

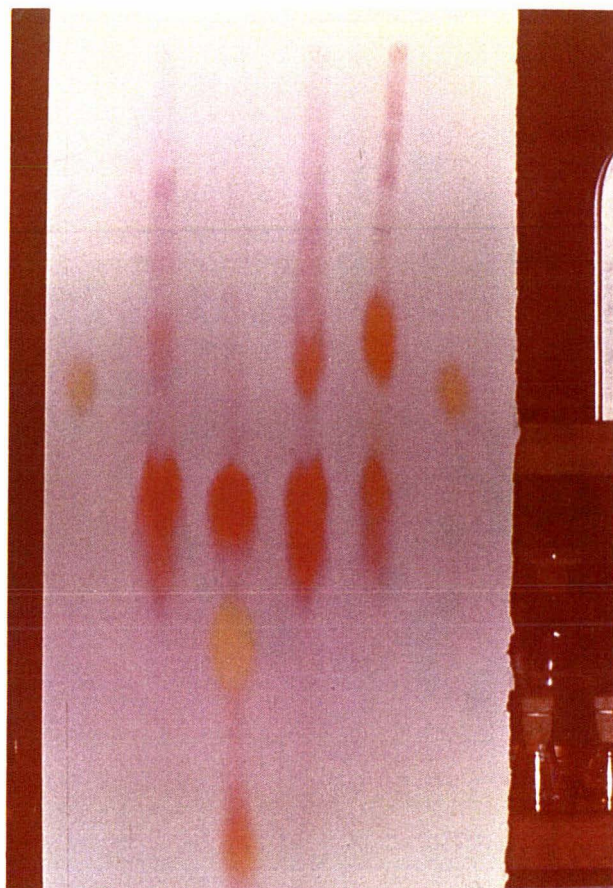
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"THE ACTION OF AMMONIA ON CARBOHYDRATES
AND RELATED CARBONYL COMPOUNDS"

A thesis
presented in partial fulfilment of the requirements
for
the degree of Doctor of Philosophy
at Massey University of Manawatu,
Palmerston North, New Zealand.

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1965



Paper Chromatogram showing Imidazoles formed from: (L to R)
(i) Imidazole (marker); (ii) Glyceraldehyde/ NH_3 ;
(iii) Pyruvaldehyde/ NH_3 ; (iv) Dihydroxyacetone/ NH_3 ;
(v) Hydroxypyruvaldehyde/ NH_3 ; and (vi) Imidazole (marker).

ACKNOWLEDGEMENTS

The author wishes to thank Dr. E.L. Richards,
Dr. R.W. Bailey and Dr. J.C. Hawke for their
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(i)

ABSTRACT

The chromatography of imidazoles has been studied and a method developed for their quantitative estimation.

The following facts have been brought to light:-

(i) Formaldehyde does not form imidazoles at room temperature in ammoniacal solution.

(ii) From the complex mixture resulting from the interaction of glyoxal with aqueous ammonia imidazole and 2,2'-bis-imidazole have been isolated and identified, while 2-formyl-imidazole has been tentatively identified.

(iii) Glycolaldehyde reacts with aqueous ammonia to form imidazole and 2-hydroxymethylimidazole.

(iv) DL-Glyceraldehyde reacts with aqueous ammonia to form a complex mixture of neutral and basic compounds. Dihydroxyacetone, glucose, fructose, mannose, arabinose, lyxose and xylose have been tentatively identified by paper chromatography while ribose was suspected in low concentration. 2-Hydroxymethyl-4(5)-methylimidazole, 4(5)-methylimidazole, 4(5)-(2-hydroxyethyl)imidazole and 4(5)-hydroxymethylimidazole have been isolated and characterised, and their orders and rates of formation studied.

(v) Pyruvaldehyde reacts exothermically with concentrated ammonia solution to form four imidazolic compounds. Three of these have been isolated and characterised as 2-acetyl-

(ii)

4(5)-methylimidazole, 2,4(5)-dimethylimidazole and 4(5)-methylimidazole. The latter two compounds were formed in approximately equimolecular proportions. These results fail to confirm Bernhauer's finding that pyruvaldehyde cannot act as a source of formaldehyde in imidazole formation.

(vi) Hydroxypyruvaldehyde browns rapidly in aqueous ammonia forming 2-hydroxymethyl-4(5)-methylimidazole, 4(5)-methylimidazole and 4(5)-hydroxymethylimidazole. The yields of the latter two compounds have been found to be higher than from a similar mixture of dihydroxyacetone with ammonia.

(vii) Both diacetyl and acetoin react with ammonia to form 2,4,5-trimethylimidazole.

(viii) 4(5)-(2-Hydroxyethyl)imidazole has been tentatively identified from the mixture resulting from the interaction of 1,4-dihydroxybutan-2-one with aqueous ammonia.

(ix) Arabinose reacts with aqueous ammonia to form a complex mixture of imidazoles from which 4(5)-methylimidazole has been isolated and identified.

(x) A chromatographic study has been carried out to determine the orders of formation of imidazoles resulting from the interactions of a number of carbohydrates and their degradation products with aqueous ammonia. Arising from this study have come the following main results:-

(a) It appears that, contrary to the findings of Komoto, a number of imidazoles with low R_f values (probably polyhydroxy-

(iii)

alkyl-substituted) are formed more rapidly than 4(5)-methyl-imidazole from hexose sugars with ammonia.

(b) Differently linked reducing disaccharides give markedly different patterns of imidazoles under ammoniacal conditions.

(xi) As a result of (b) above, a micro-method has been developed for determination of the position of the glycosidic link in reducing hexose disaccharides and homogeneously-linked oligosaccharides.

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INTRODUCTION

INTRODUCTION

Although the interaction of carbohydrates with ammonia has been intensively studied^{44, 49, 55-6, 70-1, 100-1} the mechanisms of many of the complex reactions involved remain obscure. It has proved difficult, and at times impossible, to identify sugar breakdown products in ammoniacal medium, but a knowledge of the nature of the imidazolic compounds formed in such reactions is of assistance in the prediction of the structures of these carbohydrate fragments. e.g. the identification of 4(5)-methylimidazole from the reaction of glucose with ammonia indicates that pyruvaldehyde and formaldehyde are initially formed from the hexose.⁴²

The present work was initiated with a view to studying the imidazoles formed from the interaction of ammonium hydroxide with a number of two-, three- or four- carbon α -dicarbonyl- or α -hydroxycarbonyl compounds. The results from such a study might be expected to shed some light on the complex transformations undergone by sugars in ammoniacal solution. A number of compounds (glyoxal,²⁴ glycer-aldehyde,⁷⁰ dihydroxyacetone,⁷⁰ pyruvaldehyde⁸ and diacetyl⁷⁴) had previously been studied under ammoniacal conditions, usually in the presence of metallic ions, but in most cases only the major reaction products had been elucidated. It was hoped that by the use of modern micro-methods a further study of these systems might result in the identification

of some of the minor reaction products. Reactions between ammonia and glycolaldehyde, hydroxypyruvaldehyde, 1,4-dihydroxybutan-2-one and acetoin have not been reported and were considered worthy of study.

It was proposed to isolate and identify as many imidazolic compounds as possible from these mixtures, to obtain where possible a measure of the quantities formed and to study the order of formation. As methods for the quantitative separation of small amounts of imidazoles from complex mixtures were unsuitable, it was found necessary to develop a colorimetric technique to estimate these compounds, and in order to provide a rapid, routine means of identification of imidazoles, a number of these compounds were synthesised and their chromatographic behaviour studied.

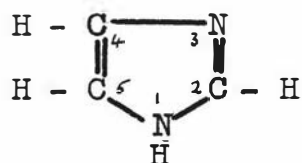
In the following sections of the introduction it is proposed to outline work which has been accomplished by a number of workers in the field of interaction of amino compounds with α -dicarbonyl and α -hydroxycarbonyl compounds. As much of the present project has been involved with imidazolic compounds, a section on "imidazole chemistry" has been included. This briefly outlines properties of the imidazole nucleus relevant to the present project i.e. nomenclature, basic and acidic nature, stability, aromatic properties and tautomerism.

The second section, dealing with the alkaline degradation of carbohydrates, is included because this reaction is of

major importance in ammoniacal solution. Sections III and IV review the reactions of carbonyl compounds with ammonia and other amino compounds.

I. CHEMISTRY OF THE IMIDAZOLES.

The term 'imidazole' refers to a five-membered heterocyclic ring containing a tertiary nitrogen atom, an imino nitrogen and two double bonds. The correct numbering of the imidazole ring is shown below.

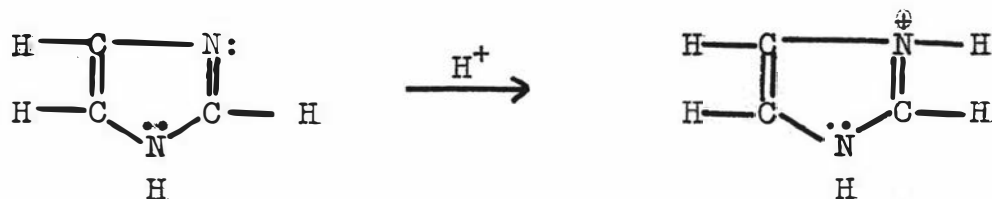


When a substituent is introduced into the 4- or 5- position the numbering becomes complex, and such a compound must be designated as 4 (or 5)-substituted because of the tautomerism exhibited by imidazoles.



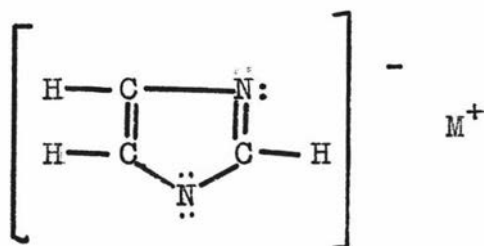
Substitution of the imino nitrogen eliminates the possibility of tautomerism.

Imidazoles are monoacidic bases having the ability to form crystalline salts with acids e.g. picrates, nitrates, chloroplatinates, oxalates etc. This basic nature of the imidazole nucleus is due to the ability of the tertiary nitrogen to accept a proton.



The electron-releasing properties of a methyl group introduced into the ring stabilises the imidazolium ion and increase the basic strength, while electron attracting groups decrease the basic strength.⁵² A comprehensive table of the known pK_a values for imidazole derivatives has been compiled by Albert.¹

Although mainly basic in character, imidazole also exhibits weakly acidic properties when it forms metal salts many of which are insoluble in water (e.g. Ag^+ , Cu^+ , and Zn^{2+}) and provide a means of precipitating imidazoles from solution.

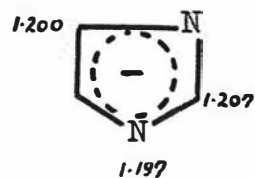
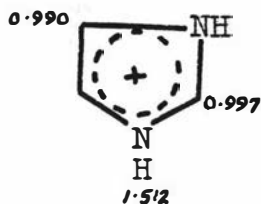
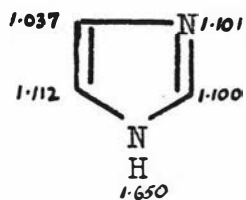


The pronounced chemical stability of the imidazole nucleus is remarkable and is due to its high degree of aromatic character. Catalytic hydrogenation is strongly resisted as is chromium trioxide oxidation.¹⁰⁴ Permanganate and

peroxide, however, readily convert imidazole to oxamide,^{81,77} while benzoyl peroxide in chloroform solution attacks the ring with the formation of urea and ammonia.¹⁰ Benzoyl chloride in strong alkali results in fission of the ring producing an α,β - dibenzamidoalkene.⁶ Acylation at a ring carbon atom does not occur readily. In this respect imidazoles resemble pyridine.

Imidazoles undergo typical aromatic substitution reactions and their ability to couple with diazotised aromatic amines provides a sensitive method of detection.⁷³

Electrophilic substitutions in imidazole can apparently involve the conjugate acid, the neutral molecule and the conjugate base.⁸⁴ Whereas nitration and sulphonation involve the conjugate acid and give 4-substitution, diazo-coupling involves the conjugate base and leads to preferential 2-substitution. The π -electron densities published by Brown and Heffernan¹⁴ for imidazole, its conjugate acid and conjugate base bear no relation to chemical activity in the conjugate acid but show a more reasonable correlation in the neutral molecule and conjugate base.



neutral molecule

conjugate acid

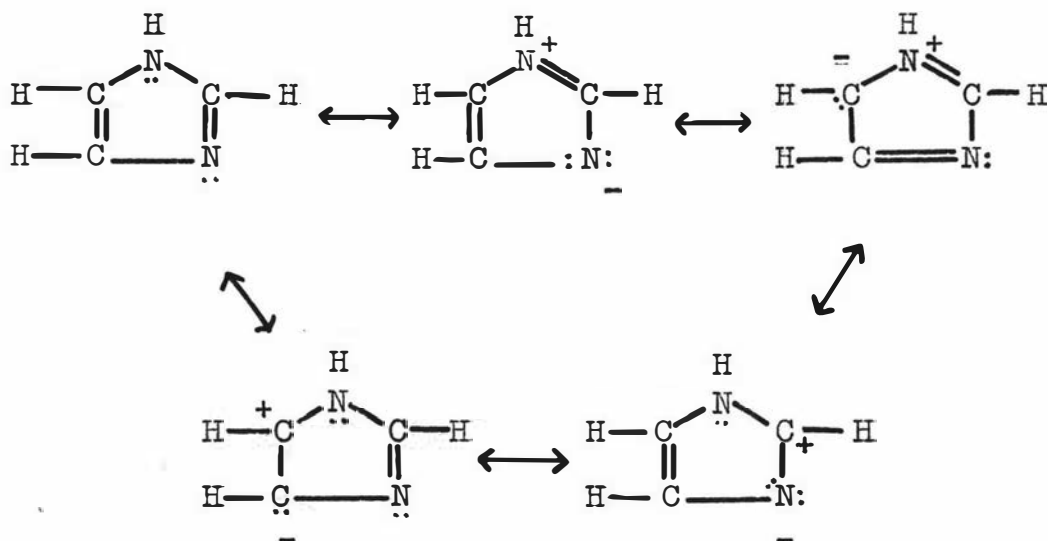
conjugate base

(uncertainty in the given values is 0.02 units)

Frontier electron densities, when established may prove more useful. Localization energies correctly assign the orientation of electrophilic attack to the 4(5)-position of the neutral molecule and conjugate acid of imidazole.^{7, 13}

In reactions of the conjugate base there is probably little difference between the reactivity of the 2- and 4- positions.

Current views on the structure of imidazole suggest that it may be represented as a resonance hybrid of the structures:-



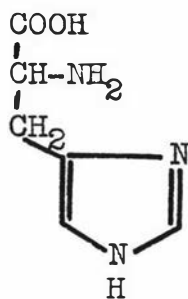
A set of similar contributions in which the functions of the nitrogens are reversed may account for the other tautomer.³⁸

This formulation could account for the acidity, aromatic character, substitution behaviour and high dipole moment of imidazole.

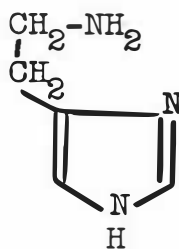
It is still not completely understood why imidazole

and many of its derivatives containing a free imino nitrogen are tautomeric. Although it is not possible to separate the isomeric forms of 4 (5)-methylimidazole, the tautomerism may be demonstrated by methylation when 1,4- and 1,5-dimethylimidazoles are formed and may be separated by distillation.⁷⁸⁻⁷⁹ Modern theories attribute the phenomenon to intermolecular reactions between two or more molecules and not to the intramolecular transfer of a proton from one nitrogen atom to the other.⁴⁰

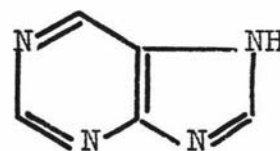
Many imidazole derivatives are of biological importance. The imidazole ring occurs in histidine (I), carnosine, histamine (II) and the purines (III). Biotin exemplifies an imidazolic vitamin, while pilocarpine is an imidazole alkaloid.



(I)



(II)



(III)

The same ring skeleton occurs in such compounds as ergothioneine (in ergot and blood), allantoin (the end product of

nitrogen metabolism in some animals), creatinine, hydantoin and parabanic acid. (The last two compounds are oxidation products of uric acid).

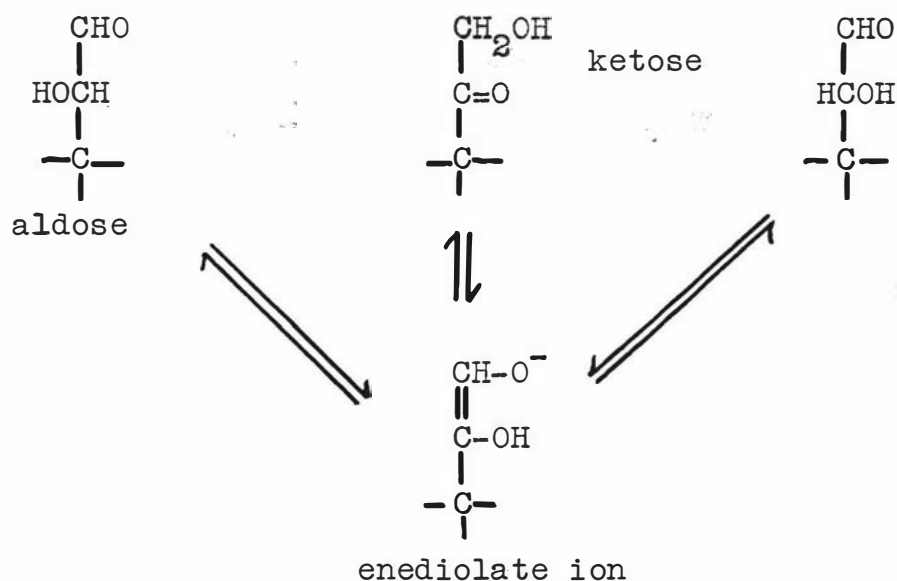
II. THE ALKALINE DEGRADATION OF CARBOHYDRATES.

Three general courses are followed in the action of alkali on carbohydrates:-

- (i) Isomerizations (mainly at the reducing end of the molecule)
- (ii) Fragmentation into substances with fewer carbon atoms and
- (iii) Internal oxidations and reductions.

(i) Isomerizations.

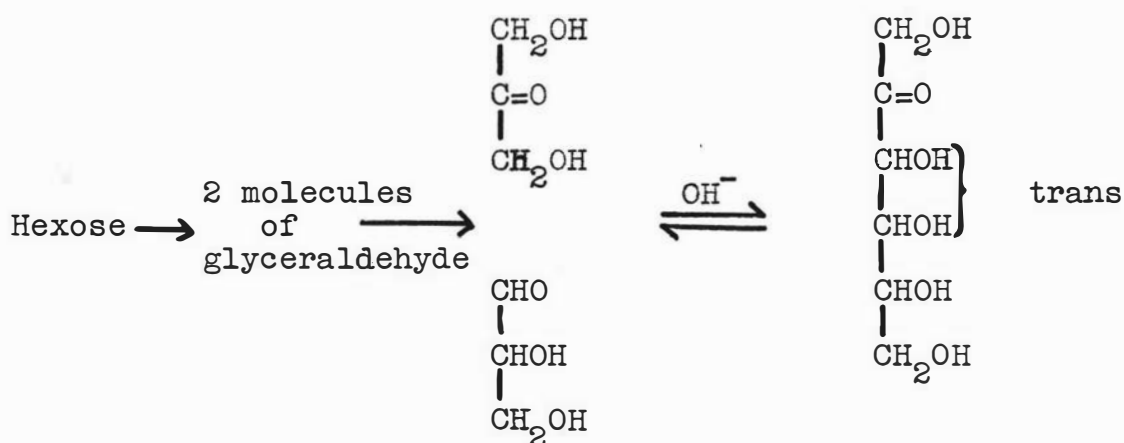
The simplest of such reactions is the Lobry de Bruyn and Alberda van Eckenstein transformation.¹⁵ When glucose is treated with dilute alkali at room temperature the optical rotation decreases and glucose, fructose and mannose can be isolated. This epimerization probably takes place via an enediol intermediate.⁶¹ Formation of the double bond destroys the asymmetry at carbon 2.



Evidence for the presence of an enediol intermediate has been obtained by noting that such mixtures consume large quantities of iodine, decolourize solutions of dichloroindophenol and that the sugars themselves are subject to oxidative cleavage between carbon atoms 1 and 2.⁷⁵ It is also possible to have epimerization of the 2-ketose (e.g. in the formation of D-psicose from D-glucose and ammonia.⁴³) or formation of ketoses with the carbonyl function at carbon 3. (e.g. "glucose" from the unfermentable fraction from fructose or glucose with dilute alkali^{15(c)}). The non-bonded interaction between two large cis substituents in a 2,3-enediolate ion is undoubtedly much greater than when one of the substituents is hydrogen as in the 1,2-enediol. It can therefore be expected that 2,3-enediolization will be slower than 1,2-enediolization.⁶¹

The products of alkaline degradation of glucose appear to depend somewhat on the reaction conditions. Hence, while Hough et al.⁴³ isolated psicose when ammonia was used as the base, Blair and Sowden⁹ used a strongly basic ion exchange resin and obtained sorbose. Wolfrom and Schumacher¹⁰³ obtained sorbose and allose among the products of reaction of fructose and potassium hydroxide solution. Clearly any hexose may be expected to be among the products from any other hexose under suitable alkali treatment. The mechanism by which D-sorbose and L-sugars are formed has yet to be

elucidated. It is conceivable that 3,4- and 4,5-enediols might form.^{89,102} There is also the possibility that the carbon chain may cleave through a reversed aldol condensation,^{75,9} e.g. cleavage of a hexose to two glyceraldehyde molecules, isomerization of glyceraldehyde to dihydroxyacetone and then condensation to give a mixture of fructose and sorbose.



(ii) Fragmentation.

Cleavage of carbon chains occurs under rather more drastic conditions than isomerizations.⁶⁶⁻⁶⁸

Fission of a hexose may result in the formation of:-

- (1) formaldehyde and aldopentoses
- (2) glycolaldehyde and aldotetroses
- (3) glyceraldehyde and dihydroxyacetone

Any of the aldoses could isomerize through the corresponding enediols to the ketoses. Oxidation of any of these isomerization products may give rise to saccharinic acids.

Short chain products of alkaline degradation of carbohydrates include oxalic acid, lactic acid, dihydroxybutyric acid, glyceraldehyde, dihydroxyacetone, methylglyoxal, formaldehyde, acetol, diacetyl, reductone (the enol of hydroxymalonic aldehyde), formic and acetic acids.⁷⁶ Under non-oxidizing conditions such cleavages of the carbon chain probably take place by means of a reversed aldol condensation (see page 12). The reverse aldolization of D-glucose is a function of alkalinity and begins at a concentration of 0.05N alkali.⁶⁸ Whereas Nef explained these fragmentations by postulating cleavage at the double bond of the enediol, studies of bond energies preclude this and instead cleavage is thought⁸⁵ to be caused by the double bond of the 1,2-enediol weakening the single bond β to it - cleavage thus occurs in the 3,4-position.

Any trioses formed from cleavage of hexoses rapidly recondense to sugars and hence the appearance of common reaction products does not necessarily establish the sequence of reactions.

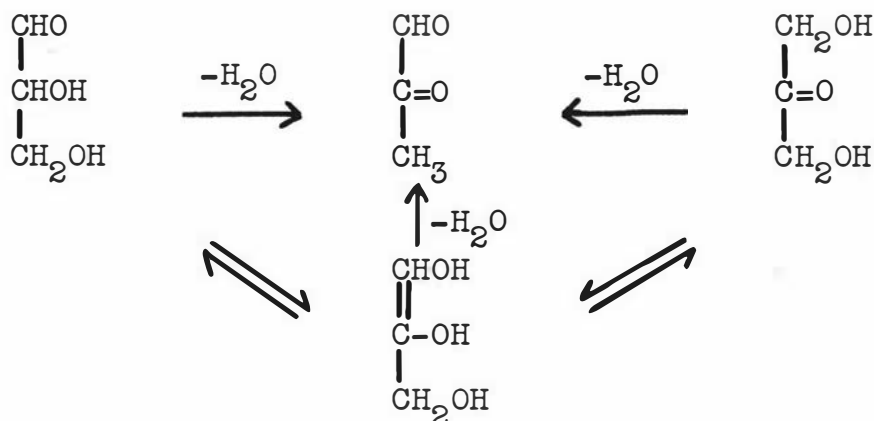
It has been shown⁵⁹ that even a simple molecule such as formaldehyde reacts in basic medium to produce glycolaldehyde, trioses and tetroses. The mechanism of this reaction has recently been studied by Breslow.¹²

(iii) Internal Oxidations, Reductions and Dehydrations.

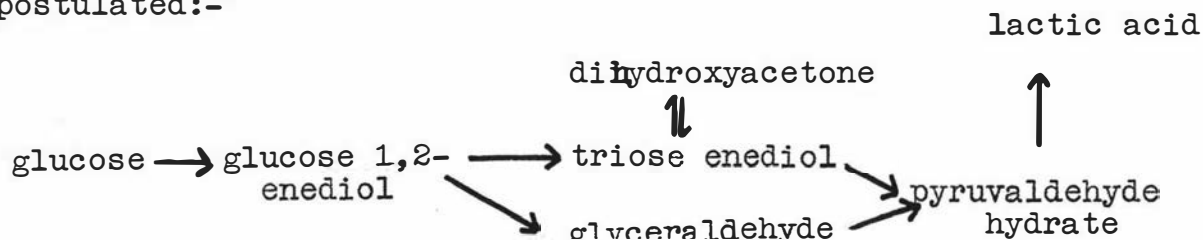
In the reaction of alkali with hexoses, small amounts

of methylglyoxal (pyruvaldehyde) and much lactic acid appear. These compounds also appear in alkali-treated solutions of trioses. In the latter case traces of acetol, lactic aldehyde and pyruvic acid have been identified.³⁰ A study of the effects of pH on the formation of methylglyoxal, diacetyl and acetol (pyruvic alcohol) from a number of sugars has been made by Lento, Underwood and Willits⁶⁰ who found that methylglyoxal predominates at acid pH while acetol and diacetyl are formed mainly above pH 8.

The formation of methylglyoxal is probably due to dehydration of glyceraldehyde, dihydroxyacetone or the triose enediol.³¹



The formation of DL-lactate from D-glucose has been studied with C¹⁴-labelled D-glucose and the following scheme postulated:³³



Whistler and BeMiller⁹⁴ state that the alkaline degradation of D-glucose to lactate by an intramolecular, oxidation-reduction Cannizzaro reaction probably proceeds through D-fructose, since the bond between carbons 2 and 3 of an aldose is less readily broken than the bond between carbons 3 and 4 of the ketose.

Various saccharinic acids are also formed under alkaline conditions - probably by internal oxidations and reductions and migrations of groups.^{34,88,95} This becomes of practical importance in polysaccharide chemistry where alkaline degradation can be used to predict the type of linkage. e.g. a 1-2 linkage is stable to dilute alkali; 2-1 is degraded with the formation of saccharinates; 1-3 gives metasaccharinates; 1-4 gives iso-saccharinates; 1-6 gives mainly lactic acid.⁹² Other acids have been found at 25-30°C when potassium hydroxide (0.2-6N) converts glycer-aldehyde to formic, acetic and lactic acids.³¹

It may thus be concluded that the action of alkalis on sugars is a complicated process. While very dilute alkalis catalyse the α,β and presumably the furanose-pyranose conversions, more concentrated alkalis bring about isomerization between the epimeric aldoses probably via an enediol intermediate. Still higher concentrations of alkali bring about conversions between all of the various sugars of the same chain length, probably partly as a result of formation

of 2,3- and 3,4-enediols and partly as a result of the recombination of cleavage fragments of the carbon chain.

Rearrangements occur in which saccharinic acids are formed from the original sugars and from their isomerization and cleavage products.

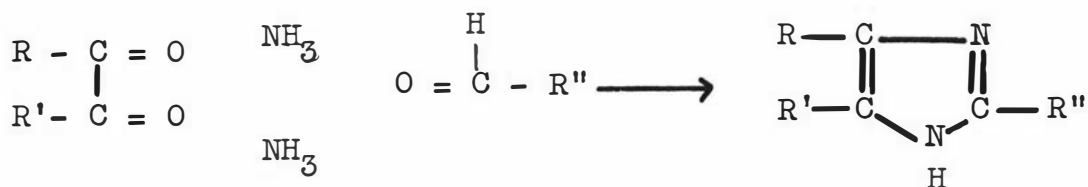
Aldopentoses would be expected to behave in the same way as they can also form pyranose and furanose rings and enediols similarly to hexoses. Ketopentoses and aldotetroses can only form furanose rings while trioses and glycolaldehyde cannot form a ring. It is known, however, that the lower sugars with two or three carbon atoms readily form dimers.^{5,31}

III. THE REACTION OF AMMONIA WITH CARBONYL COMPOUNDS.

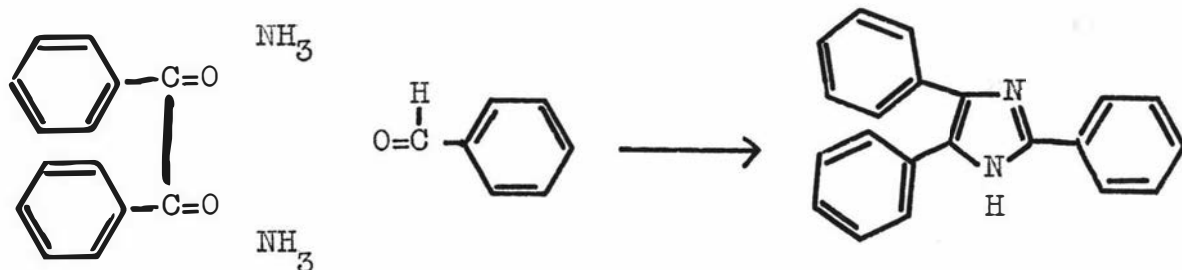
The reaction of ammonium hydroxide with carbohydrates might be expected to parallel to some extent the action of alkalis.

