



INVESTIGATIONS INTO REACTIONS
OCCURRING DURING THE
TREATMENT OF PROTEINS WITH
FORMALDEHYDE

Thesis submitted by
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for the Degree of Doctor of Philosophy
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SUMMARY

Compounds of several classes have been prepared as models for groupings that could possibly be formed during the treatment of proteins with formaldehyde.

The formation of cyclic methyleneimines from primary aliphatic amines and formaldehyde (Cambier and Brochet, Bull.Soc.Chim., 1895, (3), 13, 392) has been confirmed and a compound formulated by Kempf (Annalen, 1890, 256, 219) as a methylene bis amine has been shown to be the cyclic methyleneimine. Both the above compounds and methylene bis secondary amines are unstable to mineral acids.

Further examples of N-hydroxymethyl amides (Einhorn, Annalen, 1905, 343, 207) have been prepared and approximate dissociation constants in weak alkali measured. They have also been shown to be unstable to acids.

Several methylene bis amides have been prepared and shown to be highly stable to acid and alkaline hydrolysis.

Derivatives of N-hydroxymethyl amides in which the hydroxyl group is replaced by: (a) alkoxy, (b) thio-alkyl, (c) acyloxy, (d) primary and secondary amino groups, have been prepared. These are all unstable in various degrees to acids and alkalis and such groups are not likely to confer increased acid and alkali resistance to proteins, bearing in mind, however, that the stability of a group in a macromolecule is not necessarily of the same order as that in a compound of small molecular weight.

N-benzoyl tyrosine has been found to be less reactive to formaldehyde than tyrosine. A condensation product has been isolated from an alkaline medium but no reaction has been observed in acid, neither could condensation be brought about between hydroxymethyl amides and N-benzoyl tyrosine.

Several model compounds containing a peptide link have been synthesised by way of phthalyl-amino intermediates. Reaction with formaldehyde has been attempted under a variety of conditions, in each case with a negative result. It is, therefore, thought unlikely that the peptide group plays any part in the reaction of proteins with formaldehyde.

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I N T R O D U C T I O N
and
H I S T O R I C A L

INTRODUCTION

The reaction of formaldehyde with proteins has been of interest in the field of applied chemistry for many years since the introduction of leather tanning with formaldehyde. Theoretical interest arose from the researches of Sørensen (Biochem. Z., 1908, 7, 45) on the formol titration of amino acids and peptides and its importance today lies in its use for the preparation of diphtheria and tetanus toxoids from toxins and for hardening and conferring acid and alkali resistance on proteins for the production of plastics and fibres. This present study is mainly concerned with reactions taking place when protein fibres are hardened.

The industrial production and subsequent hardening of protein fibres is essentially the same whatever the protein and a general survey of the methods has been given by Traill (Chem. & Ind., 1945, 58).

An alkaline solution of the protein is allowed to mature, then is extruded into an acid coagulating bath and the ensuing fibres hardened by passing them through an aqueous solution of formaldehyde buffered at pH 0 followed by drying at an elevated temperature. Thus there is the possibility of reactions occurring

whilst in the hardening bath and also during the ensuing heat treatment.

On account of the number of reactive centres in a protein molecule the reaction with formaldehyde is necessarily complex since it may react with any one of a number of functional groups or, where steric conditions are favourable, form methylene bridges between two reactive groups. The extent to which these reactions take place under various conditions is by no means fully understood but it seems probable that modification of the mechanical properties of proteins is brought about by intermolecular methylene bridging.

Moreover the reactions with formaldehyde may be fast, slow, reversible or irreversible, so that the time of contact with the protein will in all probability have a major effect upon the nature of the final product.

This thesis is concerned with a study of some of the possible reactions between formaldehyde and proteins by observations on the reactivity of model compounds to formaldehyde and also the nature and stability of the ensuing reaction products. Though model compounds are undoubtedly useful for the elucidation of the complex reactions occurring, it must be borne in mind that the properties of such substances

may be appreciably different when attached to a molecule of such a size as a protein. This change in properties is illustrated by the case of polyamides (nylon) and more so in the case of polyesters (terylene) which are much more stable to all conditions of hydrolysis than are small molecular weight amides and esters.

HISTORICAL

It is well known that formaldehyde exists mainly in polymeric forms owing to the instability of the monomer. These polymeric forms range from water soluble methylene glycol and di and trioxymethylenes, $H(OCH_2)_n.OH$, $n = 1, 2$ or 3 , ^{to} insoluble polyoxymethylenes where $n = 100$ or more but physical measurements have shown that aqueous solutions of formaldehyde exist chiefly as the hydrate or methylene glycol, hence certain reactions may be considered as condensations by virtue of the two hydroxyl groups. Walker (Formaldehyde, 1944, Reinhold Pub. Corp., New York) has given a detailed account of the nature of formaldehyde and its polymeric hydrates.

The most usual reaction of formaldehyde is condensation with a reactive hydrogen atom to form an hydroxymethyl compound. Some hydroxymethyl derivatives are stable, others under certain circumstances condense with a further reactive hydrogen atom to form a methylene link:



This may take place either intermolecularly to form a methylene bridge or intramolecularly giving a cyclic compound. A further possibility is that several molecules of formaldehyde will react to form polyoxymethylenes.



Formaldehyde and Protein Functional Groups

The primary amino group

Of the functional groups present in proteins the reactions of the primary amino group have received most attention and a brief review of the work published up to 1944 has been given by Walker (*loc. cit.*)

The problem was first studied by Koltoff (*Bull. Soc. chim.*, 1885, 43, (1), 112) who treated ethylamine with aqueous formaldehyde and obtained an oil which he formulated as a Schiff's base (I, R = C₂H₅-)



(I)



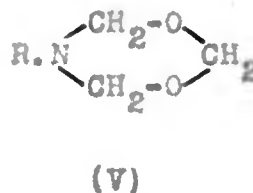
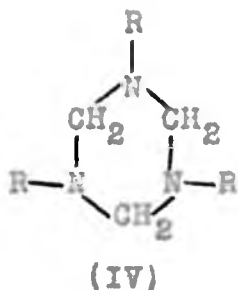
(II)



(III)

Later Henry (*Bull. Acad. roy. Belg.*, 1893, (3), 26, 200; *ibid.*, 1894 (3), 28, 355; *ibid.*, 1895, (3), 29, 23.), in a comprehensive series of experiments claimed to have obtained hydroxymethyl derivatives (II) of a number of primary amines but could not purify them since drying over caustic potash gave oils which were distillable and which analysed for Schiff's bases (I). The only crystalline derivative isolated was obtained from benzylamine and formaldehyde. This melted at 43° and was first (*ibid.*, 1894, (3), 28, 355) described as the hydroxymethyl amine (II, R = C₆H₅.CH₂-) but was later (*ibid.*, 1897, (3), 33, 407) identified as the Schiff's base (I, R = C₆H₅.CH₂-). The structure had not been finally decided until studies

by Cambier and Brochet (Bull. soc. chim. 1895, (3), 13, 392) showed that although the empirical formula given by Henry (loc. cit.) was correct, the molecular weight — measured by freezing point depression in benzene — corresponded to a trimeric molecule. They therefore suggested the cyclic structure (IV)



Duden and Scharff (Annalen, 1895, 288, 218) produced confirmatory evidence by showing these compounds to react as tertiary bases. It now seems certain that the action of equivalent quantities of formaldehyde on primary aliphatic amines is to produce cyclic trimers, however physico-chemical methods of analysis indicate that each amino group is capable of forming unstable hydroxymethyl derivatives with either one or both hydrogen atoms substituted. The formulation of the Schiff's base appears to be of little importance in the aliphatic series since it is too unstable for isolation and characterisation.

The cyclic methyleneimines (IV) are readily

prepared by adding to an aliphatic amine an equivalent quantity of formaldehyde. A vigorous exothermic reaction ensues after which the product, if one of the lower members of the series, may be separated by the addition of solid potassium carbonate. The higher members, $>C_3$, are insoluble in water and separate as an oily top layer.

When simple primary amines are treated at low temperature with a large excess of aqueous formaldehyde cyclic triformals (V) are formed. Bergman et al. (Hoppe-Seyl. Z., 1923, 131, 18. Ber., 1924, 57, 662) isolated compounds of this type by extracting the reaction mixture with ether followed by fractional distillation. They form stable salts and distillation with phosphoric acid yields the theoretical quantity of formaldehyde.

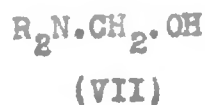
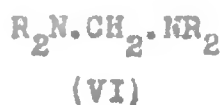
Besides condensing with primary amines, formaldehyde may also act as a methylating agent producing both secondary and tertiary amines. This was first demonstrated by Plöchl (Ber., 1888, 21, 2117) who heated a solution of ammonium sulphate with excess formaldehyde for 50 minutes and isolated trimethylamine sulphate from the reaction mixture. This reaction was confirmed by Werner (J.C.S., 1917, 111, 844) who also showed that monomethylamine and dimethylamine were produced simultaneously. The methylation of amines with

formaldehyde has been developed by Clark, Gillespie and Weissshaus (J.A.C.S., 1933, 55, 4571) who find that the presence of acids enhances the reaction.

The secondary amino group

Although the secondary amino group may occur in proteins to only a limited extent, the reaction of formaldehyde with this group may be of importance on account of the possibility of methylation of a primary amino group to a secondary methylamino group.

The first record of the isolation of the products of reaction of formaldehyde and secondary amines is given by Koltoff (Bull. Soc. chim., 1885, (1), 43, 112) who obtained methylene bis diethylamine (VI, $R = C_2H_5-$) as a distillable oil upon treatment of diethylamine with aqueous formaldehyde.

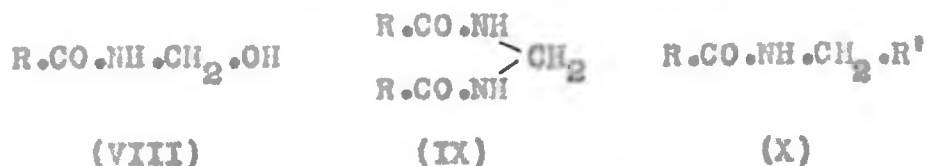


Ehrenberg (J.pr.Chem., 1887, (2), 36, 117) and Henry, (Bull. Acad. roy. Belg., 1893, (3), 26, 200) prepared a series of similar compounds and gave them the same formulation but later (ibid., 1894, (3), 28, 355) using the same method of preparation, but drying the product with potassium carbonate instead of caustic soda, produced oils which he claimed were hydroxymethyl amines (VII).

These would not distil at constant temperature, therefore they could not be purified and vapour density determinations on the crude material in aniline vapour gave values which approximated to those expected for hydroxymethyl amines. Since treatment with solid caustic soda yielded methylene big amines (VI), Henry (loc. cit.) assumed that water was eliminated from the hydroxymethyl amines (VII) by such treatment. It seems quite likely, however, that Henry's hydroxymethyl derivatives are in reality crude samples of methylene big amines, although it is not possible to decide with any certainty which of the two structures they possess.

The amide group

Extensive studies by Einhorn and his co-workers (Annalen, 1905, 343, 207. *ibid.*, 1908, 361, 113) have shown that in aqueous alkaline media one molecule of formaldehyde will condense with one molecule of an amide to form the hydroxymethyl derivative (VIII).



In acid media the product is a methylene big amide (IX). Hydroxymethyl amides, although somewhat unstable, can in most cases be isolated chemically pure, on the other hand,

methylene bis amides are high melting compounds stable to conditions of acid and basic hydrolysis.

The hydroxymethyl group shows a high degree of reactivity and condensation reactions have been brought about with aromatic nuclei (Einhorn, loc. cit., Haworth, McGillivray and Peacock, J.C.S., 1950, 1493; Downs and Lions, J.A.C.S., 1950, 72, 3053) giving compounds of type (X, R' = substituted benzenes or naphthols).

Condensation will also occur with secondary amines (X, R' = R''₂N-, Einhorn, loc. cit.), alcohols and mercaptans (X, R' = R''O- or R''S-, Kadowaki, Bull. Chem. soc. Japan, 1936, 11, 248. Sorenson, U.S. Pat., 2,201,927. Albrecht, Frei and Sallmann, Helv. chim. Acta, 1941, 24, 233E.) and in certain instances primary amines (Burke, J.A.C.S., 1947, 69, 2136. Fraenkel-Conrat and Olcott, *ibid.* 1948, 70, 2673.)

The secondary amide linkage

No formaldehyde condensation products of simple N-substituted amides have as yet been prepared. Einhorn (loc. cit.) treated N-ethyl benzamide (XI) and N-ethyl isovaleramide (XII) with formaldehyde in both aqueous acid and basic media but could only isolate the unchanged starting material. He did, however, succeed in isolating

a monohydroxymethyl $\alpha\beta$ -dimethyl urea (XIII).



(XI)



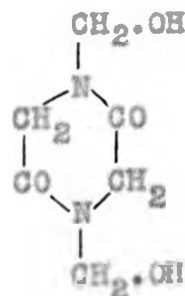
(XII)

Diketopiperazine, which contains two secondary amide groups, behaves somewhat differently and will readily form the dihydroxymethyl derivative (XIV).

(Cherbuliez and Feer, *Helv. chim. Acta.*, 1922, 5, 678).



(XIII)



(XIV)

The hydroxyl group

The primary reaction products of alcohols and formaldehyde under neutral or alkaline conditions are hemiacetals (XV) but none have been isolated owing to their instability. Evidence for their formation is based on physical measurements. Under acid conditions hemiacetals condense with a further molecule of alcohol giving formals (XVI) which are stable enough for isolation.



(XV)

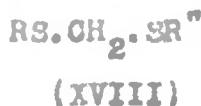
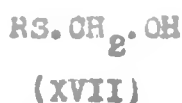


(XVI)

Formals are stable to alkali but are readily hydrolysed to formaldehyde and alcohols in the presence of acid.

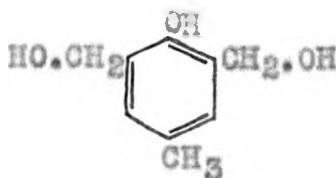
The sulphydryl group

Formaldehyde reacts with mercaptans to give thio analogues of hemiacetals and formals, these being much more stable than the oxygen analogues. The nature of the thio hemiacetal (XVII) is sufficiently definite to enable mixed mercaptans (XVIII) to be prepared from it, (Posner, Ber., 1903, 36, 296).



The phenolic residue

A large amount of literature has been published concerning the nature of the phenol-formaldehyde reaction. This has been fully reviewed by Walker (*loc. cit.*) and by Finn, Megson and Whittaker, (Chem. & Ind., 1950, 8.849). Under alkaline conditions condensation takes place in the nucleus to give benzyl alcohols, e.g. (XIX) and acid conditions cause polynuclear condensation, the final product being a thermosetting resin. There has been no reported reaction involving the phenolic hydroxyl group.



(XIX)

Other functional groups

Of the remaining reactive groups the guanidyl is known to react (Fraenkel-Conrat and Olcott, J.A.C.S., 1946, 68, 34) and although a crystalline formaldehyde derivative of methyl guanidine sulphate has been isolated, it is of a somewhat indefinite character.

The indole nucleus will react with formaldehyde in both acid and alkali, giving what are probably N-hydroxymethyl and α -methylene bis derivatives respectively. (Fraenkel-Conrat et al., J. biol. Chem., 1947, 168, 99).

The phenyl alanine aromatic nucleus may react in a similar manner to the tyrosine nucleus, the carboxylic acid group may form ethers of methylene glycol under condensing conditions and Levy (J. biol. Chem., 1935, 109, 361) has shown that the glyoxaline ring will not react under neutral conditions.

Formaldehyde and amino acids

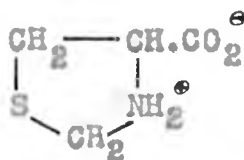
This subject has been adequately reviewed by French and Edsall (Recent advances in Protein Chemistry, Academic Press, New York, 1945, vol.II, P.285) therefore only a brief account will be given here.

Glycine

Although glycine is the simplest of the series of mono-amino mono-carboxylic acids and is to a large extent typical of the series, it shows a somewhat more complex behaviour to formaldehyde than its homologues. The isolation of numerous compounds has been reported which in most cases are the products of reactions involving the uncharged amino group.

Cysteine

Formaldehyde reacts with cysteine slowly in acid solution and rapidly in alkaline medium to give thiazolidine-4-carboxylic acid (XX) which is highly stable towards acid and alkali.



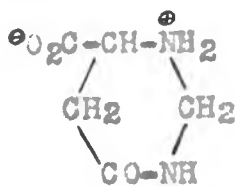
(XX)

Serine and threonine

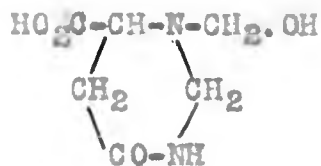
No products of reactions between formaldehyde and serine or threonine have been isolated. By analogy with cysteine, oxazolidine-4-carboxylic acid might be expected but the general instability of oxazolidines to acid and alkali suggests that should any analogous reaction take place the equilibrium will lie much further to the left than in the case of cysteine.

Asparagine

Asparagine and formaldehyde react to give 6-hydroxytetrahydropyrimidyl-4-carboxylic acid (XXI) as the primary product. A further molecule of formaldehyde will combine with this to form the methylol-imino derivative (XXII) which, however, is unstable and on standing in the presence of sulphuric acid is reconverted to the first product.



(XXI)



(XXII)

The study of aspartic and glutamic acids by optical rotation methods has indicated association of formaldehyde with the amino group in a similar manner to

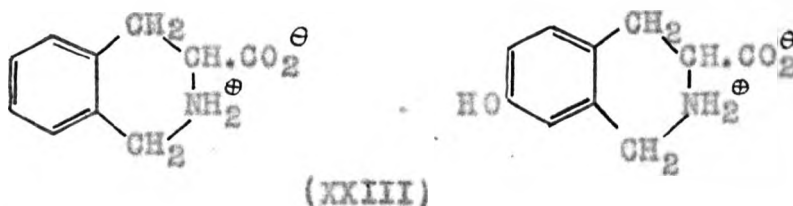
the simple amino acids. The long period required for equilibrium to be attained suggests the possibility of a methylene bridge between the amino and carboxyl groups.

