (18) obtained in ten neuroblastomas (the original nine plus one extra) expressed as μg metabolite per mg of creatinine. As before the asterisks signify results falling outside two standard deviations from the accepted normal mean value for each metabolite measured.

A study of the 18 results obtained from the ten cases (Table 31) suggests that when metabolite levels are expressed in terms of creatinine output, HVA is the metabolite most likely to exhibit an abnormal level followed by HMMA and NMA, with MA as expected, a very poor index.

In Table 32 the results are analysed in terms of how successful each

measured metabolite level, expressed as μg per mg creatinine, would be when used as a diagnostic index. HMMA and HVA appear equally suitable but their combination would have missed the individual that each failed to detect separately. NMA is markedly less useful as a diagnostic index when its level is expressed in terms of creatinine rather than in terms of time.

Figures 22 and 23 show the variation of NMA and HVA respectively, with HMMA, (all levels being expressed in terms of creatinine), in the urine samples from neuroblastoma cases in relation to normal behaviour.

8.6 Discussion

Most medical workers would agree that neuroblastoma in a young child is often initially misdiagnosed, despite the fact that this tumour produces large amounts of catecholamines. Perhaps this is because it is rare for a neuroblastoma patient to be hypertensive. It has been suggested²⁶⁶ that 'true' neuroblastomas possess virtually no storage granules for catecholamines, thus the amines which are produced are rapidly broken down intracellularly and secreted as inactive metabolites into the circulation.²⁶⁷ This should produce high urinary levels of NMA, HVA and HMMA which should make the condition simple to detect. It is also possible that the secretion of DOPA may be responsible for the relative absence of sympathetic over-activity in neuroblastoma. The amino acid DOPA has been shown to have hypotensive action in man.²⁶⁸

Hitherto, a choice has had to be made as to which urine test, or tests, to use for detecting neuroblastoma and monitoring any subsequent treatment. In recent years, the trend has been to 'screen' for excess HMMA in urine from suspected neuroblastoma patients using simple techniques, rather than to use the two-dimensional chromatographic methods previously developed^{55,269,270} in 1956-57, which are capable of detecting raised levels of both HMMA and HVA. Labrosse²⁷¹ developed a 'spot test' which consists of applying a drop of urine to a filter paper disc, followed by the addition of a drop of diazotised solution of 4-nitroaniline. An excess of purple colour

development by comparison with a normal suggests a positive result. The test uses random samples of urine and the colours produced are, to an untrained eye, extremely variable.

Bond²⁷² in 1975, after initial screening, using the Labrosse spot-test, measured HMMA quantitatively in 50 children with neuroblastoma and found seven who had normal levels of HMMA. Presumably these had been detected on clinical grounds rather than positive spot-tests. Her better diagnostic figure of 86% compared to our 67% for excess HMMA per 24 h could be due to the fact that some neuroblastomas can produce quite a large amount of 1-(4-hydroxy-3-methoxyphenyl) ethane-1,2-diol (HMPG) which would, using the method employed by her¹²⁰ be oxidised to vanillin, thus producing higher HMMA values.

Bell²⁷³ had observed in 1962 that the output of urinary NA in neuroblastoma patients, even when higher than normal, was not high enough to be easily detected except by the most sensitive fluorimeters, and he recommended as a screening test for neuroblastomas, the method of Gitlow *et al*¹¹¹ for detecting excess HMMA. Using this method four out of his sixteen cases would have been normal when expressed as the ratio of the absorptions of the ethyl acetate extract at 450 and 550 nm. In eight cases where HMMA was measured, as output per 24 h using the Pisano¹²⁰ technique, normal results were obtained in five patients. However, when his results were expressed as μg HMMA per mg creatinine all sixteen cases would have proved positive. Our results of a 90% detection rate using HMMA level, expressed as μg metabolite per mg creatinine, as a diagnostic index, but only 67% when expressed in terms of output in 24 h supports his findings.

Other workers²⁷⁴ who measured HMMA levels in seven consecutive 24 h urine collections obtained from a four year old child with neuroblastoma found all seven values to be within the normal range for HMMA expressed as mg per 24 h yet the levels of NA, and especially dopamine were high. No values for HVA or metadrenalines were reported.

Gitlow et al²⁵⁹ developed a further screening test for HMMA which consists of adding diazotised 4-nitroaniline reagent to four drops of urine at alkaline pH. The colour produced is extracted with iso-amyl alcohol and compared visually against a standard amount (5 µg) of HMMA, added to a normal urine and taken through the test procedure. Using this method, for 35 proven cases of neuroblastoma, a true diagnosis was obtained in 32. Two out of the three negative cases were shown quantitatively to have normal levels of HMMA expressed as µg per mg creatinine; however, all three had increased levels of HVA expressed as µg metabolite per mg creatinine. The quantitative results for HMMA and HVA together produced a diagnosis rate of 100% which agrees well with our present findings. These workers also found that 89% of their cases were indicated by analysis for total metadrenalines. This result is not directly comparable with the present study where individual NMA and MA measurements were carried out.