(a) Radziszewski Synthesis.

In 1882 Japp and Robinson⁴⁷ and Radziszewski⁸⁰ almost simultaneously discovered that the condensation of an α -dicarbonyl compound with ammonia and an aldehyde resulted in the formation of an imidazole.

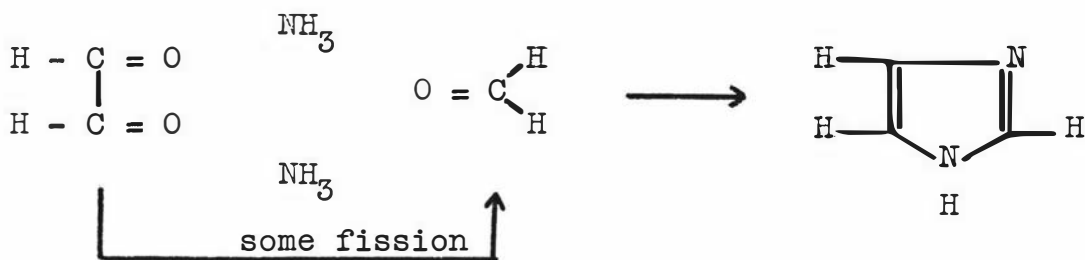


Radziszewski⁷⁹ first used this method for the synthesis of lophine from benzil and benzaldehyde.



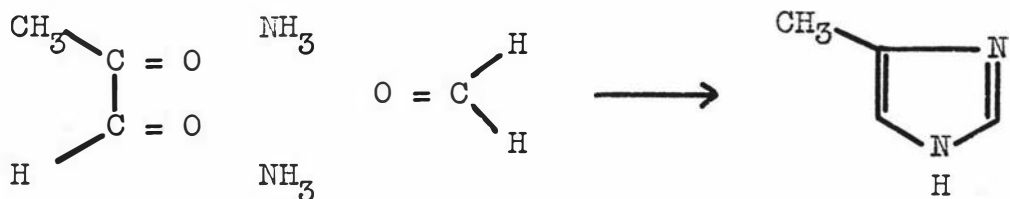
The reaction was quickly extended to simpler imidazoles when it was realized that the formation of imidazole from glyoxal and ammonia, as discovered by Debus,²⁴ might be due

to the interaction of glyoxal, formaldehyde and ammonia.



Radziszewski postulated fission of the glyoxal molecule under the influence of the ammonia and tested this by reacting glyoxal and ammonia with a variety of aldehydes, thus obtaining a number of 2-substituted imidazoles.

When an α -ketoaldehyde condenses with ammonia and formaldehyde a 4 (or 5)-substituted imidazole is produced e.g. methylglyoxal, ammonia and formaldehyde yield 4(5)-methylimidazole.⁸



When the formaldehyde is replaced by acetaldehyde the product is 2,4(or 5)-dimethylimidazole.

An α -diketone may be combined with ammonia and an aldehyde to give either a 4,5-disubstituted or a 2,4,5-trisubstituted imidazole e.g. diacetyl, ammonia and acetaldehyde give 2,4,5-trimethylimidazole as the major product.⁴⁸

Whereas the conventional method involved the reaction

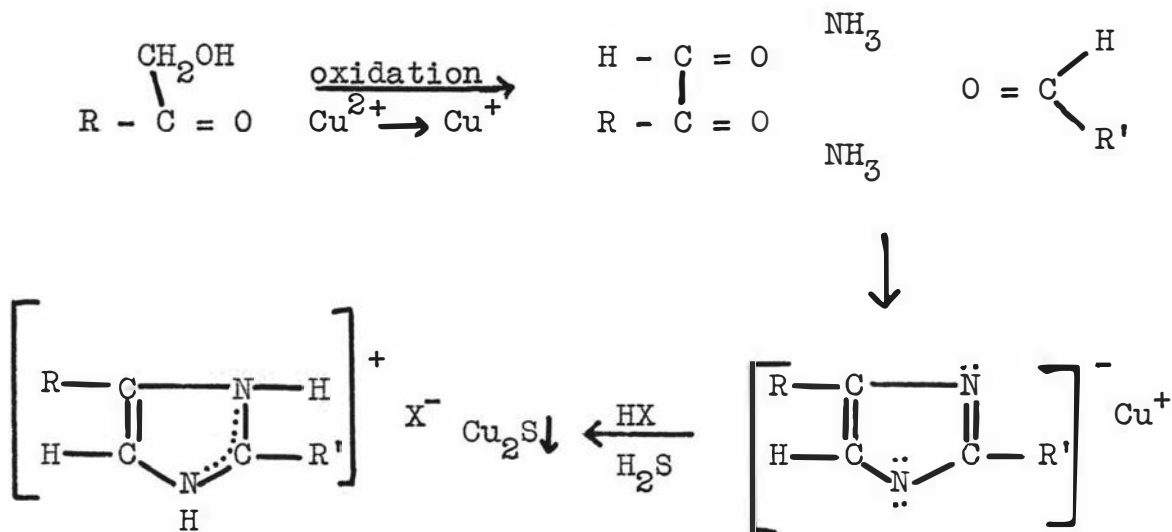
taking place in alcoholic ammonia it was found that the yield of imidazoles increased markedly when the reaction was carried out in glacial acetic acid with ammonium acetate as the source of ammonia.²³ Davidson, Weiss and Jelling²³ also found that hexamethylenetetramine provided an improved source of formaldehyde. A number of β -unsaturated aldehydes are reported to fail to give imidazoles under the same reaction conditions.²⁰

The Radziszewski synthesis of imidazoles is rather limited in scope because of :-

- (i) difficulty of synthesis of α -ketoaldehydes
- (ii) poor yields
- (iii) complex mixtures in product and consequent difficulty of separation.

(b) Weidenhagen Synthesis.

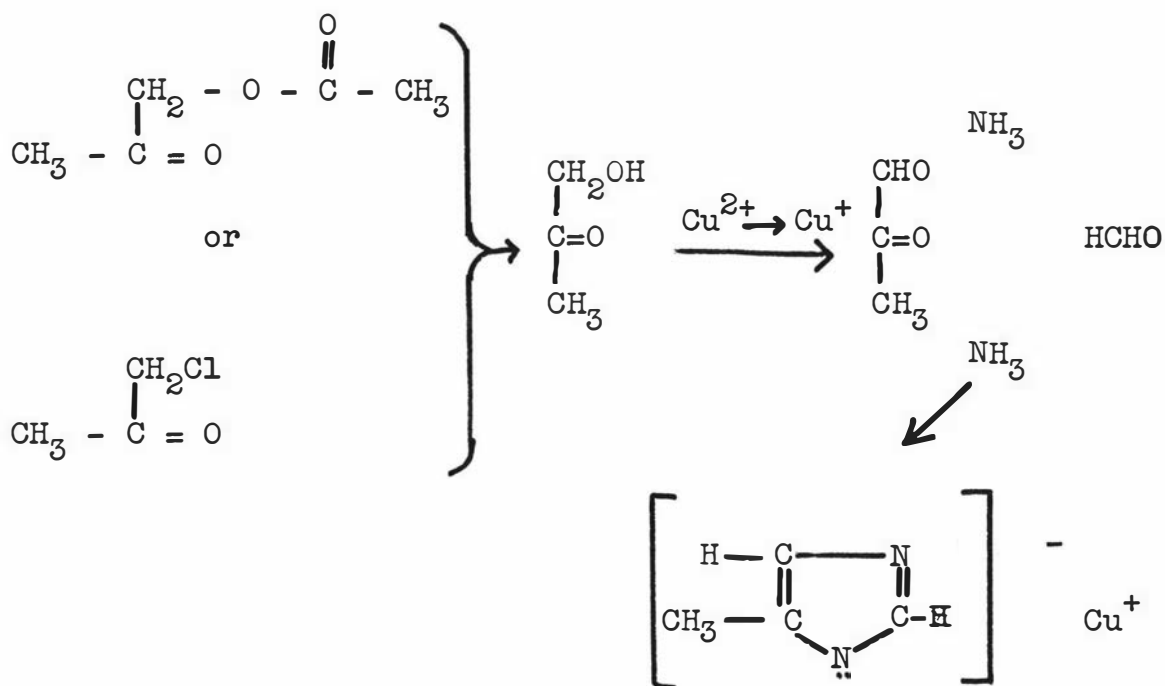
In 1935 Weidenhagen and Herrmann⁹¹ observed that α -hydroxyketones under the influence of ammoniacal cupric acetate solutions were quantitatively oxidized to the corresponding dicarbonyl compounds. When carried out in the presence of an aldehyde this reaction resulted in imidazole formation.



It is thought that cupric ions oxidize the α -hydroxyketone to the corresponding α -ketoaldehyde which then condenses with two molecules of ammonia and a molecule of aldehyde. The cuprous salt of the imidazole is precipitated and may be decomposed by the addition of hydrogen sulphide in acid solution. In some cases the acetyl derivatives of the hydroxyketones or the α -halogenoketones may be substituted for the hydroxyketones. e.g. 4(5)-methylimidazole may be formed from hydroxyacetone, acetoxyacetone or chloroacetone.⁴¹ (See reaction scheme page 21).

This imidazole synthesis is a most useful procedure often resulting in high yields. The use of such a mild oxidizing agent allows the introduction of sensitive groups. A number of undesirable side products may be formed, however, because of self-condensation of the dicarbonyl compound.

Such reactions become prominent when less reactive aldehydes are employed.



(c) Reactions of Carbohydrates with Ammonia.

(i) Formation of Imidazoles. Windaus and Knoop¹⁰⁰

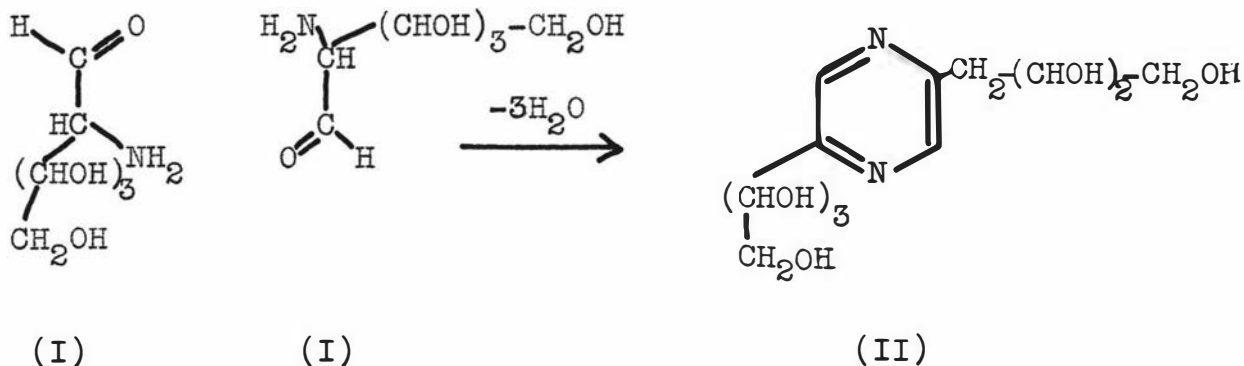
discovered that 4(5)-methylimidazole is formed from D-glucose under the influence of zinc hydroxide and ammonia. At the time this reaction was of great interest to chemists as they thought that a study of this transformation might lead to clarification of the biosynthesis of the imidazole ring, and that proteins in plants might be formed by the interaction of sugars and ammonia.

Early experiments involved the interaction of D-glucose

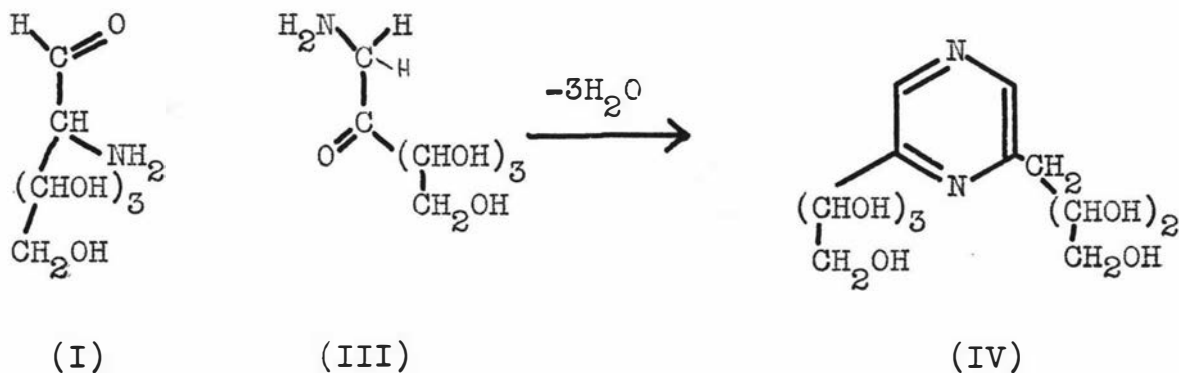
ammonium hydroxide and zinc hydroxide at room temperature for about six weeks. The resulting zinc salt was then decomposed with hydrogen sulphide and the 4(5)-methylimidazole was isolated as either the picrate or the oxalate. Since the time of Windaus and Knoop a number of chemists have studied the interaction of carbohydrates and ammonia usually in the presence of metallic salts.

Jezo⁴⁹ studied the ammonolysis of sucrose under various conditions of temperature, reagent concentration and with a number of catalysts. The best yield (25%) of nitrogenous compounds was achieved at 180° for 18 hours using 0.625% ammonium phosphate as catalyst. Distillation of ether extracts of the mixtures indicated the presence of a number of imidazolic and pyrazine compounds (See Tables 1 and 2). In the absence of a catalyst optimum yields were achieved at 220°.

In order to explain the mechanism of pyrazine formation, initial hydrolysis of the sucrose was postulated. The fructose moiety was thought to be converted to fructosylamine which could undergo a Heyns-type rearrangement⁵⁶⁻⁷ to form glucosamine (2-amino-2-deoxyglucose) (I), two molecules of which condense to form a 2,5-disubstituted pyrazine (deoxy-fructosazine) (II).⁵⁷



The glucose fragment from the hydrolysis of sucrose forms glucosylamine which undergoes an Amadori rearrangement³⁹ to form 1-amino-1-deoxy-2-fructose (III), which by condensation with a molecule of glucosamine, results in the formation of the corresponding 2,6-isomer of II (IV).



These latter compounds (II and IV), by thermal detachment of their side-chains, yield pyrazines and the fragments, by further condensation, yield imidazoles. This hypothesis is based on the fact that under identical conditions glucosamine and deoxyfructosazine yielded mixtures of products which were qualitatively and quantitatively similar to those

obtained from sucrose. A number of oligo- and poly-saccharides were also tested by Jezo and Luzac⁵⁰ under identical conditions and were found to yield identical pyrazine and imidazole fractions.

Table 1 lists the imidazoles isolated from sugar-ammonia mixtures.

TABLE 1

<u>CARBOHYDRATE</u>	<u>IMIDAZOLES IDENTIFIED</u>	<u>REF. NO.</u>
glyoxal	imidazole and bis-imidazole	24
glyceraldehyde°	4(5)-methylimidazole	70
dihydroxyacetone°	" "	70
dihydroxyacetone*°	4(5)-hydroxymethylimidazole	71
diacetyl	2,4,5-trimethylimidazole	74
arabinose°	4(5)-methylimidazole	70
L-arabinose and HCHO	4(5)-(L-erythro-trihydroxy- propyl)imidazole	2
xylose°	4(5)-methylimidazole	70
glucose	" "	70
	Imidazole-4(5)-carboxylic acid	70
	2-hydroxymethyl-4(5)-methylimidazole	55
	4(5)-(2-hydroxyethyl)imidazole	56
	4(5)-(2,3,4-trihydroxybutyl)- imidazole	56
	4(5)-(2,3-dihydroxypropyl) imidazole ⁺	56
	2,4(5)-dimethylimidazole	99
D-glucose*°	4(5)-D-arabotetrahydroxybutyl- imidazole	71
D-glucose*°	imidazole-4(5)-acetic acid	69
mannose*°	4(5)-methylimidazole	71
	4(5)-D-arabotetrahydroxy- butylimidazole	71
galactose*°	4(5)-methylimidazole (trace)	71
D-galactose*°	4(5)-D-arabotetrahydroxybutyl- imidazole	71
	4(5)-D-lyxotetrahydroxybutyl- imidazole	71
	4(5)-hydroxymethyl-2-DL-lyxo tetrahydroxybutylimidazole	71

(Table 1 contin.)

rhamnose [°]	2,4(5)-dimethylimidazole	101
	4(5)-methylimidazole	101
	2-methyl-4(5)-ethylimidazole	101
fructose [°]	4(5)-methylimidazole	70
	2-hydroxymethyl-4(5)-methylimidazole	70
	4(5)-hydroxymethylimidazole	65
fructose* [°]	" "	71
	4(5)-D-arabotetrahydroxybutylimidazole	71
fructose** [°]	imidazole-4(5)-formamide	71
sorbose [°]	4(5)-methylimidazole	70
sorbose + HCHO	4(5)-hydroxymethylimidazole	22
maltose [°]	4(5)-methylimidazole (trace)	70
lactose [°]	" "	70
sucrose* and HCHO	4(5)-hydroxymethylimidazole	58
sucrose ⁺⁺ (200°) [°]	4(5)-methylimidazole	49
	2,4-dimethylimidazole	49
	4,5-dimethylimidazole	49
	4(5)-hydroxymethylimidazole	49
molasses	4(5)-methylimidazole	97

Key:

* indicates aeration of mixture

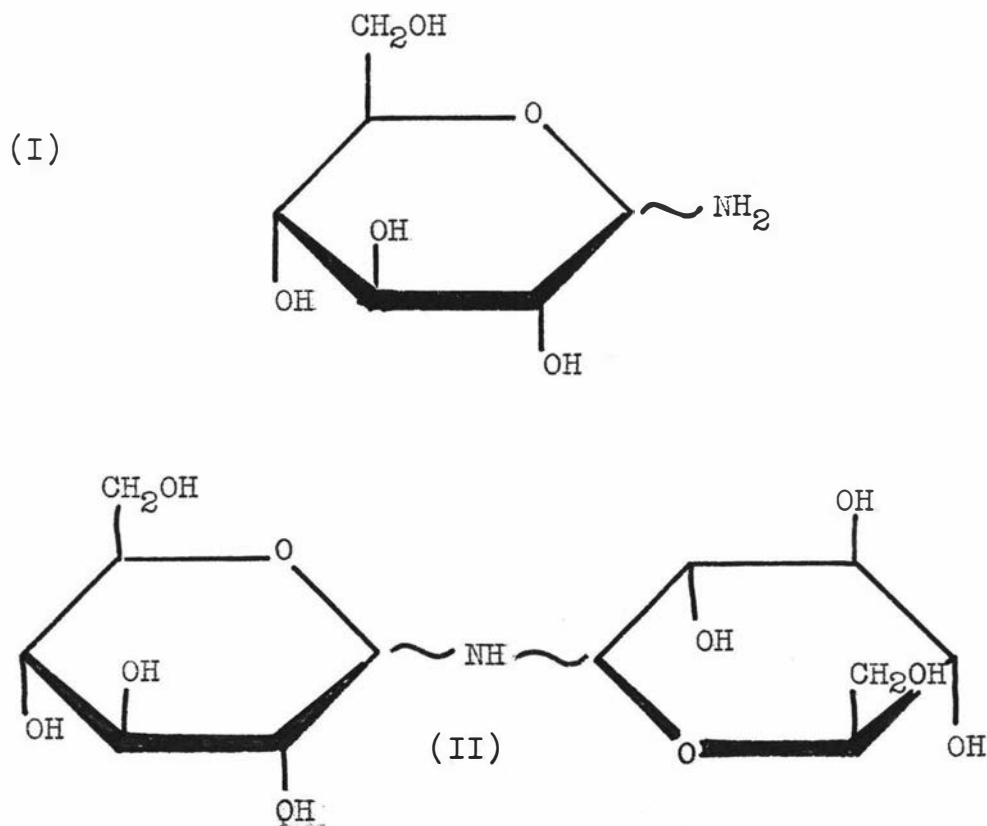
+ presumed present

** methylene blue incorporated as oxidising agent

° metallic ions present, or other catalyst

++ the same imidazoles were obtained under the same conditions from lactose, cellobiose, melase, hydrolysed starch and starch.

(ii) Formation of other Basic Compounds. From the reaction mixture, glucose-ammonia, Komoto⁵⁴ isolated glucosylamine (I) and diglucosylamine (II).



These aldosylamines may rearrange by means of the Amadori rearrangement³⁹ to ketoseamines (1-amino-1-deoxy-2-ketoses). Jezo⁴⁹ has also postulated the formation of glucosamine (2-amino-2-deoxyglucose) from the interaction of sucrose and ammonia. Heterocyclic products of such reactions include pyrazine and pyridine derivatives. See Table 2.

TABLE 2

CARBOHYDRATE	NITROGENOUS BASE ISOLATED	REF. NO.
glucose	2-methyl-5-D-arabotetrahydroxy butylpyrazine	44
	2-methyl-6-D-arabotetrahydroxy butylpyrazine	44
	2-methyl-5-(1,2-dihydroxyethyl)- pyrazine ⁺	82
	pyrazine, methylpyrazine, dimethyl- pyrazine, pyridine	11
molasses	5-hydroxymethyl-2-methylpyrazine	98
	2,6-dimethylpyrazine, 2-hydroxy- methylpyrazine	96
	2-methyl-6-arabotetrahydroxy- butylpyrazine	96
	2-methyl-5-arabotetrahydroxy- butylpyrazine	96
	2-hydroxymethylpyrazine ⁺	97
rhamnose	$C_8H_{11}O_2N$ and C_6H_7ON	70
sucrose*	2-methylpyrazine, 2,5-dimethyl- pyrazine, 2,6-dimethylpyrazine	49

Key: + presumed present

* See ** Table 1.

(iii) Formation of Acidic Compounds. Oxalic acid has been identified among the reaction products of a large number of carbohydrates and ammonia,⁷⁰ while lactic acid was also detected by Komoto⁵⁴ in the glucose-ammonia system.

Hydrogen cyanide has been detected from the reactions of a large number of sugars and their degradation products with ammoniacal cupric sulphate.⁷²

(iv) Formation of Neutral Products. A number of neutral products are formed during the interaction of carbohydrates and ammonia. Some of these probably have only transitory existence being formed as a result of alkaline degradation. (See page 13 et seq.)

(v) Conditions of Reaction. Various conditions have been employed:-

(1) Variation of Temperature and Pressure.

(a) Mixing the sugar and ammonium hydroxide at temperatures near to room temperature.^{53,45}

(b) Reaction at 100°C.⁸

(c) Reaction at 100°C and under increased pressure.^{55,56,90,}

(d) Reaction at 200°C under increased pressure.⁴⁹

Increasing the temperature and pressure has the effect of greatly speeding the reaction rate.

(2) Addition of Metal Salts. (usually Zn²⁺ or Cu²⁺ hydroxides, sulphates, carbonates or acetates).

Calcium hydroxide, ferrous and ferric sulphates and manganese

sulphate have also been added to sugar-ammonia mixtures.⁷⁰ Parrod⁷⁰ found that when air was passed slowly through an aqueous solution containing laevulose, ammonium hydroxide and the hydroxide or sulphate of a metal for one month at ordinary temperatures, 4(5)-methylimidazole was formed in all cases and 4(5)-hydroxymethylimidazole in all but with $\text{Ca}(\text{OH})_2$. When MnSO_4 was incorporated in the reaction mixture 2-hydroxymethyl-4(5)-methylimidazole was also formed. Jezo⁴⁹ found that high yields of imidazoles and pyrazines were obtained from sucrose and ammonia in the presence of catalytic amounts of zinc chloride and cupric sulphate, but that under these conditions humin formation was also favoured.

(3) Passage of Air (or Oxygen) through the Reaction Mixture. It has been noted that passage of air through the sugar-ammonia system results in an increase in oxygenated imidazoles. Hence, while fructose and ammonia stored in a stoppered flask produced mainly 4(5)-methylimidazole, when air was passed through the mixture 4(5)-hydroxymethylimidazole was also found in good yield. When a mixture of fructose, ammonia and $\text{Cu}(\text{OH})_2$ was stored in the complete absence of air for one month it was still possible to extract the same compounds as were formed in the presence of air, although in lower concentration.⁷¹ It should be noted at this stage that as chromatography was unknown at the time these observations were based on the chemist's ability to isolate

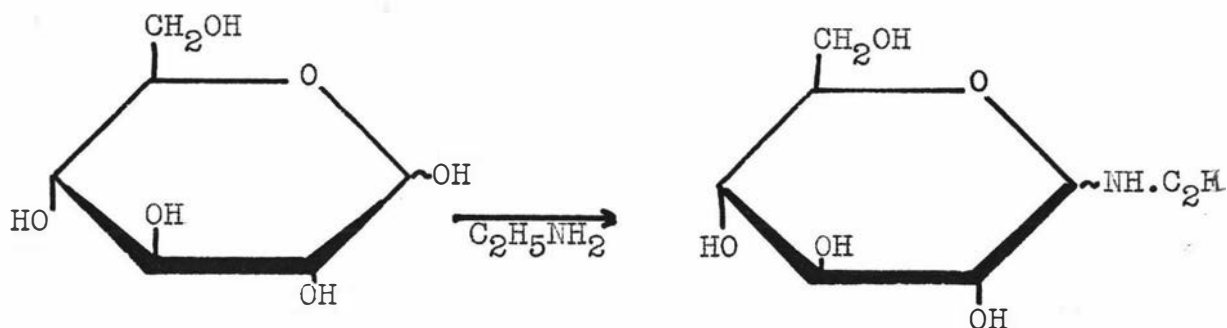
a crystalline derivative from a complex mixture. It would thus have proved very difficult to obtain an accurate quantitative estimate of the imidazoles formed under any particular set of conditions and the probability of failing to note the presence of a particular imidazole would be considerable, particularly when it was in low concentration.

(4) Variation in Reaction Medium. Reaction has been carried out in alcoholic, aqueous and glacial acetic acid medium. See page 19.

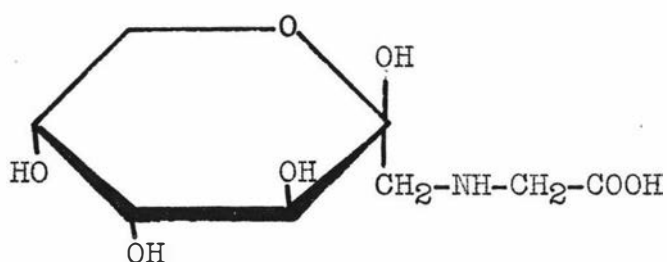
IV. THE INTERACTION OF OTHER NITROGENOUS BASES WITH SUGARS
AND THEIR FISSION PRODUCTS.

(i) Aliphatic and Alicyclic Amines.

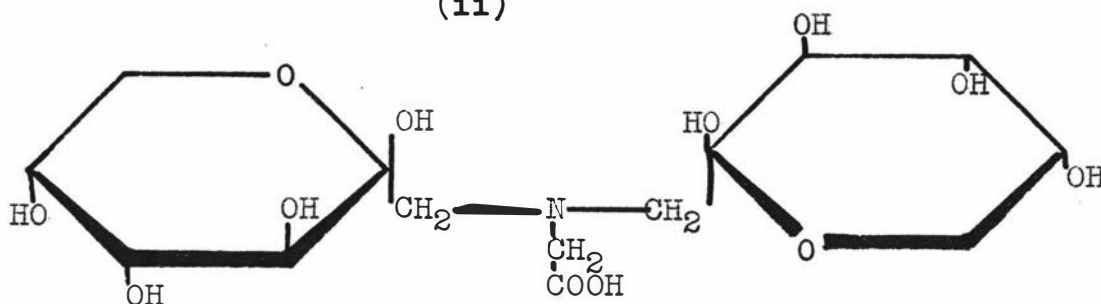
Aldose sugars react with primary or secondary amines to give aldosylamines (e.g. D-glucose and ethylamine give D-glucosylethylamine⁸² (I)) which undergo Amadori rearrangements³⁹ to form ketoseamines and



diketoseamines (e.g. D-glucose and glycine produce D-fructose-glycine (II) (1-deoxy-1-glycino-D-fructose) and di-D-fructoseglycine (III)).⁸²

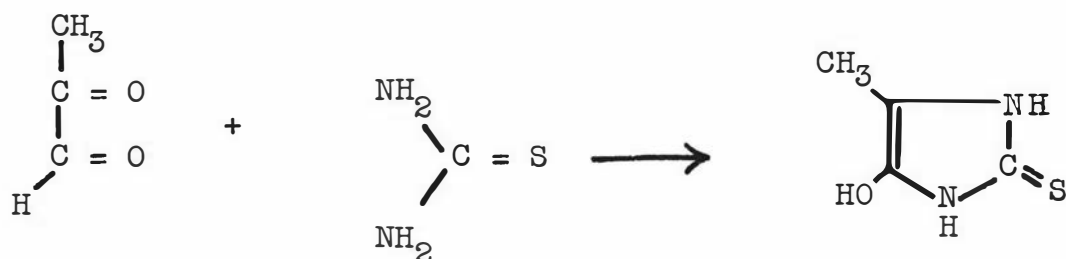


(II)

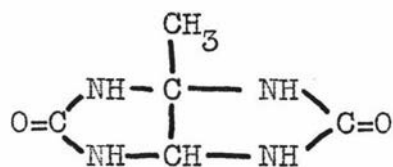
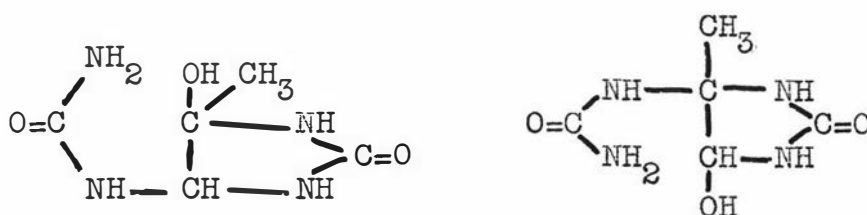


(ii) Urea and Thiourea.