Lysine

Physico-chemical methods show that both amino groups are capable of reacting with formaldehyde but no reaction products have been isolated. There is no positive evidence for methylene bridge formation.

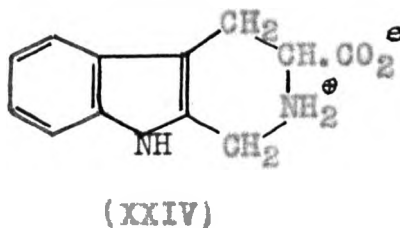
Phenylalanine and tyrosine

Tetrahydroisoquinoline derivatives (XXIII) are formed on treatment of phenylalanine and tyrosine with formaldehyde under acid conditions.



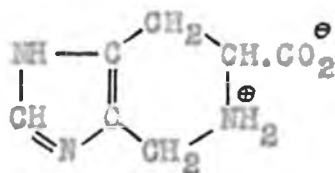
Tryptophane

With moderately acid, neutral or slightly alkaline formaldehyde, tryptophane gives 3:4:5:6-tetrahydro-4-carboline-5-carboxylic acid (XXIV).



Histidine

Histidine reacts with formaldehyde in acid or alkaline solution in an analogous manner to tryptophane forming 1:2:5:6-tetrahydropyrido-3:4-imidazole-6-carboxylic acid (XXV).



(XXV)

Further alkaline treatment with formaldehyde produces a monohydroxymethyl derivative which on heating or acidifying loses the added molecule of formaldehyde to revert back to the pyrido acid. The position of the hydroxymethyl group is not known.

Arginine

No reaction products between formaldehyde and arginine have been isolated but both pH and optical rotation measurements indicate an initial fast reaction, involving one molecule of arginine and one of formaldehyde followed by a slow reaction involving a further formaldehyde molecule. It is suggested that methylene bridge formation occurs between the amino and guanidino nitrogen atoms but conclusive evidence is lacking.

The Action of Formaldehyde on Proteins

A large amount of work has been published relating to this problem but the overall picture is still far from complete. It is almost certain that the hardening and insolubilising action that formaldehyde has on proteins is due to the formation of stable methylene cross links between the reactive residues. This was first suggested by Meyer (Biochem. Z., 1929, 208, 23) and has since received adequate confirmation from molecular weight determinations on formaldehyde treated proteins. These have shown there to be an increase in molecular weight of soluble proteins after both acid and alkaline formaldehyde treatment -- as measured by osmotic pressure determinations -- of up to eight times that of the native protein. If the formaldehyde is allowed to react further, gelling occurs which prevents any further measurements (Fraenkel-Conrat et al., J.A.C.S., 1946, 68, 34. J. biol. Chem., 1949, 177, 477).

Chemical evidence for methylene bridging was given by Nitschmann and Badorn (Helv. chim. Acta., 1944, 27, 299) who found that the increase in weight of formaldehyde treated casein was less than that expected from its formaldehyde uptake and so concluded that water had been eliminated in condensations to form methylene

bridges. No account, however, was taken of errors due to solubility of the protein in the hardening liquors and there is also an inherent weakness in all formaldehyde content analyses on proteins.

The most usual method of analysis is to digest the protein with acid and steam distil the liberated formaldehyde which may then be determined by any of the standard processes. (Walker, loc. cit., chapt.17). Alternatively the formaldehyde loss of the treatment liquors may be determined and after making a correction for absorbed formaldehyde by analysis of the wash liquors, the uptake of formaldehyde by the protein may be calculated. This latter method ignores errors due to protein solubility and for the former method there is no guarantee that all the formaldehyde is liberated.

Several workers have shown that under the conditions of acid hydrolysis the formaldehyde freed can react with certain amino acids, namely tryptophane, histidine, tyrosine and perhaps asparagine, to form cyclic derivatives which will not release formaldehyde in the presence of acids (see p.18-20). (Baudouy, C.R. Acad. Sci., 1942, 214, 692. Nitschmann and Hadorn, Helv. chim. Acta., 1943, 26, 1069. Fraenkel-Conrat et al., J. Biol. Chem., 1947, 168, 99. Middlebrook, Biochem. J., 1949, 44, 17.)

However reports are somewhat conflicting about conditions under which these stable compounds form. Nitschmann and Hadorn (loc. cit.) claimed that the best method of quantitatively liberating formaldehyde was to distil at a lower pH i.e. that of 0.1 molar phosphoric acid but there is doubt even about the accuracy of this.

On account of these facts it would therefore seem that any deductions made from quantitative estimations of formaldehyde uptake will be unreliable.

The formaldehyde binding capacity of a protein needs careful definition since it is found that the strength with which it is bound varies to a considerable extent. Some formaldehyde may be removed by washing but there remains some which is only liberated by acid. Lumiere et al. (Bull. Soc. chim., 1908., (3), 35, 872) first noted that the gelatin-formaldehyde product liberated formaldehyde with warm water and since then Nitschmann and Hadorn (Helv. chim. Acta., 1943, 26, 1075) have shown that washing with running water had to be continued for three weeks before the wash water showed a negative test for formaldehyde. Similar observations have been made by Frenkel-Conrat et al. (J.A.C.S., 1945, 67, 950) and by Traill (Chem. & Ind., 1950, 23). The latter paper reports that boiling formaldehyde hardened

ground nut protein with water for 60 minutes reduces the formaldehyde content to a constant value of 1.7%. It is considered that such treatment removes labile hydroxymethyl groups and this has received some confirmation in the work of McGillivray (Ph.D. thesis, 1950, Sheffield University) who found that unwashed formaldehyde treated ground nut protein condensed with brom- β -naphthol under the same conditions as would hydroxymethyl amides whereas when the protein had been washed very little condensation was observed.

Participation of Protein Functional Groups in the Formaldehyde Reaction

On account of the large amount of work published on this topic it is only possible to give here a brief outline of the methods used and conclusions drawn concerning the reactivity of certain groups.

The amino group

The majority of experiments designed to prove that protein amino groups react with formaldehyde are based on the difference in formaldehyde bound by native proteins and proteins deaminated with nitrous acid by the Flimmer technique (J.C.S., 1925, 2651). In the presence of acetic acid neither amide nor guanidyl groups react with nitrous acid but other reactions do occur.

Deaminated casein was shown by Nitschmann and Hadorn, (Helv. chim. Acta., 1944, 27, 299) to have a yellow-brown colour and they suggested that nitroso groups were present in the protein. One possible centre for nitrosation is the tyrosine nucleus and Carpenter and Lovelace (Industr. Engng. Chem., 1944, 36, 680) claim that the loss in formaldehyde uptake of deaminated protein is equivalent to the number of amino groups removed and the absence of reaction with tyrosine.

Many workers have observed a fall in formaldehyde binding over a large pH range after deamination, these include Bowes and Pleass (J. int. Soc. Leath. Chem., 1939, 23, 365) and Highberger and his co-workers (J. Amer. Leath. Chem. Ass., 1939, 34, 131) on scleroproteins; Nitschmann and Hadorn (loc. cit.) on casein; Eaton (J. Immunol., 1937, 33, 419) on diphtheria toxin; Ross and Stanley (J. gen. Physiol., 1939, 22, 165) on tobacco mosaic virus and Wormald and Kaye (J. Soc. Chem. Ind., 1945, 64, 75) on several proteins.

Ross and Stanley (loc. cit.) postulate two simultaneous reactions of different rate since, provided deactivation of tobacco mosaic virus has not proceeded too far, partial reactivation may be obtained by dialysis

or treatment with dimedon. This was confirmed in reaction rate studies by Wormald and Kaye (loc. cit.).

Acetylation of amino groups has been shown by Nitschmann and Hadorn to have little effect on formaldehyde uptake and Fraenkel-Conrat and Meehan (J. Biol. Chem., 1949, 177, 477) found no increase in molecular weight after such treatment. This seems to indicate that although free amino groups are necessary for cross linking there is only necessity for one free hydrogen atom on the nitrogen in order to bind formaldehyde. This latter point, however, is contrary to the findings of Einhorn (loc. cit.) and also of the work presented in this thesis.

The amide group

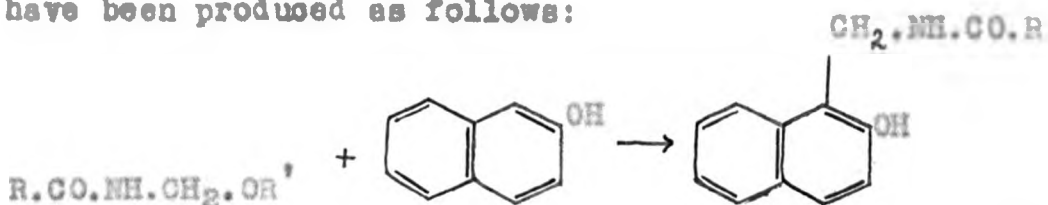
Until the comparatively recent studies of Wormald and Kaye (loc. cit.) and Fraenkel-Conrat et al. (J.A.C.S., 1945, 67, 950) little consideration had been given to the possibility of the participation of amide groups in the formaldehyde reaction.

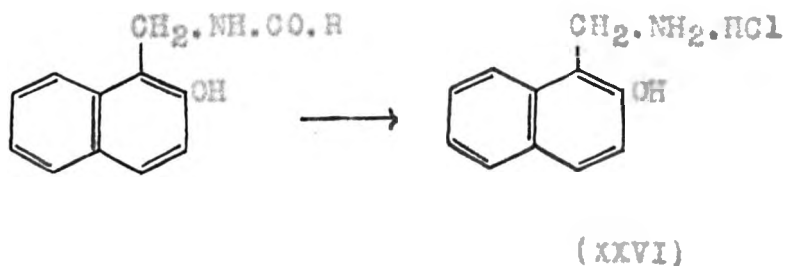
Wormald and Kaye (loc. cit.) showed that after neutral hardening of casein, a further quantity of formaldehyde may be introduced by treatment in the presence of acids and salts. Deamination prevents reaction in neutral solution but the increase in uptake

on acid treatment is observed, moreover deamidation has no effect on the neutral reaction but no further quantity is bound on salt-acid treatment.

The conclusions of Fraenkel-Conrat et al. (loc. cit.) are based on observations that formaldehyde uptake at pH 3.5 to 4 was in excess of the total number of basic groups present but found a correlation between the total basic and amide groups and the capacity to bind formaldehyde. For reasons previously given, this close correlation is thought to be coincidence but it seems quite probable that the qualitative deductions are correct. It was also found that polyglutamine, which contains a high proportion of amide groups bound more formaldehyde than any other macromolecular material.

Further evidence for participation was given by Haworth et al. (J.C.S., 1950, 1493) who condensed formaldehyde treated proteins with β -naphthol under the same conditions as required for the condensation of hydroxymethyl amides with β -naphthol (Einhorn, loc. cit.). After hydrolysis the hydrochloride of 1-aminomethyl-2-naphthol (XXVI) was isolated. This was considered to have been produced as follows:





where $R.CO.NH.CH_2.OR'$ is any derivative of a hydroxymethyl amide that will condense with β -naphthol. Bromine analyses after condensation with 6-brom-2-naphthol show that a greater number of such groups are introduced into the protein at pH 10 than at pH 0 which is to be expected since in acid solution the formation of methylene bis amides is the predominant reaction. The negligible amount of bromine introduced into silk fibroin is consistent with the absence of amide groups within the molecule.

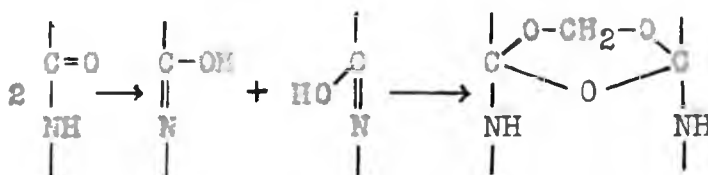
The peptide link

Evidence concerning the reactivity of the secondary amide group, $-CO.NH-$, with formaldehyde is rather conflicting but on the whole the evidence available shows that the peptide group is unlikely to participate in the reaction.

Investigations by Nitschmann and Hadorn (Helv. chim. Acta., 1944, 27, 299) have indicated both that the amino group reacts and that methylene bridging takes place on hardening casein with formaldehyde.

They suggest that one point of linking is the peptide group and give as their reasons (a) the reaction with diketopiperazine (see p.14) and, (b) the reaction takes place in two stages, an initial fast reaction followed by a slow reaction, the latter not being markedly altered by the presence of other reactive groups and the rate curve being almost linear. This means that the concentration of one of the reactive groups must be almost constant and is therefore probably the peptide link.

Carpenter and Lovelace (Industr. Engng. Chem., 1942, 34, 759) and Carpenter (Arch. Biochem., 1946, 9, 159) say that polypeptide chains are too far apart to be bridged by a methylene group attached directly to the nitrogen atoms but suggest the possibility of a trioxymethylene ring after enolisation of the peptide link.



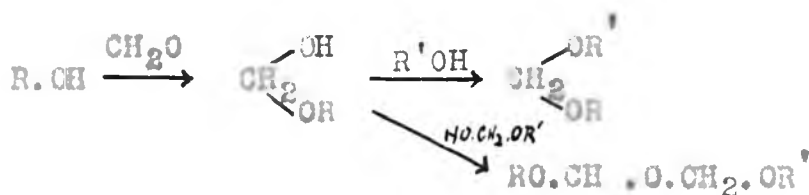
The fact that macromolecules such as silk fibroin, and polyglutamic acid which contain few functional groups other than the peptide link do not react with formaldehyde at pH 4 led Fraenkel-Conrat et al.

(J.A.C.S., 1945, 67, 950) to conclude that the peptide group took no part in reaction with formaldehyde.

The hydroxyl group

Nitschmann and Hadorn (Helv. chim. Acta., 1944, 27, 299) found that deaminated casein binds less formaldehyde than native casein at pH 5 to 6 after 48 hours' treatment but if the period of treatment is extended up to 38 days the uptake of formaldehyde reaches almost the same value as that of native casein. This is interpreted as a slow reaction taking place with the hydroxyl groups which have replaced the amino on deamination.

Carpenter and Lovelace (Industr. Engng. Chem., 1942, 34, 759) found that native casein after similar treatment bound more formaldehyde than could be accounted for by the nitrogen-containing groups and suggested formation of acetals between the hydroxyl groups of adjacent peptide chains.



Since the evidence given in both of the above papers could be explained by polyoxymethylene chain formation it is

uncertain whether or not the hydroxyl group is reactive. The reactivity is doubted by Fraenkel-Conrat et al. (JACS., 1945, 67, 950) on the grounds that silk fibroin binds so little formaldehyde.

Disulphide and sulphhydryl groups

A considerable amount of work has been published concerning the reactions of the disulphide group with formaldehyde but as yet there is no conclusive evidence in favour of reaction in proteins.

Thiazolidine-4-carboxylic acid (XX) was isolated by Middlebrook and Phillips (Biochem. J., 1947, 41, 218) from hydrolysates of formaldehyde treated wool but no account is taken of the possibility of cysteine liberated during hydrolysis reacting with the free formaldehyde. They have suggested that there is an initial hydrolysis of the disulphide link to give a thiol and a sulphenic acid group which may react with formaldehyde.

The tyrosine residues

There are several conflicting reports as to whether or not tyrosine plays any part in the reaction of proteins with formaldehyde. Middlebrook and Phillips (loc. cit.) found that acid hydrolysates of wool which had been treated at pH 1, 6.7 or 10 contained no tyrosine

identifiable chromatographically but later Middlebrook (Biochem., J., 1949, 44, 17) showed that wool so treated gave the same tyrosine analysis as for native wool.

In order to prevent any reaction occurring subsequent to treatment with formaldehyde Haworth, McGillivray and Peacock (in press) added excess resorcinol during hydrolysis which removed the formaldehyde immediately it was freed. Tyrosine was shown to be present in this case whereas it was not present after hydrolysis without added resorcinol. This indicates that reaction with tyrosine occurs during hydrolysis and not in the formaldehyde treatment.

By treatment of zein with formaldehyde in anhydrous solvents at 100°, Croston (Industr. Engng. Chem., 1950, 42, 482) was able to obtain fibres of high tensile strength and stable to conditions of acid hydrolysis. He suggested that methylene cross links had formed between tyrosine residues and gave the following points as evidence:

- (1) The intensity of Millons test is reduced.
- (2) Only a small effect on partially iodinated fibres.
- (3) Similar results are obtained with other proteins, but gelatin, which contains no tyrosine, is not affected. This last reason is hardly valid since gelatine also has a very low amide content.

Other functional groups

It is fairly certain that the guanidyl group of arginine residues and the indole nucleus of tryptophane react with formaldehyde but little is known about the glyoxaline ring of histidine or the carboxylic acid group. This latter group appears to be very unreactive, since polyglutamine binds very little formaldehyde and attempts to prepare the acid chloride of protein carboxyl groups have been unsuccessful (Traill, Chem. & Ind., 1950, 23).

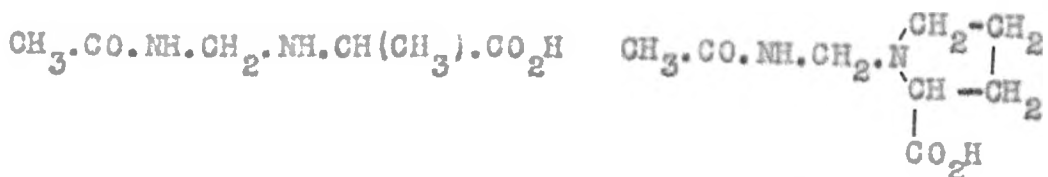
Model experiments

Extensive experiments with model compounds have been carried out by Fraenkel-Conrat and Olcott (J.A.C.S., 1948, 70, 2873. J. biol. Chem., 1948, 174, 827) in an attempt to find the groups concerned in methylene bridging. Their method was to mix the components with formaldehyde under various conditions and analyse the mixture for uncombined formaldehyde with dimedon at pH 4.6. When amines were present Van Slyke amino group determinations were made on the mixtures.

This method is of limited value since components which by themselves bind formaldehyde cannot definitely be said to have cross linked to other materials, moreover some methylene compounds may be unstable under the conditions of analysis.