Recently, Bray et al²⁷⁵ have also found two neuroblastoma patients who had normal levels of urinary HMMA, when measured using the Pisano¹²⁰ technique but both had increased levels of HVA when evaluated following two-dimensional chromatography. This result led to the conclusion that evidence for neuroblastoma is best sought by the two-dimensional chromatographic technique. However, the finding of a consistently elevated 24 h output of urinary NMA in our 9 cases of neuroblastoma might suggest that a good single test for detecting a neuroblastoma would be the measurement of this metabolite expressed as mg per 24 h. Unfortunately, in many instances only random samples of urine can be obtained from young children and our results here suggest that concurrent estimation of HMMA and HVA after two-dimensional paper chromatography and expressing the results in µg metabolite per mg creatinine would give the best chance of detecting a neuroblastoma. For correct interpretation and evaluation any result obtained must be compared

against the corresponding normal metabolite values which have been determined using the identical procedures.

Based on our limited study of a small group of neuroblastoma patients our findings could imply that screening for the presence of a neuroblastoma, using as a single test, either H₂MA, HVA or NMA might present diagnostic problems depending on whether the results are expressed as μg per mg creatinine or mg per 24 h. As the urinary excretion patterns of catecholamine metabolites are often very variable in children with neuroblastoma, our tactics should be to measure all four urinary metabolites, H₂MA, HVA, NMA and MA, in order to construct as complete a picture as possible of their urinary catecholamine metabolite levels, rather than to rely solely on any one individual metabolic assay to detect the presence of a neuroblastoma tumour.

CHAPTER 9

EXPERIMENTAL

9.1 Materials

a) Materials for extraction and chromatography

Ethyl acetate, propan-2-ol, methanol, ethanol, 2-methylbutan-2-ol, pyridine, glacial acetic acid and 0.88 ammonia were AnalaR reagents (B.D.H. Ltd.) Anisole (B.D.H. Ltd.) was technical grade. Chromatography was carried out with Whatman Grade 1 25 x 25 cm paper and Whatman Grade 2 Chroma 20 x 40 cm paper. Dowex cation exchange resin AG-50-X8 (H^+ form) 200 - 400 mesh (Cal-Biochem) was converted to the Na^+ form by stirring it overnight with 3M sodium hydroxide. The sodium hydroxide was removed by decantation and the resin was washed with distilled water until the washings were neutral (pH 7.0). The resin was then stored, prior to use, under distilled water in a dark coloured glass bottle.

b) Standard solutions

- i) HMA and HVA (Sigma Chemical Co. London) $1g\ dm^{-3}$ of each acid was dissolved in ethanol to form a combined solution.
- ii) NMA and MA (Sigma Chemical Co. London) were dissolved in 0.01 M HCl ($1g\ dm^{-3}$ of each) to form a combined solution.

c) Working Reagents

2M Aqueous ammonia, 4M alcoholic ammonia, aqueous ethanol (50% v/v), aqueous ethanol (85% v/v), aqueous sodium hydroxide (20% v/v), aqueous sodium periodate (2% w/v) and aqueous sodium metabisulphite (10% w/v) were all made up using AnalaR reagents.

d) Diazotisation

Fresh solutions of 4-nitrobenzenediazonium chloride were prepared as needed by mixing aqueous sodium nitrite (0.5% w/v), 4-nitroaniline ($2.5\ g\ dm^{-3}$) dissolved in 1M hydrochloric acid, and distilled water in the proportions 1:1:2 respectively; both reagents were kept at room temperature.

e) Equipment

Mixing and stirring was carried out using a Fisons Whirlymixer. High Voltage Electrophoresis was carried out using CAMAG HVE equipment. Shandon glass tanks (30 x 30 x 30 cm) were used for chromatography.

The chromatography papers were accommodated on Shandon all-metal chromatography frames (5 papers per frame). The papers were scanned with a Joyce-Loebl Chromoscan using a 625 nm filter. A Pye Unicam SP.600 Series II instrument was used with silica cells (matched) to estimate solutions of vanillin at 350 nm. Preliminary scanning for vanillin was carried out using a Pye-Unicam SP.800A recording spectrophotometer. All pH measurements were done using an E.I.L. Model 38B pH meter. A Technicon Autoanalyser was used to measure the creatinine levels of the urine samples using the method of Chasson et al.²⁷⁶ Statistical analysis of the results was performed using an Olivetti 101 Programmable Desk-top Calculator, and a pdp 11/34 Computer. Statistical Tables²⁷⁷ were used to calculate body surface area.

9.2 Technique for Accurate Urine Collections

Twenty four hour urine collections were obtained based on the following general instructions which were given to all participants. The volume secreted by the kidneys over the collection period is the crucial one: urine already in the bladder at the start of the test and secreted some time before should not be included; that in the bladder at the end of the test and secreted between the relevant times should be included.