A mixture of dihydroxyacetone (or pyruvaldehyde) and thiourea held for seven hours in a sealed tube at 120° gave 2-thio-4-hydroxy-5-methylimidazole (5-methylthiohydantoin) as the major product.⁸⁷



Urea and pyruvaldehyde give a mixture of products.⁸⁶



methylacetylenediurein

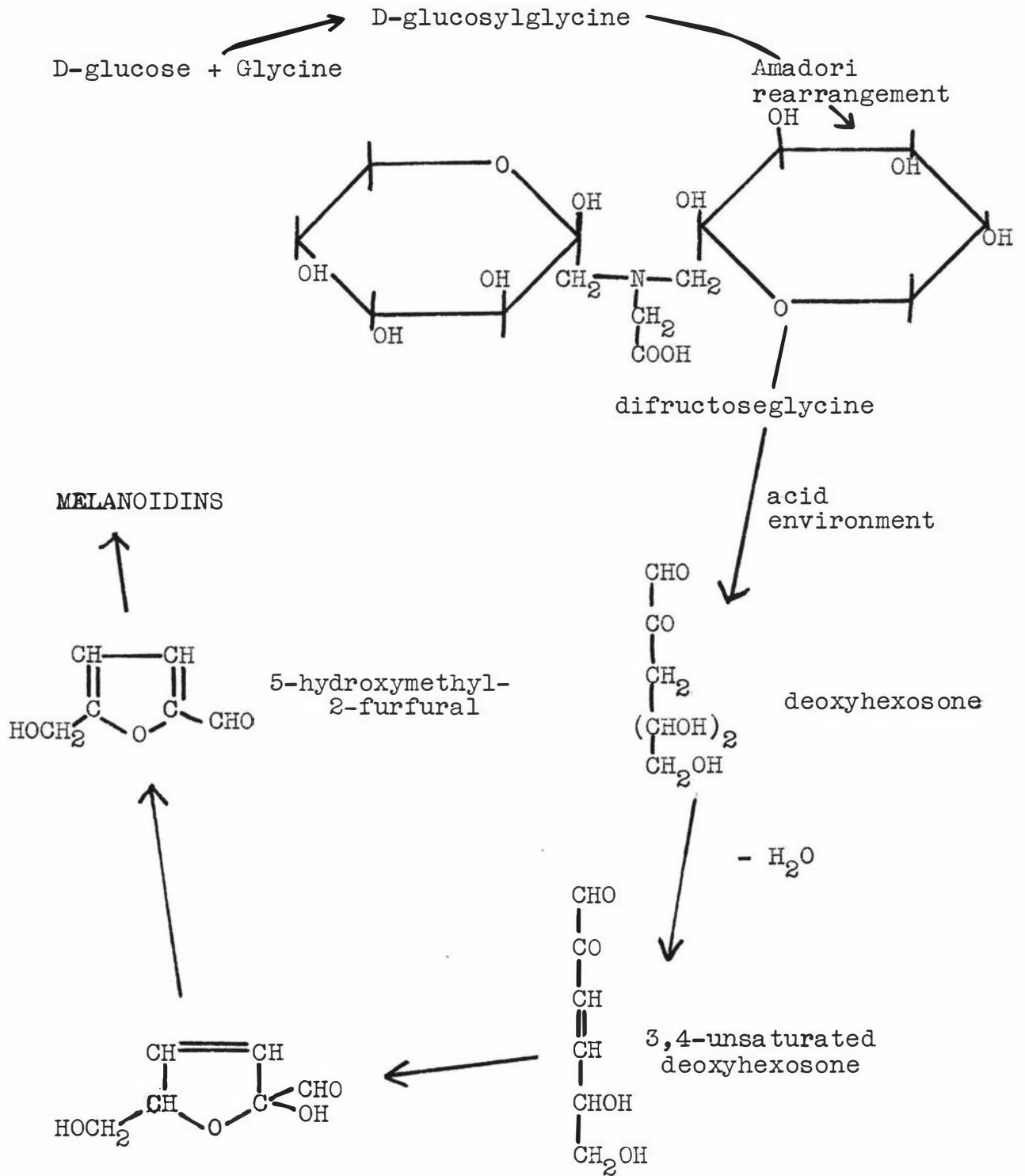
(iii) Amino Acids and Proteins.

The interaction between carbohydrates and amino acids or proteins (the Maillard reaction) has been recognized as the cause of much of the browning and loss in solubility

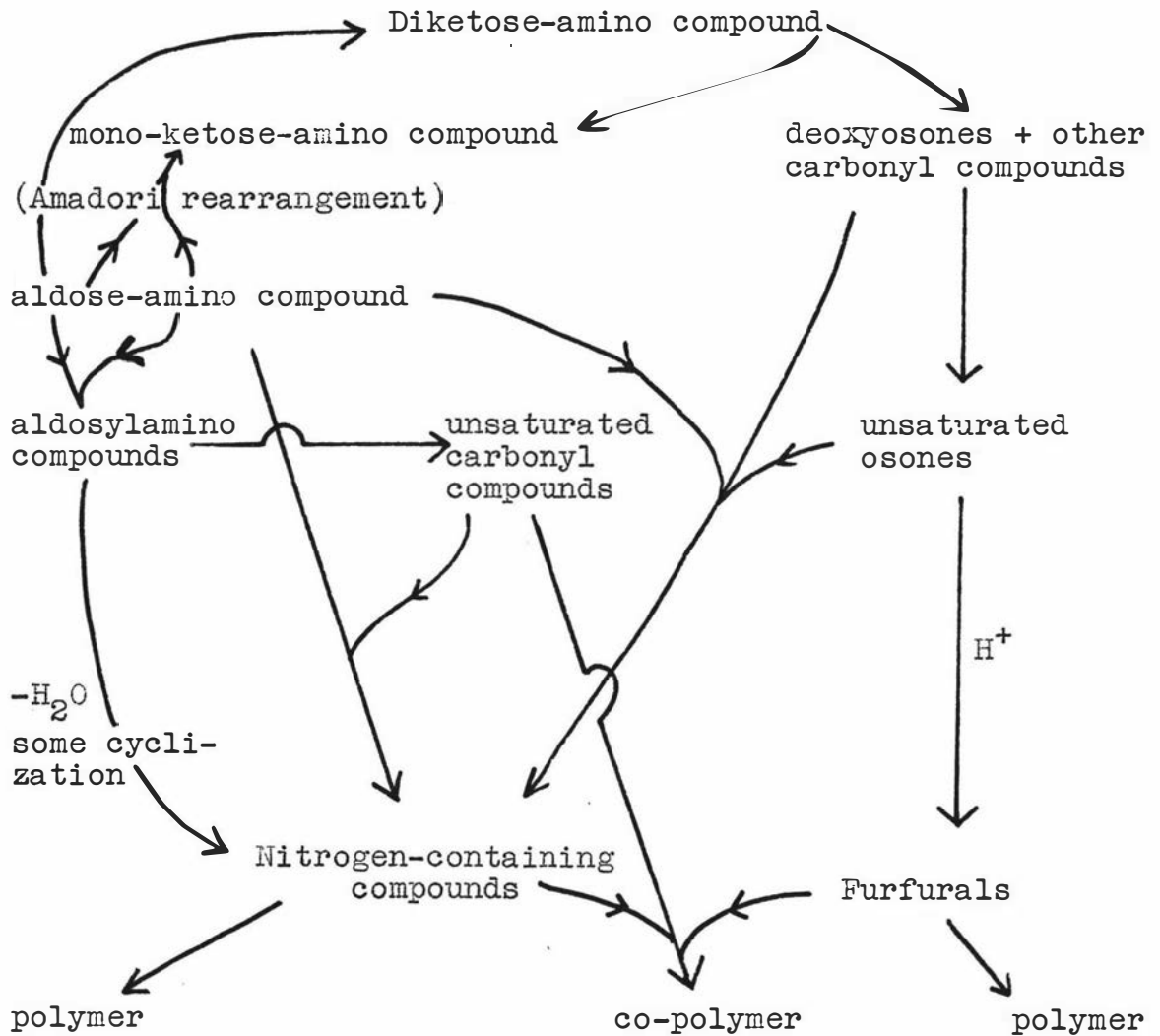
and biological value which occurs during the processing and storage of certain foodstuffs. Much of the initial research into the formation of dark-coloured products (melanoidins) from sugar-amino acid mixtures was carried out by Maillard.⁶²⁻⁴ He noted that the presence of an aldehyde group is an important factor and that the nature and extent of the reaction varies with concentration, temperature, pH, specific reactants and time.

Recently it has been claimed that the main route to melanoidins is that in which the amino function becomes doubly-substituted with the sugar component (via an Amadori rearrangement) with the subsequent elimination of a hexosone, followed by dehydration and cyclization of the latter to yield furfurals.^{3,4} It appears that although some browning may occur through furfurals (particularly in acid medium) melanoidins are formed by more than one route. Conjugated, unsaturated carbonyl compounds have been found to be formed in sugar-amino compound mixtures^{17,18} and in the presence of nitrogen functions these brown rapidly to give melanoidins.

Reaction Scheme for Melanoidin Formation According to Anet.



A complete survey of this reaction would be beyond the scope of this thesis but the modern theories of the "browning reaction" may be summarized by the following diagram.¹⁶ A hexose sugar is taken as an example.



The Formation of Melanoidins in Sugar-amino Compound Systems. According to Burton and McWeeny.¹⁶

The dark, odourous products (melanoidins) formed when reducing sugars and amino acids are heated together often resemble soil humus.²⁵ A similar group of substances (caramels) are obtained on pyrolysis of sugars. When such pyrolysis occurs in the presence of ammonia or amines the products are reported to be melanoidin in nature.²¹ Similar products are formed from the reaction of amines with methylglyoxal²⁶ and diacetyl.²⁷ The composition and properties of melanoidins vary according to the method of preparation. Enders and his co-workers have noted that as the reaction progresses the carbon and nitrogen contents of the product increase, whereas the hydrogen remains fairly constant. The water solubility of the products decreases during the reaction. It is probable that none of these melanoidins is composed entirely of a single compound. Hough, Jones and Richards⁴⁶ showed that there were many free hydroxyl groups and also reducing groups present, while hydrolysis experiments produced complex mixtures of sugars and imidazoles. Zinc dust distillation of a melanoidin from glucose/glycine yielded pyrroles and pyridines.²⁸ Carboxylic acid, hydroxy, phenolic and carbonyl groups were also identified. A high proportion of the yield in most of the systems studied in the present research project consisted of melanoidin material.

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RESULTS AND DISCUSSION

RESULTS AND DISCUSSION

I. THE CHROMATOGRAPHY OF IMIDAZOLES.

In order to facilitate identification of common imidazolic products and for routine analyses of fractions from columns, it was necessary to develop chromatographic techniques. Paper chromatography was employed in the early stages of the project, but separations were often improved and accelerated by the introduction of thin-layer chromatography on glass plates.

(i) Paper chromatography.- A number of workers^{11,33,46} have separated and identified imidazoles by paper chromatography.

In the present project chromatography was carried out by the descending method using Whatman No. 1 or 3MM papers. The latter paper was employed where the imidazoles were in high concentration and where better separation was desired than Whatman No. 1 was capable of giving.

For routine work the solvent n-butanol - acetic acid - water (4:1:1) was employed, with the parent free base, imidazole, run as a marker. All chromatographic data were then related to imidazole as R_{Im} values, where $R_{Im} = (\text{distance travelled by compound}) / (\text{distance travelled by imidazole})$. These R_{Im} values were found to be more consistent than the conventional R_f values.

Detection of spots on chromatograms was usually achieved by spraying the air-dried paper with an alkaline solution of diazotised sulphanic acid (Pauly⁶⁶ reagent) which gave yellow, orange or red dyes with imidazoles. Yellow dyes are reported by Huebner³³ to often be characteristic of 2-substituted imidazoles. On occasions, a spray consisting of a solution of iodine in carbon tetrachloride¹¹ was used to produce transient brown or yellow spots with imidazoles on chromatograms. This reagent has the advantage of being able to detect N-substituted and carboxyalkyl-substituted imidazoles which fail to react with Pauly reagent.

It has been noted by Komoto⁴⁶ that the R_f values of imidazoles increase with increasing basic strength of the compounds. The pK_a values are increased by the presence of electron-releasing groups (particularly in the 2-position), and decreased by electron-attracting substituents.⁴¹ This relationship was found to hold in a general way for the paper chromatography of the imidazoles studied in this project. Table C1 lists R_{Im} values for seventeen imidazoles in six solvents.

(ii) Thin-layer chromatography on cellulose.- In order to reduce the time spent in examining fractions from column separations an attempt was made to separate imidazoles on cellulose-coated glass plates. These were spread to a thickness of 250 μ using a Desaga apparatus and developed by the

TABLE C1

R_{1m} values for seventeen imidazoles on paper

SOLVENT WHATMAN PAPER	S ₁		S ₂		S ₃		S ₄		S ₅		S ₆		COLOUR WITH DIAZO REAGENT	PUBLISHED pKa VALUES
	No. 1	No. 3 MM	No. 1	No. 3MM	No. 1	No. 3MM	No. 1	No. 3MM	No. 1	No. 3MM	No. 1	No. 3MM		
Imidazole	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	orange	6.95
2-Methylimidazole	1.44	1.34	1.11	1.26	1.06	1.03	1.13	1.06	1.08	1.14	1.09	1.05	lemon-yellow	7.86
4(5)-Methylimidazole	1.35	1.32	1.12	1.58	1.05	1.07	1.15	1.10	1.11	1.05	1.15	1.12	red	7.52
2,4(5)-Dimethylimidazole	1.90	1.71	1.28	1.87	1.11	1.11	1.24	1.26	1.07	1.05	1.21	1.17	lemon-yellow	8.36
4,5-Dimethylimidazole	1.62	1.56	1.73	2.62	1.09	1.09	1.19	1.21	1.12	1.11	1.23	1.13	yellow	
2-Ethyl-4(5)-Methylimidazole	1.92	1.79	1.81	2.96	1.13	1.11	1.29	1.32	1.22	1.22	1.21	1.16	yellow	
2-Hydroxymethyl-4(5)-methylimidazole	1.37	1.42	1.11	1.39	1.04	1.00	1.19	1.21	1.00	0.94	1.14	1.20	red	
2-Hydroxymethylimidazole	1.12	1.12	1.02	1.28	0.96	0.91	1.06	0.99	0.95	0.94	1.04	0.97	orange	
4(5)-Hydroxymethylimidazole	0.91	0.88	0.79	0.64	0.86	0.79	0.97	0.93	0.86	0.77	0.97	0.96	orange-red	6.38
4(5)-(2-Hydroxy ethyl)-imidazole	0.99	0.99	0.80	0.60	0.93	0.91	1.03	1.02	0.92	0.85	0.94	0.94	red	
2-Acetyl-4(5)-methylimidazole	2.26	2.10	3.40	8.30	1.07	1.05	1.37	1.48	1.14	1.05	1.31	1.28	orange-yellow	
2-Mercaptoimidazole	1.61	1.54	2.80	5.00	0.90	0.82	1.06	1.04	0.95	0.95	1.08	1.05	orange	
Histamine di-HCl	0.16	0.10	0.21	0.22	0.71	0.57	0.46	0.41	0.40	0.31	0.48	0.45	red	5.98 and 9.70
Histidine HCl	0.03	0.03	0.24	0.23	0.39	0.14	0.38	0.33	0.18	0.14	0.50	0.40	red	5.08 and 9.20
Imidazole-4(5)-carboxylic acid	0.33	0.49	0.59	0.38	0.42	0.19	0.65	0.61	0.20	0.22	0.75	0.75	yellow	6.08
Imidazole-4(5)-pyruvic acid	1.25	1.21	1.48	2.39	0.94	0.80	1.00	0.97	0.74	0.73	1.07	1.03	orange	
Imidazole-4(5)-acrylic acid	1.28	1.21	1.58	2.35	0.47	0.29	1.06	1.01	0.27	0.32	1.09	1.06	orange	

Solvents: S₁ = n-butanol - acetic acid - water (4:1:1); S₂ = Ethyl acetate - acetic acid - water (3:1:3 - top layer); S₃ = Ethanol - ether - water - ammonia (28%) (4:5:1:0.1);
 S₄ = n-Propanol - acetic acid - water (4:1:1); S₅ = n-butanol - pyridine - water (2:1:1); S₆ = n-butanol - ethyl acetate - acetic acid - water (1:1:1:1)

ascending method using the same solvents as in section (i).

Compact spots were obtained in the acidic solvents, but some tailing was evident with basic solvent systems. Although it proved difficult to separate imidazoles with carboxyl substituents on alumina and silica gel layers, thin-layer chromatography on cellulose proved effective. Efficient differentiation between isomeric imidazoles, however, often proved difficult, e.g. 2- and 4(5)-methylimidazoles were difficult to separate on cellulose. Table C2 lists R_f and R_{Im} values for eighteen imidazoles in six solvent systems.

(iii) Thin-layer chromatography on aluminium oxide G and silica gel G.-

It was found that mixtures containing imidazoles could be rapidly and efficiently analysed by thin-layer chromatography on aluminium oxide G and silica gel G layers using relatively basic solvent systems. For a simple mixture, a microscope slide coated with alumina could be spotted, developed and sprayed in less than five minutes.

Compact spots were obtained with all but carboxyl substituted imidazoles. Experiments showed that there was little significant difference in R_f values between activated and unactivated alumina chromatoplates. Table C3 lists R_f and R_{Im} values for seventeen imidazoles in five solvent systems.

From the values given it may be noted that methyl-substituted imidazoles are readily differentiated from hydroxyalkylimidazoles on silica gel G chromatoplates in

TABLE C2

 R_f and R_{Im} values for eighteen imidazoles on thin-layer cellulose

SOLVENTS: COMPOUND:	S ₁		S ₂		S ₃		S ₄		S ₅		S ₆		Colour of dye with diazotized Sulphanilic acid
	R_f	R_{Im}	R_f	R_{Im}	R_f	R_{Im}	R_f	R_{Im}	R_f	R_{Im}	R_f	R_{Im}	
2-Acetyl-4(5)-methylimidazole	0.86	1.68	0.96	6.00	1.00	1.29	0.88	1.30	0.99	1.07	0.96	1.28	yellow-orange
4,5-Dimethylimidazole	0.80	1.57	0.35	2.00	0.73	0.97	0.82	1.12	1.00	1.08	0.84	1.15	yellow
2-Ethyl-4(5)-methylimidazole	0.73	1.43	0.42	2.44	0.73	0.96	0.86	1.19	0.96	1.03	0.90	1.20	yellow
2,4(5)-Dimethylimidazole	0.68	1.33	0.30	1.88	0.80	1.03	0.88	1.30	1.00	1.08	0.88	1.18	yellow
2-Hydroxymethyl-4(5)-methylimidazole	0.68	1.33	0.25	1.50	0.72	0.93	0.81	1.10	0.91	0.99	0.90	1.20	red
4(5)-Methylimidazole	0.61	1.20	0.26	1.53	0.74	0.96	0.79	1.08	0.97	1.04	0.83	1.12	red
2-Methylimidazole	0.60	1.18	0.22	1.24	0.68	0.91	0.78	1.06	0.94	1.08	0.81	1.14	yellow
2-Mercaptoimidazole	0.58	1.14	0.59	3.21	0.71	0.95	0.75	1.03	0.80	0.88	0.74	1.03	orange
Imidazole-4(5)-acrylic acid	0.57	1.09	0.38	2.08	0.33	0.43	0.76	1.03	0.26	0.29	0.75	1.04	yellow-orange
Imidazole-4(5)-pyruvic acid	0.54	1.06	0.36	2.05	0.73	0.96	0.74	1.01	0.83	0.90	0.74	1.03	yellow-orange
4(5)-(2-Hydroxyethyl)imidazole	0.54	1.05	0.12	0.75	0.57	0.74	0.68	1.00	0.86	0.80	0.78	1.05	red
Imidazole	0.53	1.00	0.17	1.00	0.76	1.00	0.68	1.00	0.92	1.00	0.75	1.00	orange
4(5)-Hydroxymethylimidazole	0.48	0.96	0.10	0.69	0.58	0.75	0.62	0.89	0.85	0.79	0.72	0.97	orange-red
Imidazole-4(5)-carboxylic acid	0.28	0.54	0.12	0.75	0.13	0.16	0.46	0.63	0.08	0.13	0.46	0.63	yellow
Imidazole-4, 5-dicarboxylic acid	0.26	0.52	0.00	0.00	0.36	0.48	0.48	0.68	0.05	0.09	0.38	0.52	yellow
4(5)-D-Arabetetrahydroxy- butylimidazole	0.24	0.50	0.02	0.13	0.23	0.30	0.53	0.72	0.32	0.34	0.46	0.64	red
Histamine dihydrochloride	0.20	0.35	0.00	0.00	0.18	0.23	streaked		0.44	0.47	streaked		red
Histidine hydrochloride	0.18	0.30	0.02	0.09	0.08	0.10	0.36	0.53	0.03	0.04	0.26	0.35	red

SOLVENTS: S₁ = n-butanol - acetic acid - water (4:1:1); S₂ = ethyl acetate - acetic acid - water (3:1:3 -, upper phase); S₃ = n-butanol - pyridine - water (2:1:1);S₄ = acetic acid - n-butanol - ethyl acetate - water (1:1:1:1); S₅ = ethanol - diethyl ether - water - 25% NH₄OH (4:5:1:0.1); S₆ = n-propanol - acetic acid - water (4:1:1)

TABLE C3

 R_F and R_{Im} values for seventeen imidazoles

COMPOUND	SILICA GEL G LAYER-ACTIVATED		ALUMINIUM OXIDE G LAYER-UNACTIVATED		ALUMINIUM OXIDE G LAYER-UNACTIVATED		ALUMINIUM OXIDE G LAYER-UNACTIVATED		ALUMINIUM OXIDE G LAYER-UNACTIVATED		PUBLISHED pKa VALUES	COLOUR OF DYE WITH DIAZOTIZED SULPHANILIC ACID SPRAY
	SOLVENT A		SOLVENT B		SOLVENT C		SOLVENT D		SOLVENT E			
	R_F	R_{Im}	R_F	R_{Im}	R_F	R_{Im}	R_F	R_{Im}	R_F	R_{Im}		
2-acetyl-4(or 5)-methylimidazole	0.84	1.50	0.56	2.55	0.78	1.97	0.69	1.13	0.77	1.14	-	yellow-orange
2-ethyl-4(or 5)-methylimidazole	0.50	0.90	0.42	1.91	0.70	1.67	0.65	1.09	0.66	1.18	-	yellow
2, 4(or 5)-dimethylimidazole	0.36	0.64	0.32	1.45	0.54	1.33	0.62	1.02	0.75	1.09	8.36	lemon-yellow
4, 5-dimethylimidazole	0.56	0.88	0.28	1.21	0.51	1.23	0.62	1.03	0.68	1.00	-	yellow
2-methylimidazole	0.40	0.71	0.24	1.09	0.61	1.45	0.63	1.05	0.76	1.09	7.86	lemon-yellow
4(or 5)-methylimidazole	0.52	0.93	0.24	1.09	0.46	1.04	0.62	1.03	0.70	1.00	7.52	red
imidazole	0.56	1.00	0.22	1.00	0.42	1.00	0.61	1.00	0.70	1.00	6.95	orange
2-hydroxymethyl-4(or 5)-methyl- imidazole	0.43	0.79	0.02	0.09	0.15	0.30	0.46	0.76	0.30	0.45	-	red
4(or 5)-(2-hydroxyethyl)imidazole	0.30	0.54	0.06	0.27	0.11	0.21	0.49	0.82	0.41	0.61	-	red
4(or 5)-hydroxymethylimidazole	0.34	0.61	0.04	0.18	0.10	0.20	0.46	0.75	0.30	0.45	6.38	orange-red
2-mercaptoimidazole	0.74	1.33	0.14	0.64	0.52	1.24	0.57	0.95	0.51	0.91	-	orange
histamine dihydrochloride	0.04	0.07	0.02	0.09	0.00	0.00	0.02	0.07	0.02	0.09	5.98	red
histidine hydrochloride	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	-	red
4(or 5)-D-arabotetrahydroxy- butylimidazole	0.03	0.07	0.01	0.04	0.01	0.02	0.00	0.00	0.00	0.00	-	red
imidazole-4(or 5)-carboxylic acid	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	6.08	yellow
imidazole-4(or 5)-acrylic acid	0.11	0.20	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	-	yellow-orange
imidazole-4(or 5)-pyruvic acid	0.01	0.14	0.00	0.00	0.01	0.02	0.01	0.01	0.00	0.00	-	yellow-orange

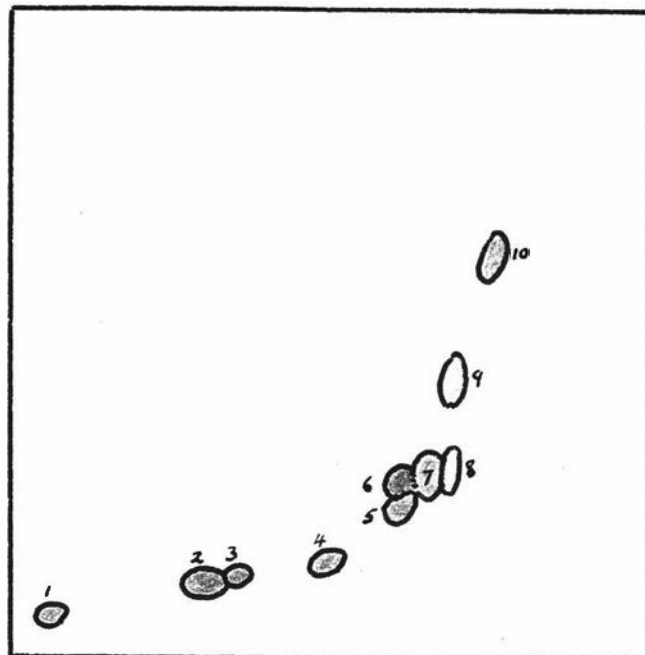
Solvents: A, chloroform/methanol/ammonia (80:25:0.1); B, toluene/ethylacetate/ammonia (1:3:0.1); C, chloroform/pyridine (2:1); D, toluene/95% ethanol (1:1); E, toluene/100% ethanol (1:1)

solvent A, and on aluminium oxide G chromatoplates in all of the solvent systems tabulated. In complex mixtures of imidazoles TLC has given good differentiation between methyl-substituted imidazoles which are difficult to separate by conventional paper chromatography. Further improvement has been achieved by using two-dimensional TLC on aluminium oxide G chromatoplates, while identification is aided by examination of the different colours of the azo-dyes produced on spraying with Pauly reagent. See Fig. C1.

For alumina chromatoplates the relationship between R_f values and pK_a values (see section (i)) appears to hold for the imidazoles of published pK_a values. Once again, R_{Im} values proved more reproducible than R_f values and usually compensated for any solvent or layer irregularities.

This successful use of alumina for the chromatography of imidazoles initiated the use of alumina column separations of reaction mixtures, thus providing an invaluable adjunct to cellulose column chromatography.

Ethyl acetate / toluene / ammonia (1:1:0.1)



Toluene/Ethanol (1:1)

Fig. C1. Two dimensional TLC on aluminium oxide G of a mixture of methyl- and hydroxyalkylimidazoles. Alkaline diazotised sulphanilic acid spray.

1. 4(5)-D-arabotetrahydroxybutylimidazole
2. 4(5)-hydroxymethylimidazole
3. 4(5)-(2-hydroxyethyl)-imidazole
4. 2-hydroxymethyl-4(5)-methylimidazole
5. imidazole
6. 4(5)-methylimidazole
7. 4,5-di-methylimidazole
8. 2-methylimidazole
9. 2,4(5)-dimethylimidazole
10. 2-acetyl-4(5)-methylimidazole.

II. SEPARATION AND DETERMINATION OF IMIDAZOLES BY COMBINED PAPER-CHROMATOGRAPHIC AND SPECTROPHOTOMETRIC TECHNIQUES.

The quantitative estimation of compounds containing the imidazole nucleus has proved of considerable interest particularly for the imidazoles of biological importance. Koessler and Hanke⁴² made use of the red colour which most imidazoles give with alkaline diazotised sulphanilic acid to estimate 10 μ g. or more of a number of imidazoles. As the dyes formed with diazotised sulphanilic acid proved to be rather unstable, some later workers^{6,23,48,54-5,70,77} have made use of non-sulphonated diazotised aniline derivatives which, with imidazoles, give dyes which can be extracted into organic solvents, in which the dyes were reported to be concentrated and stabilised.²³

Variations of these methods have been used to estimate histamine and histidine.^{21,25,57,81} A number of other methods for the colorimetric estimation of histamine and histidine exist,^{12,39,60} but these are not of universal application to imidazoles.