Other experiments were made in an attempt to bind model compounds to proteins by methylene bridges. The results may be summarised as follows:

- (1) For amide-CH₂-amine condensation, an acid medium is most favourable.
- (2) Isolated unstable condensation products of acetamide with alanine and proline (XXVII), (XXVIII).



(XXVII)

(XXVIII)

- (3) Hydroxymethyl amides would not condense with amines.
- (4) Cyclic methyleneimines condensed with amides at pH 4 but not at pH 8.3.
- (5) Methyl guanidine can be bound by proteins rich in amino groups by treatment with formaldehyde over a pH range 4.2 to 8.5. A mixture of methyl guanidine sulphate and alanine bound more formaldehyde above pH 7 and below pH 5 than did the components alone.
- (6) Amide-CH₂-guanidine links will not form under these conditions.
- (7) Amides can be bound by proteins and formaldehyde.
- (8) No evidence for methylene bridging between thioglycol and an amine or amide at pH 4.3 to 7.6. At pH 1.5 half an equivalent of formaldehyde is bound per -SH group. This may be due to the formation of HO.(CH₂)₂.S.CH₂.S.(CH₂)₂.OH.
- (9) Proteins rich in amide groups will cross link with ammonia, primary amines and secondary diamines

in the presence of formaldehyde to form gels. Fraenkel-Conrat and Mecham (J. Biol. Chem., 1949, 177, 477) showed that there is a molecular weight increase on such treatment. Similarly when excess small molecular weight amides or secondary amines are added these are bound in preference to methylene bridging between protein chains.

- (10) Neither threonine nor *p*-cresol react with formaldehyde alone but together they take up 1 mole of formaldehyde at pH 3.9, 5.5 and 6.8.
- (11) Proteins will bind phenols, indoles and imidazoles on treatment with formaldehyde in alkaline solution.
- (12) Gramicidin and formaldehyde will bind amines, alanine, proline and lysine over a wide pH range but the greatest binding takes place in alkaline solution. It is thought that the methylene bridge is attached to the tryptophane nitrogen atom.

THEORETICAL



THEORETICALThe Reaction of Formaldehyde with Amines

The products of the reaction of amines and formaldehyde were said by Henry (Bull. Acad. roy. Belg., 1894, (3), 28, 355) to be hydroxymethyl amines (I) (see p. 8). These he obtained as colourless oils which on dehydration with solid caustic potash gave Schiff's bases (II) which were also colourless oils.



(I)



(II)



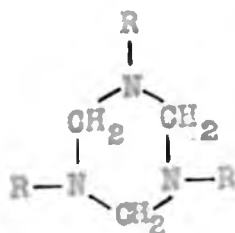
(III)

The only crystalline product isolated was that from benzylamine which melted at 43° and which he (ibid., 1897, (3), 33, 407) considered to be the Schiff's base (II).

Kempf (Annalen, 1890, 256, 219) had previously obtained a base by heating benzylamine with methylene dichloride in a sealed tube which he claimed to be the methylene bis amine (III), melting at 45 - 46°. The preparation and analytical figures of several of its organic and mineral salts were given in confirmation of this structure.

The close proximity of the melting points of these two supposedly different compounds has not hitherto received comment but there is a possibility that they are

one and the same substance. Accordingly the preparations were performed as reported in the literature and both products after crystallisation from dilute alcohol melted at 50° and were shown to be the same substance by mixed melting point. Elementary analysis and molecular weight measurements — by melting point depression in camphor — were in agreement with formulation as the trimer of the Schiff's base (IV), ($R = C_6H_5.CH_2.$) as proposed by Cambier and Brochet (Bull. Soc. chim., 1895, (3), 13, 392).



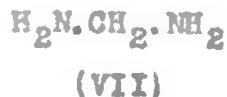
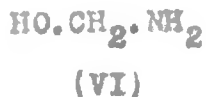
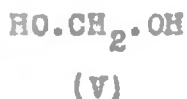
(IV)

The salts prepared by Kempf (*loc. cit.*) melted at the same temperature as the corresponding salts of benzylamine and the analyses quoted, although somewhat outside the limits of experimental error, are in all cases near to the values calculated for benzylamine salts. It therefore appears that treatment with organic or mineral acids breaks down the cyclic methylenesimines to the amine and formaldehyde with the production of amine salts and it has been shown that the conditions used for preparation of the

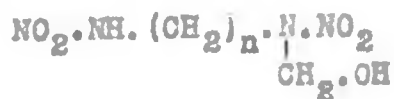
hydrochloride, dilute acid, break down the trimer with release of formaldehyde.

It seems, therefore, that the only obtainable products from the reaction of primary amines and formaldehyde are cyclic methylenesimines and that hydroxymethyl and methylene bis amines if they do exist under any conditions, are unstable.

This instability is probably of the same type as that of methylene glycol (V), aldehydeammonia (VI) and methylenediamine (VII).



No reason is known but it may be due to close proximity to two -I substituents i.e. -OH and -NH₂ thus it might be expected that any substituent on the nitrogen atom capable of reducing its electron releasing effect should help to stabilise these compounds. Hence although R.NH.CH₂.OH is unstable the hydroxymethyl derivatives of nitroamines (VIII) may be isolated (Woodcock, J.C.S., 1949, 1635), the nitro group acting as a cationoid substituent.



(VIII)

This would also account for the greater stability of hydroxymethylanides (IX) and methylene bis amides (X). It would not, however, account for the greater stability of cyclic methyleneimines (IV) or methylene bis secondary amines (XI).



(IX)



(X)

Secondary amines react with formaldehyde under the same conditions to give methylene bis amines (XI)



(XI)



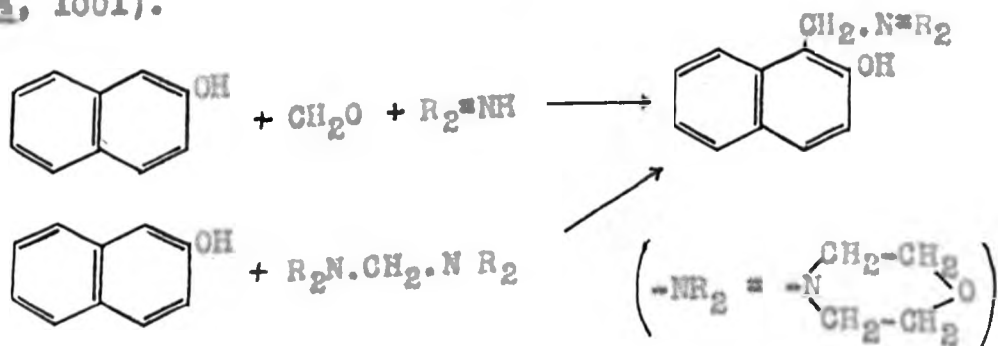
(XII)

Henry (loc. cit.) however, claims to have obtained unstable hydroxymethyl amines (XII) which eliminate water and formaldehyde on dehydration with caustic potash, to form methylene bis amines. Since these substances cannot be distilled at constant temperature, distillation giving (XI), it seems quite likely that these hydroxymethyl amines are in reality crude samples of methylene bis amines.

The non-isolation of hydroxymethyl secondary amines could be explained by the reasoning given for instability of such derivatives of primary amines and

since it is generally accepted that methylene glycol and methylene diamine exist in aqueous solution there is no apparent reason why analogous solutions of hydroxymethyl amines should not exist. However the sparing solubility of all but the low homologues make it unlikely that more than a very small quantity could exist as such in solution.

Condensation reactions that would be expected to occur with hydroxymethyl amines have been shown by Feldman and Wagner (J. org. Chem., 1942, 7, 31) to take place with methylene bis amines and the products of the Mannich reaction between amines, formaldehyde and reactive groups can also be obtained from methylene bis amines and the appropriate reactive centre. Thus, for example, β -naphthol, formaldehyde and morpholine give the same product under comparable conditions as is obtained by reacting β -naphthol and methylene bis morpholine (Lieberman and Wagner, J. org. Chem., 1949, 14, 1001).



Mannich reactions will also take place with cyclic methyleneimines of aromatic amines (IV, R = aryl group).

This reactivity of methylene bis amines has been confirmed by condensing methylene bis piperidine (XI, R₂N = piperidyl) with phenylacetyl glycol amide to give the same product as obtained by inter-reaction of the amide, formaldehyde and piperidine (XIII).



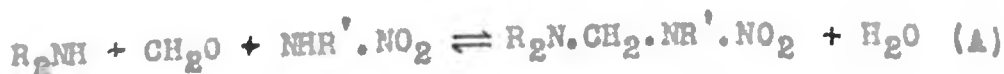
(XIII)

Cyclic trimers and methylene bis amines are stable to alkalis and standing for a week at ordinary temperatures with N caustic soda has no effect on these substances. In acid solution they are unstable and break down to give free formaldehyde which may be estimated as the 2:4-dinitrophenylhydrazone by precipitation from Brady's reagent. Lamberton et al. (J.C.S., 1949, 1641) showed that on rapid addition of 30 or more equivalents of at least 1 molar hydrochloric acid to methylene bis morpholine (XI, -NR₂ = morpholyl) followed by addition of Brady's reagent, precipitation only commenced after 10 minutes and was still slight even after 24 hours. If, however, the acid is added slowly

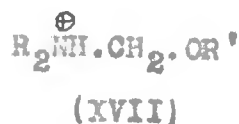
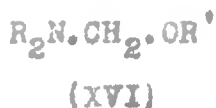
to an aqueous solution -- two equivalents in 35 minutes -- or if set aside with two equivalents of acid, precipitation commenced after 15-35 seconds under comparable final conditions of acidity. They also observed this phenomenon with hydroxymethyl nitroamines (VIII) and aminomethyl nitroamines (XIV) when on slow addition of acid 80-100% precipitation of formaldehyde was obtained, whereas rapid addition of excess acid led to only 10-20% precipitation in 24 hours. This was said to be due to formation of a stable cation (XV) which only slowly decomposed in the presence of acid, and confirmation was obtained from



osmotic determination of molecular weight in hydrochloric acid. The high apparent value in acid suggested that the compound existed mainly as the cation (XV) but the value obtained in water was one third of the formula weight which indicated decomposition in accordance with equation (A). On slow acidification the stronger base, (R_2NH) , reacts with the limited amount of acid and so upsets the equilibrium.

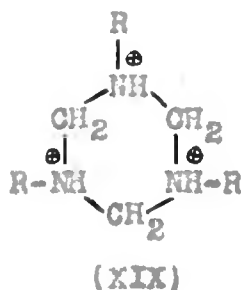
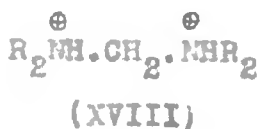


This stability is analogous to that of the quaternary salts of dialkylaminomethyl ethers (XVII) which were shown by Robinson and Robinson (J.C.S., 1921, 1470) to be more stable than the free bases (XVI)



This stability has been confirmed in the case of methylene bis dibenzylamine (XI, R = C₆H₅.CH₂-) and shown also to apply to cyclic methyleneimines (IV) by a similar procedure with Brady's reagent. The reagent is a saturated solution of 2:4-dinitrophenylhydrazine in 2N hydrochloric acid which is also saturated with formaldehyde-2:4-dinitrophenylhydrazone so as to make solubility errors negligible. The cyclic trimer is slightly more stable than the methylene bis amine since only 60% of its available formaldehyde was released after the addition of two equivalents of acid in 30 minutes whereas similar treatment of methylene bis dibenzylamine gave an 86% dissociation.

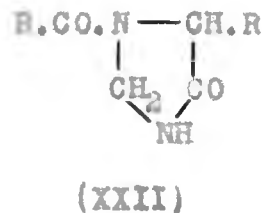
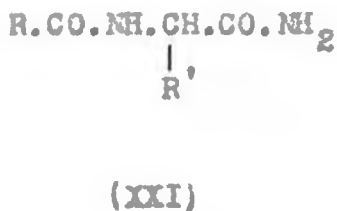
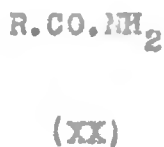
Stability of the cations under discussion might be explained by the fact that the quaternary ammonium groups (XVIII) and (XIX) are no longer strongly anionic (see p.40).



The amide group

Of the two condensation products of formaldehyde and amides very little has been published concerning their stabilities to acid and alkali. Further examples of hydroxymethyl amides and methylene bis amides have been prepared by the method first used by Einhorn (Annalen, 1905, 343, 207. 1908, 361, 113) and investigations into the stability towards acid and alkali have been made.

The types of derivatives studied are those of simple amides (XX) and those of amides containing a peptide link within the molecule (XXI) the latter type



being used to investigate whether the inclusion of a peptide link had any effect on the reactivity of the

amide group or conversely whether the amide group had any activating effect on the peptide link. It was thought that formaldehyde treatment may give a cyclic compound (XXII) by reaction with both the amide and the peptide nitrogen atoms. This, however, was not obtained, the only products being hydroxymethyl amides (XXIV) in alkaline medium and methylene bis amides (XXVI) in acid medium, the peptide group apparently not reacting.



(XXIII)



(XXIV)



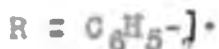
(XXV)



(XXVI)

The hydroxymethyl and methylene bis amides studied were those of:

(a) benzamide [Einhorn, loc. cit., (XXIII) and (XXV),



(b) phenylacetamide [Haworth et al., J.C.S., 1950, 1493,



(c) phenylpropionamide [(XXIII) and (XXV), R = C₆H₅.CH₂.CH₂-].

(d) hippuramide, [(XXIV) and (XXVI), $R = C_6H_5$, $R' = H$].

(e) phenylacetyl-glycyl amide, [(XXIV) and (XXVI),

$R = C_6H_5 \cdot CH_2-$, $R' = H$].

(f) phenylacetyl-dl-alanyl amide [(XXIV) and (XXVI),

$R = C_6H_5 \cdot CH_2-$, $R' = CH_3-$].

The hydroxymethyl derivatives were prepared by the standard method of Einhorn (*loc. cit.*), by dissolving the amide in a warm aqueous 40% formaldehyde solution containing a little potassium carbonate and allowing the hydroxymethyl amide to crystallise on cooling.

Methylene bis amides were prepared by warming the amide with an acid-salt buffer solution at pH 0 similar in composition to that used for hardening fibrous proteins. This method tended to give low yields probably due to hydrolysis of the amide link.

Although most of the hydroxymethyl amides were stable enough to be isolated in a pure state, those of type (XXIV) melted over a temperature range which suggests that they decompose at their melting points. That of hippuramide (XXIV), ($R = C_6H_5$, $R' = H$) was the most unstable, inasmuch as all attempts at recrystallisation resulted in reversion to the amide. The crude product, however, took part in the usual condensation reactions without any difficulty.

Hydroxymethyl amides have been found to be stable in neutral aqueous solution at room temperature but to give up formaldehyde on warming and in the presence of acids and alkalis. The position of equilibrium could be found from the formaldehyde freed in solution. Since acid causes the methylene bis amide to be precipitated, no dissociation constant can be found, but that in alkaline solution has been measured.

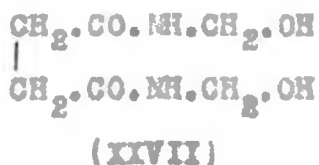
Formaldehyde was determined gravimetrically as its 2:4-dinitrophenylhydrazone by quantitative precipitation with Brady's reagent (see p.44). Analyses were performed upon aliquot samples from standard solutions of hydroxymethyl amides and potassium carbonate in water. The equilibrium constant was calculated from the free formaldehyde content at equilibrium.

The constant could also be calculated from the formaldehyde present in an equilibrium mixture when alkaline formaldehyde was added to the amide.

Both of these methods gave comparable results but the latter method caused equilibrium to be reached more rapidly. The alkali present acts only catalytically since increase in quantity has no effect on the final

position of equilibrium, it only affects the rate of dissociation.

Results show that at the concentrations used all hydroxymethyl amides studied reached equilibrium in the region of 100 hours at ordinary temperatures. The numerical values for monohydroxymethyl amides were of the same order, the values for each group of compounds, i.e. (XXIII) and (XXIV), being approximately the same. Dihydroxymethyl succinamide (XXVII), however, gave a much smaller percentage of formaldehyde but since the mechanism of dissociation is not known it is not possible to calculate a dissociation constant.



Dissociation in acid took place slowly along with the gradual crystallisation of the methylene bis amide. Theoretically this reaction should go to completion and 50% of the available formaldehyde be freed but this value was only slowly approached and after several weeks' standing at room temperature the reaction was not complete. Warming the hydroxymethyl amide with Brady's reagent was found to give only 91% of the

formaldehyde, the remainder probably having reacted to form the methylene bis amide which is stable under these conditions.

Similar experiments on neutral hydroxymethyl amide solutions gave no precipitate even after having stood for several days but boiling with water immediately released formaldehyde which was detected, after cooling, with Brady's reagent.

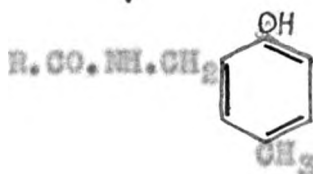
In view of these results it seems that hydroxymethyl groups would neither be part of the irreversibly bound formaldehyde, since they would not be stable to washing, nor are they likely to confer acid and alkali stability to formaldehyde treated proteins.

Methylene bis amides are, in contrast, highly stable compounds. Their stability to acids is shown by the work of Haworth et. al. (loc. cit.) who found that 10 hours boiling with 4% alcoholic hydrochloric acid was needed in order to remove the formaldehyde with β -naphthol giving methylene bis β -naphthol. They are also stable to treatment with alkalis. Normal caustic soda has no effect on methylene bis phenylacetamide after being in contact for one hour at 90°. The methylene bis amide link therefore is undoubtedly one such link which is irreversible to washing and stable to acids and alkalis

and therefore probably has an appreciable effect on the degree of hardening of proteins.

Condensation of hydroxymethyl amides with aromatic compounds

The ready condensation which occurs between hydroxymethyl amides and activated aromatic nuclei (Kilhorn, loc. cit.) would suggest the possibility of methylene bridges being formed between amide groups and aromatic type nuclei during formaldehyde treatment of proteins. One such possibility is the tyrosine nucleus and as a model for tyrosine, *p*-cresol was used giving the expected condensation product (XVIII).