The procedure is as follows:-

8 a.m.	Day 1.	Empty bladder completely. Discard specimen. Collect all urine passed until
8 a.m.	Day 2.	Empty bladder completely. Add this urine to the total collection.

Timed overnight collections were obtained from normotensive adults, University students and hypertensive patients using the same technique.

The 24 h urine samples obtained from babies were collected by attaching plastic collecting bags to them; timing was started after the passing of the first urine sample, which was discarded; all urine passed over the next 24 h was collected. The walkers emptied their bladders before and at the completion of the race and these samples were collected. All urines were acidified to pH 3.0 with concentrated hydrochloric acid as soon as possible after collection and if analysis was not done next day were stored deep frozen at -20°C .

9.3 Extraction Procedure for Estimation of HMMA and HVA in Urine

After mixing the urine, its volume was measured and a portion was removed and adjusted to pH 3. An aliquot (3 cm^3) was then placed in a C19 test-tube with 15 cm^3 ethyl acetate. The tube was stoppered and the contents mixed vigorously with a Whirlymixer for exactly 2 min before centrifuging at 2000 r.p.m. for 15 min.

An aliquot of the organic supernatant liquid equivalent to a 'one minute volume*' was removed and placed in a small conical tube. This aliquot was carefully reduced under vacuum at room temperature and the residue dissolved by adding three drops of methanol down the sides of the tube to ensure maximum recovery. The methanol solution was applied as a small spot at a distance of 2.5 cm from two adjacent sides of a sheet of chromatography paper.

9.4 Chromatography of HMMA and HVA

The chromatography papers, consisting of four tests and one standard, were placed in a Shandon metal frame prior to chromatography. The standard chromatogram comprised 3 spots ($3\text{ }\mu\text{l}$ of a standard solution containing $3\text{ }\mu\text{g}$ each of HMMA and HVA) applied at 4 cm intervals on a diagonal line drawn from the lower left-hand corner to the upper right-hand corner of the paper. The chromatography papers were developed overnight by ascending chromatography (16 h) using as first solvent (propan-2-ol/ammonia/water, 80:10:10). The chromatograms were removed from the first solvent tank and allowed to dry, before being re-chromatographed at right angles to the first solvent run, in the solvent system (anisole/acetic acid (70:30)

for 7 hours. The chromatograms were removed from the frame and allowed to dry before being dipped in a 10% solution of sodium carbonate, blotted and hung up to dry completely. Finally they were dipped in a solution of freshly prepared diazotised 4-nitroaniline to develop the phenolic acids. The surplus solution was removed by a clean sheet of blotting paper.

9.5 Estimation of HMMA and HVA on Chromatograms by Scanning

The well separated purple and grey spots of HMMA and HVA, respectively, were delineated by means of a blue wax pencil mark (which served as a useful guide in the correct alignment of the spots on the slide holder of the Chromoscan) drawn 0.25 cm above and below the spots, at right angles to the direction of the first solvent front. The spots were then carefully cut out as (2.5 x 1.0 cm) oblong sections with the long side at right angles to the direction of the second solvent system and placed whilst still damp on the slide holder of the Joyce-Loebl Chromoscan. The oblong sections containing the HMMA and HVA spots were then covered with a strip of clear sellotape (2.5 cm wide) to hold them in position and the slide holder placed in the Chromoscan. The recording pen was zeroed using a blank section of the chromatogram and the spots were scanned using a red filter (625 nm). The integrated numbers for each peak were recorded.

* An aliquot of supernatant equivalent to a 'one-minute volume' was calculated from the formula:

$$\frac{\text{Total volume of urine (cm}^3\text{)}}{\text{Total period of collection (min)}} \times 5 = \text{'one-minute volume'}$$

9.6 Expression of Results and Standard Curve for HMMA and HVA

The chromoscan estimation was standardised using 1, 2, 3, 4 and 5 μg quantities in methanol of HMMA and HVA. Each amount was chromatographed in triplicate and scanned. It was found that the mean values of the recorded peaks for each triplicate determination were linearly related to the amount of material in the standard. Therefore, the HMMA and HVA present on the chromatograms of urine extracts were estimated by comparison with the mean of three (3 μg) standard amounts of HMMA and HVA chromatographed with the test samples.

The results are expressed as follows:-

$$\frac{A_t}{A_s} \times \frac{c \times d}{v} \times 1000 = \text{mg/24 h}$$

Where

A_t	=	Integrated value recorded for test
A_s	=	" mean value of 3 (3 μg) replicate standards
c	=	Concentration of standard (3 μg)
d	=	1440 min/day
v	=	1 min urine volume used.

Any test which gave a reading higher than the 5 μg standard value was re-chromatographed using half quantities, as the linearity of the standard graph tended to decrease at higher concentrations.