In the course of the investigation of the reaction between glyceraldehyde and ammonia it became necessary to separate and determine the individual imidazoles formed. As almost all imidazoles couple with diazotised aniline derivatives to give red, orange or yellow dyes²⁸ it was decided, after a study of a number of such derivatives, to

use diazotised sulphanilic acid as the colour-producing reagent. In order to obtain consistent results it proved necessary to make a study of the reaction conditions with this reagent and subsequently to modify the original Koessler and Hanke procedure. Colours produced with diazotised sulphanilic acid are prone to interference from phenols, transition metal ions, ammonium salts and a number of other compounds, but none of these species was expected to be present in the mixtures under study. Should any such species have been present the chromatographic separation carried out prior to the spectrophotometric estimation would have been expected to eliminate most of such interference. Mixtures of up to five imidazoles were readily separated by chromatography, using Whatman No. 3MM chromatography paper and one or more of the solvents listed in Table EI (page 170) (It was not possible to achieve a perfectly clean separation of 2-methylimidazole and 4(5)-methylimidazole by this method). The separated imidazoles could be eluted quantitatively from the paper for spectrophotometric estimation. Optimum working conditions have been established for the determination of imidazoles and standard curves plotted for imidazole, 4(5)-methylimidazole, 2-methylimidazole, 4(5)-hydroxymethylimidazole, 4(5)-(2-hydroxyethyl)-imidazole, 2-hydroxymethyl-4(5)-methylimidazole, L-histidine hydrochloride and histamine dihydrochloride. See Fig. 1, page 172.

These standard curves were found to obey Beer's Law and to be highly reproducible within the range of concentrations studied (See Fig. 2 page 173). The intensities of the azo dyes were found to vary with time, usually rising to a maximum in 2-8 minutes and then slowly falling off, and thus it was necessary to stipulate suitable times for reading the absorbances of the colours formed with the individual imidazoles. Although the wavelengths of the absorption maxima varied somewhat (See Fig. 3 page 175) all readings were standardised at 480 m μ . As reported by Macpherson⁵⁷ it was found that reproducible results could be achieved without careful temperature control.

The standard method described in the experimental section was found to give accurate and reproducible results within the concentration range studied.

It was thought that the use of non-sulphonated aniline derivatives such as p-bromoaniline and p-toluidine might offer some advantages as the dyes produced are soluble and stabilised in organic solvents.²³ Standard curves were therefore determined with a number of imidazoles using these compounds in the place of sulphanilic acid. However, when the colour was extracted into iso-butanol it was found that the accurate estimation of concentrations less than 10 μ g./ml. (4 μ g.) of imidazole was prevented by the high and rather variable blank obtained. Imidazoles could be extracted

with isobutanol, from paper chromatograms which had been sprayed with alkaline diazo-p-bromoaniline more rapidly than by the standard procedure. However, because of the large volume (approx. 20 ml.) of organic solvent required for complete extraction of the dye from the paper, and also the high blank, this method was only useful for the determination of quantities of imidazole greater than 10 μg (3-10% error).

The standard procedure using diazotised sulphanic acid, while requiring more care and time, gave more accurate and consistent results. The paper-chromatographic technique of separation should prove applicable to many mixtures containing imidazolic materials, while careful choice of solvent should eliminate most of the species which are known to interfere with the diazo reaction.

Use has been made of the combined paper-chromatographic and spectrophotometric technique for the separation and estimation of imidazoles:

- (i) In a comparison of the yields of 4(5)-methylimidazole from pyruvaldehyde and ammonia under different reaction conditions,
- (ii) In a survey of the rates of formation of the component imidazoles formed in the glyceraldehyde-ammonia system,
- (iii) In a comparison of yields of 4(5)-methylimidazole and 4(5)-hydroxymethylimidazole formed from aqueous ammoniacal solutions of dihydroxyacetone and hydroxypyruvaldehyde.

III. THE REACTION OF α -DICARBONYL AND α -HYDROXYCARBONYL COMPOUNDS WITH AQUEOUS AMMONIA.

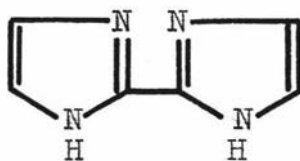
(i) The Reaction of Formaldehyde with Aqueous Ammonia.

When formaldehyde is treated with a mild base, sugar-like compounds are formed.^{48b} Generally a heterogeneous mixture of products is obtained but under certain conditions as much as 50% of the original formaldehyde has been converted to glycolaldehyde^{48a} which may then undergo aldol condensations to form trioses and tetroses. It has also been found that the reaction is very dependent on the metal ion involved in the base^{8a} e.g. reaction is very rapid with thallium or lead hydroxides, fast with calcium hydroxide and very slow with sodium or potassium hydroxides. Trace amounts of glycolaldehyde, glyceraldehyde or dihydroxyacetone also catalyse the reaction.

Reaction mixtures of formaldehyde in concentrated aqueous ammonia, with and without a trace of glycolaldehyde as catalyst, failed to produce imidazolic compounds even when heated for twelve hours at 100°. It is possible that the "formose" reaction has a very long induction time in the presence of ammonium hydroxide. Formation of glycolaldehyde and higher sugars may be prevented by formation of the formaldehyde-ammonia addition compound.

(ii) The Reaction of Glyoxal with Aqueous Ammonia.

This reaction was first studied in 1858 by Debus¹⁶ who heated a solution of glyoxal with three times its volume of concentrated ammonia at 60-70°. He noted that the reaction mixture rapidly became brown with the evolution of heat and the separation of colourless needles of a compound, of empirical formula $C_3H_4N_2$, which he named "glycosine". From the dark solution remaining after removal of this compound Debus isolated imidazole in high yield. The same reaction mixture was further studied by Lehmstedt⁵⁰ who suggested a structure for glycosine corresponding to 2,2'-bis-imidazole (I)



(I)

As this reaction mixture appeared to merit further attention, a 30% solution of glyoxal was treated at room temperature with concentrated ammonia solution. The mixture rapidly darkened with the evolution of heat and the separation of some solid material. Chromatography indicated the presence of at least seven imidazolic compounds, although only two were present in high concentration. After six weeks the solid was removed and purified by sublimation, followed by crystallization from 95% ethanol. The cream-white crystals obtained had properties and derivatives identical

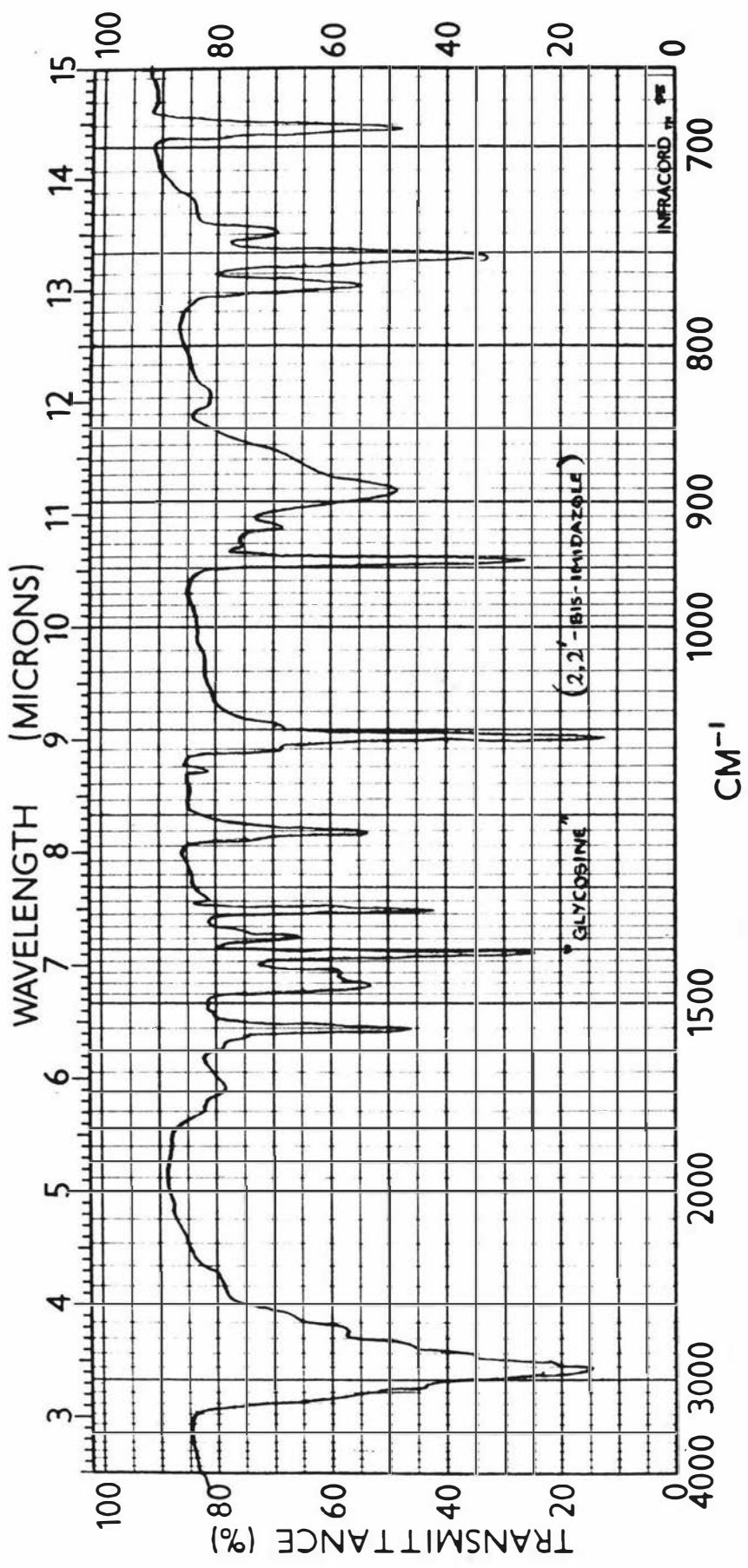
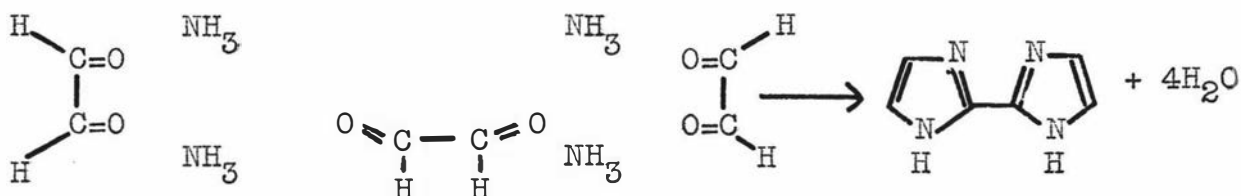


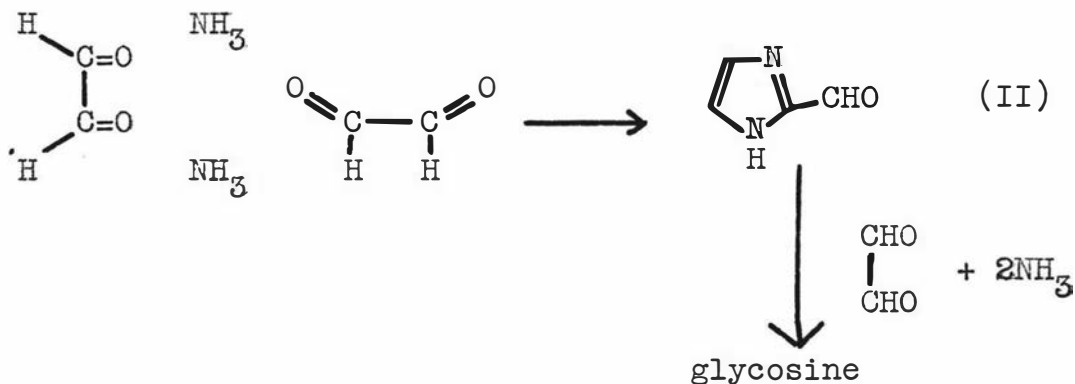
Fig. 1

to Lehmstedt's "glycosine". The orange colour given with diazotised sulphanilic acid indicated an imidazolic compound, while a proton magnetic resonance spectrum, determined in glacial acetic acid, gave a single peak at 451 c./sec. Such a result indicated that all non-exchangeable protons were equivalent and would tend to confirm the presence of the 2,2'-bond. The infrared spectrum (paraffin mull) resembled that of imidazole. See Fig. 1.

The formation of glycosine probably occurs by condensation of three molecules of glyoxal with four molecules of ammonia.



It is possible that the reaction may proceed via 2-formylimidazole (II) which subsequently condenses with a further molecule of glyoxal and two molecules of ammonia.



Attempts to isolate 2-formylimidazole (imidazole-2-aldehyde) from the glyoxal-ammonia mixture were not successful owing to the extremely high concentration of imidazole in the reaction product. The chromic acid oxidation of 2-hydroxymethylimidazole afforded a compound which gave a brown colour with the diazo spray reagent and moved to R_{Im} 0.53 (Whatman No. 3MM paper; n-butanol-acetic acid-water (4:1:1)). It did not prove possible to further purify this oxidation product or to obtain a crystalline derivative, but an infrared spectrum, although generally ill-defined, showed a broad band in the region $1600-1690\text{ cm}^{-1}$. The oxidation product was probably 2-formylimidazole as Turner⁸⁴ reported the carbonyl stretching band for 4(5)-formylimidazole at $1654-1696\text{ cm}^{-1}$. The brownish-orange colour and weak intensity of the azo dye is a known characteristic of imidazoles with an aldehyde group attached directly to the ring¹².

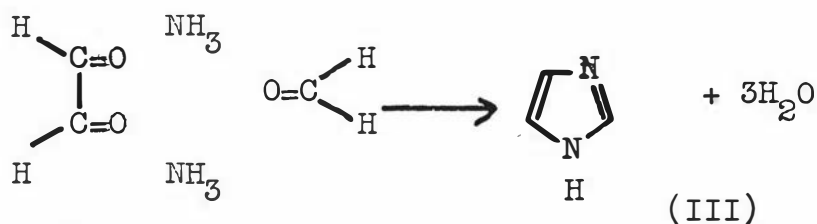
The oxidation product of 2-hydroxymethylimidazole was chromatographically identical, in a number of solvents, to one of the products, from the glyoxal-ammonia mixture, which also gave an orange-brown colour with the diazo reagent at R_{Im} 0.53 on Whatman No. 3MM paper in n-butanol-acetic acid-water (4:1:1). Although the evidence is somewhat tenuous, it seems that this compound was probably 2-formylimidazole.

A nitric acid oxidation of 2-hydroxymethylimidazole

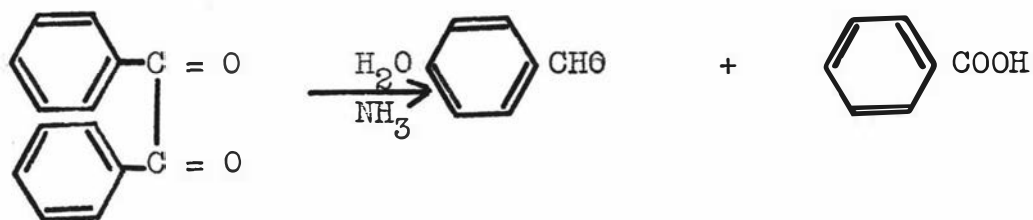
afforded mainly starting material with only traces of the compound at R_{Im} 0.53.

After filtration of the 2,2'-bisimidazole from the glyoxal-ammonia solution, the ammonia was removed and the aqueous solution continuously extracted with ether. Paper chromatography showed that only the imidazolic compounds with R_{Im} values of 1.00 or greater had been extracted to any extent. Vacuum distillation of a portion of this extract gave the parent base, imidazole (III), identified by melting point, infrared spectrum, and comparison of the picrate with an authentic sample.

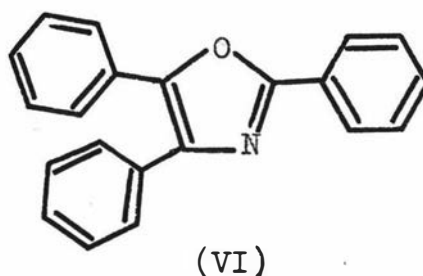
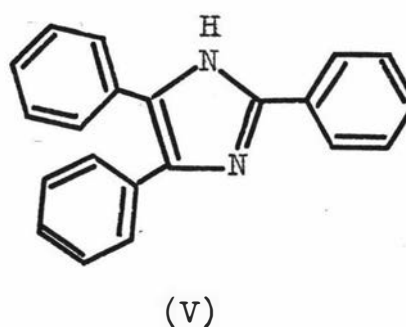
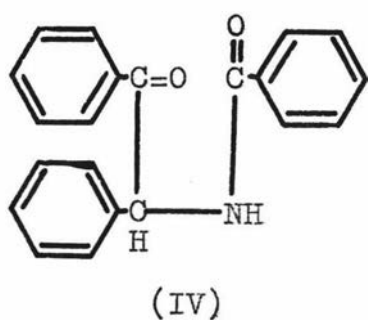
It is considered⁷² that imidazole is formed by the condensation of a molecule of glyoxal with two molecules of ammonia and one of formaldehyde, and it has been suggested



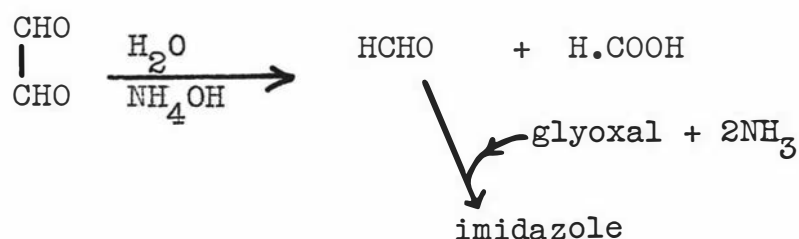
by Radziszewski⁷² that the formaldehyde results from fission of glyoxal under the influence of ammonia. Possibly this cleavage occurs as suggested by Japp³⁶ for the fission of benzil under similar conditions.



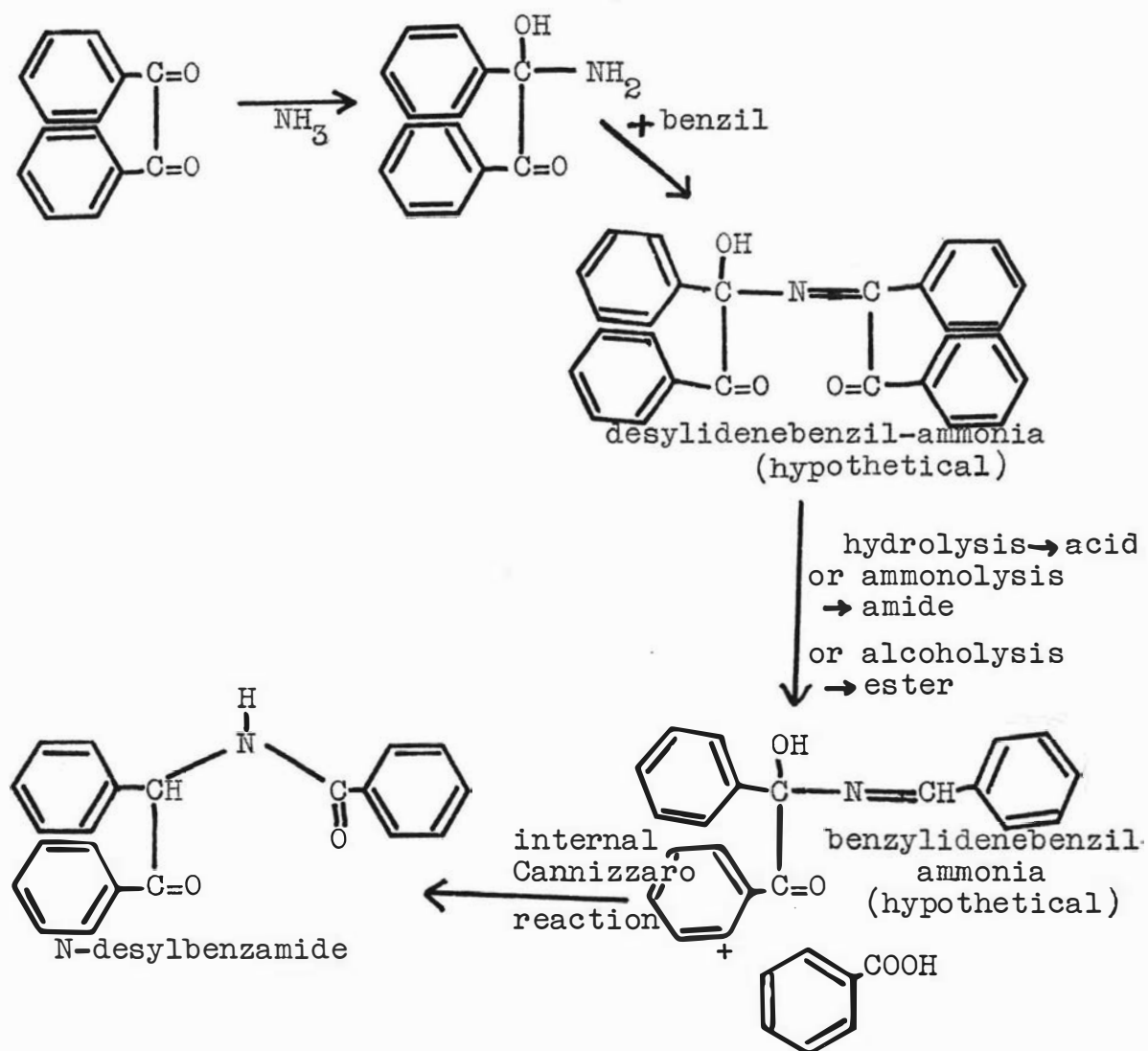
The benzoic acid may appear either as ethyl benzoate or as the amide, depending on conditions. Support for this mechanism came from Schönberg⁷⁶ who isolated ammonium benzoate from benzil in aqueous ammonia at 120°, and from Zinin⁹⁸ who found that the action of ammonia on benzil in ethanol at 70° gave ethyl benzoate. Benzil might then react with a molecule of benzaldehyde and one or two molecules of ammonia to produce N-desylbenzamide (IV) or lophine (2,4,5-triphenylimidazole) (V), both of which had been isolated from such a mixture by Laurent⁴⁹ along with 2,4,5-triphenyloxazole (VI).



This would give a mechanism for the fission of glyoxal similar to the following scheme:

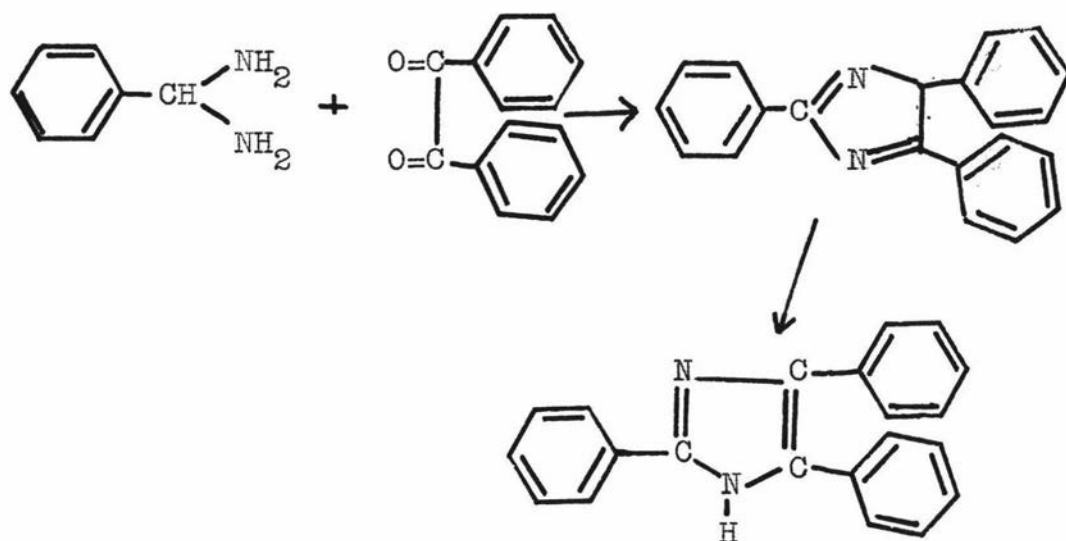


According to Davidson et al.¹⁵ there is an inherent weakness in Japp's mechanism for the cleavage of benzil. Radziszewski⁷² had shown that a mixture of benzaldehyde and benzil treated with ammonia in ethanol at 40° gave lophine as the exclusive product. Furthermore Zincke⁹⁷ had found that benzil did not undergo scission when treated with primary aliphatic amines. Davidson et al. therefore devised the following scheme which did not involve benzaldehyde:

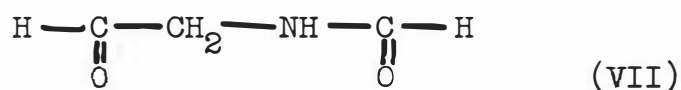


From N-desylbenzamide (a known reaction product) can be obtained 2,4,5-triphenyloxazole by cyclodehydration, or lophine by condensation with a molecule of ammonia.

The formation of lophine from the interaction of benzil, benzaldehyde and ammonia was explained by Davidson et al.¹⁵ by initial conversion of the benzaldehyde to the diamine, condensation with benzil, and then a Cannizzaro reaction:-



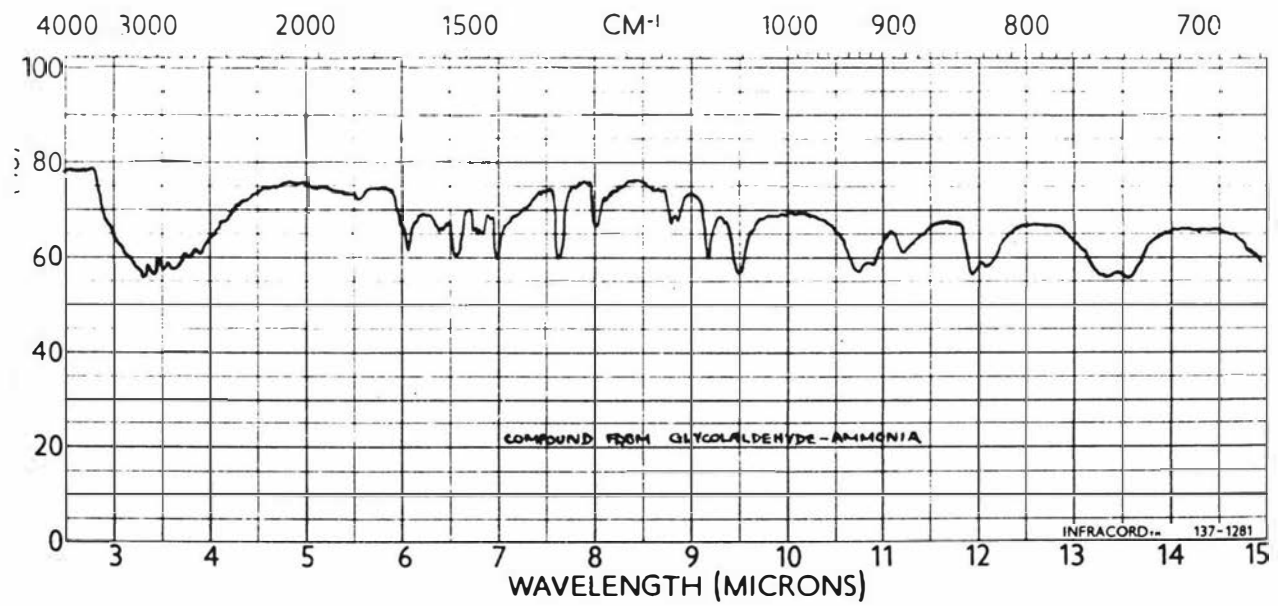
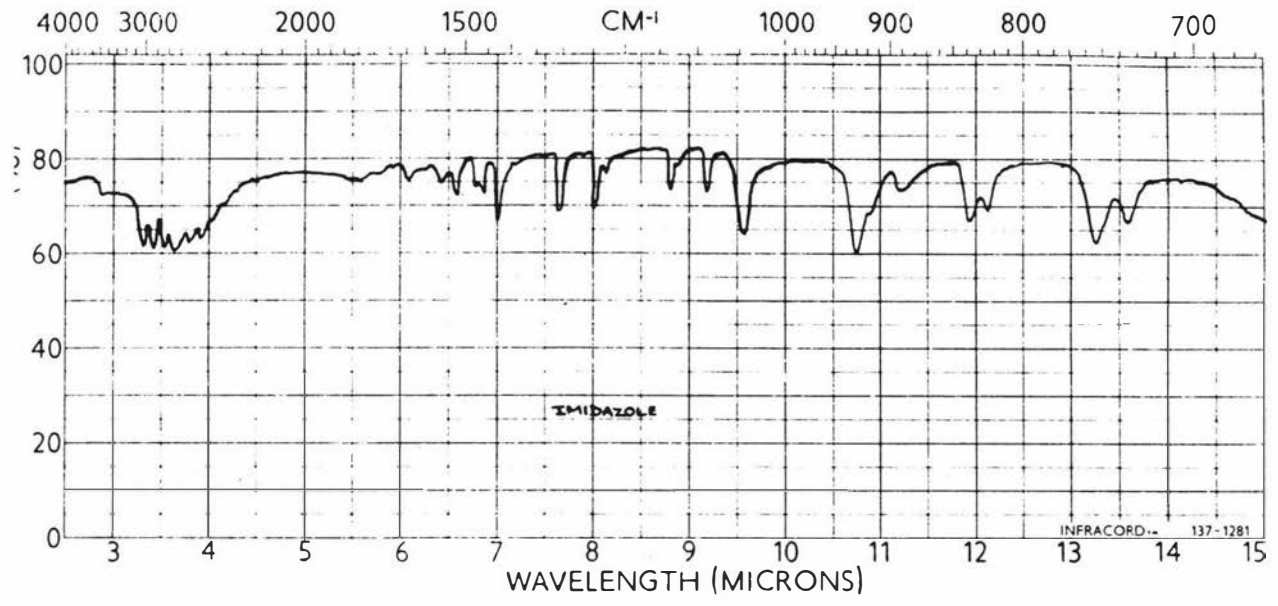
If the formation of imidazole from glyoxal was postulated to follow the benzil-ammonia pathway both formic acid and the compound of structure (VII) would be expected in the reaction mixture. There has been no mention in the literature of this compound having been identified.