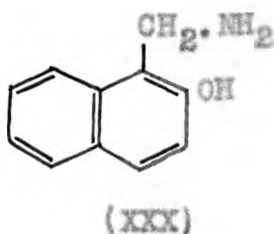
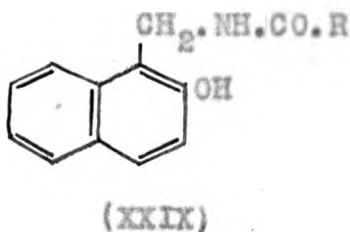
(XVIII)

Condensation can either be brought about by boiling the reactants in acidified alcoholic solution or by dissolving the hydroxymethyl amide in warm acidified *p*-cresol followed by distillation of the excess *p*-cresol under reduced pressure. Although no proof of the above structure has been sought, it is reasonable to expect that substitution takes place in the ortho position to the hydroxyl group.

Two such condensation products have been prepared. These are:

3-benzamidomethyl-*p*-cresol (XXVIII, $R = C_6H_5-$), and
3-phenylacetylglycylamidomethyl-*p*-cresol (XXVIII,
 $R = C_6H_5 \cdot CH_2 \cdot CO \cdot NH \cdot CH_2-$).

In addition to this, all the hydroxymethyl amides prepared have been condensed with β -naphthol to give *N*-substituted-1-aminomethyl-2-naphthols (XXIX) by heating acidified alcoholic solutions of the reactants (Einhorn, loc. cit.). Acid hydrolysis gives the parent 1-aminomethyl-2-naphthol (XXX).

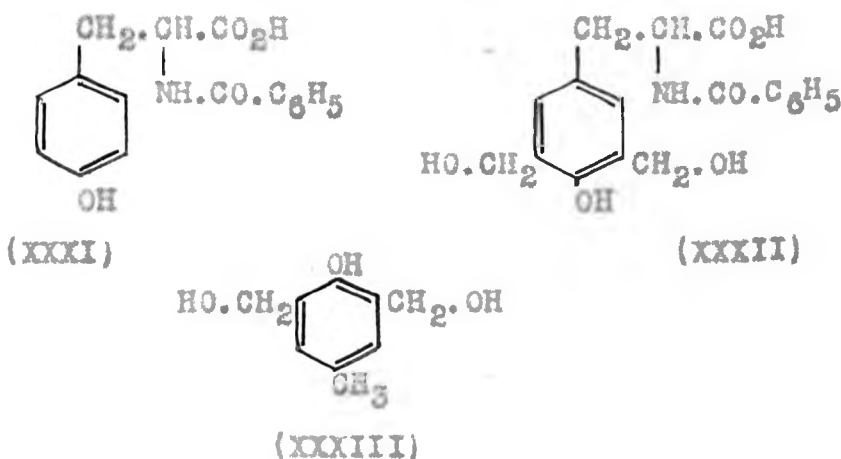


Attempting this condensation with *N*-benzoyl tyrosine (XXXI) as a model for protein tyrosine residues, under all conditions used gave unreacted benzoyl tyrosine, the methylene bis amide and some ethyl ether of the hydroxymethyl amide (XXXVIII, $R' = C_2H_5-$) formed from the solvent, alcohol. Neither *N*-hydroxymethyl benzamide (XXVIII, $R = C_6H_5-$) nor *N*-hydroxymethyl phenylacetylglycylamide (XXIV, $R = C_6H_5 \cdot CH_2-$, $R' = H$) would react even after boiling for up to 5½ hours.

N-benzoyl tyrosine and formaldehyde

Since N-benzoyl tyrosine (XXXI), did not condense with hydroxymethyl amides in an analogous manner to p-cresol or β -naphthol, its reactions with formaldehyde were investigated.

It was found that treatment with formaldehyde in the presence of acids under conditions which gave methylene bis p-cresol and methylene bis β -naphthol had no effect on N-benzoyl tyrosine. In the presence of caustic soda a condensation product was obtained, the analysis of which corresponded to a dihydroxymethyl derivative possibly (XXXII) but no further investigations into its structure or its reactivity were made. Under the latter conditions, p-cresol gives an analogous derivative (XXXIII). (Ullmann and Brittner, Ber., 1909, 42, 2539.)

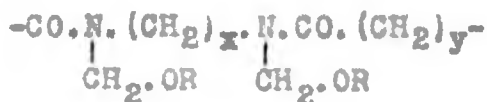


Condensation of hydroxymethyl amides with alcohols

Formation of ethers by condensing hydroxymethyl amides with alcohols was first reported by Kadowaki (Bull. chem. Soc. Japan, 1938, 11, 248) who prepared a series of ethers and thio ethers of dihydroxymethyl urea (XXXIV) by warming the reactants in the presence of hydrochloric acid.



Further analogues were reported by Sorenson (U.S. Pat., 2,201,927) who used phosphoric acid to catalyse the reaction and Albrecht et al. (Helv. chim. Acta., 1941, 24, 233E) prepared several benzyl ethers (XXXV) by treating the hydroxymethyl amide with benzyl alcohol in the presence of formic acid. This method was further used by Haworth et al. (J.C.S., 1950, 1493) to prepare benzyl ethers of simple hydroxymethyl amides (XXXV). Polyamides (nylon) react with formaldehyde and alcohols in the presence of acid catalysts to give ethers contained in units of type (XXXVI). (Lewis and McCreath, B.Pat., 582,517; 582,520. Cairns et al., J.A.C.S., 1949, 71, 651, 655).



(XXXVI)

It seems, therefore, that formaldehyde treatment of proteins could cause methylene bridging between alcoholic hydroxyl groups on one hand and either amides or the peptide link on the other.

Several ethers of types (XXXVII) and (XXXVIII) have been found to form readily when a suspension of a hydroxymethyl amide in an alcohol acidified with a little hydrochloric acid is allowed to stand at ordinary temperature for about 24 hours. More rapid formation is brought about by dissolving the hydroxymethyl amide in the warm acidified alcohol and allowing to stand, whereupon crystals of the ether are deposited on cooling.

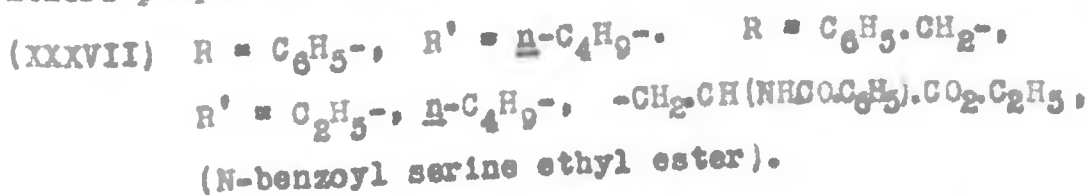


(XXXVII)



(XXXVIII)

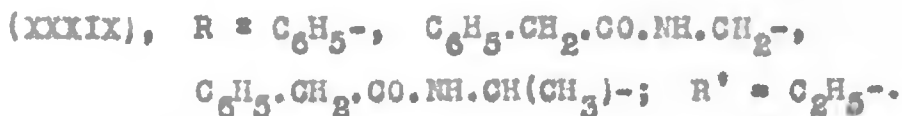
Ethers prepared in this way from primary alcohols include:



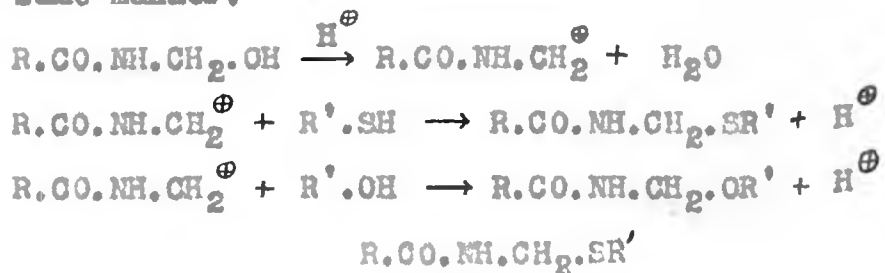
The formation of ethers from N-benzoyl serine ethyl ester indicates that the primary alcoholic group of the serine residues in proteins could link to an amide group via a methylene bridge.

All attempts to prepare analogous ethers of secondary and tertiary alcohols resulted in isolation of only the methylene bis amide, hence it appears that such links would not form between amide groups and threonine or other secondary alcoholic residues.

These ethers (XXXIX) have been found to form as readily their oxygen analogues and members of this series which were prepared include:



On account of the formation of these compounds, it may be concluded that a proton is eliminated from the mercaptan in the course of the reaction. This would be brought about from attack by the cation, $\text{R.CO.NH.CH}_2^{\oplus}$, formed after elimination of an hydroxyl ion in the presence of acid. The reaction with alcohols should occur in the same manner.



(XXXIX)

This explanation of the reaction mechanism accounts for the non-reactivity of secondary and tertiary alcohols since the two -I substituents close to the hydroxyl group would hinder the removal of a proton and so the reaction to form the methylene bis amide would predominate.

The ethers condense with β -naphthol under the same conditions and with the same ease as do the parent hydroxymethyl amides. They also undergo alcoholysis when treated with alcohols in the presence of a little concentrated hydrochloric acid, either at ordinary temperature or more rapidly on warming. Thus the benzyl ether (XXXVIII, $R = R'' = C_6H_5 \cdot CH_2-$, $R' = H$), when dissolved in warm acidified ethyl alcohol deposits crystals of the ethyl ether (XXXVIII, $R = C_6H_5 \cdot CH_2-$, $R' = H$, $R'' = C_2H_5-$) on cooling.

Although stable in neutral solution even on boiling — no positive reaction to Schiff's reagent — the ethers are slowly hydrolysed when in contact with acids and alkalis at room temperature although they are more stable than hydroxymethyl amides. Whereas hydroxymethyl amides will dissociate in the presence of a small quantity of potassium carbonate, no such dissociation was observed with the ethers — no formaldehyde detectable

with Brady's reagent. A slow dissociation was, however, observed in the presence of molar caustic soda but quantitative measurements of the dissociation constant could not be made because of their sparing solubility and because formaldehyde undergoes a Cannizzaro reaction at this pH. The final product of acid hydrolysis is the methylene bis amide along with liberation of formaldehyde.

A considerably greater stability is shown by these ethers than by the ethers of hydroxymethyl secondary amines (XVI) prepared by Robinson and McLeod (J.C.S., 1921, 1470) which may be accounted for in the same way as for the parent hydroxymethyl compounds (p.40).

The stability of the ethers of hydroxymethyl amides increases with the higher members of the series, this being illustrated by the inability to isolate N-ethoxymethyl benzamide (XXXVII, $R = C_6H_5-$, $R' = C_2H_5-$), the methylene bis amide being the only product obtained. Although the butyl ether (XXXVII, $R = C_6H_5-$, $R' = n-C_4H_9-$) may be isolated and purified, it is an oil which decomposes to the methylene bis amide on standing at ordinary temperatures over a period of a few weeks. All the other ethers prepared were unchanged even after many months.

Thio ethers are more stable to both acid and alkali than their oxygen analogues. The greater stability to acids is shown by the appreciably longer time of boiling necessary for them to condense with β -naphthol. Oxygen ethers will condense in 20 minutes but the thio ethers will only condense in the same yield after 3 hours boiling under similar conditions of acidity. After shorter periods of treatment the unchanged material has been recovered. The relative stability to alkali of the sulphur and oxygen ethers has been shown by the dissociation of suspensions in N caustic soda (p.118).

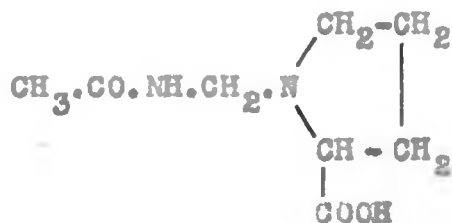
In light of these results it appears that the presence of ethereal and sulphide links in proteins would neither confer acid nor alkali stability to the molecules but the formaldehyde contained therein may be stable to prolonged washing.

Condensation of hydroxymethyl amides with amines

The only known acyclic compounds containing a methylene link between an amide and a primary amino group have been reported by Fraenkel-Conrat and Olcott (J.A.C.S., 1948, 70, 2673) who prepared (XL) and (XLI) by treatment of acetamide and alanine or proline with aqueous formaldehyde for 3 days at ordinary temperatures.



(XL)



(XLI)

These were found to be very unstable compounds which decomposed on attempted recrystallisation and could only be purified by precipitation with chilled alcohol from ice-cold aqueous solutions.

In a patent specification on textile finishers Sallmann and Graenacher (U.S.Pat., 2,448, 125) reported the production of new basic materials by treating hydroxymethyl amides with aliphatic amines and paraformaldehyde in glacial acetic acid as solvent.

They suggested that their compounds had structure (XLII) but no experimental proof or even methods of purification were given.

Similar conditions were used in an attempt to condense hydroxymethyl benzamide with methylamine to give (XLII, $R = C_6H_5-$, $R' = CH_3-$) and a basic compound was obtained in small yield, the major product being benzamide. The hydroxymethyl compound was found to condense without the necessity of paraformaldehyde being present, by the following method. A solution of hydroxymethyl amide and methylamine in acetic acid was warmed to 75° for 30 minutes after which the solvent was evaporated under reduced pressure. Water precipitated benzamide from the oily residue and caustic soda on the filtrate caused the base to separate.

Analysis and molecular weight determination — by melting point depression of camphor — indicated that the structure of the base was not (XLII) but was (XLIII, $R = C_6H_5-$, $R' = CH_3-$) and preparation of the n-butyl homologue gave a compound whose analysis agreed with this formulation (XLIII, $R = C_6H_5-$, $R' = n-C_4H_9-$). Further members of this series were prepared from hydroxymethyl phenylacetamide and methylamine (XLIII, $R = C_6H_5.CH_2-$, $R' = CH_3-$)



(XLII)



(XLIII)

and hydroxymethyl propionamide and methylamine (XLIII, $R = C_6H_5 \cdot CH_2 \cdot CH_2-$, $R' = CH_3-$).

During these last two preparations a reaction between the hydroxymethyl amides and the acetic acid solvent was observed. Dilution of the oily residue obtained after removal of the solvent gave a copious white crystalline material which was sparingly soluble in hot water and very soluble in cold alcohol. This was therefore not the amide as was produced in the case of hydroxymethyl benzamide.

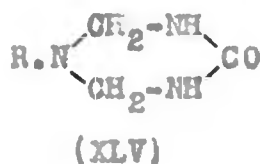
Analytical figures suggested the structures (XLIV, $R = C_6H_5 \cdot CH_2-$ or $C_6H_5 \cdot CH_2 \cdot CH_2-$), acetyl esters of the hydroxymethyl amides. Confirmation was obtained by synthesis from the hydroxymethyl amides and acetic acid under the same mild conditions as used for condensation with amines.

No esters of hydroxymethylamides have previously been reported and since such stable compounds can be prepared ester links must be considered as possible bridges in formaldehyde treated proteins. These esters condense with β -naphthol with the same ease as do hydroxymethyl amides, both at room temperature and on boiling. Their stability to acids and alkalis is intermediate between hydroxymethyl amides and ethers of



hydroxymethyl amides. They are stable to boiling water but formaldehyde is liberated in the presence of acids and alkalis. It would seem, therefore, that these ester links in proteins would be water stable but would not confer acid and alkali resistance.

Condensations between amines and dihydroxymethyl urea to give triazines (XLV) have been reported by



Burke (J.A.C.S., 1947, 69, 2136) who found that good yields were obtained by boiling aqueous solutions of the reactants for two hours. These conditions also suffice for the reaction between hydroxymethyl amides and amines but as before only poor yields are obtained. Hydroxymethyl benzamide was condensed with n-butylamine by this method.

All attempts to prepare n-butyl homologues of phenylacetamide (XLIII, $R = C_6H_5.CH_2-$, $R' = \underline{n}\text{-C}_4H_9-$) and phenylpropionamide (XLIII, $R = C_6H_5.CH_2.CH_2-$, $R' = \underline{n}\text{-C}_4H_9-$) were unsuccessful. Treatment in aqueous solution gave the free amide; in acetic acid the amide and acetyl esters (XLIV); and in formic acid the only product obtained was the methylene bis amide. The reason for this absence of reaction is not apparent.

Einhorn (Annalen, 1905, 343, 207; 1908, 361, 113) was the first to obtain condensation products of hydroxymethyl amides and secondary amines (XLVI) and he found that the same compounds were formed



(XLVI)

by the treatment of alcoholic solutions of amines and amides with formaldehyde. The latter method was the one commonly used although no information is given as to which gave the better yield.

It has been found that the latter method gives much better yields than the former and this has been used to prepare some further representatives of this class of compound. The condensation of methylene bis amines with amides has been considered in the section on amines (p.42). Compounds prepared include:

dibenzylaminomethyl benzamide (XLVI, $R = C_6H_5-$,



dibenzylaminomethyl phenylacetamide (XLVI,



piperidylmethyl phenylacetylglycylamide (XLVI,

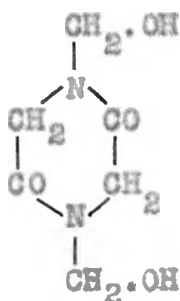


Condensation products of hydroxymethyl amides with primary and secondary amines are fairly stable to alkalis at room temperature but they slowly decompose in the presence of acids, the products from primary amines (XLIII) being appreciably less stable than those from secondary amines (XLVI). The latter are stable to boiling water but the former decompose rapidly, and neither of these compounds will react with β -naphthol under the same conditions as do the hydroxymethyl amides.

It seems, therefore, that the primary amino group takes no part in stable binding of formaldehyde. Secondary amino groups only occur to a limited extent in proteins but there is a possibility of formaldehyde causing methylation of primary amino to secondary amino groups. These may bind formaldehyde which would be stable to boiling water but which would be unlikely to confer acid stability to proteins.

The peptide group

Although no formaldehyde condensation products of N-substituted amides have been prepared, derivatives of a similar type have been isolated in certain exceptional cases. The most notable example is hydroxymethyl diketopiperazine (XLVII) prepared by Cherbuliez and Feer (Helv. chim. Acta., 1922, 5, 678). Polyamides, polyhexamethylene adipamide (XLVIII, $x = 6$, $y = 4$; nylon 66) and polyhexamethylene sebacamide (XLVIII, $x = 6$, $y = 8$; nylon 610) have been shown to give

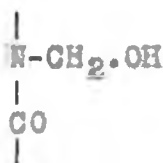


(XLVII)

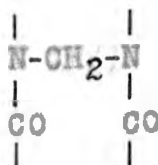


(XLVIII)

hydroxymethyl derivatives (XLIX) under both acid and alkaline conditions and methylene bridged nylons (L) in acid solution. Almost complete amide substitutions can be obtained.