9.7 Evaluation of Experimental Technique

a) Recovery of HMMA and HVA from distilled water

Triplicate estimations of HMMA and HVA were carried out using 1, 3 and 5 μg amounts in distilled water (3 cm^3). The pH was adjusted to 3.0 with HCl and the solutions were mixed with ethyl acetate (15 cm^3), centrifuged, chromatographed and scanned as described above (9.3 - 9.6).

b) Recovery of HMMA and HVA from urine

To samples of urine (3 cm^3) at pH 3.0 were added three drops of 2% sodium metaperiodate solution and the solutions were allowed to stand for 30 mins at room temperature. The urine samples were extracted and chromatographed in the normal way (9.3 - 9.4). Treatment with diazotised 4-nitroaniline showed that HMMA and HVA had been removed.

The above procedure was repeated, but with the addition, after the 30 minute oxidation period, of five drops of 10% sodium metabisulphate solution to neutralise any excess periodate remaining, followed by the addition of 10 cm³ amounts containing 10 µg of HMA and HVA standards. The urines were then taken through the complete extraction, chromatography and scanning procedure (Chapter 9, 3 - 5)

9.8. Preliminary Attempts at Extracting NMA and MA from distilled water

a) Solvent extraction (modified Yoshinaga et al¹⁴² technique)

NMA (10 µg) and MA (10 µg) were added to distilled water (20 cm³), the pH was adjusted exactly to pH 0.9 with the aid of a pH meter and the mixture boiled for 20 mins, cooled and adjusted carefully with sodium hydroxide (20%) to pH10. Sodium chloride (10g) was dissolved in the mixture, which was then extracted by shaking with two successive amounts (2 x 20 cm³) of ethyl acetate. The ethyl acetate layer was removed and the pooled extracts evaporated to dryness. The residue remaining was re-dissolved in methanol (3 cm³), transferred to a small conical test-tube and carefully concentrated under reduced pressure to approximately (0.5 cm³) before being spotted onto a 25 x 25 cm Whatman No. 1 chromatography paper. The paper was subjected to two-dimensional chromatography using 2-methylbutan-2-ol/0.88 ammonia (4:1) as the first overnight solvent system, followed by butan-2-ol/pyridine acetate buffer (4:1): (the buffer of pH4 comprised water/pyridine/acetic acid in the proportions (100:10:41) as the second overnight solvent. The NMA and MA spots were visualised using diazotised 4-nitroaniline and the purple spots were eluted with 4 cm³ of a 2% sodium carbonate/methanol (2:1) mixture and measured colorimetrically at a wavelength of 520 nm.

b) Extraction using ion-exchange resin Dowex AG-50W-X8, Na⁺ form

i) Percentage recovery of the metadrenalines from the resin after different extraction times

Six samples (20 cm³) of distilled water were placed in glass beakers

(50 cm³) and adjusted to pH 0.9; to the first three beakers 20 µg of NMA, to the second three beakers 20 µg of MA were added. The solutions were transferred to boiling tubes and boiled for 20 mins, cooled and adjusted to pH 7.0 before 10 cm³ of Dowex resin was pipetted into each sample. The mixtures were then stirred continuously for 15 mins. After the resin had settled the supernatant was decanted and the resin washed successively with water (10 cm³), 50% ethanol (5 cm³) and 85% ethanol (10 cm³) followed by decantation. The resin was finally stirred with 4M alcoholic ammonia (10 cm³) for 1 min. The resin mixture was filtered through a sintered glass filter funnel and the eluate divided into two portions - blank and test. The test portion was oxidised to vanillin with sodium periodate and estimated spectrophotometrically (as in Chapter 9, 9c). The whole process was repeated four times with different elution times for the NMA and MA from the resin with 4M alcoholic ammonia, namely 3, 5, 10 and 15 mins.

ii) Percentage recovery of the metadrenalines from the resin after varying the concentration of alcoholic ammonia

Duplicate samples of distilled water (10 cm³) containing NMA (10 µg) and MA (10 µg) were treated as in (Chapter 9. 8 b above). The resin samples were mixed (3 mins) with 1, 2, 4 and 8M alcoholic ammonia solutions (10 cm³) and the supernatants were filtered and worked up for vanillin (as in Chapter 9, 9 c).

9.9 Procedure Used for Extraction and Estimation of NMA and MA in Urine

a) Extraction

24 h urine collections, acidified with 10 cm³ of concentrated acid were measured. An aliquot equal to the amount passed in 30 mins was removed and acidified with conc. HCl to pH 0.9 using a pH meter. The urine was transferred to a Pyrex boiling tube, capped with aluminium foil to prevent evaporation and heated in a boiling water bath for 20 min to hydrolyse the sulphate conjugates of the metadrenalines. The tubes were then cooled to room temperature by immersing them in cold water and the pH of the urine was carefully adjusted to 7.0 using 20 % sodium hydroxide. The bottle of