It may be concluded that the reaction is rather complex and both Japp's and Davidson's mechanisms may apply.

Separation of further imidazolic compounds from either the ether extract or aqueous fraction of the glyoxal-ammonia mixture did not prove possible. As imidazoles with an amino substituent attached directly to the ring in the 4(5) position are reported²⁷ to give blue dyes with diazotised sulphanilic acid it is possible that the compound present giving such a reaction could be an imidazole of this type.

Fig. 2

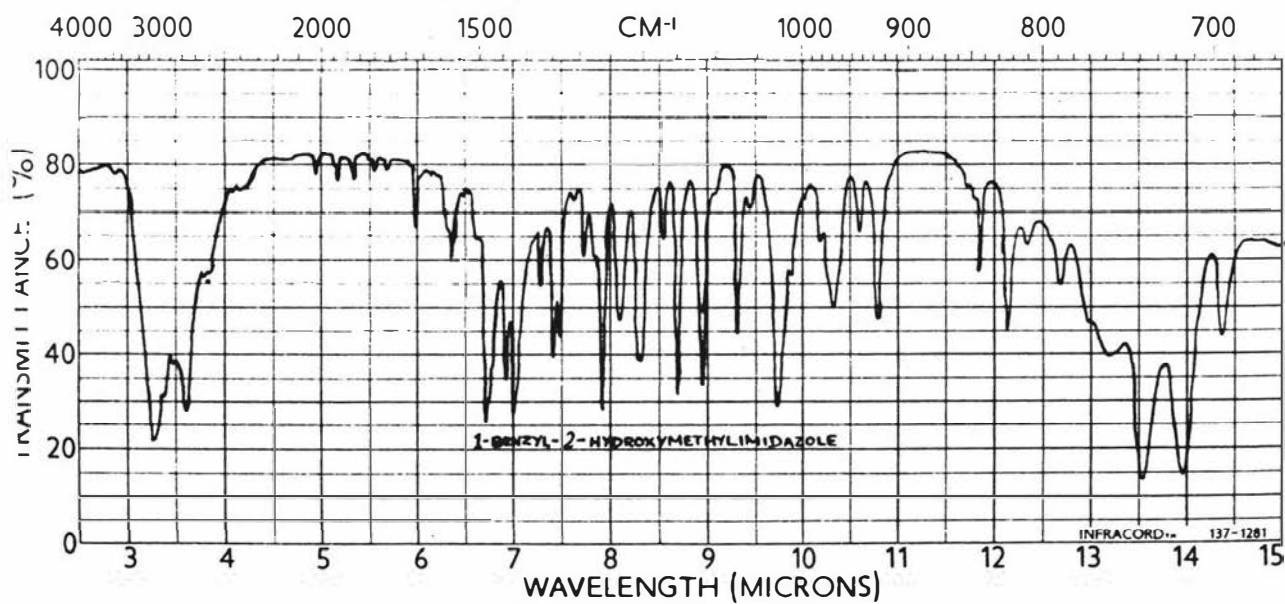
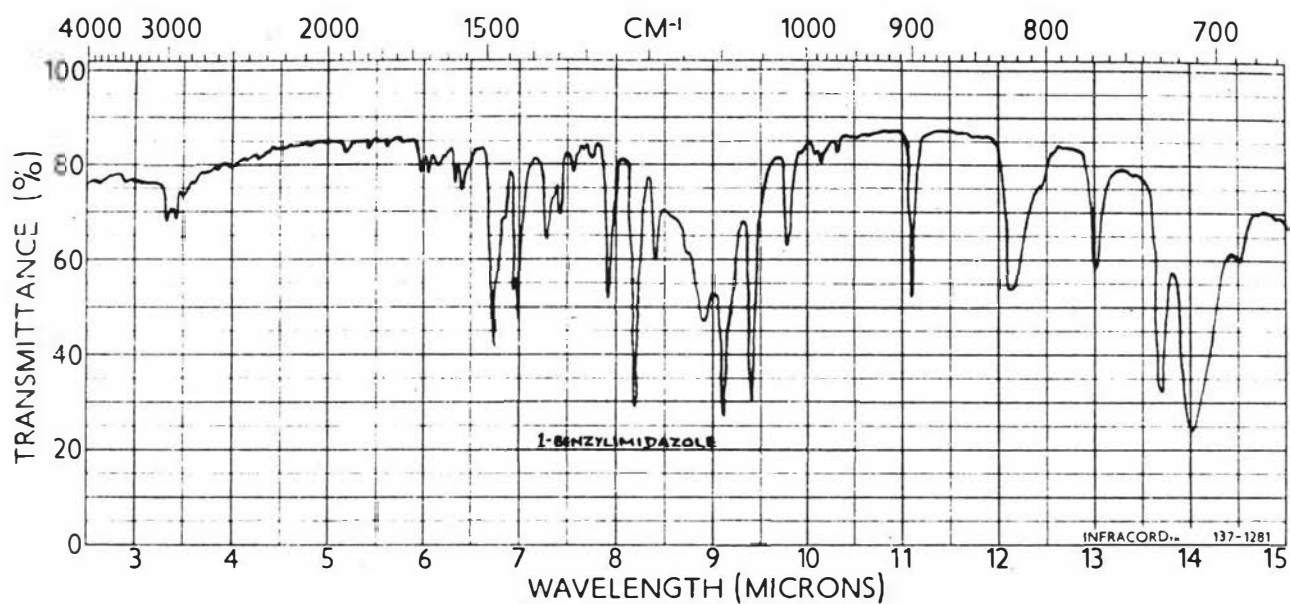


(iii) The Reaction of Glycolaldehyde with Ammonia.

A mixture of glycolaldehyde and aqueous ammonia, stored at room temperature in a stoppered flask, slowly assumed a pale-yellow colour. Paper chromatography at the end of eight weeks showed the presence of two major products giving orange dyes with the diazotised sulphanilic acid spray, and a number of minor imidazolic components travelling behind these two on chromatograms. One of the major components travelled to the same position as imidazole on paper chromatograms, while the other moved somewhat faster. The solution was evaporated to a syrup which was fractionated on an alumina column using chloroform, with increasing amounts of methanol and ammonia as eluant. The compound with chromatographic behaviour resembling imidazole was eluted with chloroform/methanol (1:1) while the other major component was much more strongly adsorbed and required methanol ammonia for elution. The infrared spectrum (film) of the compound with R_{Im} 1.00 was identical to that of an authentic sample of imidazole (see Fig. 2) while a picrate derivative proved identical in melting-point and microanalysis to imidazole picrate.

Passage of chloroform/methanol/ammonia and then methanol/ammonia through the alumina column removed the more strongly adsorbed imidazolic compound. When the syrup obtained on evaporation of the solvent was treated

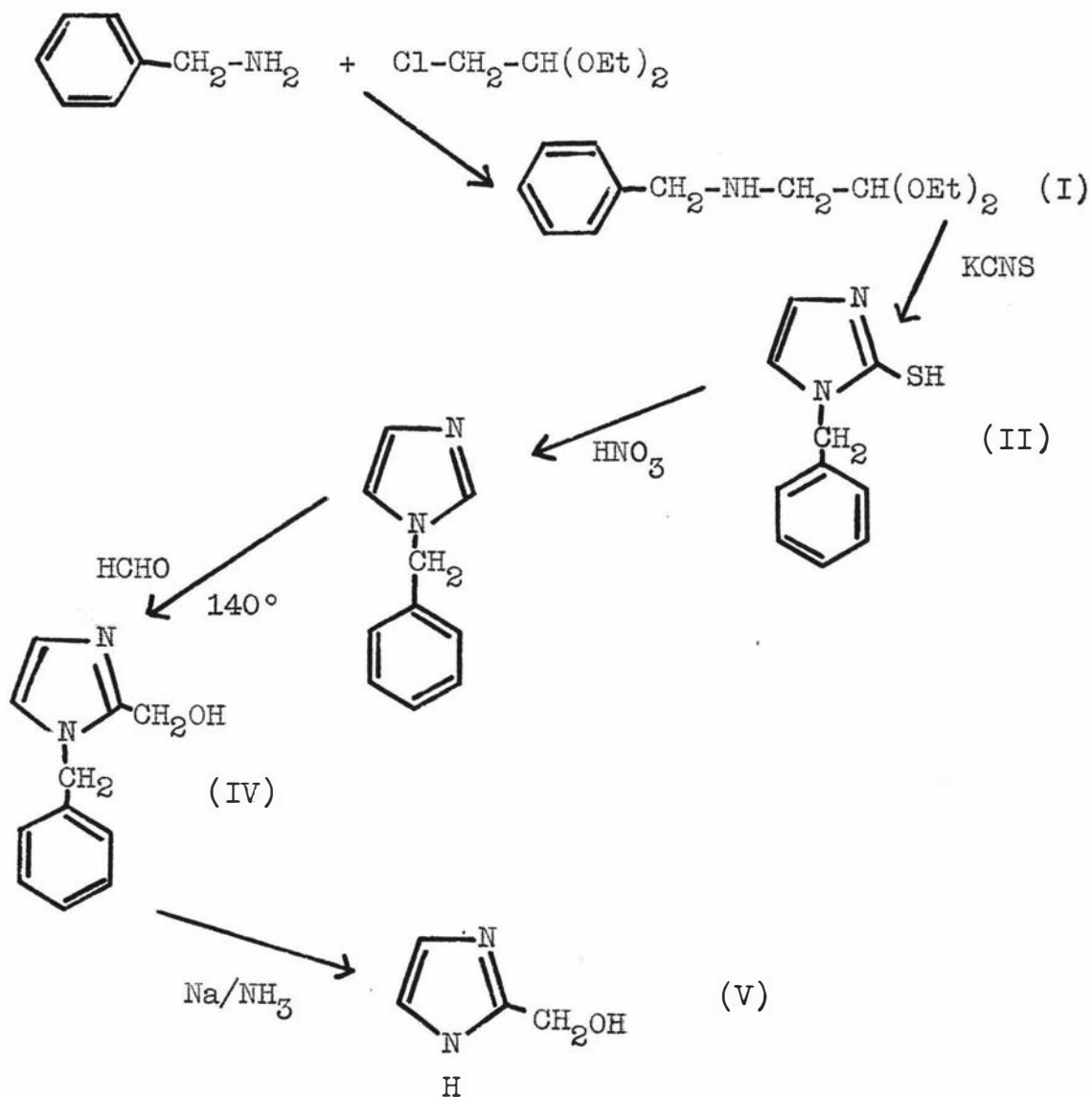
Fig. 3



with picric acid, a picrate was obtained analysing for $C_{10}H_9N_5O_8$. This corresponded to the picrate of a hydroxymethyl-substituted imidazole, while the melting point was the same as that of 2-hydroxymethylimidazole picrate.³⁸

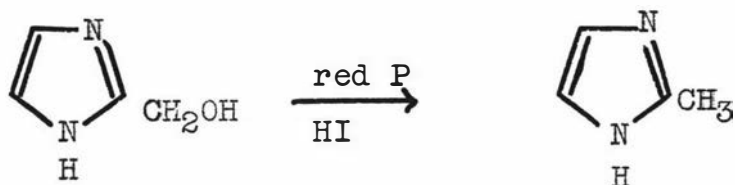
In order to confirm the presence of 2-hydroxymethylimidazole in the reaction mixture, a quantity of the authentic compound was prepared by some modification of the method described by Jones.³⁸ N-Benzylaminoacetal (I) was prepared from the reaction of chloroacetal with benzylamine, and then converted to 1-benzyl-2-mercaptoimidazole (II) using potassium thiocyanate in ethanol. The mercapto-group was removed by treatment with concentrated nitric acid yielding 1-benzylimidazole (III). Hydroxy-methylation of the 1-benzylimidazole following Jones' conditions resulted in a mixture of products, but when the reaction time was increased the yield of 1-benzyl-2-hydroxymethylimidazole (IV) improved. Purification of this product was achieved via the hydrochloride, which was decomposed with barium carbonate, allowing isolation of 1-benzyl-2-hydroxymethylimidazole in the crystalline form (Jones had only reported its presence as a syrup). Microanalysis and infrared spectroscopy (See Fig. 3) confirmed the identity of this compound. Reaction of 1-benzyl-2-hydroxymethylimidazole with sodium in liquid ammonia removed the benzyl group and gave (on ethanol extraction of the product) a

syrup yielding 2-hydroxymethylimidazole picrate. Purification of the free base (V) by the use of alumina and cellulose column chromatography permitted its isolation in the hitherto unreported crystalline form.

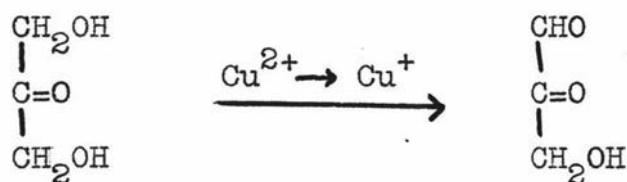


There was a marked similarity between the infrared spectra of the authentic 2-hydroxymethylimidazole and the compound isolated from the glycolaldehyde-ammonia

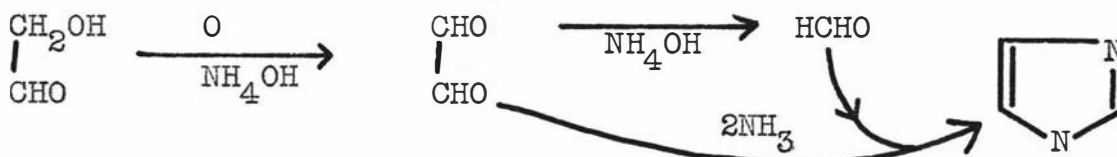
mixture. The structure was confirmed by a mixed-melting point determination on the picrate, comparison of chromatographic data, and finally by reduction of the compound to 2-methylimidazole.



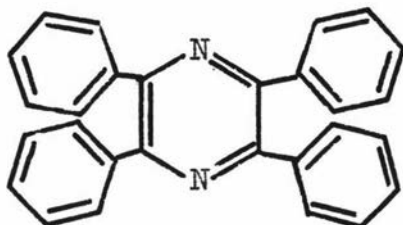
The formation of imidazolic compounds from glycolaldehyde and ammonia would seem to require some oxidation of glycolaldehyde to glyoxal. Such oxidations of α -hydroxy-carbonyl compounds occur readily in the presence of such oxidizing agents as the cupric ion e.g. oxidation of dihydroxyacetone to hydroxypyruvaldehyde,¹⁸



but osone formation by air oxidation does not appear to have been reported. Nevertheless, if imidazole is formed by the condensation of a molecule of glyoxal with one of formaldehyde and two of ammonia it is necessary to postulate formation of glyoxal from glycolaldehyde under concentrated ammoniacal conditions.



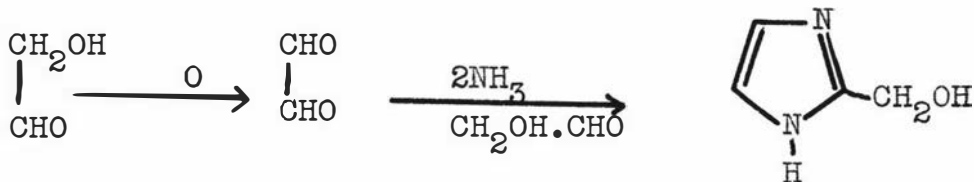
That such an oxidation may occur (at least to a slight extent) is supported by Leukart⁵³ who found that from the interaction of benzoin and ammonium formate at 230° he obtained mainly amarone (2,3,5,6-tetraphenylpyrazine)(I)



(I)

but also traces of lophine (2,4,5-triphenylimidazole) and benzaldehyde.

Formation of 2-hydroxymethylimidazole from glycolaldehyde in ammoniacal solution probably proceeds by the following mechanism:-



BROWNING OF GLYCERALDEHYDE IN
AMMONIA AT 37°C.

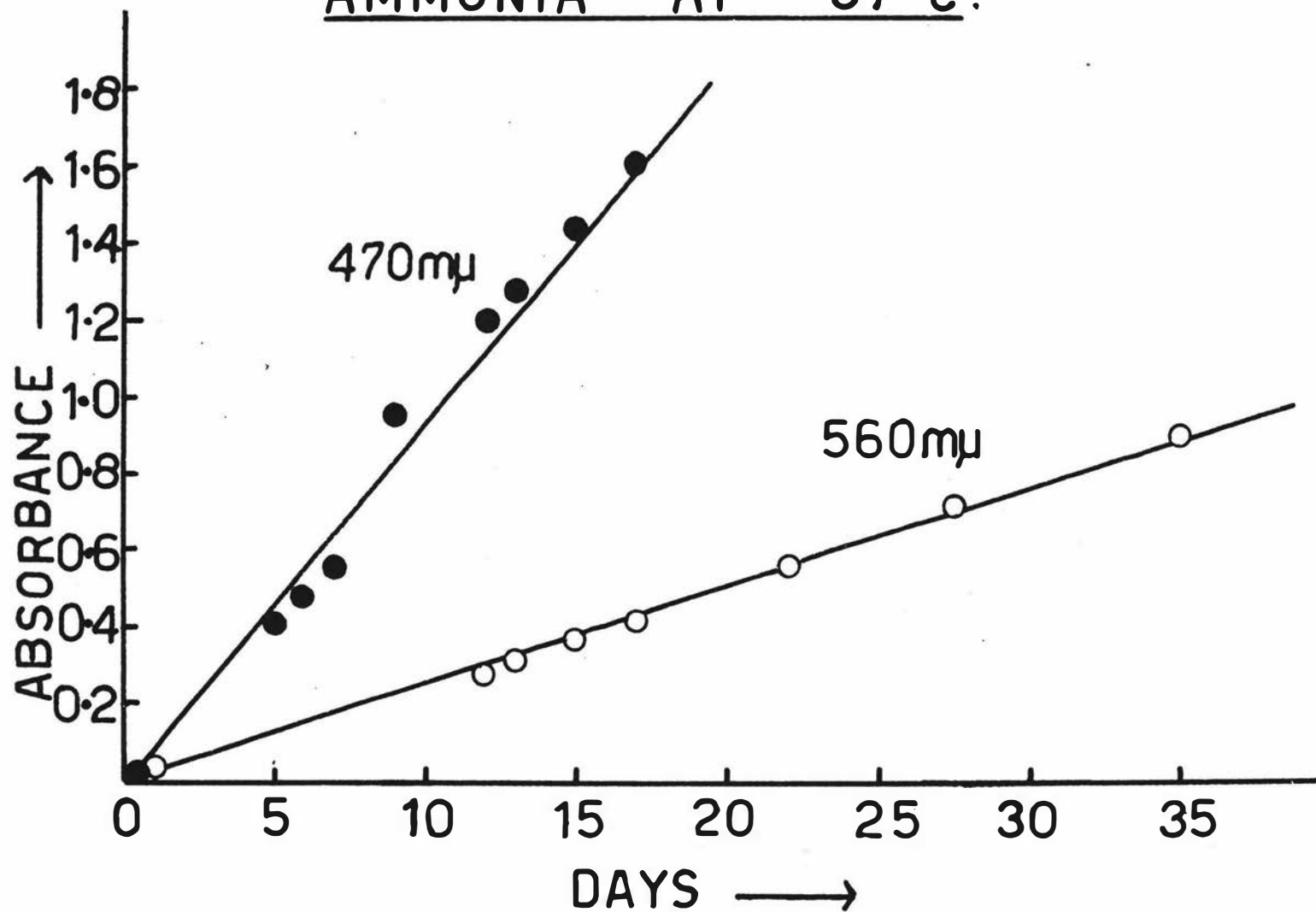


Fig. 4

(iv) The Reaction of DL-Glyceraldehyde with Aqueous Ammonia.

In 1933 Parrod⁶³ isolated 4(5)-methylimidazole from a reaction mixture containing glyceraldehyde, aqueous ammonia and a metallic salt as catalyst. Since that time no further interest appears to have been taken in this reaction, although a number of workers^{47,62} have stated that glyceraldehyde is a degradation product of hexose sugars in ammoniacal solution.

It was thought that a study of the glyceraldehyde-ammonia system might help in the clarification of the complex mechanism involved in the reaction of sugars in ammoniacal solution.

In a preliminary experiment, glyceraldehyde was dissolved in aqueous ammonia and stored at 37°. The solution became dark brown in colour, the absorbance of which was measured at regular intervals (See Fig. 4). Paper chromatography, and the use of a wide range of detection reagents for sugars, amines, and imidazoles showed that an extremely complex mixture had been formed (See Table E5. page 187).

A large scale mixture was therefore prepared and stored in a stoppered flask at 37° for eight weeks. The basic compounds were separated from the neutrals by ion-exchange chromatography and the latter fractionated by partition chromatography on a cellulose column. The fractions from the column were examined by paper-chromatography and combined into five main fractions which were found to contain

a ketotriose, aldopentoses and aldo- and ketohexoses. Identification on the chromatograms was achieved by use of the p-anisidine hydrochloride spray reagent³¹ (which gives yellow or brown colours with hexose sugars and pink colours with pentose sugars) and urea phosphate spray³¹ (which gives a blue colour with ketoses). By use of these location reagents, and chromatography in a number of solvents (see Table 1) it proved possible to tentively identify dihydroxyacetone, lyxose, xylose, arabinose, fructose, mannose and glucose. The first-named compound was also isolated in the form of the 2,4-dinitrophenylhydrazone. A further compound in very low concentration chromatographically resembled ribose.

The formation of these products from glyceraldehyde in ammoniacal solution undoubtedly follows the general courses of alkaline degradation discussed in the Introduction. Dihydroxyacetone would be formed by isomerization, the various hexoses by condensation of two triose units and then epimerisation, and the pentoses either by reverse aldolization of the hexoses or by the recombination of smaller fragments.

The basic compounds were stripped from the ion-exchange resin with hydrochloric acid, evaporated to a syrup and then fractionated on a cellulose column into a number of fractions containing imidazoles.

TABLE 1

SUGARS PRESENT IN GLYCERALDEHYDE/AMMONIA MIXTURE.

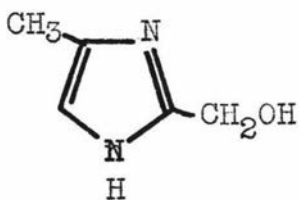
Fraction	Sugars Present	Solvents used for Identification	p-Anisidine HCl Spray	Urea Phosphate Spray	Aniline Phosphate Spray
1.	dihydroxy-acetone	I, VII*	brown	brown	nil
2.	lyxose ribose?	I, III, IV, V I, V	pink pink	nil	red-brown
3.	xylose arabinose	III, IV, VI I	pink pink	nil nil	red-brown red-brown
4.	arabinose fructose mannose	I, III, V, VI, I, III, IV I, III, V	pink brown brown	nil blue nil	red-brown nil red-brown
5.	glucose	I, III, V	brown	nil	brown

* 2,4-Dinitrophenylhydrazone, m.p. 276° (decomp.) Lit. m.p. 277-278° (decomp).

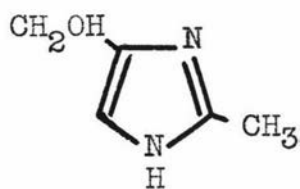
Solvents: (I) n-butanol/glacial acetic acid/water (4:1:1); (II) acetone/chloroform/water/28% ammonia (30:5:4:0.2); (III) ethyl acetate/pyridine/water (2:1:2); (IV) n-butanol/ethanol/water/28% ammonia (4:1:4.9:0.1); (v) phenol/water (4:1); (VI) ethyl acetate/glacial acetic acid/formic acid/water (18:3:1:4); (VII) n-butanol/pyridine/water (8:3:3).

The first fraction from the column contained a very small amount of a compound giving a yellow colour with the diazo spray, perhaps indicating a 2-substituted imidazole, but although a small amount of a picrate could be formed, there was insufficient for chemical analysis.

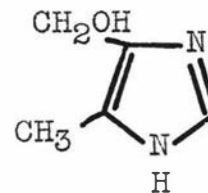
The second fraction comprised a compound giving a red colour with diazotised sulphanilic acid and moving to R_{Im} 1.37 on Whatman No. 3MM paper chromatograms in n-butanol/acetic acid/water (4:1:1). Treatment of a portion of the syrup with picric acid yielded a picrate with melting point (78-80°) and nitrogen analysis corresponding to the sesquihydrate of 2-hydroxymethyl-4(5)-methylimidazole (I) picrate. This compound had been isolated by Komoto⁴³ from an ammoniacal solution of glucose. As reported by Komoto, it was found that the picrate could be dehydrated by heating in vacuo for 48 hours. From a close study of the literature it appeared that previous workers^{43,63} had failed to confirm that the structure of this compound conformed to I rather than II (physical data available for 4-hydroxymethyl-5-methylimidazole (III) allowed this further possible structure to be ruled out). In order to confirm one of these



(I)

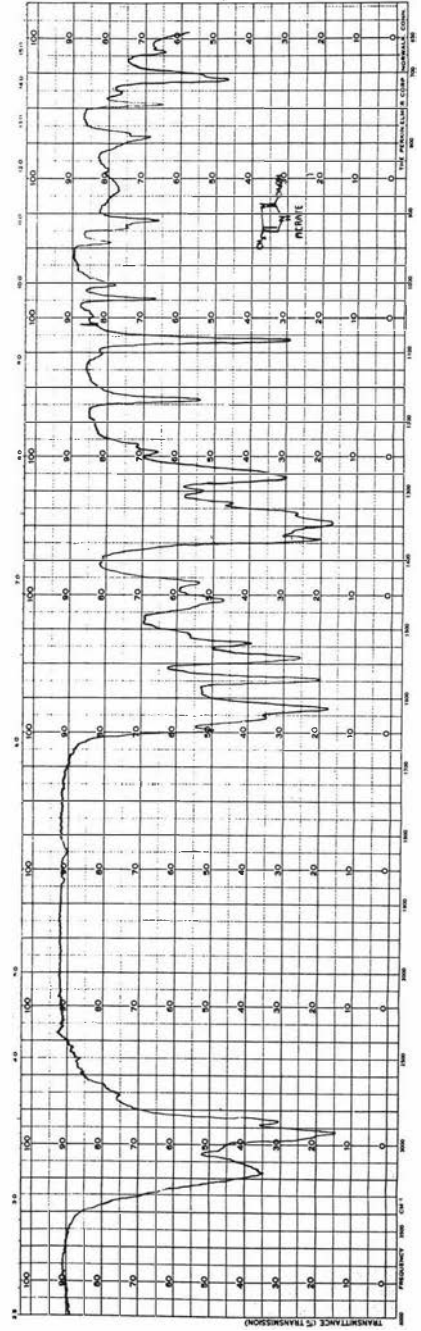
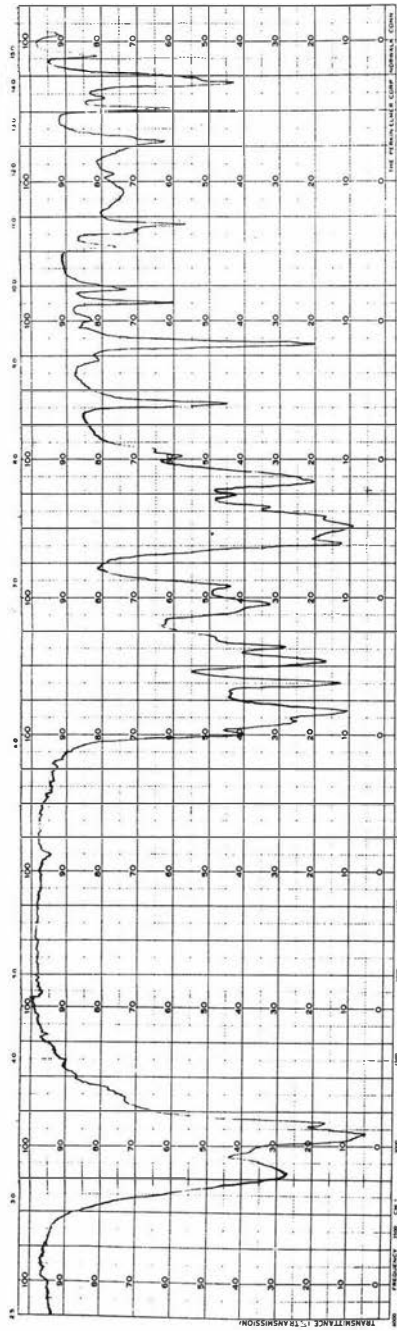
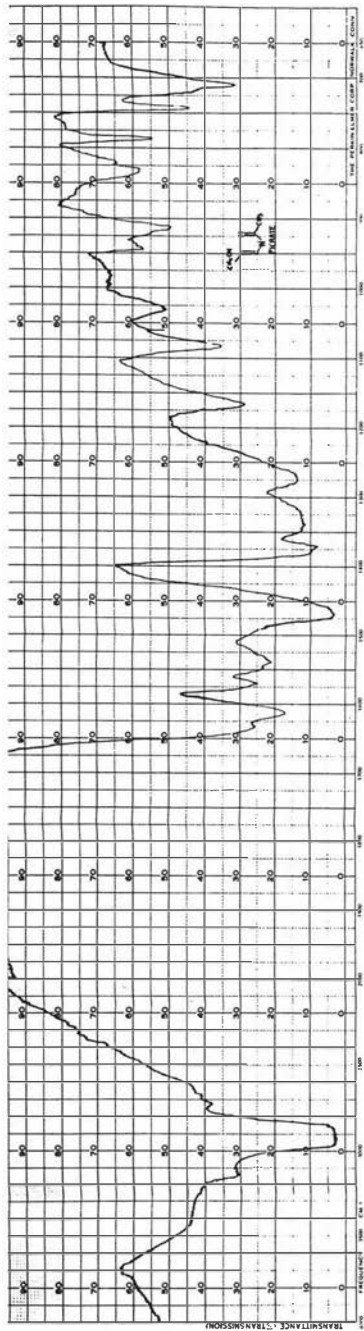


(II)



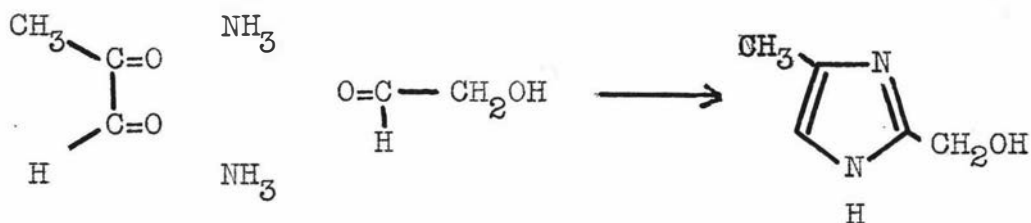
(III)

Fig. 5

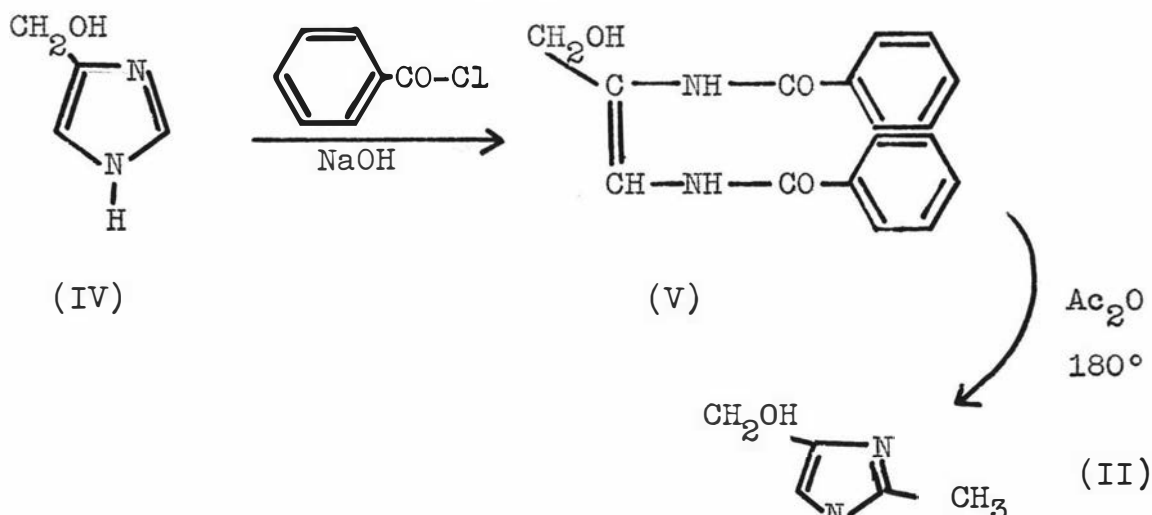


structures it was decided to synthesise isomers I and II by non-ambiguous methods.