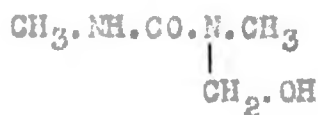


(XLIX)



(L)

(Lewis and McCreath, B.Pat., 582, 517. Cairns et al., J.A.C.S., 1949, 71, 651). A further example of reactivity of this type of linkage is sym-dimethyl urea which gives the monohydroxymethyl derivative (LI).

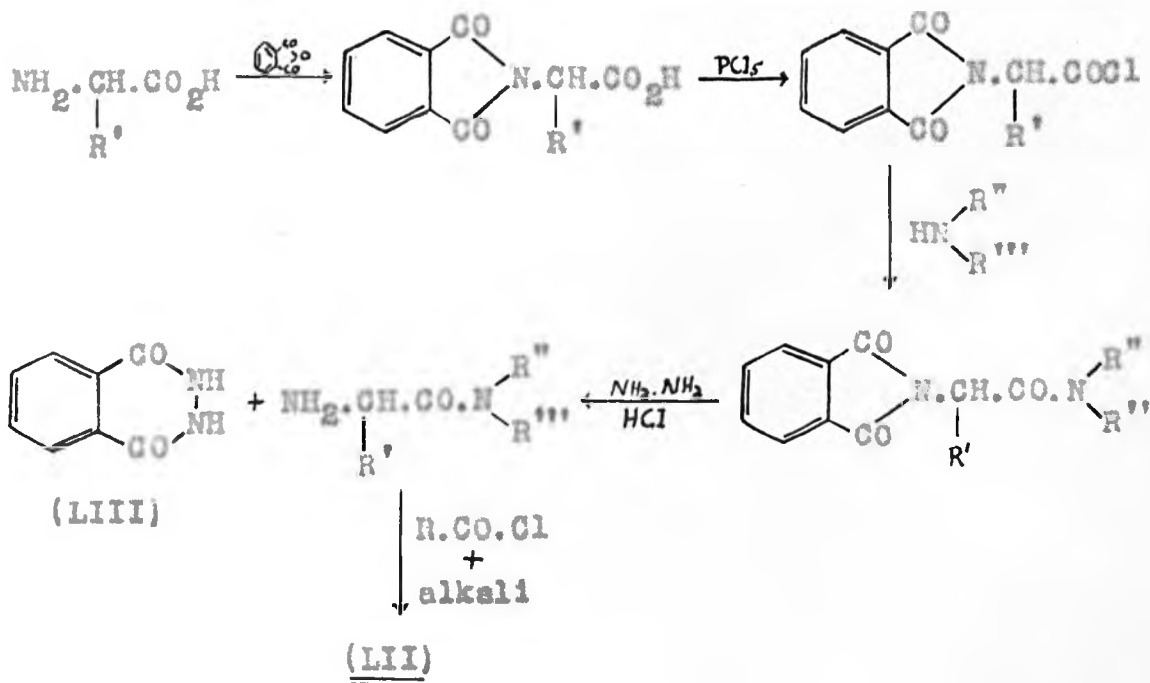


(LI)



(LII)

Attempts have been made to prepare similar derivatives of simple mono-peptides (LII) these having been synthesised by the following scheme. (Sheehan and Frank, J.A.C.S., 1949, 71, 1856).



The method of blocking the amino group with the phthalyl group was first used by Gabriel (Ber., 1907, 40, 2648) who subsequently removed this group by prolonged hydrolysis. Treatment with hydrazine followed by hydrochloric acid (Ing and Manske, J.C.S., 1926, 2348) is a much simpler method and gives good yields. Phthalhydrazide (LIII) which is formed along with the amine is a highly insoluble material and may be completely removed from the amine solution. Final treatment with the acid chloride gave the required peptide. Compounds prepared by this method include:

benzoylglycyl morpholide (LII, $R = C_6H_5-$, $R' = H$,



phenylacetylglycyl ethylamide (LII, $R = C_6H_5.CH_2-$,



phenylacetylglycyl diethylamide (LII, $R = C_6H_5.CH_2-$,



phenylacetylglycyl morpholide (LII, $R = C_6H_5.CH_2-$,



phenylacetyl-dl-alanyl diethylamide (LII, $R = C_6H_5.CH_2-$,



Condensations of formaldehyde with these peptides have been attempted under the following conditions:

Alkaline

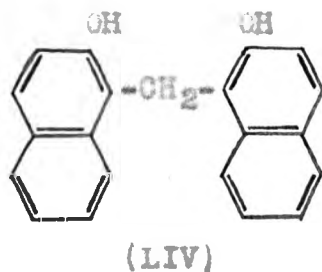
(a) An aqueous solution with the addition of potassium carbonate using the method whereby Einhorn (loc. cit.) obtained hydroxymethyl derivatives of unsubstituted amides.

(b) Using potassium carbonate with dioxan as an inert solvent. Cairns et al. (loc. cit.) found that these conditions were the most advantageous for substituting hydroxymethyl groups into nylon.

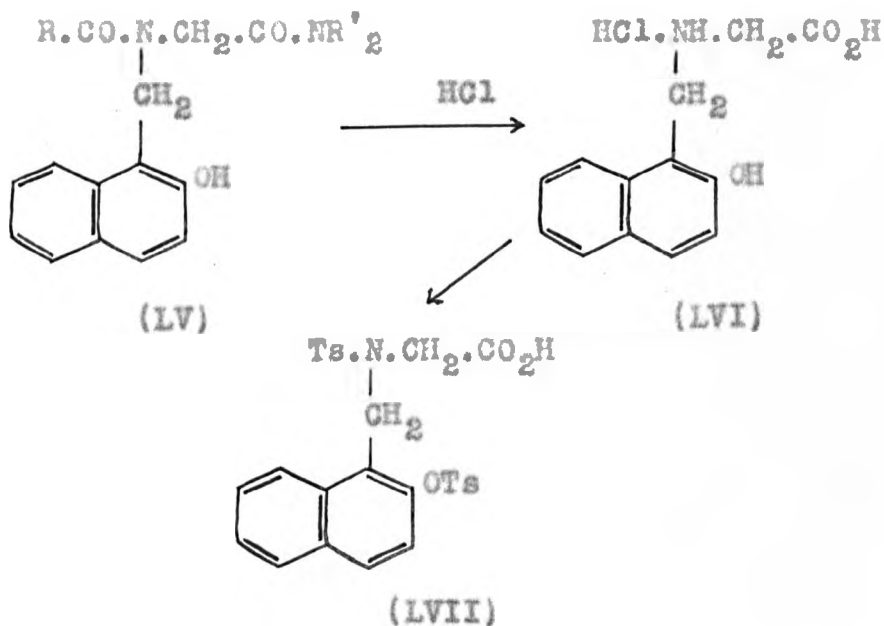
(c) Pyridine as solvent and basic catalyst in which polyoxymethylene is dissolved. This method also brings about the reaction of formaldehyde with nylon. (Cairns et al., loc. cit.).

Under all the above conditions the only product isolated was the unchanged starting material.

If reaction with formaldehyde had occurred there was a possibility that the hydroxymethyl group had split off during the attempted isolation. If reaction could be brought about with β -naphthol in an analogous manner to hydroxymethyl primary amides the product (LV) should be capable of isolation. Consequently this was attempted but, as before, the starting material was recovered unchanged and in addition, methylene bis β -naphthol (LIV).



Hydrolysis of any such condensation product would yield a naphthol amine (LVI) which would be capable of reacting with p-toluenesulphonyl chloride and be isolated as an amphoteric material (LVII).



No such product was isolated.

The same series of reactions was attempted with ethyl benzamide, also with negative results.

Acid

(a) Aqueous formaldehyde with hydrochloric acid (Einhorn, loc. cit.) showed no tendency to cause reaction, nor did an alcoholic solution, which Cairns et al. (loc. cit.) found to give ethoxymethyl derivatives.

(b) Dioxan, an inert solvent, and hydrochloric acid did not bring about condensation.

The above methods of treatment were performed for varying time periods at 60° and with amounts of formaldehyde of up to 20 equivalents. A certain amount of hydrolysis is probably obtained by this acid treatment since the odour of phenylacetic acid is evident after treatment and caustic soda liberates the odour of an amine from the residues after the final concentration.

(c) Formic acid and formaldehyde, which rapidly causes gelling of nylon has no effect on the model peptides.

Analytical determinations of formaldehyde present in a mixture with model peptides were made with Brady's reagent but no formaldehyde uptake was observed even after having been in contact for 21 days at ordinary temperatures. Owing to the low water solubility of these peptides it was only possible to treat the most soluble model compounds in this manner. These were

phenylacetyl glycol diethylamide (LII, $R = C_6H_5 \cdot CH_2-$,
 $R' = H$, $R'' = R''' = C_2H_5-$) and N-ethyl benzamide (LVIII).



(LVIII)

It is not possible to conclude from these experiments whether or not formaldehyde derivatives of the peptide group are formed since they may be so unstable as to give up their formaldehyde with Brady's reagent in 2N hydrochloric acid and during attempted isolation. It seems, therefore, that low molecular weight compounds containing the group $-CO.NH-$ will not react with formaldehyde to give stable products. There is no apparent reason for this.

EXPERIMENTAL



EXPERIMENTALTreatment of benzylamine with aqueous formaldehyde

40% aqueous formaldehyde (0.75 c.c.) was added slowly with cooling and shaking to benzylamine (1 g.). This produced a colourless viscous oil which was separated by decanting the water layer. Standing in vacuo over concentrated sulphuric acid and periodic scratching caused the oil to crystallise into long prisms (1 g.), m.p. 38-42°. Distillation at atmospheric pressure caused decomposition; Henry (Bull. Acad. roy. Belg., 1895, (3), 29, 23) gives b.p. 245° without decomposition. Distilled at 100°/0.005 m.m. with slight decomposition giving a pale yellow oil which crystallised on standing to pale yellow prisms m.p. 49°. Recrystallisation from dilute methylated spirits removed the colour and raised the melting point to 80°. (Found: C, 80.7; H, 7.8; N, 11.7; M.Wt., 378. Calc. for $(C_8H_9N)_3$, C, 80.7; H, 7.6; N, 11.8; M.Wt., 357).

Treatment of benzylamine with methylene chloride

The method given by Kempff (Annalen, 1890, 256, 219) was followed; Benzylamine (2.5 c.c.) and methylene chloride (1 c.c.) were heated together in a sealed tube in a water bath for

8 hours. Crystals were observed to form which on pouring into water dissolved, leaving an oil which was extracted with ether and the ethereal solution dried over fused calcium chloride. Evaporation of the ether left a colourless oil which gradually solidified and on recrystallisation from dilute methylated spirits yielded long colourless prisms; m.p. 50° which was not depressed on mixing with a sample of the previously prepared tribenzyl trimethylene triamine.

N-hydroxymethyl phenylacetyl glycol amide (A).

(XXIV, $R = C_6H_5-CH_2-$, $R' = H$)

(1) Phenylacetyl glycol amide was prepared by the treatment of phenylacetyl glycol ethyl ester with concentrated ammonia at room temperature. (Hotter, J. pr. Chem., 1888, (2), 38, 97).

A mixture of phenylacetyl glycol amide (10 g.), anhydrous potassium carbonate (0.4 g.), water (10 c.c.) and 40% aqueous formaldehyde solution (10 c.c.) was warmed in a water bath until complete solution occurred. On standing, the hydroxymethyl derivative (10 g.) crystallised. This was filtered, washed with dilute hydrochloric acid and water and dried in vacuo over concentrated sulphuric acid. This melted at $140-148^{\circ}$. Several recrystallisations from water gave colourless plates, m.p. $144-148^{\circ}$.

(Found: C, 59.4; H, 6.2; N, 12.6. $C_{11}H_{14}O_3N_2$ requires C, 59.5; H, 6.3; N, 12.6).

(2) A solution of phenylacetyl-glycylamide (0.5 g.) and polyoxymethylene (0.2 g.) in pyridine (2 c.c.) was boiled under reflux for $1\frac{1}{2}$ hours and on cooling a solid crystallised (0.35 g.), which was filtered and washed with ether. M.p. 132-138°. Ether on the mother liquors gave a further quantity (0.1 g.) of m.p. 132-142°. Both of these materials gave good yields of ethoxymethyl phenylacetyl-glycylamide (XXXVIII, $R = C_6H_5 \cdot CH_2-$, $R' = C_2H_5-$) on treatment with acidified ethyl alcohol.

Phenylacetyl-dl-alanyl ethyl ester

dl-Alanine ethyl ester hydrochloride (25 g.) was dissolved in water (250 c.c.) and magnesium oxide (20 g.) added. To the cooled and stirred mixture was added, over a period of two hours, phenylacetyl chloride (30.7 g.), the mixture being stirred for a further half hour after all had been added. The solid was filtered, suspended in water (70 c.c.) and the solution cooled and made acid to Congo red with concentrated hydrochloric acid. The product was filtered and washed with water. Recrystallisation from alcohol or cyclohexane gave colourless needles (25.5 g.) of m.p. 162°.

(Found: C, 66.3; H, 7.3; N, 6.2. $C_{15}H_{17}O_3N$ requires C, 66.4; H, 7.2; N, 6.0).

Phenylacetyl-dl-alanyl amide

Phenylacetyl-dl-alanyl ethyl ester (20 g.) was stirred with .880 ammonia (150 c.c.) for three and a half hours. The liquid was then saturated with ammonia gas and the stirring continued for a further two hours, the mixture afterwards being left to stand overnight at room temperature. The product (12.6 g.), after filtering and washing with water, melted at 153-155°. This was recrystallised from water in colourless prisms of m.p. 154-155°. (Found: C, 64.4; H, 6.9; N, 13.4. $C_{11}H_{14}N_2O_2$ requires c, 64.1; H, 6.8; N, 13.3).

N-hydroxymethyl phenylacetyl-dl-alanyl amide (B).

Prepared similarly to (A) in 87% yield.

Colourless plates from water, m.p. 137-140°. (Found: C, 61.2; H, 6.7; N, 11.7. $C_{12}H_{16}O_3N_2$ requires C, 61.0; H, 6.8; N, 11.9.)

N-hydroxymethyl hippuramide (C). (XXIV, R = C_6H_5- , R' = H)

Hippuramide was prepared by stirring a suspension of ethyl hippurate in .880 ammonia (Conrad, J. pr. Chem., 1880, (2), 15, 248).

The hydroxymethyl derivative was prepared similarly to (A) in 86% yield. Attempted recrystallisation from alcohol resulted in decomposition to the amide. The compound was soluble in warm water and warm alcohol;

insoluble in benzene; light petroleum, ether, acetone and chloroform.

For the preparation of the derivatives crude hydroxymethyl hippuramide was used.

N-hydroxymethyl phenylpropionamide (D).

(XXIII, R = C₆H₅.CH₂.CH₂-)

Prepared in the usual manner in 84% yield, was recrystallised from benzene or ethyl acetate giving colourless prisms m.p. 98°. (Found: C, 67.4; H, 7.0; C₁₀H₁₃O₂N requires C, 67.1; H, 7.3).

1-phenylacetylglycolamidomethyl-2-naphthol

(XXIX, R = C₆H₅.CH₂.CO.NH.CH₂-)

A mixture of hydroxymethyl amide (A), (1 g.), β-naphthol, (1 g.), absolute alcohol, (10 c.c.) and concentrated hydrochloric acid (0.2 c.c.) was boiled under reflux for twenty minutes. On cooling, the condensation product (1 g.) crystallised and was filtered and washed with alcohol. The crude material melted at 187-189° and recrystallisation from alcohol gave colourless needles m.p. 189°. (Found: C, 72.2; H, 5.7; N, 7.9. C₂₁H₂₀O₃N₂ requires C, 72.4; H, 5.8; N, 8.1).

1-phenylacetyl-dl-alanylamidomethyl-2-naphthol(XXIX, R = C₆H₅.CH₂.CO.NH.CH(CH₃)-)

Hydroxymethyl amide (B) was condensed with β-naphthol in a similar manner in 68% yield. Recrystallisation from alcohol gave colourless needles m.p. 200°. (Found: C, 72.6; H, 5.9; N, 7.9. C₂₂H₂₂O₃N₂ requires C, 72.9; H, 6.1; N, 7.7).

1-hippurylamidomethyl-2-naphthol(XXIX, R = C₆H₅.CO.NH.CH₂-)

This was obtained from hydroxymethyl amide (C) and β-naphthol, after concentration of the solution, in 25% yield, m.p. 185-186°; colourless prisms. (Found: C, 71.6; H, 5.6; N, 8.1. C₂₀H₁₈O₃N₂ requires C, 71.9; H, 5.4; N, 8.4).

1-phenylpropionamidomethyl-2-naphthol(XXIX, R = C₆H₅.CH₂.CH₂-)

Hydroxymethyl amide (D) condensed with β-naphthol in 75% yield and the product was recrystallised from alcohol giving colourless prisms m.p. 121-122°. (Found: C, 78.3; H, 6.2; N, 5.1. C₂₀H₁₉O₂N requires C, 78.6; H, 6.2; N, 4.6).

Hydrolysis of β -naphthol condensation products

The above acylamidomethyl-2-naphthols (0.5 g.) were hydrolysed by boiling for 3 hours in a solution of alcohol (10 c.c.) acidified with concentrated hydrochloric acid (3 c.c.). On standing overnight, 1-aminomethyl-2-naphthol (XXX) hydrochloride crystallised in yields varying between 59 and 69%. Recrystallisation from water acidified with a drop of concentrated hydrochloric acid gave small pale red prisms, m.p. 220° which was not depressed on mixing with an authentic sample.