prepared cation exchange resin was shaken and 10 cm^3 of the resin slurry pipetted into the neutral urine and the mixture stirred rapidly for exactly 15 min before the resin was allowed to settle and the supernatant decanted. The resin plus adsorbed amines was stirred for 2 min with a solvent and then decanted. The following solvents were used successively: water (10 cm^3), 50% ethanol (5 cm^3) and 85% ethanol (10 cm^3). The resin was then stirred vigorously for exactly 5 min with aqueous 2M ammonia (10 cm^3) to elute the amines. The mixture was filtered through a sintered glass filter funnel and the eluate shaken and divided into two equal portions which were placed in glass Kober tubes (20 cm^3) capacity, labelled 'test' and 'control'. Each aliquot represented a 15 min volume of urine. These extracts were carefully reduced under vacuum to about 0.5 cm^3 , keeping the temperature below 60°C , before two drops of glacial acetic acid were added and the samples taken to dryness.

b) Chromatography

Chromatography was carried out on 25 cm square Whatman No. 1 paper specially cut for this purpose (Fig.1). Three slots were cut out of the paper to produce two strips 2.5 cm wide. These two strips were labelled 'test' and 'control'. The metadrenaline extracts, test 1, control 1; test 2, control 2; etc. were dissolved in a small amount of methanol (1 cm^3) added a few drops at a time, and the methanol extract was applied evenly over the rectangles, marked in pencil at the bottom of the strip corresponding to the tube number. Care was taken to ensure that all the extract was applied to the strip. The papers were dried and NMA and MA ($1 \mu\text{l}$ of standard solution) was applied to the right of the rectangle on the control strip. This helped to locate the urine NMA and MA bands on the control strip. The chromatogram was suspended with the edge AB just under the surface of methanol contained in a narrow plastic trough, the ascending methanol concentrated the amines spread over the rectangles into a narrow and compact band. The chromatogram was removed from the trough when the solvent front reached the line (X-X) after approx 5 - 10 min.

The chromatograms were removed and allowed to dry before being placed on a Shandon metal chromatography frame. A standard chromatogram was developed with each frame of tests. This consisted of known amounts of NMA and MA (10 μ l of standard solution) applied to 'control' and 'test' strips.

The chromatograms were allowed to develop overnight (16 h) in the solvent system 2-methylbutan-2-ol/0.88 ammonia (4:1). Next morning, the chromatograms were removed from the solvent tank and dried in a fume cupboard. The control strip (Fig.1 Chap. 2) was then carefully dipped in 10% sodium carbonate solution, blotted dry and re-dipped in freshly prepared diazotised 4-nitroaniline solution to develop the purple marker spots of NMA and MA which were cleanly separated using this technique.

c) Elution of Metadrenalines and Quantitation Spectrophotometrically

The areas of the test strip which corresponded in Rf with the control NMA and MA bands were then delineated and cut out of the test strip. Each portion was placed in a glass stoppered tube (10 cm³) containing 4M alcoholic ammonia (8 cm³). The tubes were capped for 1 h to allow elution of the NMA and MA to take place. The chromatography paper pieces were then carefully removed from each tube and the residual solution accurately divided into two halves; one portion (4 cm³) was placed in a test tube labelled 'blank'; the remaining portion into a tube labelled 'test'. Distilled water (2.1 cm³) and (2.0 cm³) were added respectively to blank and test followed by 2% sodium periodate (0.1 cm³) to the test solution alone. This converted the NMA and MA in the test solutions to vanillin. Each test was then measured spectrophotometrically against its own unoxidised blank at 350 nm.

Standard amounts of NMA and MA (6, 10, 20 and 30 μ g) were added to 4 cm³ of 4M alcoholic ammonia and converted to vanillin as above. Graphs of absorbance versus amounts added were found to be linear (Fig.2 Chapter 2). Test results could be calculated directly from the calibration graph or the standard amounts chromatographed alongside the

test chromatograms could be eluted, oxidised and quantitated.

In practice the amount extracted from the standard chromatogram was found to be extremely reproducible over the whole of the research period. The outputs of the NMA and MA per 24 h were calculated as follows:

$$\frac{A_t}{A_s} \times c \times \frac{d}{v} = M$$

Where

A_t	=	Absorbance of test NMA or MA at 350 nm
A_s	=	Absorbance of Standard NMA or MA at 350 nm
c	=	Concentration of Standard (5 μg)
d	=	1440 min/day
v	=	7.5 min urine volume used
M	=	output of NMA or MA μg per 24 h

a) Empirical Normal Ranges

Due to the degree of skewness found in our normal adult sample interpretation of our data as to what might be considered normal and what abnormal presents difficulties. If our data had been normally distributed then values falling outside 2 S.D. from the mean would be considered as suspicious and values greater than 3 S.D. would be treated as abnormal.