2-Hydroxymethyl-4(5)-methylimidazole was prepared by the reaction of pyruvaldehyde, glycolaldehyde and aqueous ammonia. The imidazolic product was isolated from the reaction mixture in the form of a picrate which had melting point, mixed melting point and infrared spectrum identical to the compound isolated from the glyceraldehyde-ammonia mixture. (See Fig. 5)

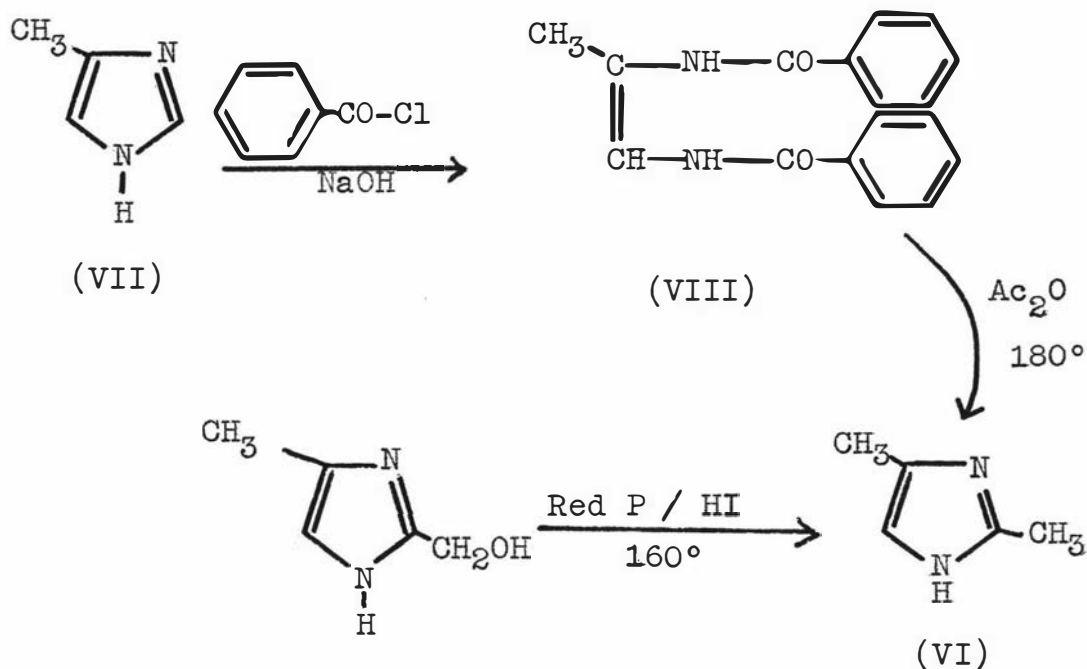


2-Methyl-4(5)-hydroxymethylimidazole was prepared from 4(5)-hydroxymethylimidazole (IV) by a Bamberger⁵ degradation to 1,2-dibenzamido-3-hydroxy-1-propene (V) and then by reaction of this product with acetic anhydride at 180°. The compound, II, was isolated

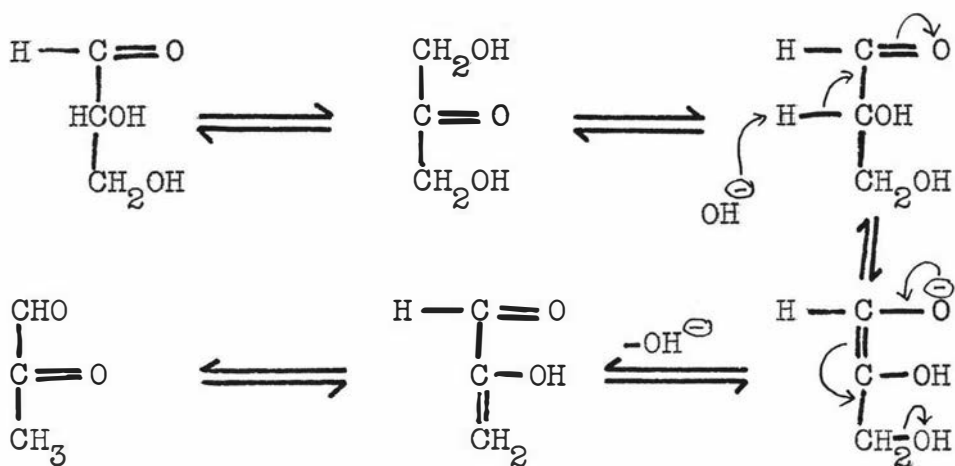


as a picrate with melting point identical to that of the compound prepared by Mackay and Shepherd⁵⁶ from dihydroxyacetone, acetaldehyde and ammoniacal cupric carbonate. A comparison of the infrared spectra of the picrates is shown in Fig. 5. From the top the picrates are those of 2-methyl-4(5)-hydroxymethylimidazole, the compound from glyceraldehyde-ammonia and 2-hydroxymethyl-4(5)-methylimidazole.

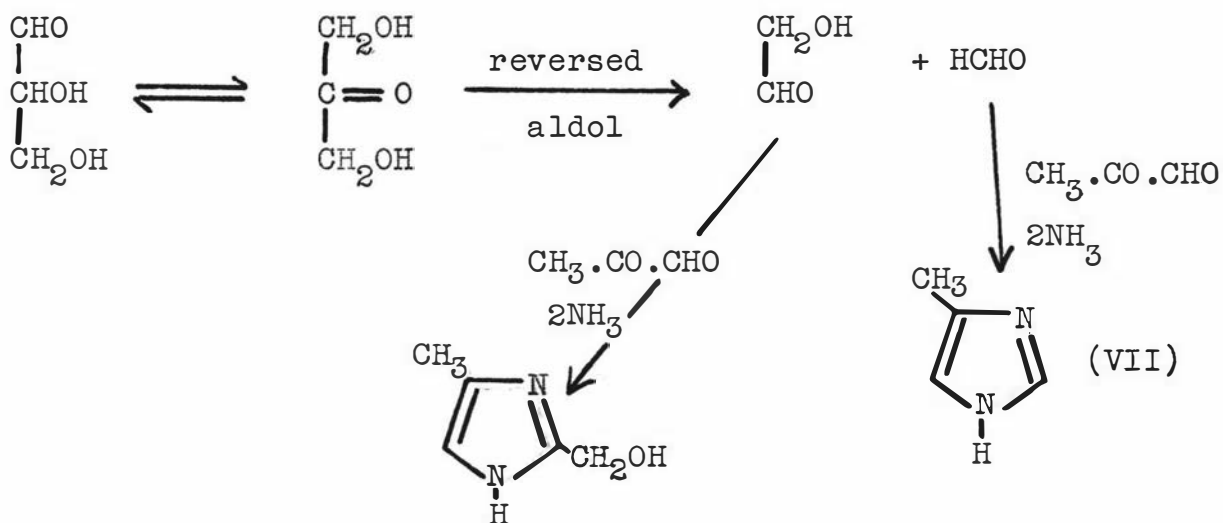
Further confirmatory experiments carried out on the compound isolated from the glyceraldehyde--ammonia system included comparison of chromatographic behaviour in a number of solvents and reduction with red phosphorus and hydriodic acid to 2,4(5)-dimethylimidazole (VI). This latter compound was prepared for comparison by the method of Windaus and Langenbeck⁹⁰ from 4(5)-methylimidazole (VII) via 1,2-dibenzamido-1-propene (VIII).



The mechanism of formation of 2-hydroxymethyl-4(5)-methylimidazole involves preformation of pyruvaldehyde and glycolaldehyde from glyceraldehyde. It is well known that pyruvaldehyde is formed from trioses under alkaline conditions by a rearrangement reaction involving loss of the elements of water. The mechanism reported by Sowden and Pohlen⁸⁰ for this transformation is as follows:



Glycolaldehyde is probably formed by a reversed aldol-ization of the triose, and when it combines with a molecule of pyruvaldehyde and two molecules of ammonia 2-hydroxy-methyl-4(5)-methylimidazole is formed.



A further fraction from the cellulose column separation was found to contain traces of an imidazole travelling slightly behind 2-hydroxymethyl-4(5)-methylimidazole on paper chromatograms. It did not prove possible to separate the unknown compound in a sufficiently pure state to allow meaningful structural analysis, but an n.m.r. spectrum in deuterium oxide pointed to this compound having an hydroxy-methyl group directly attached to an aromatic ring, while a further signal resembled a 4- or 5-proton in an imidazole ring.

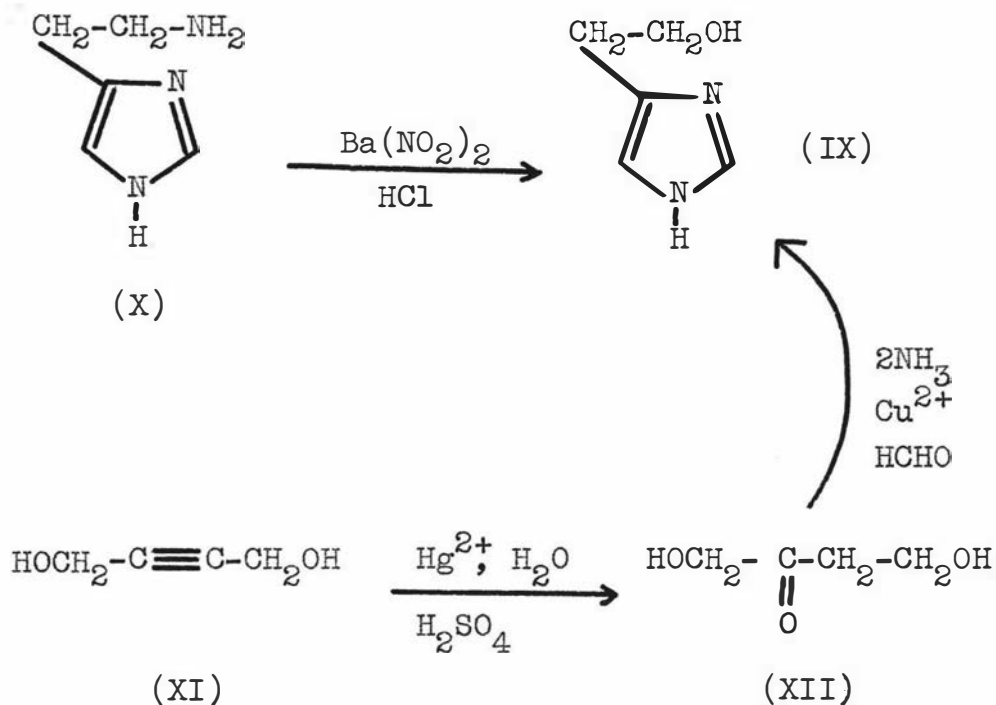
Fraction 4 from the column proved to contain 4(5)-methylimidazole (VII), the structure of which was confirmed by comparison of the physical properties of a number of derivatives with those obtained from a sample of 4(5)-methylimidazole prepared by the method of Koessler and Hanke.⁴² Treatment of the isolated compound at 0° with

benzoyl chloride in concentrated aqueous sodium hydroxide gave 1,2-dibenzamido-1-propene (VIII).

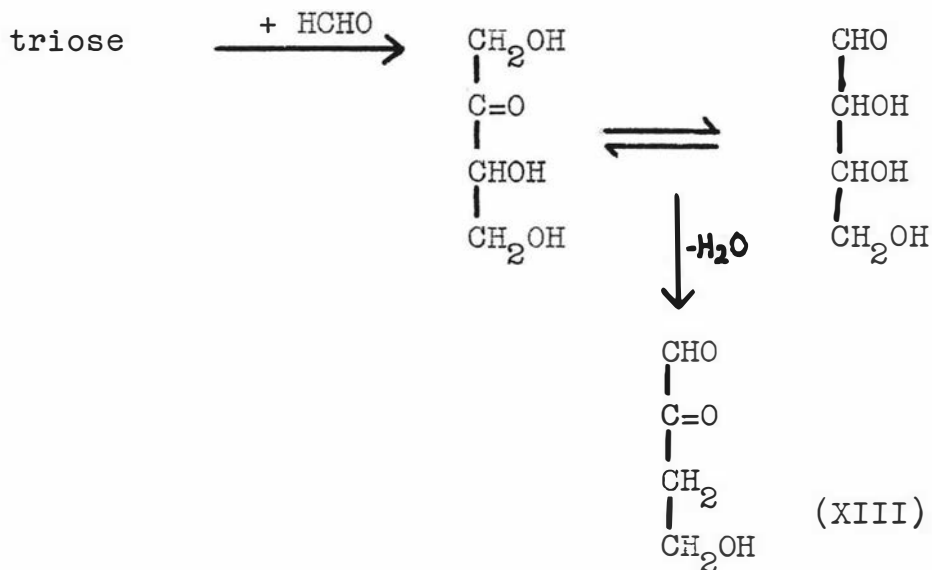
Formation of 4(5)-methylimidazole from glyceraldehyde undoubtedly proceeds by condensation of a molecule of pyruvaldehyde with a molecule of formaldehyde and two molecules of ammonia (see reaction scheme, page 76).

Whether the formaldehyde originates from reverse aldolization of triose or hexose is uncertain. It is possible that both pathways may contribute. The presence of glycolaldehyde in the mixture is evidence for the former, while the identification of pentose components points to the latter (if it is assumed that they are formed by degradation of hexoses rather than recombination of smaller fragments). It is even possible that some alkaline fission of pyruvaldehyde may occur, although this has been denied by Bernhauer.⁷

Paper-chromatographic examination of fraction 5 from the cellulose column separation showed the presence of an imidazole moving to R_{Im} 0.99 in n-butanol/acetic acid/water on Whatman No. 3MM paper. The compound was purified by chromatography in the same solvent on sheets of thick (No.3MM) paper and yielded a picrate which proved identical in melting point and mixed melting point to a sample of the picrate of 4(5)-(2-hydroxyethyl)imidazole (IX) prepared from histamine (X) by the method of Erlenmeyer et al.¹⁷ (See Experimental Section, page 158).

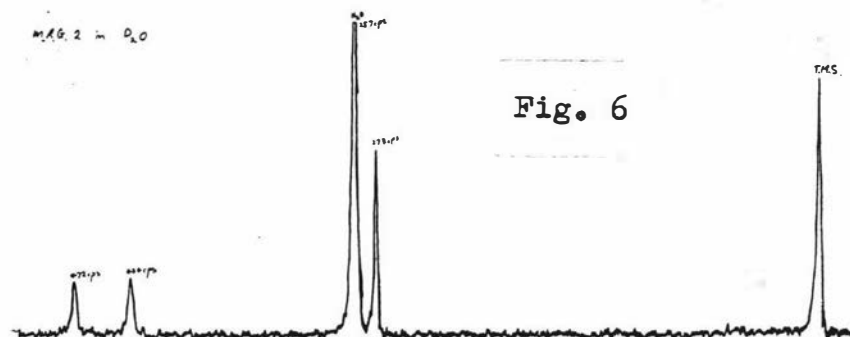


The same compound was also prepared by the method of Turner⁸⁵ in which 2-butyne-1,4-diol (XI) is hydrated to 1,4-dihydroxybutan-2-one (XII) which may be converted, with ammoniacal cupric acetate and formaldehyde, to 4(5)-(2-hydroxyethyl)imidazole. Komoto⁴⁷ explained the formation of 4(5)-(2-hydroxyethyl)imidazole from glucose in ammoniacal solution by postulating the formation of the required intermediate, 4-hydroxybutan-1,2-dione (XIII), through the following reaction scheme:-

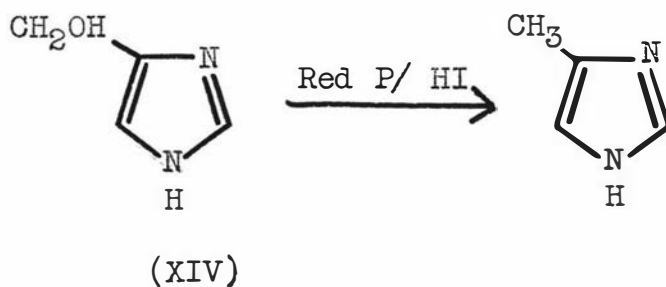


The sixth fraction was found to contain an imidazolic compound giving an orange-red colour with alkaline diazotised sulphanic acid. From an aqueous solution of this compound, was isolated in the form of a picrate, a crystalline compound analysing for a hydroxymethyl-substituted imidazole picrate, and having a melting point (and mixed melting point) identical to that of a sample of 4(5)-hydroxymethylimidazole (XIV) picrate, prepared from fructose by the method of Totter and Darby.⁸³ A proton magnetic resonance spectrum determined in deuterium oxide for the isolated free base (See Fig. 6) had peaks at 424, 472, and 273 c./sec. (from TMS) in the ratio, 1:1:2. When the spectrum was compared with that of imidazole⁸⁶ the singlet at 424 c./sec. was assigned to a 5(4)-proton, the singlet at 472 c./sec. to a 2-proton, and the two proton singlet at 273 c./sec. to a CH_2OH group attached directly to an aromatic ring (cf benzyl

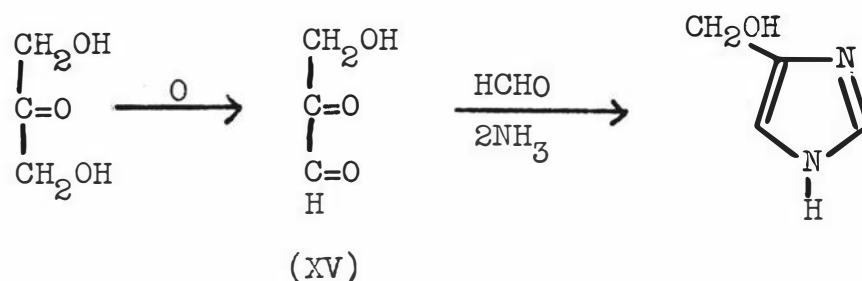
alcohol⁸⁷ -CH₂OH at 274 c./sec.).



Because of the relative insolubility of the compound in deuteriochloroform it was not possible to obtain confirmation of -OH or -NH protons. On reduction with red phosphorus and hydriodic acid a further quantity of the syrup from fraction 6 gave 4(5)-methylimidazole, confirming the structure of the compound as 4(5)-hydroxymethylimidazole.



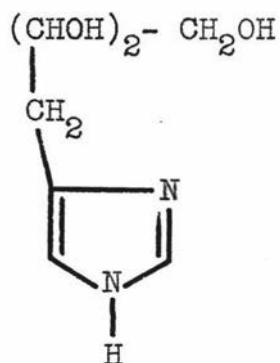
4(5)-Hydroxymethylimidazole is probably formed by the condensation of hydroxypyruvaldehyde (XV), formaldehyde and ammonia. The origin of the hydroxypyruvaldehyde in the mixture of glyceraldehyde and ammonia is uncertain but it probably arises by oxidation of dihydroxyacetone. Some



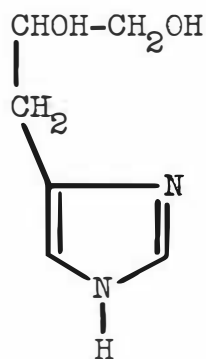
evidence in favour of this oxidation came from a study of two mixtures of ammoniacal glyceraldehyde, one tightly stoppered and the other undergoing aeration. Examination of the areas and intensities of spots on paper chromatograms showed that whereas 4(5)-methylimidazole and 4(5)-hydroxymethylimidazole were formed in both cases, the ratio of amounts of 4(5)-methylimidazole to the amounts of 4(5)-hydroxymethylimidazole was greater than one in the case of the stoppered vessel, and less than one in the case of the aerated mixture.

It did not prove possible to identify a number of minor fractions containing imidazolic compounds with R_f values lower than 4(5)-hydroxymethylimidazole. It is possible that these compounds are polyhydroxyalkylimidazoles of the type 4(5)-(2,3,4-trihydroxybutyl)imidazole (XVI) and 4(5)-(2,3-dihydroxypropyl)imidazole (XVII) isolated by Komoto⁴⁵

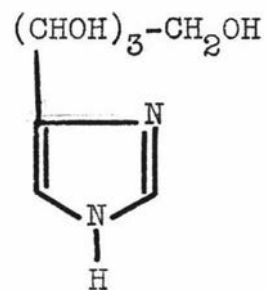
from the products of interaction of glucose and ammonia. If the oxidation of hexoses or pentoses is possible under the reaction conditions compounds of the type 4(5)-tetrahydroxybutylimidazole (XVIII), as isolated by Parrod⁶⁴ from sugars and cuprammonium solution with formaldehyde, might also be expected.



(XVI)



(XVII)



(XVIII)

An experiment to find the rates of formation of the major imidazolic products was carried out on the glyceraldehyde-ammonia mixture, by taking aliquots of the solution at regular intervals during the time of reaction and estimating the amounts of imidazoles present by the combined chromatographic and spectrophotometric technique (See Section 2, page 51 et seq.) It was noticeable that all of the identified imidazoles increased rapidly in concentration during the first week of reaction, and then more slowly during the latter period. (See Fig. 7)

Fig. 7.

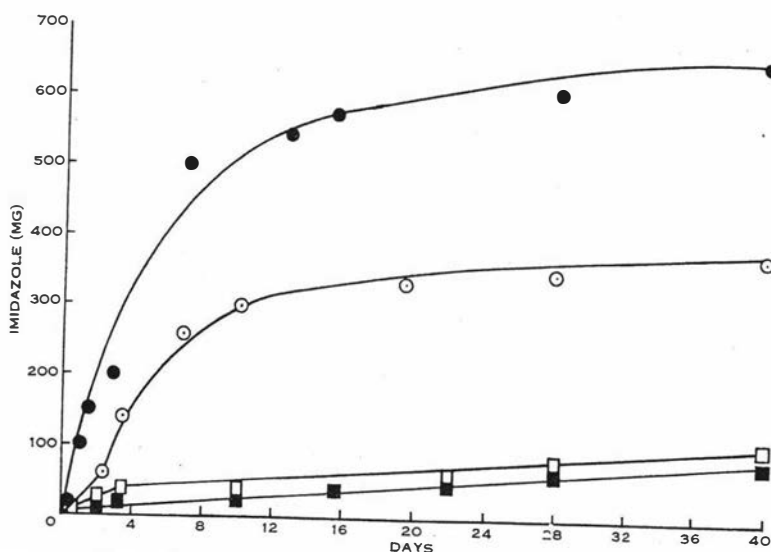
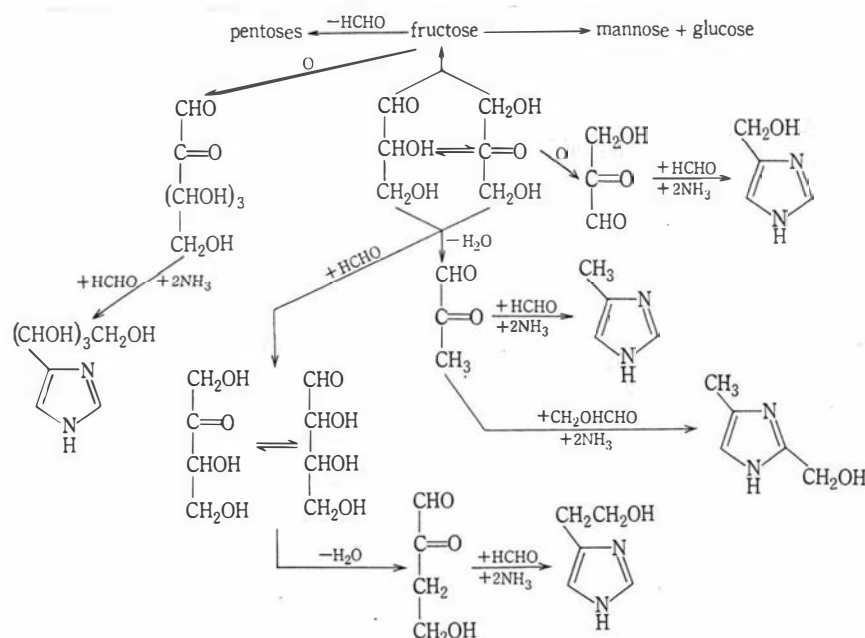


Fig. 7.—Rate of formation of imidazoles during first 6 weeks of reaction. ●, 4(5)-Methylimidazole; ○, 2-hydroxymethyl-4(5)-methylimidazole; □, 4(5)-hydroxymethylimidazole; ■, 4(5)-(2-hydroxyethyl)imidazole.

A small quantity of the reaction mixture of glyceraldehyde and ammonia was stored at room temperature (to slow down the reaction rate) to determine the order of formation of imidazoles. This was accomplished by spotting chromatograms with the reaction mixture at regular intervals and then after development and spraying with the diazo reagent, examining the papers for the imidazoles formed. Both 4(5)-methylimidazole and 4(5)-hydroxymethylimidazole could be detected immediately after mixing indicating that the first

reactions to occur are isomerisation of the glyceraldehyde, dehydration of trioses to pyruvaldehyde and oxidation of dihydroxyacetone to hydroxypyruvaldehyde. Some formaldehyde must also be formed rapidly. After 17 hours it was possible to detect 2-hydroxymethyl-4(5)-methylimidazole, but as this compound is not well separated from 4(5)-methylimidazole on chromatograms, it may have been formed earlier. Certainly, at least by this stage, formaldehyde has been formed by reverse aldolization of triose. After 32 hours an unidentified imidazole appeared, followed by 4(5)-(2-hydroxyethyl)-imidazole (33 hours) and then a number of unknown imidazoles.

The mechanism of formation of imidazoles in an ammoniacal solution of formaldehyde can be represented as follows:-



(v) The Reaction of Pyruvaldehyde with Aqueous Ammonia.