Methylene bis phenylacetyl glycol amide

(XXVI, $R = C_6H_5.CH_2-$, $R' = H$)

(1) Phenylacetyl glycol amide (0.5 g.) and a salt hardening liquor (5 c.c.) [a buffer solution at pH 0 containing saturated sodium sulphate (860 c.c.), concentrated sulphuric acid (258 c.c.) and 40% aqueous formaldehyde solution (56 c.c.)] were warmed in a water bath until complete solution had occurred. On cooling the methylene bis amide crystallised. Recrystallisation from glacial acetic acid gave colourless plates (0.1 g.) m.p. 260° . (Found: C, 63.5; H, 6.1; N, 14.1. $C_{21}H_{24}O_4N_4$ requires C, 63.6; H, 6.1; N, 14.2).

(2) A suspension of *N*-hydroxymethylphenylacetyl-glycyl amide (0.2 g.) in water (2 c.c.) acidified with concentrated hydrochloric acid (0.1 c.c.) was warmed until complete solution had taken place. On standing overnight, colourless plates separated (0.15 g.), m.p. 230-240°. Recrystallisation from glacial acetic acid raised the melting point to 259° which was not depressed on mixing with a sample from (1).

Methylene bis phenylacetyl-dl-alanine amide



Prepared as above from formaldehyde buffer solution at pH 0. The product was washed with benzene and alcohol and recrystallised from glacial acetic acid giving colourless prisms, m.p. 280° with decomposition. (Found: C, 64.9; H, 6.9; N, 13.3. $C_{25}H_{28}O_4N_4$ requires C, 65.1; H, 6.6; N, 13.2).

Methylene bis hippuramide. (XXVI, $R = C_6H_5-$, $R' = H$)

Prepared as above in 28% yield. Recrystallised from glacial acetic acid in colourless plates of m.p. 258°. (Found: C, 61.9; H, 5.8; N, 15.7. $C_{19}H_{20}O_4N_4$ requires C, 62.0; H, 5.5; N, 15.2).

The following ethers were prepared by warming a mixture of hydroxymethyl amide and the appropriate

alcohol with a little concentrated hydrochloric acid until solution occurred. On standing and cooling, the product crystallised and was washed with ether and dried. The ethers can also be prepared by allowing the reaction mixture to stand overnight at room temperature, the yields being approximately the same by either method.

N-methoxymethyl phenylacetylalcyoyl amide

(XXXVIII, $R = C_6H_5 \cdot CH_2-$, $R' = H$, $R'' = CH_3-$)

Hydroxymethyl amide (A), (0.5 g.), methyl alcohol (5 c.c.) and concentrated hydrochloric acid (0.1 c.c.). The product (0.3 g.) was recrystallised from water in colourless plates, m.p. 146° .

(Found: C, 60.9, H, 6.7; N, 11.8. $C_{12}H_{16}O_3N_2$ requires C, 61.0; H, 6.8; N, 11.9).

N-ethoxymethyl phenylacetylalcyoyl amide

(XXXVIII, $R = C_6H_5 \cdot CH_2-$, $R' = H$, $R'' = C_2H_5-$)

Prepared similarly from (A), (0.5 g.), ethyl alcohol (5 c.c.) and concentrated hydrochloric acid (0.1 c.c.). Recrystallised from water in colourless plates (0.3 g.), m.p. 159° . (Found: C, 62.2; H, 7.2; N, 11.7. $C_{13}H_{18}O_3N_2$ requires C, 62.4; H, 7.2; N, 11.2).

Soluble in alcohol, hot acetone, benzene, chloroform, hot ethyl acetate, pyridine. Fairly soluble

in dioxan; sparingly soluble in hot light petroleum.
Insoluble in cold acetone, cold light petroleum,
carbon tetrachloride and ether.

N-n-propoxymethyl phenylacetylglycyl amide

(XXXVIII, $R = C_6H_5 \cdot CH_2-$, $R' = H$, $R'' = C_3H_7-$)

Prepared from (A), (0.5 g.), n-propyl alcohol
(2 c.c.) and concentrated hydrochloric acid (0.05 c.c.).
Recrystallised from water in colourless plates (0.5 g.),
m.p. 145°. (Found: C, 63.4; H, 7.3; N, 10.8.

$C_{14}H_{20}O_3N_2$ requires C, 63.6; H, 7.6; N, 10.6).

N-iso-butoxymethyl phenylacetylglycyl amide

(XXXVIII, $R = C_6H_5 \cdot CH_2-$, $R' = H$, $R'' = C_4H_9-$)

Prepared from (A), (0.5 g.), iso-butyl alcohol
(2 c.c.) and concentrated hydrochloric acid (0.05 c.c.).
Recrystallised from water in colourless plates (0.25 g.),
m.p. 120-121°. (Found: C, 64.9; H, 7.9; N, 9.9.

$C_{15}H_{22}O_3N_2$ requires C, 64.8; H, 7.9; N, 10.1).

N-benzoxymethyl phenylacetylglycyl amide

(XXXVIII, $R = R'' = C_6H_5 \cdot CH_2-$, $R' = H$)

Prepared from (A), (0.5 g.), benzyl alcohol
(3 c.c.) and concentrated hydrochloric acid (0.1 c.c.).
Recrystallised from dilute methylated spirits in
colourless leaflets (0.35 g.), m.p. 138°. (Found:
C, 68.9; H, 6.2; N, 8.6. $C_{18}H_{20}O_3N_2$ requires
C, 69.2; H, 6.4; N, 9.0).

N-ethoxymethyl phenylacetyl-dl-alanyl amide

(XXXVIII, $R = C_6H_5.CH_2-$, $R' = CH_3-$, $R'' = C_2H_5-$)

Prepared from (B), (0.3 g.), ethyl alcohol (2 c.c.) and concentrated hydrochloric acid (0.1 c.c.). Recrystallised from water in colourless prisms (0.2 g.), m.p. 128° . (Found: C, 63.7; H, 7.5; N, 10.6. $C_{14}H_{20}O_3N_2$ requires C, 63.6; H, 7.6; N, 10.6).

N-benzoxymethyl phenylacetyl-dl-alanyl amide

(XXXVIII, $R = R'' = C_6H_5.CH_2-$, $R' = CH_3-$)

Hydroxymethyl amide (B) (0.3 g.), benzyl alcohol (2 c.c.), concentrated hydrochloric acid (0.05 c.c.) were warmed until dissolved. On standing overnight nothing crystallised. The solution was neutralised by adding solid potassium carbonate, concentrated under vacuum, ($98^\circ/15$ m.m.) and allowed to stand in the refrigerator overnight. The product (0.25 g.), which crystallised, melted at $98-99^\circ$ after washing with ether. Recrystallisation from methylated spirits gave colourless leaflets, m.p. 101° . (Found: C, 69.8; H, 6.8; N, 8.3. $C_{19}H_{22}O_3N_2$ requires C, 69.9; H, 6.8; N, 8.6).

N-methoxymethyl hippuramide. (XXXVIII, $R = C_6H_5-$,

$R' = H$, $R'' = CH_3-$)

Prepared by the usual method from hydroxy-

methyl amide (C), (0.4 g.), methyl alcohol (1 c.c.)
and concentrated hydrochloric acid (0.1 c.c.).

Recrystallisation from water gave colourless plates
(0.3 g.), m.p. 138-139°. (Found: C, 59.1; H, 6.1;
N, 12.8. $C_{11}H_{14}O_3N_2$ requires C, 59.4; H, 6.3; N, 12.6).

N-ethoxymethyl hippuramide (XXXVIII, R = C_2H_5- ,



Prepared from (C), (0.5 g.), ethyl alcohol
(5 c.c.) and concentrated hydrochloric acid (0.1 c.c.).

Recrystallisation from water gave colourless leaflets
(0.4 g.), m.p. 166°. (Found: C, 60.7; H, 7.0;
N, 11.8. $C_{12}H_{16}O_3N_2$ requires C, 61.0; H, 6.8; N, 11.9).

N-benzoxymethyl hippuramide (XXXVIII, R = C_6H_5- ,



Hydroxymethyl amide (C), (1 g.), benzyl alcohol
(2 c.c.) and concentrated hydrochloric acid (0.1 c.c.).
Yield 0.3 g. Ether added to the filtrate precipitated
a further quantity (0.2 g.), both fractions melting at
129-131°. Crystallisation from methylated spirits gave
colourless leaflets, m.p. 133°. (Found: C, 68.3;
H, 6.1; N, 9.8. $C_{17}H_{18}O_3N_2$ requires C, 68.5; H, 6.0;
N, 9.4).

N-ethoxymethyl benzamide. (XXXVII, R = C₆H₅-,

R' = C₂H₅-)

Could not be isolated owing to its rapid conversion into methylene bis benzamide even under very mild conditions. (Standing at room temperature for a day in N/5 alcoholic hydrochloric acid.)

N-ethoxymethyl phenylacetamide (XXXVII, R = C₆H₅.CH₂-,

R' = C₂H₅-)

A mixture of N-hydroxymethyl phenylacetamide (0.5 g.), (Haworth, McGillivray and Peacock, J.C.S., 1950, 1493), ethyl alcohol (3 c.c.) and concentrated hydrochloric acid (0.05 c.c.) after standing for a day at room temperature was evaporated under reduced pressure at 30°. A colourless tar remained which on recrystallisation from light petroleum (b.p. 60-80°) gave colourless leaflets (0.35 g.), m.p. 55-56°. (Found: C, 68.8; H, 7.6; N, 7.3. C₁₁H₁₅O₂N requires C, 68.4; H, 7.3; N, 7.3).

N-n-butoxymethyl benzamide. (XXXVII, R = C₆H₅-,

R' = C₄H₉-)

Prepared as above from N-hydroxymethyl benzamide (0.5 g.), (Einhorn, Annalen, 1905, 343, 207) n-butyl alcohol (1 c.c.) and 50% butanolic hydrochloric acid (0.05 c.c.). The residue after evaporation was a

liquid which distilled at $180^{\circ}/3$ m.m. pressure and which changed to methylene bis benzamide on standing in a corked tube over a period of several weeks.

(Found: C, 69.3; H, 7.9; N, 7.2. $C_{12}H_{17}O_2N$ requires C, 69.6; H, 8.2; N, 6.8).

N-n-butoxymethyl phenylacetamide. (XXXVII, R = $C_6H_5.CH_2-$)
R' = C_4H_9-)

Prepared as above from N-hydroxymethyl phenylacetamide (0.5 g.), n-butyl alcohol (2 c.c.) and 50% butanolic hydrochloric acid (0.05 c.c.). The crystalline residue was recrystallised from light petroleum (b.p. $60-80^{\circ}$) giving colourless leaflets (0.5 g.), m.p. 56° . (Found: C, 70.7; H, 8.4; N, 6.4. $C_{13}H_{19}O_2N$ requires C, 70.6; H, 8.6; N, 6.4).

Ether of (A) and N-benzoyl serine ethyl ester.

(XXXVIII, R = $C_6H_5.CH_2-$, R' = H,

R'' = $-CH_2.CH(NH.CO.C_6H_5).CO_2.C_2H_5$)

A mixture of N-benzoyl serine ethyl ester (1 g.), (Erlenmeyer, Annalen, 1904, 337, 251), hydroxymethyl amide (A) (0.8 g.), acetone (4 c.c.) and concentrated hydrochloric acid (0.05 c.c.) was warmed in a water bath until complete solution occurred. On standing overnight a solid crystallized (0.43 g.) which melted over the range $155-220^{\circ}$.

Fractional crystallisation yielded the ether; colourless leaflets, m.p. 182-183°. (Found: C, 62.7; H, 6.0; N, 10.2. $C_{23}H_{27}O_6N_3$ requires C, 62.6; H, 6.1; N, 9.5).

Ether of N-hydroxymethyl phenylacetamide and N-benzoyl serine ethyl ester. (XXXVII, $R = C_6H_5.CH_2-$,



A mixture of N-benzoyl serine ethyl ester (2 g.) and N-hydroxymethyl phenylacetamide (1.6 g.) was warmed until dissolved with acetone (10 c.c.) acidified with concentrated hydrochloric acid (0.05 c.c.). Removal of the solvent under reduced pressure at room temperature left a greasy solid residue which was recrystallised from dilute alcohol and washed with ether, giving colourless needles (1.05 g.), m.p. 110°. (Found: C, 65.3; H, 6.3; N, 6.9. $C_{21}H_{24}O_5N_2$ requires C, 65.6; H, 6.3; N, 7.3).

Alcoholysis of a benzyl ether to an ethyl ether

A mixture of the benzyl ether (XXXVIII, $R = R'' = C_6H_5.CH_2-$, $R' = H$) (0.2 g.), ethyl alcohol (2 c.c.) and concentrated hydrochloric acid (0.1 c.c.) was warmed until solution occurred. On cooling a solid crystallised (0.1 g.) which was filtered and washed with ether. M.p. 157°, raised to 158° on crystallising from

water. This did not depress the melting point of a sample of the ethyl ether (XXXVIII, $R = C_6H_5.CH_2-$, $R' = H$, $R'' = C_2H_5-$).

Acid treatment of ethyl ether (XXXVIII, $R = C_6H_5.CH_2-$, $R' = H$, $R'' = C_2H_5-$) in a similar manner to that described for hydroxymethyl phenylacetyl glycol amide (p.80) gave methylene bis phenylacetyl glycol amide in 50% yield.

Reaction of the ethers with β -naphthol.

The above ethers condensed with β -naphthol in yields of about 50% under the same conditions as for the hydroxymethyl amides (p.77).

N-ethylthiomethyl phenylacetyl glycol amide.

(XXXIX, $R = C_6H_5.CH_2.CO.NH.CH_2-$, $R' = C_2H_5-$)

Ethyl mercaptan (5 c.c.), hydroxymethyl amide (A), (1.5 g.) and concentrated hydrochloric acid (0.05 c.c.) were warmed to 60-70° in a sealed tube for fifteen minutes. During this time the solid did not dissolve. After three hours the mixture was filtered and washed with ether. Recrystallisation from water gave colourless plates (1.75 g.) with a faint garlic odour, m.p. 127°. (Found: C, 58.9; H, 6.6; N, 10.7; S, 11.6. $C_{13}H_{18}O_2N_2S$ requires C, 58.6; H, 6.8; N, 10.5; S, 12.0).

N-ethylthiomethyl phenylacetyl-dl-alanylamine.

(XXXIX, $R = C_6H_5.CH_2.CO.NH.CH(CH_3)-$, $R' = C_2H_5-$)

A solution of hydroxymethyl amide (B) (0.5 g.) and concentrated hydrochloric acid (0.1 c.c.) in ethyl mercaptan (7 c.c.) was allowed to stand at room temperature for 2 days. After evaporation of excess ethyl mercaptan a solid remained (0.5 g.), m.p. 120-125°, which on recrystallisation from dilute methanol gave colourless prisms m.p. 124-125°. (Found: C, 59.8; H, 7.3; N, 9.6; S, 11.3. $C_{14}H_{20}O_2N_2S$ requires C, 60.0; H, 7.1; N, 10.0; S, 11.4).

N-ethylthiomethyl benzamide.

(XXXIX, $R = C_6H_5-$, $R' = C_2H_5-$)

Prepared as above from hydroxymethyl benzamide (1 g.), concentrated hydrochloric acid (0.1 c.c.) and ethyl mercaptan (7 c.c.). The product was extracted with ether and on evaporation gave a solid (1 g.), m.p. 57°. Recrystallisation from dilute methanol gave colourless prisms, m.p. 74-75°. (Found: C, 61.6; H, 6.7; N, 7.2; S, 16.3. $C_{10}H_{13}ONS$ requires C, 61.6; H, 6.7; N, 7.2; S, 16.4).

The thio ethers condensed with β -naphthol to give 1-acylamino-2-naphthols (XXIX) after 3 hours boiling in acidified alcohol.

3-benzamidomethyl-p-cresol. (XXVIII, R = C₆H₅-)

- (1) A solution of N-hydroxymethyl benzamide (0.5 g.) and p-cresol (0.5 g.) in alcohol (2 c.c.) acidified with concentrated hydrochloric acid (0.05 c.c.) was boiled under reflux for 20 minutes. On standing overnight, colourless crystals separated (0.3 g.).
- The mother liquors after standing a further 5 days deposited a second crop of crystals (0.1 g.). These were added to the first crop and together recrystallised from methylated spirits, giving colourless prisms m.p. 160°. (Found: C, 74.5; H, 6.0; N, 6.1. C₁₅H₁₅O₂N requires C, 74.7; H, 6.2; N, 5.8).

(2) Also prepared by warming a mixture of N-hydroxymethyl benzamide (0.5 g.), p-cresol (2 g.) and concentrated hydrochloric acid (0.05 c.c.) until a clear solution was obtained; this was allowed to stand at room temperature overnight. The addition of ether to the solution precipitated colourless crystals (0.5 g.), m.p. 160° undepressed in a mixture with a sample prepared by method (1).

3-phenylacetylglucylamidomethyl-p-cresol.



- (1) A solution of N-hydroxymethyl phenylacetylglucyl amide (0.5 g.) and p-cresol (0.5 g.) in alcohol (1 c.c.)

acidified with concentrated hydrochloric acid (0.1 c.c.) was boiled under reflux for 20 minutes. The solution, which on cooling did not deposit any solid material, was distilled in steam until the p-cresol had been completely removed. The residue, an oil, was separated from the aqueous layer, taken up in a little alcohol and allowed to stand at room temperature for a day. The crystals which separated (.35 g.), m.p. 115-140°, gave colourless prisms, m.p. 154-155°, after several recrystallisations from methylated spirits. (Found: C, 68.9; H, 6.3; N, 8.6. $C_{16}H_{20}O_3N_2$ requires C, 69.2; H, 6.4; N, 8.9).

(2) Also prepared by warming N-hydroxymethyl phenylacetyl glycol amide (0.2 g.), p-cresol (2 g.) and concentrated hydrochloric acid (0.05 c.c.) until all in solution. The p-cresol was removed by distillation under reduced pressure and the tarry residue (0.2 g.) solidified by scratching and warming with benzene; m.p. 140° raised to 155° by recrystallisation from methylated spirits and not depressed on mixing with a sample from (1).

Attempted condensations between N-hydroxymethyl amides
and N-benzoyl tyrosine.

N-benzoyl tyrosine was prepared as described by Erlenmeyer and Halsey (Annalen, 1899, 307, 138).