Empirical limits, however, could be placed on our skew data by considering the frequency distribution plots for HMMA, HVA, NMA and MA where certain values are found to lie at the higher end of the distribution curves. These high values in the light of clinical experience could be viewed with suspicion. If we consider each distribution curve in turn then we find in the case of HMMA output per 24 h that a result of 7.5 mg/24 h would be classed as suspicious, as would two results lying between 6 - 6.5 mg/24 h. Values similar to these levels can be found in patients who are known to be producing excess amounts of catecholamines (e.g. phaeochromocytoma). If we eliminate these three values from our 123 'normal' results, we could then define an arbitrary upper limit of normal at 6.0 mg/24 h for HMMA. Similarly, there are two values of 8.8 and 8.6 mg/24 h in the 123 normal HVA results which clinically would be considered as suspicious. By removing these two values we could now set our upper limit of normal at a value of 8.2 mg/24 h.

A study of the output of NMA suggests that two results falling between 700 - 750 μ g/24 h would be classed as suspicious. Eliminating these two values would allow the upper limit to be fixed at 650 μ g/24 h. Similarly setting the upper limit at 600 μ g/24 h for MA would include all 123 results.

Results obtained from our abnormal groups could be judged against our empirical normal levels, in the light of previous clinical experience gained in the application of the tests to various groups of patients. Measuring increases which take place within the same group of individuals should not be affected as in this case each subject would act as his or her base-line control.

b) Urine metabolites vs age

A visual inspection of the data found for the metabolite output of HMMA, HVA, NMA and MA in babies and young children (Chapter 3, Fig 3) suggests that the urinary output of each metabolite increases with increasing age, a view which has been put forward by other workers.^{167,169,170}

However, when the outputs of HMMA, HVA, NMA and MA obtained from the 123 adults (aged 18 - 78) were plotted against age, there was no obvious increase or decrease in the catecholamine metabolite output with increasing age.

This was confirmed by deriving the correlation coefficients for each metabolite against age. The correlation coefficients were as follows:-

	r	p
HMMA	0.01	0.5
HVA	0.05	0.5
NMA	0.07	0.5 > P > 0.1
MA	0.09	0.5 > P > 0.1

It would appear from our results that the output of the catecholamine metabolites increase with age from birth to puberty, a finding which presumably reflects the increase in size and activity of the growing organism, but no further increase takes place with increasing age after puberty.

c) Student Group

The results of the data in Chapter 4 (Table 12) suggest that the mean overall output of MA shows a significant increase in Groups 2, 3 and 4 when compared to the Control (Group I) mean value. In order to try and improve the specificity of the test results, each student's values were

Table 33

Indicates the significance of the results when the differences between paired samples for the overnight outputs of HMMA, HVA, NMA and MA in various groups from the 20 University students are compared

	Significance HMMA <u>µg/min</u>	Significance HVA <u>µg/min</u>	Significance NMA <u>µg/min</u>	Significance MA <u>µg/min</u>
Group 1 compared to <u>Group 2</u>	0.1 > p > 0.05	0.5 > p > 0.1	N/S	N/S
<u>Group 3</u>	0.1 > p > 0.05	0.5	N/S	N/S
<u>Group 4</u>	N/S	N/S	N/S	N/S
Group 2 compared to <u>Group 3</u>	N/S	N/S	N/S	N/S
Group 3 compared to <u>Group 4</u>	N/S	N/S	N/S	N/S

compared against his or her own control value using a paired 't' test in order to measure the difference between the means of the paired samples. Using this test we calculate the mean of the difference between the observations \bar{d} and then the standard error of the mean. To find 't', the mean of the differences \bar{d} is divided by the S.E.M.

$$t = \frac{\bar{d}}{\sqrt{\frac{SD^2}{n}}}$$

The table of distribution is entered at $n - 1$ degrees of freedom in the case of the student group ($n = 20$)

From the results (Table 33) we can see that a comparison of Group I (Control) with Groups 2, 3 and 4, Group 2 compared with Group 3, and Group 3 compared with Group 4, indicates that there is apparently no significant statistical differences between the outputs of HMMA, HVA, NMA and MA for any of the Groups studied over the selected time periods. This finding could in part be due to the small sample sizes and the imprecision of the methodology. Further work using larger samples and matched controls would be useful.

d) Systemè International Units (S.I.)

This system is based on expressing 'actual biological activity' in a sample and is defined by two parameters. The first of these is the 'mole'. All substances whose Atomic or Molecular weights are known can be expressed in terms of 'numbers of molecular units', that is an 'amount of substance' per unit volume. The 'accepted' unit of volume, the litre (1000 cm^3) has been adopted for laboratory work.

Thus moles per litre gives Molar concentration, unlike the conventional system, mg per 100 cm^3 , which is Mass concentration.

To convert the conventional units for HMMA, HVA, NMA and MA expressed as mg/24 h to μ moles per 24 h, we can multiply the conventional

unit by a factor, derived by dividing the molecular weight of the compound into 1000.