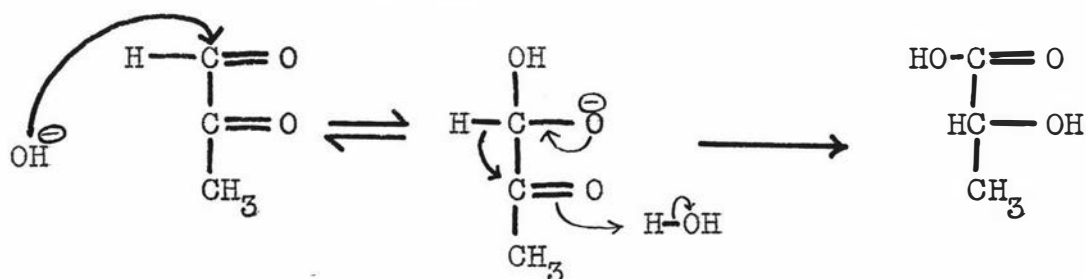
Pyruvaldehyde has been named as one of the important intermediates in alkaline reactions of sugars e.g. in the formation of 4(5)-methylimidazole under ammoniacal conditions⁸⁹ and in the formation of lactic acid with caustic alkali.³⁴ Bernhauer⁷ reported that pyruvaldehyde failed to yield 4(5)-methylimidazole when treated with ammonia in the absence of added formaldehyde, and therefore concluded that pyruvaldehyde could not act as a source of formaldehyde. That this conclusion was based on a single experiment with a one gram quantity of pyruvaldehyde in the days before chromatography, does not appear to have prevented its complete acceptance and use in the formulation of mechanisms of sugar breakdown in ammoniacal medium.²⁶ Also, in spite of the fact that similar dicarbonyl compounds (e.g. glyoxal,¹⁶ diacetyl⁶⁷ and benzil³⁶) are known to give imidazoles, after fission, when treated with ammonia, the belief has persisted that pyruvaldehyde does not undergo ammoniacal fission of the bond linking the carbonyl functions.

Addition of aqueous ammonia to a small amount of 25% solution of pyruvaldehyde produced a mixture which rapidly darkened in colour with evolution of heat. Paper-chromatography and thin-layer chromatography on alumina showed the immediate formation of at least four imidazolic compounds, one of which had the same R_{Im} values as 4(5)-

methylimidazole. (See Table E6, p.199).

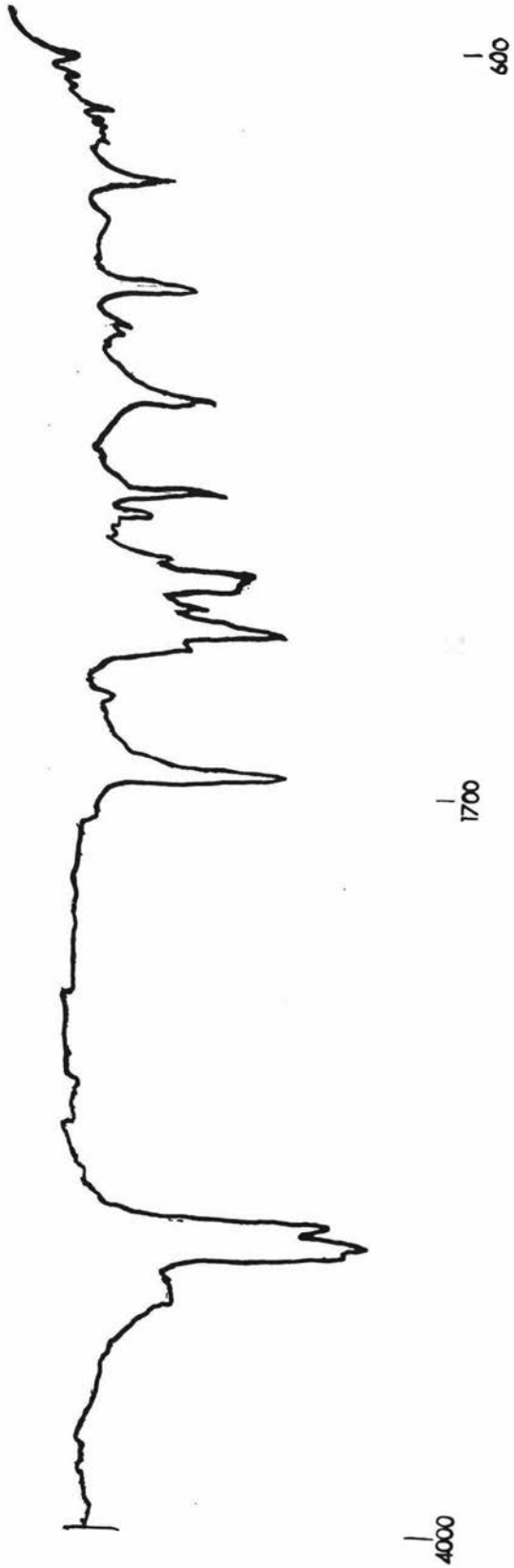
A large-scale reaction mixture was therefore prepared using a 25% aqueous solution of pyruvaldehyde, obtained by purification⁵² of a commercial solution. The starting material was shown by gas chromatography and thin layer chromatography of the 2,4-dinitrophenylhydrazone to be free of other carbonyls. After the reaction mixture had stood in a stoppered flask for five weeks, the ammonia and water were removed in vacuo, and the resulting dark-brown oil, in aqueous solution, passed through a column containing cation-exchange resin and the resin washed with water.

It was not possible to detect aldose or ketose components in this neutral fraction although chromatography and use of the aniline-xylose spray⁷⁵ indicated traces of a compound running to the same position as lactic acid. This result would seem to imply that (i) pyruvaldehyde cannot be rehydrated in ammoniacal solution to form trioses, and (ii) a small amount of hydration of pyruvaldehyde may occur to form lactic acid.



(Mechanism according to Sowden and Pohlen⁸⁰)

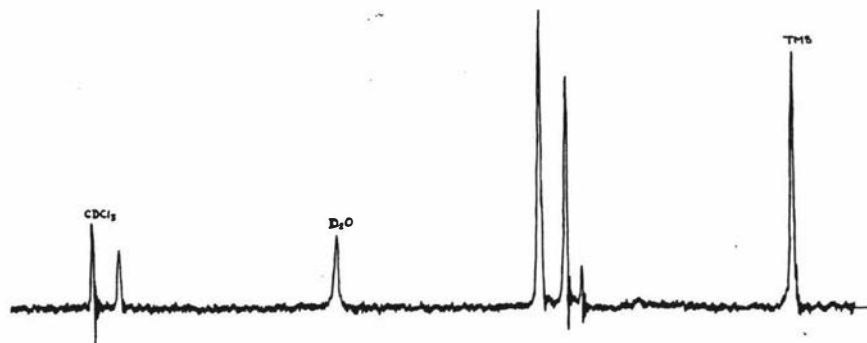
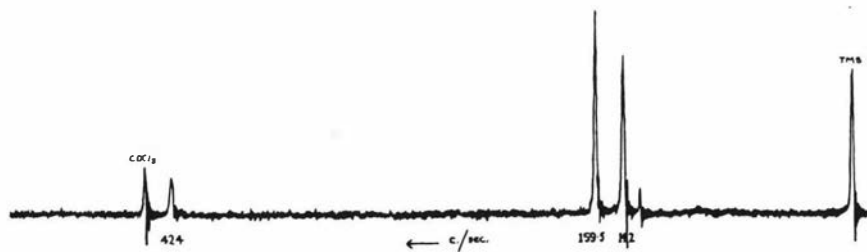
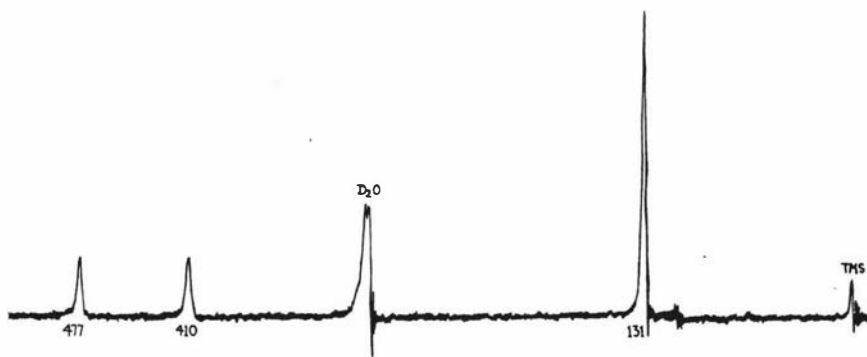
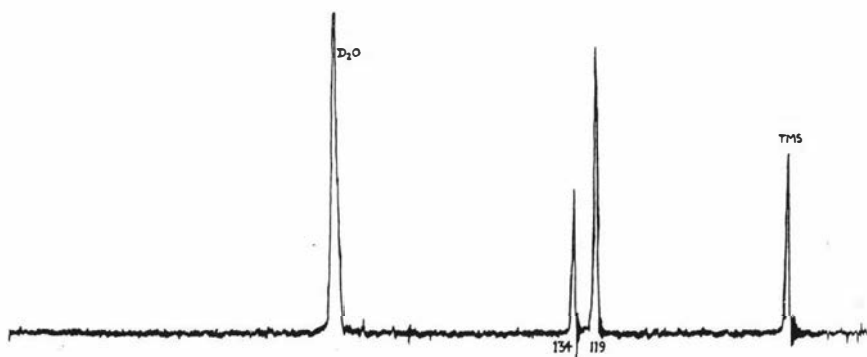
Fig. 8



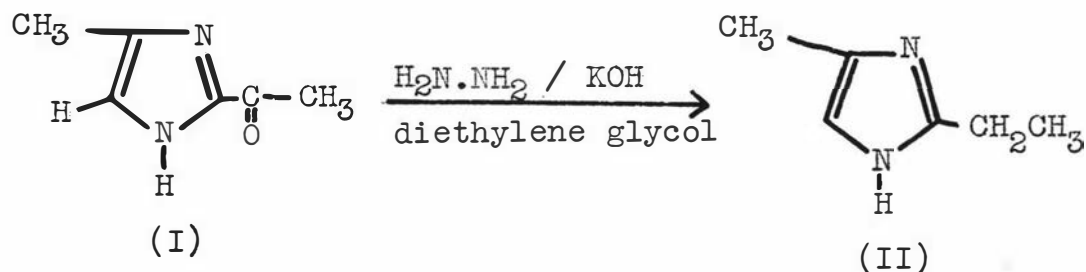
The basic material was removed from the resin with 4N ammonium hydroxide and evaporated to a syrup which contained four Pauly⁶⁶ positive compounds. On Whatman No.1 paper in n-butanol/acetic acid/water (4:1:1) these compounds appeared at R_{Im} values 1.29 (red), 1.58 (lemon-yellow), 1.87 (red) and 2.27 (orange-yellow). A portion of the syrup was fractionated on a cellulose column using n-butanol half-saturated with water and slightly acidified with acetic acid. On the basis of a chromatographic survey the fractions from the column were combined into three main fractions.

The first of these, after purification by chloroform extraction, and then by alumina column chromatography using the same solvent, gave white crystals analysing for $C_6H_8N_2O$. Both ultraviolet and infrared spectra (See Fig. 8) indicated an aromatic methyl ketone, confirmed by formation of a crystalline 2,4-dinitrophenylhydrazone and a positive iodoform test. A 60 Mc. nuclear magnetic resonance spectrum in deuteriochloroform (See Fig. 9) had singlets at 790 c./sec. (H), 424 c./sec. (H), 160 c./sec. (3H), 142 c./sec. (3H). The low field peak was rather broad and disappeared on addition of a drop of deuterium oxide. It resembled the NH proton of imidazole (803 c./sec.).⁸⁶ When the spectrum was compared with similar spectra for 4(5)-methylimidazole and 2,4,5-trimethylimidazole (See Fig. 9) it proved possible to assign the signal at 424 c./sec. to a 5(4)-proton and

Fig. 9



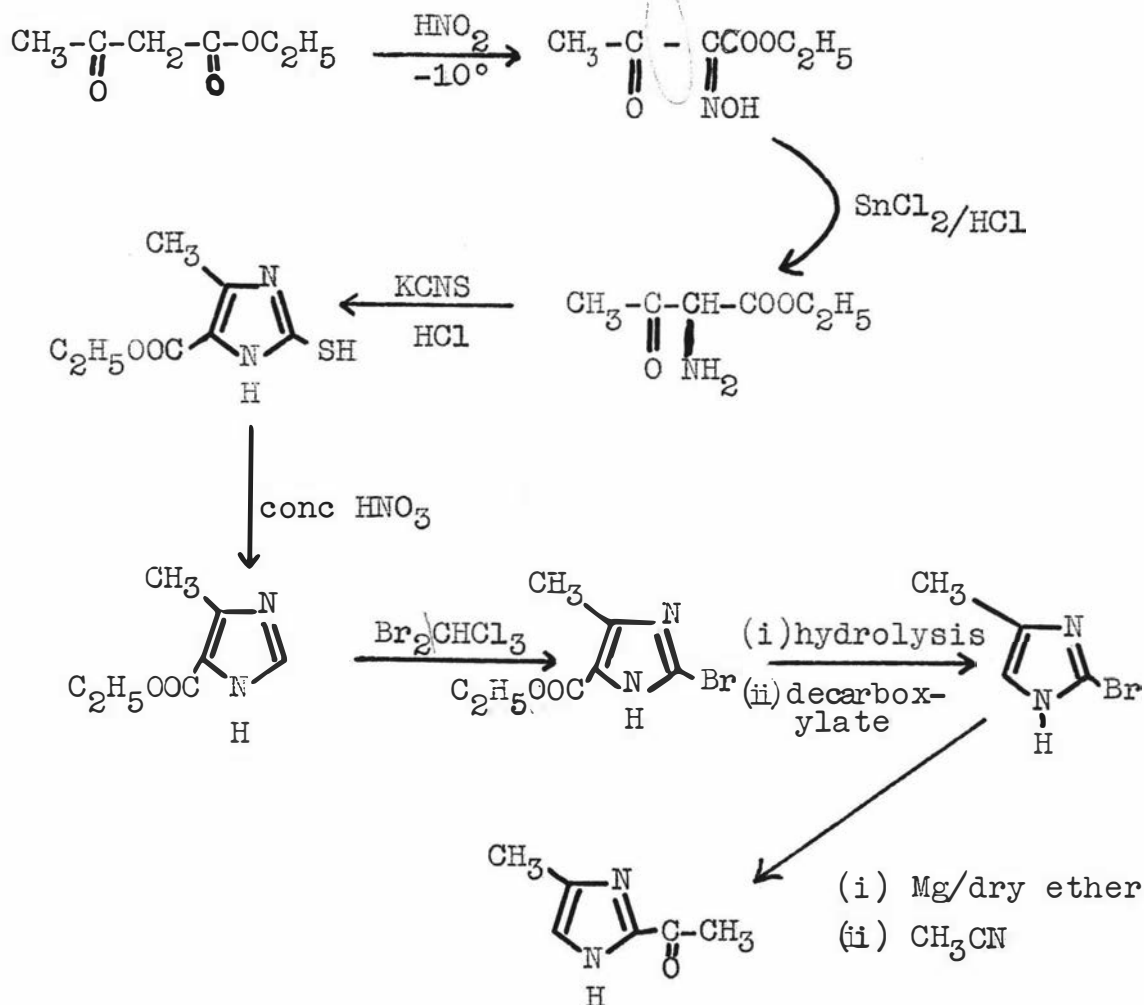
that at 160 c./sec. to a 4(5)-methyl group. There was no signal corresponding to an imidazolic 2-proton but the three-proton peak at 160 c./sec. can be assigned to a $\begin{array}{c} -\text{C}-\text{CH}_3 \\ \parallel \\ \text{O} \end{array}$ group attached directly to an aromatic ring (cf. $-\text{C}:\text{O}-\text{CH}_3$ of acetophenone has a three-proton signal at 157 c./sec.⁷⁹) This evidence gives a structure for the compound corresponding to 2-acetyl-4(5)-methylimidazole (I) (Fig. 9 shows from top to bottom p.m.r. spectra of (i) 2,4,5-trimethylimidazole (D_2O), (ii) 4(5)-methylimidazole (D_2O), (iii) and (iv) 2-acetyl-4(5)-methylimidazole (CDCl_3 and D_2O))



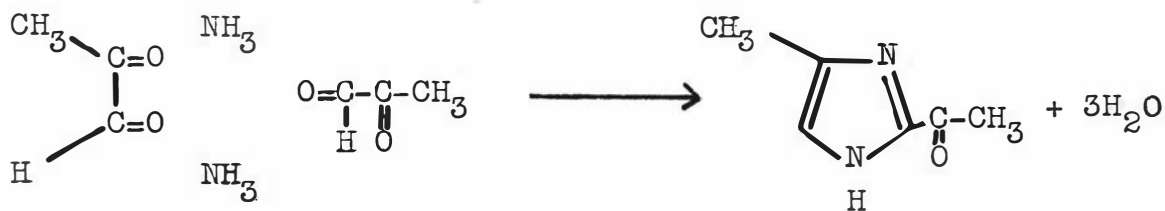
A Wolff-Kischner reduction converted the compound to 2-ethyl-4(5)-methylimidazole (II) although a previous attempt at reduction under Clemmensen conditions gave an unidentified imidazolic product. Such a result could be explained by the known tendency of α -aminoketones to undergo rearrangements during Clemmensen reductions.⁹

An attempt to synthesise 2-acetyl-4(5)-methylimidazole via the following reaction scheme was only partially successful. Successively diminishing yields through the

many stages finally permitted only tentative chromatographic evidence for the final product.



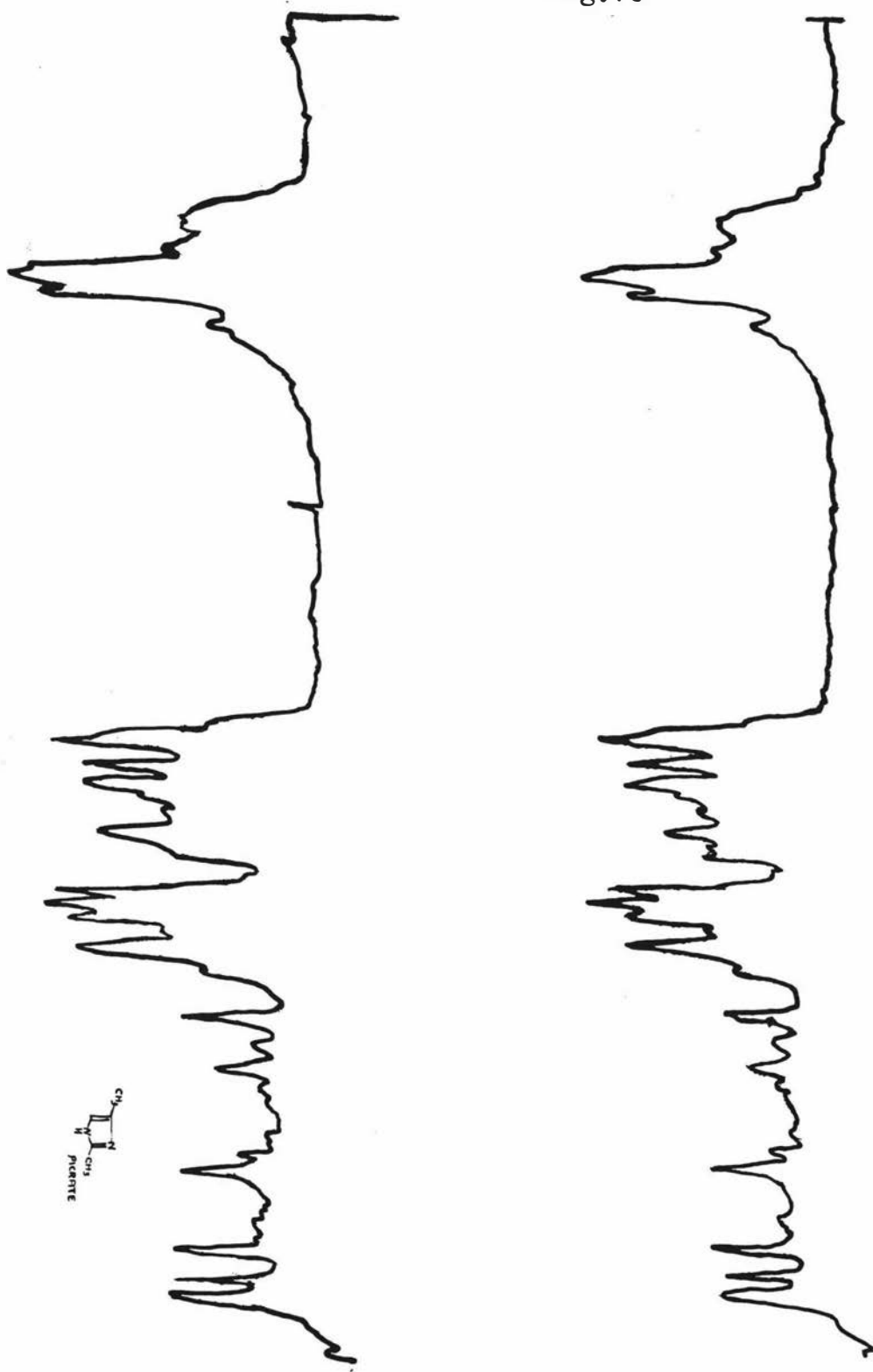
2-Acetyl-4(5)-methylimidazole is probably formed in the pyruvaldehyde-ammonia mixture by condensation of two molecules of pyruvaldehyde with two of ammonia.



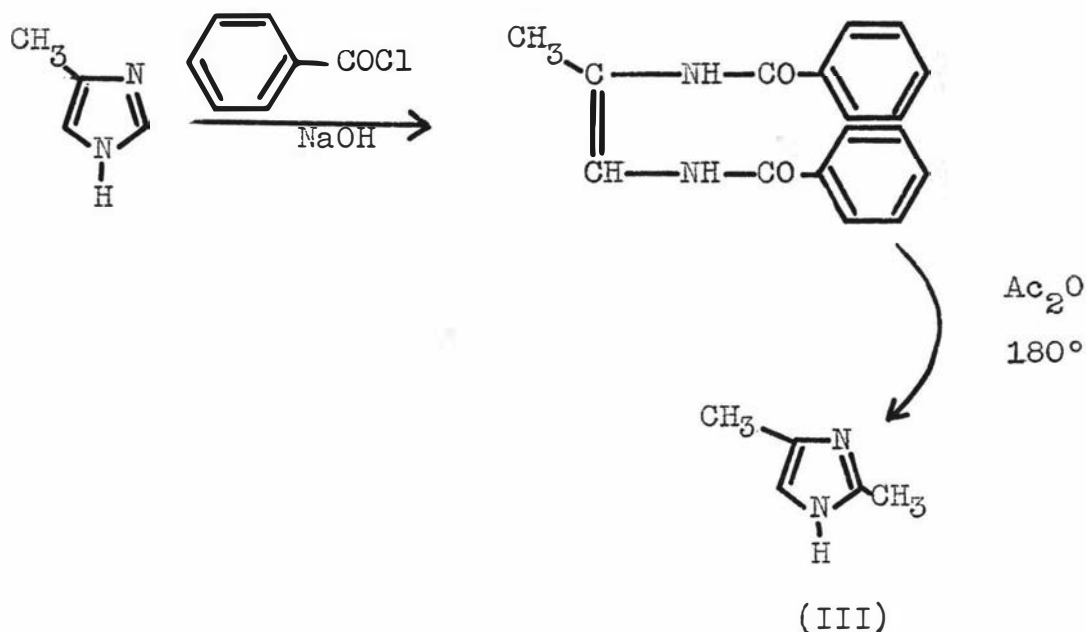
Fraction 2 from the cellulose column was found to contain small quantities of two imidazolic compounds, one of which appeared to be the previously identified 2-acetyl-4(5)-methylimidazole. The other compound, which gave a red colour with a diazo spray, could not be identified although an infra red spectrum indicated the probable presence of hydroxyl and carbonyl groups. A 60 Mc. proton magnetic resonance spectrum showed a relatively complex pattern of signals. Among these were peaks which could have corresponded to NH, a 4(5)-proton, and a 4(5)-methyl group. However three other peaks corresponding to three protons, one peak corresponding to two protons and a further single proton peak appeared to be present. Attempts to apply spin-decoupling to this spectrum produced little change in the signals. It did not prove possible to isolate a derivative of this compound suitable for elemental analysis.

Vacuum distillation of the third fraction yielded a mixture of two imidazoles which were efficiently separated by use of an alumina column with chloroform/pyridine (4:1) as solvent. The first compound, which was obtained crystalline, gave a lemon yellow colour with diazotised sulph-anilic acid (this is often characteristic of 2-substituted imidazoles³³) and had melting point identical to that reported by Bernhauer⁷ for 2,4(5)-dimethylimidazole (III). It formed a picrate derivative with melting point, mixed melting point

Fig.10



and infrared spectrum (See Fig. 10) identical to the corresponding properties of a sample of 2,4(5)-dimethylimidazole picrate prepared from 4(5)-methylimidazole by the method of Windaus and Langenbeck.⁹⁰



The compound giving the red dye with the diazo reagent gave a picrate identical with that of 4(5)-methylimidazole in infrared spectrum (See Fig. 11), melting point and mixed melting point.

As it had been noted with glyceraldehyde-ammonia that aeration increased the yield of 4(5)-hydroxymethylimidazole, it was decided to aerate a pyruvaldehyde-ammonia mixture to determine if any oxidation could occur in this system. As Davidson et al.¹⁵ had reported improved yields of imidazoles when reaction was carried out in glacial acetic acid

Fig.11



using ammonium acetate as the source of ammonia, it was decided to examine the yield of 4(5)-methylimidazole under these conditions. Accordingly mixtures of pyruvaldehyde in ammoniacal solution were prepared as described in Table 2, and the 4(5)-methylimidazole estimated colorimetrically.²⁴

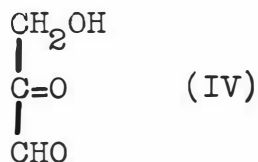
TABLE 2

Yields (grams) of 4(5)-methylimidazole per gram of pyruvaldehyde
under various conditions.

1. Pyruvaldehyde/ammonia in stoppered flask.....	0.124
2. Pyruvaldehyde/ammonia aerated continuously.....	0.121
3. Pyruvaldehyde/ammonia nitrogenated continuously....	0.110
4. Pyruvaldehyde/ammonium acetate/glacial acetic acid.	0.124

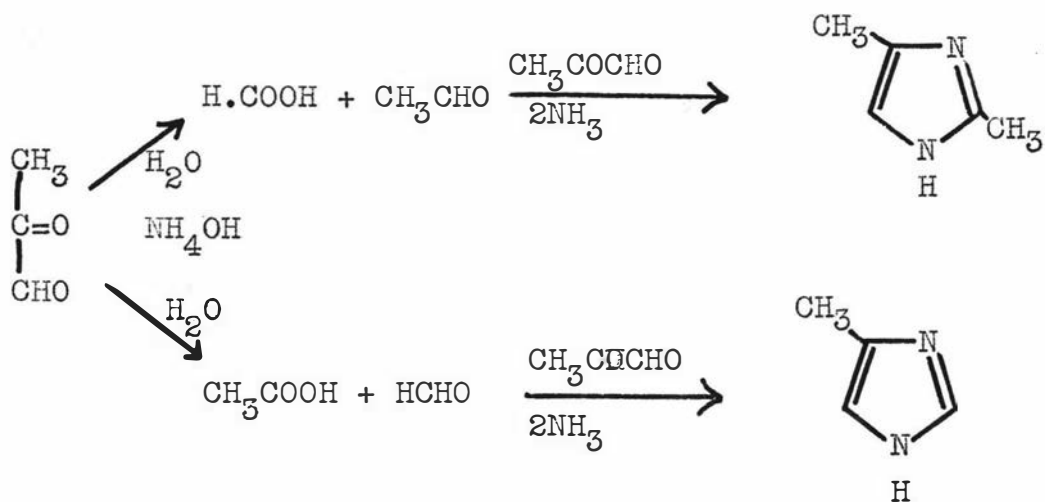
It was found that aeration neither affected the yield of 4(5)-methylimidazole, nor resulted in formation of 4(5)-hydroxymethylimidazole. Nitrogenation lowered the yield to a small extent and use of Davidson's conditions had little effect. Because of the very low colour intensity of the azo dye formed with 2,4(5)-dimethylimidazole, it was not possible to use the colorimetric method to give an accurate estimation of its concentration. Even slight traces of 4(5)-methylimidazole resulted in erroneous results. The main conclusion which can be drawn from the results in the above table is that pyruvaldehyde cannot be converted to

hydroxypyruvaldehyde(IV) in ammoniacal solution and hence the 4(5)-hydroxymethylimidazole present in the glyceraldehyde-



ammonia system (see page 79) cannot be derived from pyruvaldehyde.

With an alumina column and by careful use of solvents it proved possible to achieve clean separations of 4(5)-methylimidazole and 2,4(5)-dimethylimidazole from the mixture of bases. Duplicate measurements showed that these imidazoles had been formed in approximately equimolecular proportions, and this would seem to indicate that if Japp's mechanism³⁶ is correct, pyruvaldehyde forms formaldehyde and acetaldehyde at almost equal rates under ammoniacal conditions. However, the possibility of a benzil-type

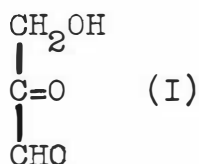


rearrangement reaction¹⁵ should not be ruled out.

Attempts to identify formaldehyde and acetaldehyde in the reaction mixture were somewhat inconclusive although spectroscopic measurements and thin-layer chromatography of a mixture of 2,4-dinitrophenylhydrazones (obtained by collecting the carbonyls in a stream of nitrogen and trapping them in chilled 2,4-dinitrophenylhydrazine solution) indicated their possible presence in very low concentration. It is possible that if formaldehyde and acetaldehyde are formed from pyruvaldehyde in aqueous ammonia, their existence in the free aldehyde form must be extremely brief.

(vi) The Reaction of Hydroxypyruvaldehyde with Aqueous Ammonia.