(1) At room temperature. A solution of N-benzoyl tyrosine (0.2 g.) and N-hydroxymethyl benzamide (0.2 g.) in ethyl alcohol (1 c.c.) acidified with concentrated hydrochloric acid (0.05 c.c.) was left to stand at room temperature for two weeks during which time the solution remained clear. The addition of water (2 c.c.) to the alcoholic solution caused precipitation of an oil which gave a white crystalline material (A) on making alkaline with 2N caustic soda. The crystals (0.15 g.) were removed by filtration and the filtrate made acid to Congo red with 2N hydrochloric acid. This caused an oil to separate which solidified on scratching (B), (0.15 g.)

(A) Recrystallised from methylated spirits gave colourless needles melting at 210° , undepressed in a mixture with methylene bis benzamide.

(B) Recrystallisation from water gave colourless prisms m.p. 189° which was not depressed on mixing with a sample of N-benzoyl tyrosine.

(2) Boiled for 20 minutes. The above experiment was repeated but the treatment modified to 20 minutes boiling under reflux in place of standing at room temperature. Similar results were obtained.

(3) Boiling for 20 minutes a solution of N-hydroxymethyl phenylacetyl glycol amide (0.5 g.) and N-benzoyl tyrosine (0.5 g.) in alcohol (2 c.c.) acidified with concentrated hydrochloric acid (0.1 c.c.) yielded on cooling a solid which melted over the range 154-230°. Recrystallisation from water yielded an insoluble portion melting at 260° undepressed in a mixture with a sample of methylene bis phenylacetyl glycol amide, and a soluble portion which crystallized on cooling to colourless plates m.p. 159° undepressed on mixing with N-ethoxymethyl phenylacetyl glycol amide (XXXVIII, $R = C_6H_5 \cdot CH_2-$, $R' = H$, $R'' = C_2H_5-$).

(4) Boiling for 5½ hours a repeat of (3) gave a similar result.

Treatment of N-benzoyl tyrosine with formaldehyde

(A) Acid solution

(1) The unchanged starting material was recovered after boiling a suspension of N-benzoyl tyrosine (0.2 g.) in a salt hardening liquor (see p.79) (2 c.c.) for a quarter of a minute and then allowing to stand at room temperature for 24 hours.

(2) A solution of N-benzoyl tyrosine (0.5 g.) and concentrated hydrochloric acid (0.2 c.c.) in methylated spirits (2 c.c.) after boiling for 20 minutes gave no solid material on standing. The unchanged starting material (0.45 g.) was recovered as a tar after evaporation of the solvent and was purified by dissolving in sodium carbonate solution and precipitating with dilute hydrochloric acid.

(B) Alkaline solution

N-benzoyl tyrosine (1 g.) was dissolved in a solution of caustic soda (0.2 g.) in water (1 c.c.) and 40% formaldehyde was added. After standing at room temperature for two weeks no solid material had crystallised. Evaporation of the solvent in vacuo over concentrated sulphuric acid left a sticky residue which was taken up in a little water, the solution filtered to remove a trace of solid material, and hydrochloric acid

added to the filtrate until acid to Congo red. This caused precipitation of a white solid (0.6 g.), m.p. 160-165° (decomp.). Recrystallisation several times from water gave small plates m.p. 175-176° with decomposition. (Found: C, 62.8; H, 5.5; N, 4.2. $C_{18}H_{19}O_6N$ requires C, 62.6; H, 5.5; N, 4.1).

Di-N-benzamidomethyl methylamine.(XLIII, R = C₆H₅-, R' = CH₃-)

N-hydroxymethyl benzamide (5 g.) and a 23% solution of methylamine in glacial acetic acid (5 g.) were dissolved in glacial acetic acid (50 c.c.) and kept at a temperature of 75° for 45 minutes, after which the solvent was removed under reduced pressure at 40°. Water (20 c.c.) was added to the oily residue and a small amount of insoluble matter removed (0.3 g.); this was shown to be benzamide. The solution was made alkaline to litmus with 2N caustic soda and stood overnight at 0°, this causing a colourless oil to separate. The oil was taken up in a little alcohol and water added until the solution became turbid. On standing, crystals were deposited (1 g.), m.p. 120-124°. Recrystallisation from dilute alcohol gave long colourless prisms m.p. 127-128°. (Found: C, 68.8; H, 7.0; N, 14.1; molecular wt., 311, (in camphor). C₁₇H₁₉O₂N₃ requires C, 68.7; H, 6.4; N, 14.1; molecular wt., 297).

Soluble in acetone, alcohol, benzene, hot carbon tetrachloride, ether and ethyl acetate.

Insoluble in light petroleum and water.

Di-N-benzamidomethyl-n-butylamine.(XLIII, $R = C_6H_5-$, $R' = C_4H_9-$)

(1) Prepared as above from N-hydroxymethyl benzamide (2 g.) and n-butylamine (1 g.) in glacial acetic acid (20 c.c.). Caustic soda produced a soft greasy mass (2.25 g.) which after several recrystallisations from alcohol gave colourless prisms (0.5 g.), m.p. 115° . (Found: C, 71.1; H, 7.5; N, 12.4. $C_{20}H_{25}O_2N_3$ requires C, 70.8; H, 7.4; N, 12.4).

(2) A suspension of N-hydroxymethyl benzamide (2 g.) and n-butylamine (0.5 g.) in water (5 c.c.) was heated to $90-100^{\circ}$ on a water bath for 12 hours. Throughout this time two phases were present. On standing, the lower oily phase solidified, giving a greasy material (2 g.). Recrystallisation twice from alcohol yielded colourless prisms (0.6 g.), m.p. $114-115^{\circ}$ undepressed on mixing with a sample prepared by method (1).

Di-N-phenylacetamidomethyl methylamine.(XLIII, $R = C_6H_5 \cdot CH_2-$, $R' = CH_3-$)

To a 23% solution of methylamine in acetic acid (2 g.) were added N-hydroxymethyl phenylacetamide (2 g.) and glacial acetic acid (20 c.c.) and the solution kept at 75° for 45 minutes. Evaporation of

the solvent gave a colourless oil which on the addition of water (50 c.c.) yielded a colourless crystalline material (A), (1.2 g.). The crystals were removed and the filtrate made alkaline to litmus with 2N caustic soda. Standing at 0° overnight caused the formation of a crystalline material (B), (0.2 g.) which melted at 140-145°. Recrystallisation from dilute methylated spirits gave colourless prisms, m.p. 144°. (Found: C, 70.3; H, 7.1; N, 13.0. $C_{19}H_{23}O_2N_3$ requires C, 70.2; H, 7.1; N, 12.9).

Compound (A) melted at 90-95°; recrystallisation from benzene yielded colourless needles, m.p. 98°. (Found: C, 63.8; H, 6.4; N, 7.0; molecular wt., 198 (in camphor). $C_{11}H_{13}O_3N$ requires C, 63.8; H, 6.3; N, 6.8; molecular wt., 207).

N-acetoxymethyl phenylacetamide. (XLIV, R = $C_6H_5.CH_2-$)

A solution of N-hydroxymethyl phenylacetamide (2 g.) in glacial acetic acid (20 c.c.) was heated to 75° for 30 minutes. Evaporation of the solvent at 40° under reduced pressure left a colourless oily residue which on the addition of water gave colourless prisms (1.5 g.), m.p. 90-95°. Recrystallisation from benzene raised the melting point to 98° which remained undepressed in a mixture with compound (A) of the previous experiment.

Condensation with β -naphthol under the usual conditions gave 1-phenylacetamidomethyl-2-naphthol in good yield.

Di-N-phenylpropionamidomethyl methylamine.

(XLIII, R = $C_6H_5 \cdot CH_2 \cdot CH_2-$, R' = CH_3-)

This was prepared in a similar manner to the phenylacetyl homologue from the N-hydroxymethyl phenylpropionamide (2 g.), a 20% solution of methylamine in acetic acid (0.86 g.) and acetic acid (20 c.c.). After evaporation, water precipitated solid (A), (1.2 g.). The filtrate on making alkaline gave (B), (0.3 g.), a greasy crystalline solid which on recrystallisation from dilute methylated spirits gave colourless plates melting at 118-119°. (Found: C, 71.2; H, 7.6; N, 12.1.

$C_{21}H_{27}O_2N_3$ requires C, 71.4; H, 7.7; N, 11.9).

Compound (A) melted at 55-62° and several crystallisations from a mixture of benzene and cyclohexane gave colourless prisms, m.p. 68-69°. (Found: C, 65.5; H, 6.8; N, 6.5. $C_{12}H_{15}O_3N$ requires C, 65.2; H, 6.9; N, 6.3). This is N-acetoxymethyl phenylpropionamide which may also be prepared by treating hydroxymethyl phenylpropionamide with acetic acid. (XLIV R = $C_6H_5 \cdot CH_2 \cdot CH_2-$)

All attempts to prepare di-N-phenylacetamidomethyl and di-N-phenylpropionamido methyl-butylamine (XLIII, R = $C_6H_5 \cdot CH_2-$; $C_6H_5 \cdot CH_2 \cdot CH_2-$, R' = C_4H_9-)

in either aqueous suspension or in acetic acid solution by methods previously described resulted in the isolation of only the free amide and the N-acetoxymethyl derivative.

Methylene bis piperidine. (XI, $-NR_2 = -N(CH_2)_5$)

40% aqueous formaldehyde (0.75 c.c.) was slowly added to piperidine (0.8 c.c.) whilst cooling in water. When the addition was complete, fused potassium carbonate was added which caused an oily layer to separate above the aqueous layer. This was removed, dried over fused potassium carbonate and distilled under reduced pressure giving a pale yellow oil (0.5 g.), b.p. $114^\circ/10$ m.m. Braun and Röver (Ber., 1903, 36, 1196) give $131^\circ/30$ m.m. Ehrenberg (J. pr. Chem., 1887, (2), 36, 117) gives $230^\circ/760$ m.m. (Found: N, 15.0. Calc. for $C_{11}H_{22}N_2$, N, 15.4).

Phenylacetylglycyl amidomethyl piperidine.

(XLVI, $R = C_6H_5.CH_2.CO.NH.CH_2-$, $-NR'_2 = -N(CH_2)_5$)
 (1) 40% aqueous formaldehyde solution (0.3 c.c.) was slowly added to piperidine (0.3 c.c.) cooled in water. To the solution was added phenylacetylglycyl amide (0.5 g.) and alcohol (1.5 c.c.) and the mixture boiled for 4 hours under reflux. On standing overnight the crystalline product separated which was filtered and washed with ether. Recrystallisation from water yielded

colourless plates (0.35 g.), m.p. 148° . (Found: C, 66.1; H, 7.8; N, 14.5. $C_{16}H_{23}O_2N_3$ requires C, 66.4; H, 8.0; N, 14.5).

(2) Also prepared from methylene bia piperidine (0.5 g.) and phenylacetyl glycol amide (0.5 g.) in alcohol (1.5 c.c.) in a similar manner, (0.35 g.); m.p. 148° , undepressed in a mixture with compound prepared by method (1).

Benzamidomethyl dibenzylamine.



Prepared as above from dibenzylamine (2.5 g.), 40% formaldehyde (1.5 g.), benzamide (1 g.) and alcohol (5 c.c.). The crude product was a colourless oil which turned to a semi-solid material (3.2 g.) on scratching and standing in vacuo over concentrated sulphuric acid. Several recrystallisations from methylated spirits gave long colourless prisms (1.3 g.), m.p. $145-146^{\circ}$. (Found: C, 80.1; H, 6.8; N, 8.9. $C_{22}H_{22}ON_2$ requires C, 80.0; H, 6.7; N, 8.5).

Phenylacetamidomethyl dibenzylamine.



Prepared similarly from 40% formaldehyde (0.8 g.), dibenzylamine (2 g.) and phenylacetamide (1 g.) in alcohol (2 c.c.). Greasy crystals (2 g.) were obtained which were recrystallised from methylated

spirits as colourless prisms, m.p. 99° . (Found: N, 8.0. $C_{23}H_{24}ON_2$ requires N, 8.1).

Preparation of model peptides

Phthalylglycyl chloride was prepared using a modification of Gabriel's method. (Ber., 1907, 40, 2648).

A suspension of powdered phthalyl glycine (40 g.) and phosphorus pentachloride (40 g.) in dry carbon tetrachloride (300 c.c.) was heated on a steam bath for 30 minutes until the evolution of hydrogen chloride had ceased. The remaining solid, a little phthalyl glycine, was filtered and the filtrate evaporated under vacuum leaving a light brown oil which solidified on cooling. The crude product (37 g.) was crystallized from a mixture of benzene and light petroleum (b.p. $60-80^{\circ}$) giving light brown needles, m.p. 83° . Gabriel (loc. cit.) gives m.p. $84-85^{\circ}$.

Phthalyl-dl-alanyl chloride was prepared by a similar method from phthalyl-dl-alanine (Gabriel, Ber., 1905, 38, 634) in 93% yield, m.p. $71-72^{\circ}$. Gabriel (Ber., 1908, 41, 247) gives m.p. 73° .

The following general methods are given by McGillivray (Ph.D. Thesis, Sheffield University, 1951):

Phthalylglycyl morpholide

Powdered phthalylglycyl chloride (29 g.) was added portionwise over a period of 20 minutes to an ice cooled and stirred solution of morpholine (50 g.) in dry carbon tetrachloride (300 c.c.). The contents of the flask were allowed to stand at room temperature for 20 minutes before filtering and washing with water to remove morpholine hydrochloride. The product (29 g.) was recrystallised from methylated spirits, yielding colourless prisms, m.p. 199-200° which was not depressed on mixing with an authentic sample.

Similarly prepared were:

Phthalylglycyl diethylamide from phthalylglycyl chloride (45 g.) and diethylamine (43 g.) in dry carbon tetrachloride (300 c.c.) yielding colourless prisms (21.5 g.) from methylated spirits, m.p. 150° undepressed in a mixture with an authentic sample.

Phthalylglycyl ethylamide from phthalylglycyl chloride (10 g.) and ethylamine (8 g.) in dry carbon tetrachloride (300 c.c.). Recrystallisation from methylated spirits yielded colourless needles (7.3 g.), m.p. 240°.

(Found: C, 62.2; H, 5.0; N, 12.0. $C_{12}H_{12}O_3N_2$ requires C, 62.1; H, 5.2; N, 12.1).

Phthalyl-dl-alanyl morpholide from phthalyl-dl-alanyl chloride (10 g.) and morpholine (8 g.) in dry carbon tetrachloride (100 c.c.). Recrystallisation from dilute methylated spirits gave colourless prisms (12.2 g.), m.p. 172°. (Found: C, 62.3; H, 5.5; N, 9.9. $C_{15}H_{16}O_4N_2$ requires C, 62.5; H, 5.6; N, 9.7).

Phthalyl-dl-alanyl diethylamide

Phthalyl-dl-alanyl chloride (12.4 g.) reacted in the usual manner with diethylamine (11 g.) in dry carbon tetrachloride (100 c.c.) gave a precipitate of diethylamine hydrochloride which was filtered and the filtrate concentrated to small bulk under reduced pressure. Cooling caused the product to crystallise from the solution. Recrystallisation from dilute methylated spirits yielded colourless hexagonal prisms (10 g.) melting at 85°. (Found: C, 65.5; H, 6.2; N, 10.2. $C_{15}H_{18}O_3N$ requires C, 65.7; H, 6.5; N, 10.2).

Removal of the phthalyl group was brought about as follows: (Ing & Manske, J.C.S., 1926, 2349; Sheehan and Frank, J.A.C.S., 1949, 71, 1856.)

Glycyl morpholide hydrochloride

A solution of phthalylglycyl morpholide (5.9 g.) and 1.09 N alcoholic hydrazine (90% hydrazine hydrate in

absolute alcohol), (21.5 c.c.) in absolute alcohol (100 c.c.) was boiled under reflux for 4½ hours. After being allowed to cool to 40° the solvent was removed under reduced pressure, leaving a greasy residue. Acidification of the residue with 2N hydrochloric acid (50 c.c.) caused precipitation of phthalhydrazide, the reaction being completed by warming at a temperature of 50-55° for 10 minutes. On cooling, the white precipitate was removed and the filtrate evaporated to dryness at 45° under reduced pressure. The crude product, which was hygroscopic, was recrystallised from absolute alcohol yielding colourless prisms (2.6 g.), m.p. 103-104°. McGillivray (loc. cit.) gives 106°.

The same method was applied in the following preparations:

Glycyl diethylamide hydrochloride

Phthalylglycyl diethylamide (31 g.) and 1.04 N alcoholic hydrazine (135 c.c.) in absolute alcohol (670 c.c.) on boiling for 6 hours gave colourless prisms (17 g.) melting at 97-98°. McGillivray (loc. cit.) gives 101°.

Glycyl ethylamide hydrochloride

Phthalylglycyl ethylamide (10 g.) and 1.05 N alcoholic hydrazine (43 c.c.) in alcohol (200 c.c.) after 7 hours boiling gave colourless prisms of glycine ethylamide hydrochloride (4.1 g.), m.p. 128-130° after recrystallisation from alcohol. von Braun and Münch (Ber., 60, 350) give m.p. 134°. (Found: Cl., 26.0. Calc. for $C_4H_{11}ON_2Cl$, Cl., 25.6).

dl-Alanyl diethylamide hydrochloride

Phthalyl-dl-alanyl diethylamide (9.8 g.) and 1.05 N alcoholic hydrazine (36 c.c.) in alcohol (100 c.c.) on boiling for 2 hours gave a greasy material which was purified by precipitation with ether from a concentrated alcoholic solution. The product (4.8 g.), colourless needles, melted at 136°. (Found: Cl., 20.5. $C_7H_{17}ON_2Cl$ requires Cl., 19.7).

Phenylacetylglycyl morpholide. (McGillivray, loc. cit.)

Phenylacetyl chloride (10.6 g.) was added portionwise over a period of 30 minutes to an ice cooled and stirred mixture of glycyl morpholide hydrochloride (9 g.), magnesium oxide (9 g.) and water (80 c.c.). When the addition was complete the stirring was continued for a further 15 minutes followed by standing at room

temperature for half an hour. The solid material, consisting of excess magnesium oxide and the reaction product, was filtered, stirred with water and the slurry made acid to Congo red with concentrated hydrochloric acid. The insoluble product was removed and washed with water. Recrystallisation from alcohol yielded colourless needles (8.2 g.), m.p. 170° , not depressed on mixing with an authentic sample.