	Observed Normal Range Conventional Unit <u>mg/24 h</u>	<u>Factor</u>	Observed Normal Range S.I. Units <u>μ mol/24 h</u>
HMMA	1.5 - 7.5	X 5.06	7.6 - 38
HVA	2.4 - 8.8	X 5.50	13.2 - 48
NMA	0.20 - 0.71	X 5.35	1.1 - 3.8
MA	0.20 - 0.60	X 4.90	1.0 - 2.9

	Empirical Normal Range Conventional Unit <u>mg/24 h</u>	<u>Factor</u>	Empirical Normal Range S.I. Unit <u>μ mol/24 h</u>
HMMA	1.5 - 6.0	X 5.06	7.6 - 30
HVA	2.4 - 8.2	X 5.50	13.2 - 45
NMA	0.2 - 0.65	X 5.35	1.1 - 3.5
MA	0.2 - 0.60	X 4.90	1.0 - 2.9

A P P E N D I X

Statistics

a) Mean and Standard Deviation

Mean

To calculate the mean we add up the observed values and divide by the number of them.

$$\bar{x} = \frac{\sum x}{n}$$

In addition to knowing the mean value of a series of measurements it is informative to have some idea of their range about the mean as this gives the figures at the top and bottom of the range which are furthest away from the generality. However, these figures do not give any indication as to the spread of the deviations about the mean. This problem is solved by the Standard deviation.²⁷⁸ A point to note is that whether the calculation is done on the whole 'population' of data or on a sample drawn from it.

The population itself should approximate to a so called 'normal' (or Gaussian) distribution.

The standard deviation therefore is a measure of the dispersion of a set of observations from their mean. If the distribution is Gaussian, 1, 2 and 3 standard deviations above and below the mean include 68%, 96% and 99.7% of the observations respectively, for a large sample size.

The formula for calculating S.D. is derived from:-

i)

$$\text{Variance} = \frac{\sum (x - \bar{x})^2}{n - 1}$$

where \bar{x} = mean

n = number of observations.

ii) The square root of the variance provides the standard deviation.

$$\text{S.D.} = \sqrt{\frac{\sum (x - \bar{x})^2}{n - 1}}$$

The coefficient of variation from the mean is given by :

$$\text{coeff. of variation} = \frac{100 \times \text{S.D.}}{\bar{x}}$$

However, if we are dealing with sample sizes below 30, then we must apply Student's 't' test to determine the probability that a certain range round the sample mean includes the population mean. Thus having calculated the mean and S.D. of our sample we then determine the standard error of the mean.

$$\text{S.E.M.} = \frac{\text{S.D.}}{\sqrt{n}}$$

To find the 95% confidence limits above and below the mean we now have to find a multiple of the S.E.M. (for large sample sizes this is 1.96) but for small sample sizes we resort to the use of tables for 't'.

To find the number by which we must multiply the standard error to give 95% confidence limits, we enter the 't' table at (n - 1) degrees of freedom and read across to the column headed 0.05. The S.E.M. value is then multiplied by this figure and the resultant value is added or subtracted from the sample mean. We can now state with a 95% chance of being correct, that the range we have determined includes the population mean.

b) Reproducibility

The standard deviation of the day to day reproducibility for the method was calculated using the formulae:-

$$R = \sqrt{\frac{\sum D^2}{2n - 1}}$$

Where D is difference between duplicates

n is number of pairs of duplicates

c) Students' 't' Test

²⁷⁹
This test is used to determine the significance of the difference between the means of two samples whose means and S.D. are known or can be calculated. We assume a 'null hypothesis' that there is no difference between the means of our two samples in that they are derived from the same large population. We calculate first the standard error of the

difference between the means for each sample

$$\text{S.D. Diff.} = \sqrt{\frac{SD^2}{n_1} + \frac{SD^2}{n_2}}$$

Where SD^2 is the square of the standard deviation for the two samples, n_1, n_2 are the number of observations in Groups 1 and 2.

When the difference between the means ($\bar{x}_1 - \bar{x}_2$) is divided by the standard error the result is 't'

$$\text{Thus 't'} = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{\frac{SD^2}{n_1} + \frac{SD^2}{n_2}}}$$

d) Probability

To find the probability level of a calculated 't' value a table of 't' distribution is used²⁸⁰ which is entered at $(n_1 - 1) + (n_2 - 1)$ degrees of freedom. If the 't' value for a given degree of freedom is larger than the 't' value given in the 0.05 Probability (5%) column then this would suggest that the 'null hypothesis' (no difference between the means of our two samples) is probably unlikely and that the samples do not come from the same population.

e) Skew distribution

An assumption was made in this Thesis that the output of the catecholamine metabolites HMMA, HVA, NMA and MA in the general population conformed to a normal Gaussian distribution pattern and therefore our normal sample of 123 adults would also be normally distributed so that mean, S.D. and 't' tests could be applied to the data.

However, inspection of the data (Chapter 2) obtained from our normal sample, when the frequency distribution curves of the outputs for each metabolite are drawn, suggests that a moderate degree of positive skewness occurs in our normal sample, which may also be expected in the general population. If this is correct then application of Gaussian curve attributes, mean, S.D. and Students' 't' test would be invalid. To overcome a degree of

skewness our data would have to be 'transformed' in order to try and normalise this degree of skewness.