The above method was used for the following model peptides:

Phenylacetylglycyl diethylamide. (McGillivray, loc. cit.)

(LII, $R = C_6H_5 \cdot CH_2-$, $R' = H$, $R'' = C_2H_5-$ = R''')

Glycyl diethylamide hydrochloride (6.2 g.); phenylacetyl chloride (7 g.); magnesium oxide (6 g.); water (30 c.c.). The product (4.8 g.) was recrystallised from dilute methyl alcohol giving colourless prisms, m.p. $87-89^{\circ}$ which was not depressed on mixing with an authentic sample.

Phenylacetylglycyl ethylamide.

(LII, $R = C_6H_5 \cdot CH_2-$, $R' = R'' = H$, $R''' = C_2H_5-$)

Phenylacetyl chloride (4.3 g.); glycyl ethylamide hydrochloride (3.6 g.); magnesium oxide (3.2 g.); water (25 c.c.). The product (5.6 g.) was

recrystallised from dilute methyl alcohol, giving colourless plates, m.p. 170° . (Found: C, 65.2; H, 7.3; N, 12.5. $C_{12}H_{16}O_2N_2$ requires C, 65.5; H, 7.3; N, 12.7).

Phenylacetyl-dl-alanyl diethylamide.

(LII, $R = C_6H_5 \cdot CH_2-$, $R' = CH_3-$, $R'' = R''' = C_2H_5-$)

Phenylacetyl chloride (4 c.c.); dl-alanyl diethylamide hydrochloride (4.3 g.); magnesium oxide (3 g.); water (25 c.c.). The product (5.7 g.) was recrystallised from dilute methyl alcohol in colourless prisms, m.p. 116° . (Found: C, 68.7; H, 8.5; N, 10.3. $C_{15}H_{22}O_2N_2$ requires C, 68.7; H, 8.4; N, 10.7).

Benzoylglycyl morpholide. (LII, $R = C_6H_5-$, $R' = H$,
 $-N(R'')R''' = -N(CH_2)_4=O$)

A mixture of glycyl morpholide hydrochloride (2.3 g.); benzoyl chloride (2.8 g.) and sodium bicarbonate (42 g.) in water (25 c.c.) was stirred until a solid material separated. The product was recrystallised from dilute methanol in colourless needles (1 g.), m.p. 150° . (Found: C, 63.2; H, 6.6; N, 10.9. $C_{13}H_{16}O_3N_2$ requires C, 62.9; H, 6.5; N, 11.3).

Treatment of model peptides with formaldehyde

(1) Aqueous potassium carbonate.

Conditions which gave hydroxymethyl primary amides (Einhorn, Annalen, 1905, 343, 207) had no effect on the model peptides.

(2) Potassium carbonate in dioxan.

A solution of phenylacetylglycyl morpholide (0.5 g.) and potassium carbonate (0.2 g.) in 40% aqueous formaldehyde (3 c.c.) and dioxan (4 c.c.) was kept in a water bath at 60° for half an hour. After standing for 9 hours at room temperature no solid material had crystallised. Concentration under vacuum yielded the starting product.

(3) Aqueous hydrochloric acid.

A suspension of phenylacetylglycyl morpholide (0.5 g.) in 40% formaldehyde (1 c.c.), water (2 c.c.) and concentrated hydrochloric acid (0.2 c.c.) was warmed in a water bath. After 5 minutes some undissolved material was filtered and the filtrate on cooling deposited colourless crystals. Evaporation of the mother liquors under reduced pressure gave a third crop. Each fraction was found to be the starting material.

(4) Alcoholic hydrochloric acid.

A solution of phenylacetyl glycol morpholide (0.5 g.) in 40% formaldehyde (3 c.c.), absolute alcohol (4 c.c.) and concentrated hydrochloric acid (0.2 c.c.) was kept at 60° for 5 hours. On standing overnight, crystals of the starting material were deposited. Evaporation of the mother liquors to dryness gave a small quantity of a light brown oil from which a solid separated. The solid on washing with alcohol was found to be a further quantity of starting material; the oil smelt strongly of phenyl acetic acid and when dissolved in 2N caustic soda, had the odour of an amine.

(5) A similar treatment to (4) using dry dioxan in place of alcohol, gave the same result. In some cases the solid material formed as a gelatinous mass which had to be separated by centrifuge. Increasing the quantity of formaldehyde up to five times had no effect on the result.

(6) Formic acid.

40% aqueous formaldehyde (1.2 g.) at 60° was added to a solution of phenylacetyl glycol morpholide (0.5 g.) in formic acid (3 g.) also at 60° and the mixture kept at that temperature for 1½ hours. The clear solution was then poured into water (25 c.c.)

which on standing did not deposit any solid material. Concentration under vacuum at 40° produced, on standing overnight, a crop of crystals (0.45 g.) which was found to be the pure starting material.

(7) Treatment with polyoxymethylene in pyridine.

Phenylacetylglycyl morpholide (0.5 g.) was heated in an open tube with polyoxymethylene (0.1 g.) and pyridine (1.5 c.c.) to $120-130^{\circ}$ for 30 minutes. On cooling, a solid separated which was removed and washed with ether. Addition of ether to the mother liquors gave a second fraction. A third fraction was obtained after removal of the ether by making the pyridine solution acid to Congo red with dilute hydrochloric acid and concentrating under reduced pressure. The solid which separated was filtered and washed with ether. Mixed melting points showed these three fractions to be the starting material (total weight, 0.48 g.).

The same material was recovered even after boiling under reflux for 5 hours.

(8) Alkaline treatment followed by β -naphthol.

The solution from (1), ^{CONTAINING 0.5% MODEL PEPTIDE} after warming, was treated with β -naphthol (0.5 g.) and concentrated hydrochloric acid (0.2 c.c.) and the mixture boiled

in alcohol (3 c.c.) for 20 minutes. On cooling, two layers separated and the top layer was removed. Further standing caused solids to crystallise from both layers. Top layer (A); bottom layer (B).

(A) Recrystallisation from alcohol gave the starting material. Water on the mother liquors produced a crystalline solid melting at 190° , undepressed in a mixture with methylene bis β -naphthol.

(B) Recrystallisation from alcohol gave the starting material and water on the filtrate gave methylene bis β -naphthol.

(9) Alkaline treatment followed by β -naphthol, hydrolysis and treatment with p-toluene sulphonyl chloride.

Repeated as for experiment (8) up to boiling with β -naphthol. On cooling, a dark red oil separated which solidified on standing overnight. The dark red crystals were dissolved in alcohol (5 c.c.), concentrated hydrochloric acid (2 c.c.) was added and the solution boiled for 3 hours. Cooling caused a further dark red oil to separate which, on standing overnight, solidified to a glass. (Solid A). The supernatant liquid was decanted and diluted with water; this precipitated an oil which solidified on standing. (Solid B). The supernatant liquid was again decanted and the addition of

2N caustic soda to the acid solution did not produce a precipitate. p-Toluene sulphonyl chloride (0.5 g.) in a few c.c. acetone was now added and the solution warmed in a water bath for a minute and kept alkaline to litmus by the addition of 2N caustic soda. Standing a day caused a few needle-like crystals of p-toluene sulphonyl chloride to separate; acidification produced no further material.

Solid A. Dissolved in 2N caustic soda and re-precipitated with acid. Sodium fusion test on the solid gives negative result for nitrogen. The acid filtrate was treated with p-toluene sulphonyl chloride but yielded no derivative.

Solid B. Treated in a similar way to Solid A likewise gave a negative result.

(10) Experiments (1) to (6) were performed on other model peptides which included: phenylacetylglucyl diethylamide, phenylacetylglucyl ethylamide and phenylacetyl-dl-alanyl diethylamide. In no case was any positive evidence of reaction obtained.

(11) Experiments (1), (8) and (9) were repeated using N-ethyl benzamide in place of phenylacetylglucyl morpholide, also with negative results.

Estimation of free formaldehyde present after treatment
of model peptides.

In acid solution. The model peptide (0.5 g.) was dissolved in water (about 50 c.c.) by warming. To the cooled solution was added standard aqueous formaldehyde (10 c.c., containing 0.1905 g. formaldehyde) and concentrated hydrochloric acid (5 c.c.) and the volume made up to 100 c.c. with water. Samples (10 c.c.) were analysed periodically for free formaldehyde using Brady's reagent (see p.116).

In alkaline solution. To a solution of the model peptide (0.5 g.) in water (about 80 c.c.) was added standard formaldehyde solution (10 c.c., containing 0.1905 g. formaldehyde) and potassium carbonate (0.02 g.) and the volume made up to 100 c.c. Aliquot portions (10 c.c.) were analysed as before for formaldehyde after various time intervals.

Compounds treated in this way included ethyl benzamide and phenylacetyl glycol diethylamide and in neither case was there any uptake of formaldehyde detected even after having stood in contact at room temperature for 21 days.

Stability of formaldehyde derivatives.

Brady's reagent (Brady, J.C.S., 1931, 757) was prepared by dissolving 2:4-dinitrophenyl hydrazine (4 g.) in warm 2N hydrochloric acid (1 litre). On cooling, a trace of formaldehyde was added to give a slight precipitate of the 2:4-dinitrophenyl hydrazone (D.N.P.). The reagent was allowed to stand at room temperature for 24 hours and after removal of the solid matter, was ready for use.

Determinations of the rate of precipitation of the D.N.P. by 0.2% formaldehyde (10 c.c.) from Brady's reagent (50 c.c.) showed that after being in contact for 15 minutes 99.9% of the total formaldehyde obtainable after 24 hours was precipitated as the D.N.P., hence it was considered that 15 minutes would be sufficient time to allow for precipitation in the following determinations.

It was found that prolonged contact (7 days) of a 0.2% formaldehyde solution with 2N hydrochloric acid or a 0.08% solution of potassium carbonate had no effect on the amount of D.N.P. precipitated after 15 minutes. In the presence of N caustic soda, a decrease in the formaldehyde detectable is observed after 48 hours.

It was also found that the solubility of the D.N.P. in 2N hydrochloric acid at room temperature is 0.5 m.g./10 c.c., hence errors due to solubility will be negligible.

Dissoiation of formaldehyde derivatives in alkali.

(1) Hydroxymethyl amides.

A weighed sample of hydroxymethyl amide (0.5 g.) was dissolved in water (40 c.c.) by warming. After cooling, potassium carbonate (0.02 g.) was added and the volume made up to 50 c.c. Aliquot samples (10 c.c.) were periodically removed and analysed for free formaldehyde with Brady's reagent. The precipitated D.N.P. was allowed to stand for 15 minutes, then filtered in a sintered glass crucible and dried at 80° to constant weight. Free formaldehyde was expressed as a percentage of the total available and the equilibrium constant was calculated from the amount present at equilibrium.

The following is a sample result for hydroxymethyl benzamide:

Weight of hydroxymethyl amide	=	0.5429 g.
Weight of potassium carbonate	=	0.02 g.
Volume of solution	=	50 c.c.
Samples taken	=	10 c.c.

Time after mixing (hrs.)	5	17	51	120	15 days
Wt. of D.N.F. precipitated (g.)	0.0387	0.0624	0.0709	0.0731	0.0734
% Total formaldehyde	23.6	36.1	43.3	44.5	44.8

1 g. D.N.F. \equiv 0.1316 g. formaldehyde

% formaldehyde =

$$\frac{\text{Wt. of D.N.F.} \times 0.1316 \times \text{molecular wt. of hydroxymethyl amide}}{\text{Wt. of hydroxymethyl amide in solution} \times \text{molecular wt. of formaldehyde}}$$

$$\text{Equilibrium constant } K_0 = \frac{1}{V} \cdot \frac{\frac{w_f^2}{MW_f^2}}{\frac{w_a}{MW_a} - \frac{w_f}{MW_f}} = \underline{\underline{2.8 \times 10^{-5}}}$$

Where V = volume of sample (10 c.c.)

w_f = wt. of formaldehyde in equilibrium mixture.

w_a = wt. of hydroxymethyl amide originally in sample.

MW_f = molecular wt. of formaldehyde.

MW_a = molecular wt. of hydroxymethyl amide.

(2) Ethers and thio ethers of hydroxymethyl amides and N-acetoxymethyl phenylacetamide.

A weighed quantity of material (0.3 - 0.5 g.) was suspended in N caustic soda (50 c.c.) and the suspension shaken periodically. Aliquot samples (10 c.c.) were made acid with concentrated hydrochloric acid, then analysed for free formaldehyde in the usual manner. Owing to the insolubility of the derivatives and the occurrence of the Cannizzaro reaction of formaldehyde at this pH, the above method will only give a rough comparison of the stability of the compounds used.

(3) Alkali has no effect on cyclic methyleneimines, methylene big secondary amines and amine-hydroxymethyl amide condensation products since complete recovery of the above compounds was obtained after having been in contact with N caustic soda at room temperature for 3 days.

Uptake of formaldehyde by amides in alkaline solution.

To a solution of an amide (0.5 g.) and potassium carbonate (0.02 g.) in water (about 80 c.c.) was added standard formaldehyde solution (10 c.c. containing 0.255 g. formaldehyde as estimated by Brady's reagent) and the total volume made up to 100 c.c.. Samples (10 c.c.) were taken after varying intervals and analysed for free

formaldehyde as before. From the value obtained when the solution had reached equilibrium, the reciprocal of the equilibrium constant was calculated.

$$\frac{1}{K_c'} = \frac{1}{V} \cdot \frac{w_f'' \left[\frac{w_a}{MW_a} - \left(\frac{w_f' - w_f''}{MW_f} \right) \right]}{w_f' - w_f''}$$

Where V = volume of sample (10 c.c.)

w_a = wt. of amide originally in sample.

w_f' = wt. of formaldehyde originally in sample.

w_f'' = wt. of free formaldehyde in sample.

MW_a = molecular wt. of amide.

MW_f = molecular wt. of formaldehyde.

Dissociation of formaldehyde derivatives in acid.

(1) Hydroxymethyl amides and acetoxymethyl

phenylacetamide.

A solution (50 c.c.) of hydroxymethyl amide (0.5 g.) was made up in 2N hydrochloric acid and aliquot samples (10 c.c.) were analysed for formaldehyde as previously described. The estimation was complicated by precipitation of the methylene bis amide which was filtered before sampling.

A freshly prepared acid solution was heated for 15 minutes on a water bath with Brady's reagent and the D.N.P. which crystallised on cooling was weighed. This gave a value of about 90% of the total formaldehyde available.

The determination at room temperature was repeated with acetoxymethyl phenylacetamide which took about 3 hours to completely dissolve and which precipitated only a trace of methylene bis amide even after 16 days.

(2) Methylene bis amines, cyclic methyleneimines and hydroxymethyl amide-amine condensation products.

(A) To a weighed sample (0.1 g.), 2N hydrochloric acid was rapidly added and after standing for a specified time period, Brady's reagent was added (50 c.c.) and free formaldehyde determined.

(B) To a weighed sample (0.1 g.) suspended in water (10 c.c.), 2 equivalents of hydrochloric acid (10 c.c.) were added dropwise over a period of 30 minutes followed by Brady's reagent (50 c.c.). Free formaldehyde was estimated as the D.N.P. as usual.

Dissociation of formaldehyde derivatives in neutral solution.

Ethers, acetoxy derivatives and secondary amine

derivatives of hydroxymethyl amides do not give formaldehyde detectable with Brady's reagent on boiling with water. Hydroxymethyl amide-primary amine derivatives rapidly release formaldehyde on boiling with water.

Results

Dissociation of hydroxymethyl amides in alkaline solution.

Age of total formaldehyde

Hydroxymethyl amide	5 hrs.	17 hrs.	51 hrs.	120 hrs.	212 hrs.	15 days	K_0
Benzamide	23.6	38.1	43.3	44.5	-	44.8	2.8×10^{-5}
Benzamide (0.04 g. K_2CO_3)	33.5	39.9	40.4	42.3	-	42.2	-
Phenyl-acetamide	-	37.7	-	42.5	42.2	-	2.3×10^{-5}

	54 hrs.	98 hrs.	142 hrs.	21 days	K_0
Phenylacetyl glycol amide	56.1	59.5	59.8	59.5	8.1×10^{-5}
Phenylacetyl alanyl amide	49.5	53.3	53.8	53.2	7.3×10^{-5}

	26 hrs.	68 hrs.	115 hrs.	21 days
Succinamide	6.8	9.4	9.9	10.0

Amides and formaldehyde in alkaline solution

	Time taken to reach equilibrium	$\frac{1}{K_0}$
Benzamide	48 hrs.	1.6×10^{-5}
Phenylacetyl-glycyl amide	70 hrs.	4.5×10^{-5}

In suspension at room temperature with caustic soda

	48 hrs.	9 days
R.CO.NH.CH ₂ .O.CO.CH ₃ (N. NaOH)	19.1	22.3
R'.CO.NH.CH ₂ .O.C ₂ H ₅ (N. NaOH)	14.9	55.8
R'.CO.NH.CH ₂ .S.C ₂ H ₅ (N. NaOH)	none	19.0
R'.CO.NH.CH ₂ .S.C ₂ H ₅ (2N. NaOH)	15.1	-

In acid solution

Hydroxymethyl amide	20 hrs.	50 hrs.	112 hrs.	250 hrs.	16 days	21 days	Heating
Benzamide	18.3	35.4	41.6	43.1	-	-	91.2
Phenylacetyl glycyl amide	8.8	-	30.4	40.7	-	-	91.8
Succinamide	10.1	19.1	24.5	-	-	37.8	-
Acetoxymethyl phenyl-acetamide	13.8	24.3	44.8	65.0	73.0	-	-



	2N hydrochloric acid rapidly			2 equivalents of hydrochloric acid over 30 minutes
	5 mins.	1 hr.	5 hrs.	
$(C_6H_5 \cdot CH_2 \cdot N=CH_2)_3$	18.3	27.4	-	59.5
$[(C_6H_5 \cdot CH_2)_2N]_2CH_2$	-	20.5	-	86.4
$(C_6H_5 \cdot CO \cdot NH \cdot CH_2)_2N \cdot C_4H_9$	none	-	16.5	none
$C_6H_5 \cdot CO \cdot NH \cdot CH_2 \cdot N \cdot (C_7H_7)_2$	none	-	none	none