The common measure of skewness is defined by

$$\text{Skewness} = \frac{\text{Mean} - \text{Mode}}{\text{Standard Deviation}}$$

or for distributions which are not too skew we can say

$$\text{Coefficient of skewness} = \frac{3 (\text{Mean} - \text{Median})}{\text{Standard deviation}}$$

The range lying between +3 and -3.

The W test for normality

To confirm mathematically the earlier suggestion (Chapter 2, 5) that our data was positively skewed, the raw data from the 123 normal adults for each of the specific catecholamine metabolites was subjected to computer (pdp 11/34) analysis using the W test of Shapiro and Wilk.²⁸¹

Basically this test is designed to produce a variance figure (W) which by use of special tables can be used to test the data for 'degree of skewness' or normal distribution.

The W figures obtained from our 123 normal adults for HMMA, HVA, NMA and MA were as follows:-

	<u>W</u>	<u>Interpretation from W Tables</u>
HMMA	0.911	Value indicates skew distribution
HVA	0.942	" " " "
NMA	0.937	" " " "
MA	0.912	" " " "

These results confirm that our chosen sample has in fact a positive skew distribution. The input data was then 'transformed' by conversion to log base₁₀ values to try to correct the 'skewness' and W tests were recalculated on the transformed data, with the following results:-

HMA	0.933	Improved figure but data still skew
HVA	0.940	Data still skew
NMA	0.821	Greater degree of skewness
MA	0.944	Improved figure but data still skew

These results confirm that our normal sample of 123 adults show a positively skew distribution concerning the output of HMA, HVA, NMA and MA and that log base₁₀ transformation of the data, whilst improving in some cases the degree of skewness would not produce a 'normal distribution' curve. Thus, in order to define rigid statistical limits for our data, non-parametric statistical tests would have to be employed.

The finding of a skew distribution for the output of the catecholamine metabolites has not, to my knowledge, been reported before. Normal results quoted previously in the literature have all been measured on smaller sample sizes and the means and S.D. values have been determined assuming a normal Gaussian distribution.

One possible explanation of our skew distribution could be that our sample of 123 'normal' adult results does in fact contain a proportion of 'abnormal' results, from people who, whilst apparently well, have a biochemical abnormality and that our so called 'normal group' is in fact a combination of two distinct populations.

In a large sample we might therefore expect to find more 'abnormal' normal values, and hence a larger degree of skewness. A small sample size perhaps would not exhibit as marked a degree of skewness and in fact when our first thirty results were tested for skewness, the W-test indicated a normal distribution, however, increasing the sample size above fifty results in marked skewness.

It would appear that selecting small sample sizes below 30, tends to produce a more normal distribution and as most of the work on catecholamine metabolites in the past has been done on small sample sizes, the skew distribution found in the present work might not have been as apparent.

When our data from the twelve babies and fourteen young children were subjected to the W-skew test, the results were as follows:-

<u>Babies</u>	<u>W</u>	<u>Raw data</u> <u>Distribution</u>	<u>W</u>	<u>Log base</u> ₁₀ <u>Distribution</u>
HMMA	0.777	Skew	0.893	Normal
HVA	0.846	Skew	0.907	Normal
NMA	0.951	Normal	0.983	Normal
MA	0.779	Skew	0.876	Normal

<u>Children</u>	<u>W</u>	<u>Raw data</u> <u>Distribution</u>
HMMA	1.0	Normal
HVA	1.0	Normal
NMA	1.0	Normal
MA	1.0	Normal

Results for our babies and children's group indicate a more normal distribution but are based on small sample sizes.

f) Correlation coefficient 'r'

The correlation coefficient²⁸² is measured on a scale that varies from +1 through 0 to -1 . Perfect correlation between two variable is expressed by a correlation coefficient of 1. When one variable increases as the other increases the correlation coefficient is positive; when one decreases as the other increases the correlation coefficient is negative. Complete absence of correlation is represented by a correlation coefficient of 0.

A coefficient of correlation is a single number that tells us to what extent two characteristics are related. It tells us to what extent variations in the incidence of one characteristic of a population go with variations in another characteristic of the same population. However, in interpreting correlation it is important to remember that correlation is not causation. There may or may not be a causative correlation between the two correlated variables and if there is a connection it may be indirect.

The probability of these differences arising by chance is exceedingly low and the hypothesis that these samples came from the same population is very unlikely. It would appear therefore from (Figs 3, 5, 8, 9 - Chapter 3) that although good correlation between the different parameters is apparent when the two groups are combined as one, correlations should be calculated separately for each group. However, because of the small sample sizes involved, one would expect to find a lower degree of correlation. For a better correlation of output with age in babies and young children, larger sample sizes with smaller age intervals between the selected groups should be studied in order to obtain meaningful results.

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