A further possible product of the alkaline degradation of carbohydrates is hydroxypyruvaldehyde (I). This compound



is probably the intermediate dicarbonyl required for the formation of 4(5)-hydroxymethylimidazole when aerated mixtures of fructose, or dihydroxyacetone react with ammoniacal cupric acetate.⁶⁵ Hydroxypyruvaldehyde has been prepared by oxidation of dihydroxyacetone^{19,22} and in small quantities by the photochemical decomposition of glyoxal.⁶¹ Most of the methods involving oxidation of dihydroxyacetone removed excess oxidising agent (usually cupric ion) by precipitation with hydrogen sulphide, but this process often resulted in the production of toxic sulphur compounds. To avoid this hazard the method described by Evans et al.,¹⁸ for the preparation and purification of the alcoholate of hydroxypyruvaldehyde trimer was used. In this procedure, dihydroxyacetone was oxidized in aqueous solution with cupric acetate, the excess copper removed by precipitation as an oxalate and the hydroxypyruvaldehyde isolated as the semicrystalline, trimer alcoholate, $(\text{C}_3\text{H}_4\text{O}_3)_3\text{C}_2\text{H}_5\text{OH}$. It was thus possible to obtain the desired compound in an

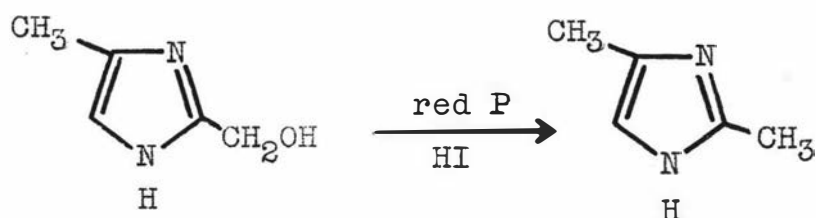
analytically pure form for reaction with ammonia. However, to effect depolymerisation, an aqueous solution of the trimer was heated for a time before reaction.

On reaction with aqueous ammonia, hydroxypyruvaldehyde rapidly formed a dark-coloured solution with concurrent formation of a number of imidazolic compounds which travelled to R_{Im} values 0.00, 0.25, 0.37, 0.91*, 1.20, 1.30* and 1.38* on Whatman No. 1 paper using n-butanol/acetic acid/water (4:1:1) as solvent. (The major components are marked with an asterisk). From a paper and thin-layer chromatographic survey these major components appeared to be (in order of increasing R_{Im} values) 4(5)-hydroxymethylimidazole, 4(5)-methylimidazole and 2-hydroxymethyl-4(5)-methylimidazole.

After the mixture had remained at room temperature for six weeks it was evaporated to a syrup, the basic material separated by ion-exchange, and the neutral fraction examined for the presence of aldoses or ketoses. In contrast to the glyceraldehyde-ammonia system, no such compounds were evident, and thus it seems unlikely that hydroxypyruvaldehyde is converted to trioses in aqueous ammoniacal medium.

The basic material was removed from the ion-exchange resin and was found to contain all of the imidazolic compounds from the original mixture. Extraction of the syrup with boiling anhydrous acetone appeared to remove only those compounds with R_{Im} 0.91 and greater, and even these were

sparingly soluble. Absolute ethanol proved to be a more efficient extractant but rather less selective as it removed all of the imidazoles present in the original mixture. Fractionation of the acetone extract on a cellulose column gave two main imidazolic fractions - one containing the compounds with R_{Im} 1.30 and 1.38; the other containing the compound with R_{Im} 0.91 with traces of faster-moving compounds. Further fractionation of the first fraction on an alumina column yielded the imidazolic compounds, 4(5)-methylimidazole (identified by analysis, infrared spectrum and mixed melting point of a picrate) and 2-hydroxymethyl-4(5)-methylimidazole (identified by isolation of the picrate sesquihydrate of known melting point, and by a red phosphorus/hydrionic acid reduction producing 2,4(5)-dimethylimidazole).



Purification of the other main fraction from the cellulose column by alumina column chromatography yielded a further compound chromatographically identical to 4(5)-hydroxymethylimidazole. Confirmation of its structure came from microanalysis, an infrared spectrum and a mixed melting point determination on a picrate.

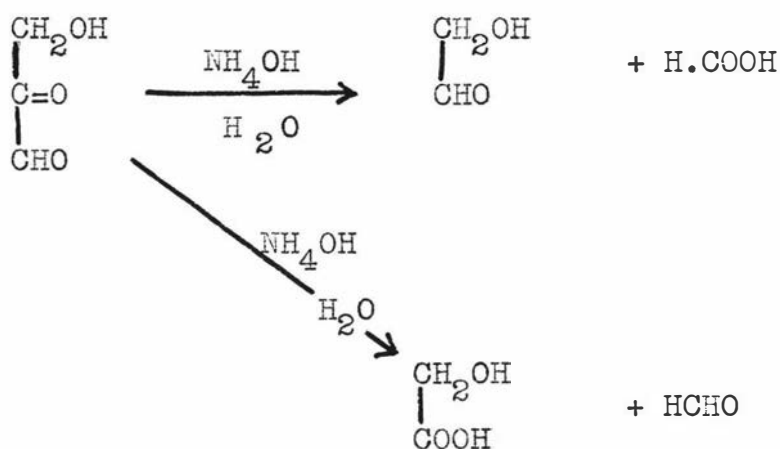
A number of minor imidazolic compounds, present in the ethanol extract, were not investigated.

As dihydroxyacetone, in ammoniacal solution, appeared to give a similar mixture of imidazoles to hydroxypyruvaldehyde, equivalent mixtures of these two carbonyl compounds were prepared and stored at room temperature for four weeks. It was most apparent that the rate of browning of the hydroxypyruvaldehyde-ammonia mixture far exceeded that of the dihydroxyacetone-ammonia mixture. At the end of the four-week period aliquots of each mixture were taken and 4(5)-methylimidazole and 4(5)-hydroxymethylimidazole were separated and estimated using the method developed for this purpose (see page 51). Results obtained for duplicate determinations were in close agreement and indicated a much higher yield of imidazoles from hydroxypyruvaldehyde than from the ketotriose. The results are shown in Table 3. The observation from Table 3 that slightly less 4(5)-methylimidazole was formed from the dihydroxyacetone-ammonia mixture could be explained by the probable occurrence of competing condensation reactions converting some of the dihydroxyacetone to hexoses (cf. formation of hexoses and pentoses from glyceraldehyde in aqueous ammonia). 4(5)-Methylimidazole would be formed from ammoniacal dihydroxyacetone by a mechanism similar to that followed by glyceraldehyde (see page 75). It is rather more difficult to account for the formation of 4(5)-methylimidazole from

TABLE 3

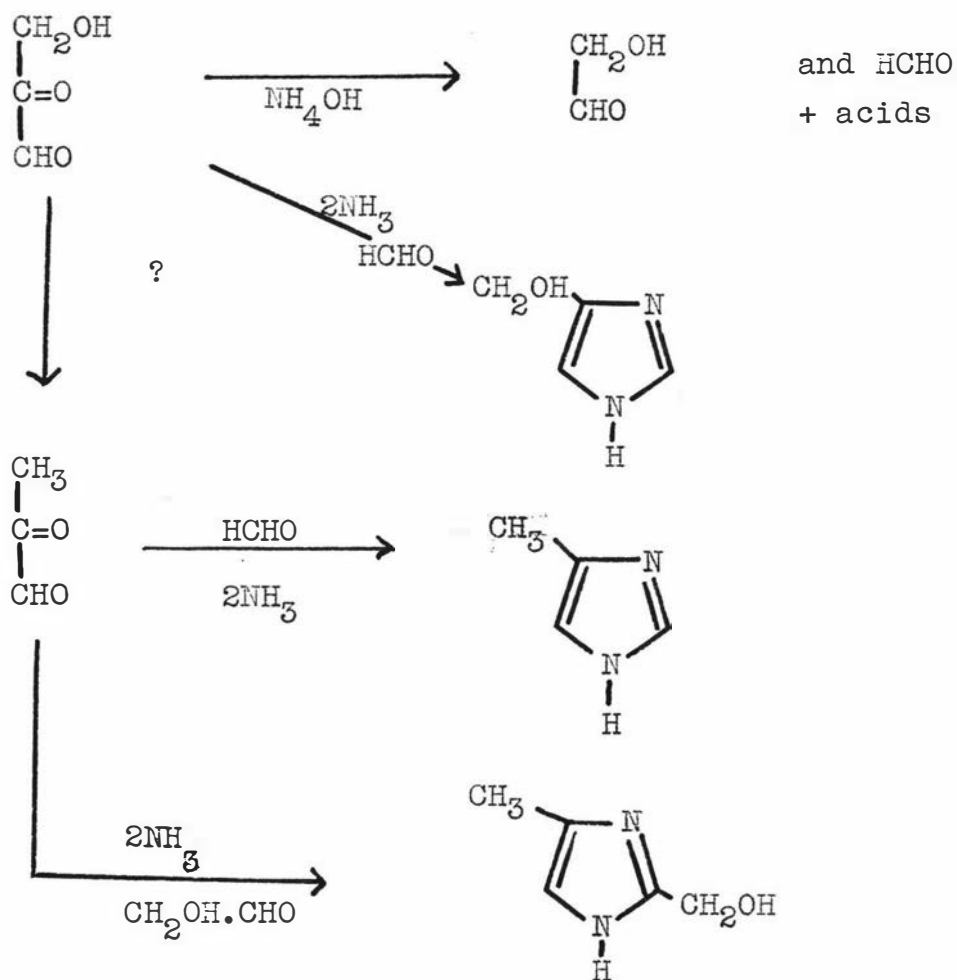
Compound	mg. per ml. of reaction mixture	
	Hydroxypyruvaldehyde/NH ₃	Dihydroxyacetone/NH ₃
4(5)-Methylimidazole	4.40	3.85
4(5)-Hydroxymethylimidazole	24.60	8.70
Compound	gm. per mole of reaction mixture	
	Hydroxypyruvaldehyde/NH ₃	Dihydroxyacetone/NH ₃
4(5)-Methylimidazole	5.50	4.80
4(5)-Hydroxymethylimidazole	30.75	10.88
Compound	moles per mole of reaction mixture	
	Hydroxypyruvaldehyde/NH ₃	Dihydroxyacetone/NH ₃
4(5)-Methylimidazole	0.066	0.058
4(5)-Hydroxymethylimidazole	0.310	0.109

hydroxypyruvaldehyde. Conceivably alkaline fission of the molecule could occur yielding, among acidic products, formaldehyde and glycolaldehyde.



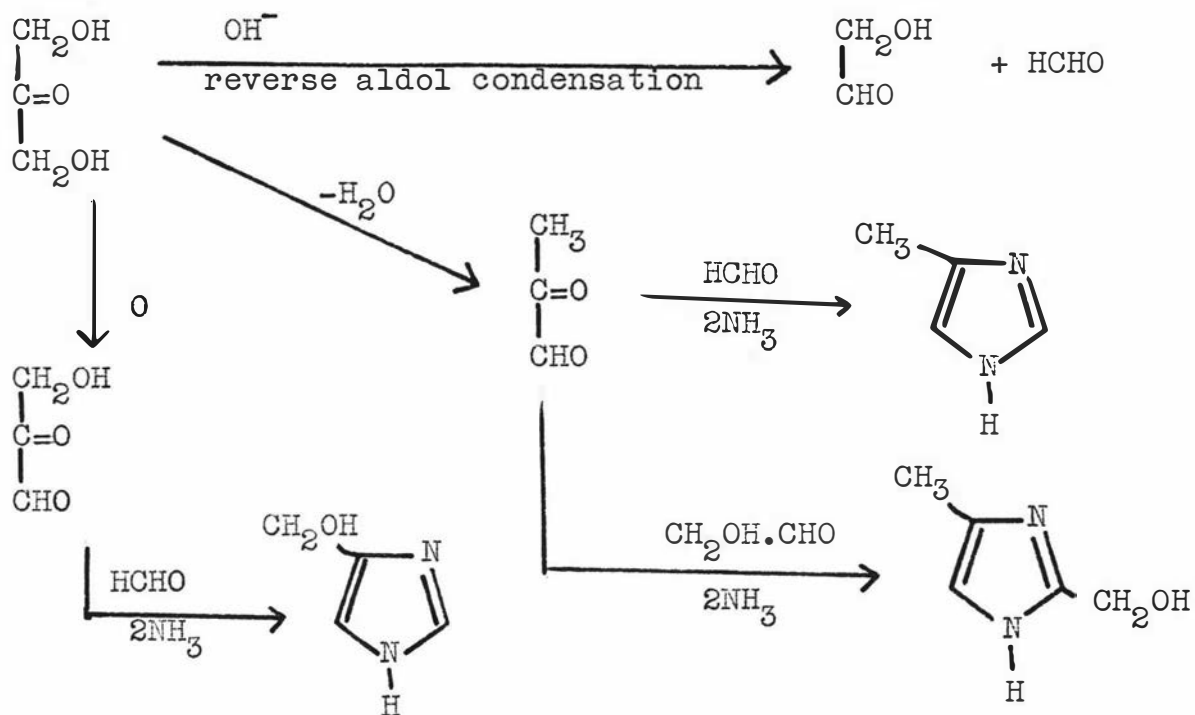
For 4(5)-methylimidazole and 2-hydroxymethyl-4(5)-methylimidazole to be formed it would also be necessary to have prior formation of pyruvaldehyde. Whether this involves reduction of the hydroxymethyl group of hydroxypyruvaldehyde or formation of pyruvaldehyde through recombination of smaller fragments is uncertain. It seems that direct reduction of hydroxypyruvaldehyde to pyruvaldehyde would be unlikely, and it may be that the reaction involves reduction in the first instance to dihydroxyacetone (or glyceraldehyde), and then dehydration of the triose to pyruvaldehyde. This last scheme, however, is not supported by the observation that hydroxypyruvaldehyde forms slightly more 4(5)-methylimidazole than dihydroxyacetone does under the same conditions.

The very high yield of 4(5)-hydroxymethylimidazole from the hydroxypyruvaldehyde-ammonia system was expected but the amount formed from the dihydroxyacetone-ammonia seems rather large in view of the fact that no conscious effort was made to aerate the mixture. An incomplete reaction scheme for the formation of the individual imidazoles from hydroxypyruvaldehyde in aqueous ammonia is shown below:-



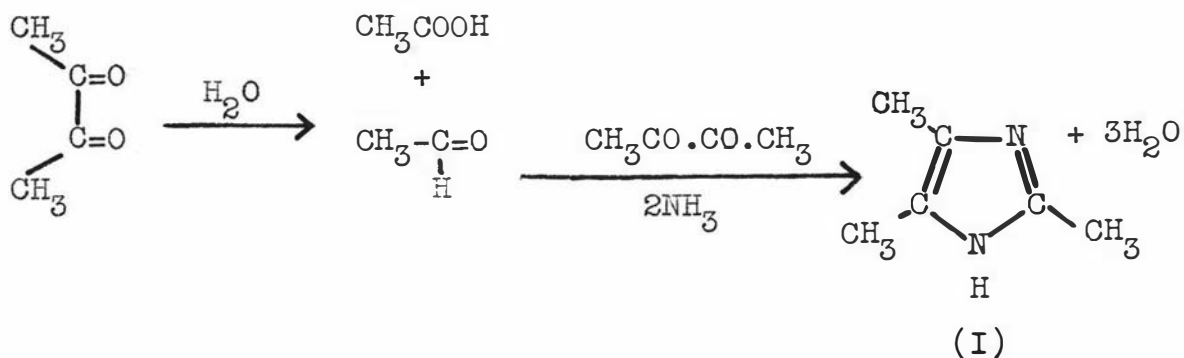
The reaction of dihydroxyacetone in aqueous ammonia could be represented in part by the following reaction

scheme, although the complete reaction is probably similar in complexity to that of glyceraldehyde (the tautomer of dihydroxyacetone).



(vii) The Reaction of Diacetyl with Ammonia

In 1888, von Pechmann,⁶⁷ obtained 2,4,5-trimethylimidazole (I) from the ammoniation of an ethanolic solution of diacetyl. Such a reaction product must involve either alkaline "fission" of the bond linking the carbonyl groups, or follow the type of reaction mechanism described by Davidson¹⁵ for the formation of lophine from benzil and ammonia. If the former is true the following reaction scheme might apply:-



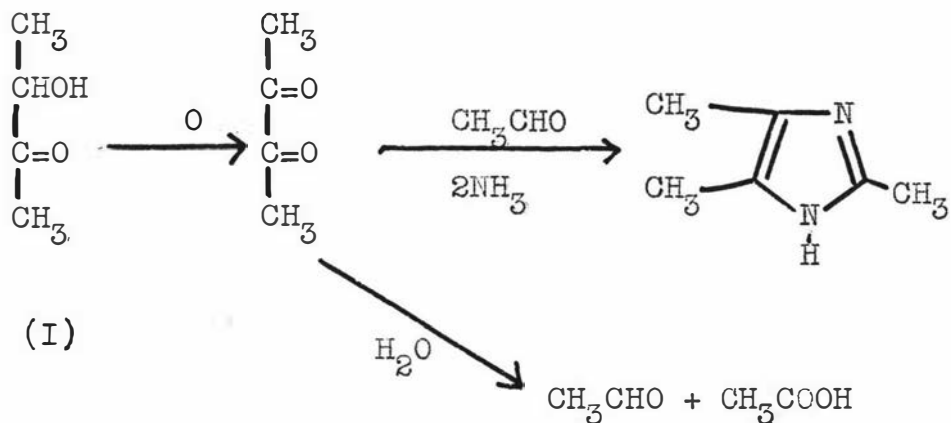
A solution of diacetyl in absolute ethanol was treated with dry ammonia and the resulting syrup, after evaporation of the solvent, added to a column containing Dowex 50 ion exchange resin. When 2N hydrochloric acid was passed through the column and the eluate evaporated to dryness, a white crystalline hydrochloride was obtained identical to 2,4,5-trimethylimidazole hydrochloride. A sample of the free base, prepared by neutralization of the hydrochloride, and vacuum distillation gave an n.m.r. spectrum (see Fig. 9 before page 88) containing only two peaks in the ratio 2:1. These

were assigned to 4-CH₃, 5-CH₃ (119 c./sec.) and 2-CH₃ (134 c./sec.).

No pyrazines were detected in the mixture.

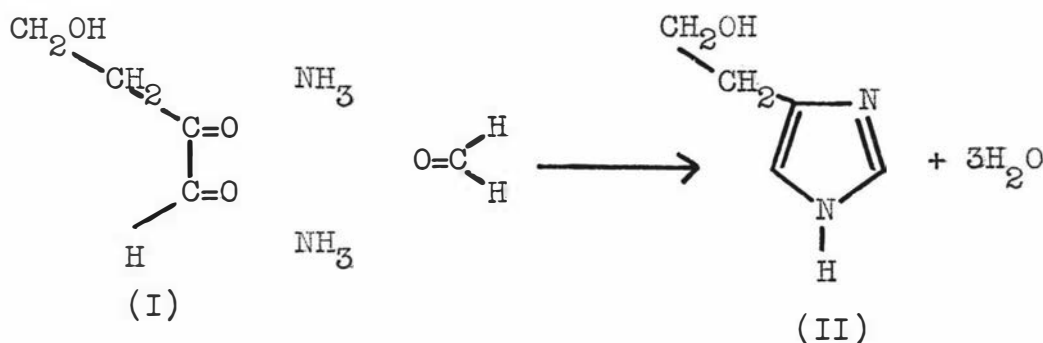
(viii) The Reaction of Acetoin with Aqueous Ammonia.

The reaction of acetoin (I), formaldehyde and ammoniacal cupric acetate forms 4,5-dimethylimidazole.⁸⁸ When the formaldehyde is replaced by acetaldehyde, the main product is 2,4,5-trimethylimidazole. It was expected that acetoin should be able to undergo oxidation to diacetyl in ammoniacal solution in an analogous fashion to that in which glycolaldehyde is thought to be converted to glyoxal (see page 67). Therefore, 2,4,5-trimethylimidazole should be a product of the reaction between acetoin and aqueous ammonia. From such a mixture this imidazole was isolated and characterised by the formation of a picrate and a hydrochloride.

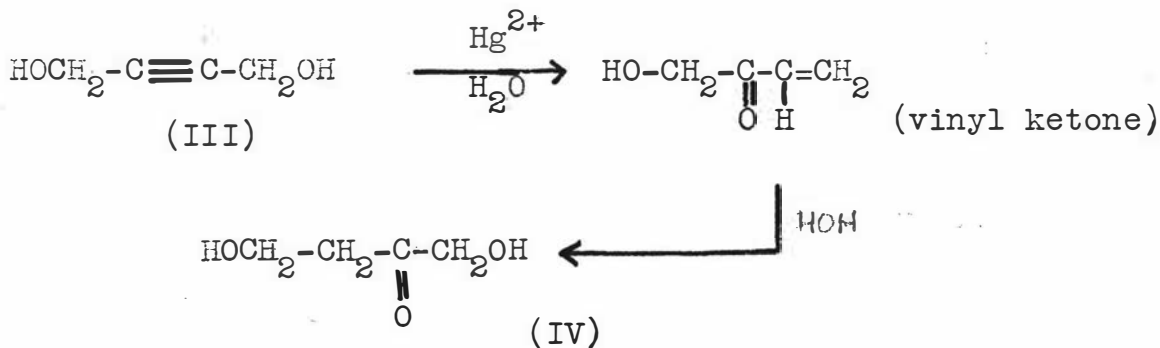


(ix) The Reaction of 1,4-Dihydroxy-2-butanone with Aqueous Ammonia.

Komoto⁴⁴ explained the formation of 4(5)-(2-hydroxyethyl)-imidazole (II) from the interaction of glucose and ammonia by combination of an intermediate deoxytetrosone (I) with ammonia and formaldehyde.



As a number of α -hydroxycarbonyl compounds had been found, during the present project, to undergo some oxidation to the corresponding dicarbonyl in aqueous ammonia, it was decided to test the action of ammonia on 1,4-dihydroxy-2-butanone (IV). The synthetic method followed was essentially that of Reppe⁷³, involving catalytic hydration of the commercially-available 2-butyne-1,4-diol (III). Although



some workers^{58,74} reported that the ketone may be purified

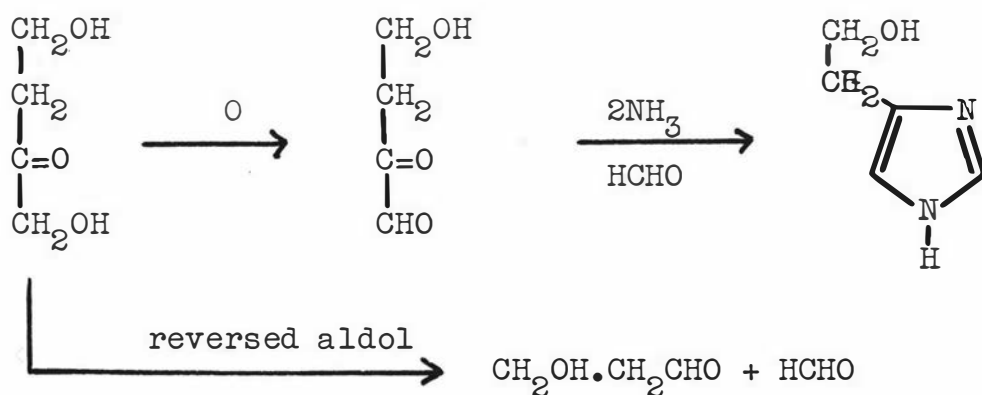
by vacuum distillation, any such attempt resulted in polymerisation and yielded mainly starting product. It was, however, possible to prepare a solution containing the required carbonyl compound, (see page 217) and to obtain a 2,4-dinitrophenylhydrazone corresponding to that reported³⁵ for 1,4-dihydroxy-2-butanone.

From the rather impure reaction mixture obtained by mixture of this solution with concentrated ammonium hydroxide it was possible to detect a number of imidazolic compounds in very low concentrations. The main components appeared on chromatograms (Whatman No. 1 paper; n-butanol/acetic acid/water (4:1:1)) at R_{Im} 0.99 and 2.10 giving red colours with the diazo spray reagent. Minor imidazolic components were also present at R_{Im} 0.00 and 0.73. After the reaction had proceeded four weeks at room temperature, the basic compounds were separated from the mixture by use of an ion-exchange column and evaporated to a syrup. A combination of cellulose and alumina column chromatography allowed purification of the compound of R_{Im} 2.10 to a chromatographically homogeneous syrup from which it did not prove possible to obtain any crystalline derivatives. The infrared spectrum was relatively featureless. As the compound was fairly soluble in deuteriochloroform an n.m.r. spectrum was determined in this solvent, but the complex pattern of signals seemed to indicate a non-homogeneous sample. Further attempts

at purification were not successful.

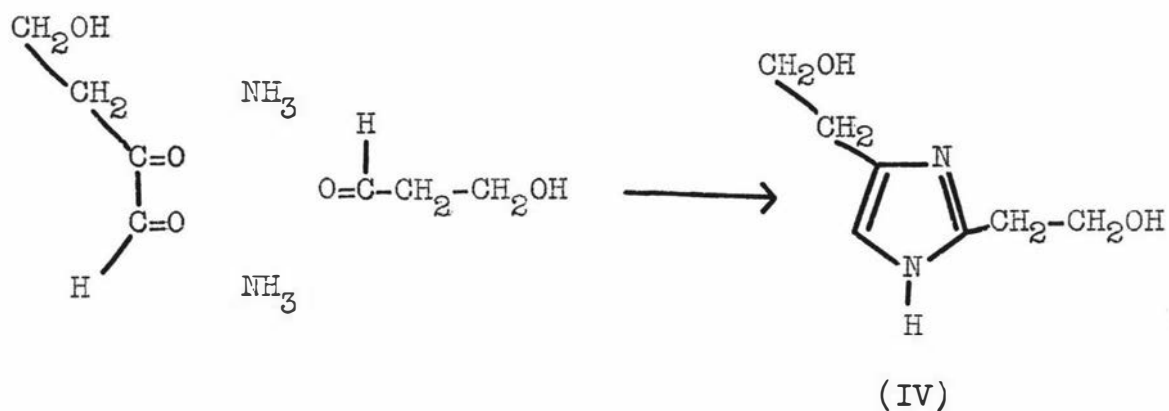
The compound with R_{Im} 0.99 was purified by column chromatography in turn on cellulose, alumina and then again cellulose. the resultant syrup was chromatographically identical to a sample of 4(5)-(2-hydroxyethyl)imidazole which had been prepared by the method of Erlenmeyer et al.¹⁷ (See page 158) Further evidence for the structure of this compound was obtained from infrared spectroscopy, which showed a broad hydroxyl band at 3400 cm^{-1} and by the isolation of a small quantity of crystalline picrate melting a few degrees below that reported by Komoto⁴⁴ for 4(5)-(2-hydroxyethyl)imidazole picrate.

The formation of this compound may be explained in much the same way as the formation of 2,4,5-tri-methylimidazole from an ammoniacal solution of acetoin.



When an analogy is taken with the formation of 2,4(5)-^{di} methylimidazole from pyruvaldehyde and ammonia, one

could speculate that the identity of one of the other reaction products of the dihydroxybutanone-ammonia mixture could correspond to 2,4(5)-di-(2'-hydroxyethyl)imidazole (IV) as a consequence of 3-hydroxypropionaldehyde competing (to a minor degree) with formaldehyde for the 2-position in the imidazole ring.



(x) The Reaction of L-Arabinose with Aqueous Ammonia.

Work was started on this reaction mixture early in the project, but was later abandoned when it was felt that more useful results could be obtained by a study of the effects of ammonia on some of the smaller fragments of alkaline degradation of sugars. By this stage, however, 4(5)-methylimidazole had been isolated and identified from the chloroform extract of the reaction mixture. It was noticeable from a study of paper chromatograms that an extremely complex pattern of imidazoles had been formed.

(xi) Reactions of Carbohydrates and a Number of Their Suspected Alkaline Degradation Products with Aqueous Ammonia.

In an endeavour to compare the reactions of a number of carbohydrates and their degradation products in ammoniacal solution, mixtures in the ratio of one mole of carbonyl compound to three moles of ammonia were stored in stoppered vials at room temperature, but trisaccharides and polysaccharides were dissolved in the proportions of 0.9 g. of carbohydrate to 1.5 ml. of concentrated ammonia solution.

Chromatography of these reaction mixtures at selected time intervals allowed the formation of the imidazolic products to be studied. In order to limit the entrainment of extra air in the reactions duplicate mixtures were prepared in each case, and each of these was used for approximately one half of the sampling. At the end of approximately 220 hours at room temperature the reaction mixtures were studied for a further six hours at 75° and eighteen hours at 110°.

For these experiments to be meaningful it was necessary to stop reaction at the instant of sampling (or very soon thereafter). Although it was not possible to "kill" the reaction, spotting the sample on chromatography paper was found to be effective as the ammonia rapidly evaporated in the air. There will undoubtedly be a small error (a matter of a minute or even two minutes) in the absolute values of "time of appearance" of the imidazoles, but this would not be ex-

pected to affect the validity of any comparisons since all reactions were carried out under identical conditions. After the first hour of reaction any error in absolute times would be very small. Table 4 summarises the results obtained:-

TABLE 4

Compound	R _{Im}	Diazo Colour	Time of First Appearance
Glyoxal	0.40	orange	50h.
	0.53	"	1 min.
	0.82	"	50h.
	1.00*(Im)	"	1 min.
	1.21	purple→blue	3 min.
	1.31	orange-red	50h.
	1.64*(bis-Im)	orange	1 min.
Glycol-aldehyde	0.68	orange	10h.
	1.00*(Im)	"	1 min.
	1.12*(2-OHMe)	"	1 min.
	1.40	"	10 h.
Pyruvaldehyde	1.25*(4-Me)	red	1 min.
	1.58*(2,4-diMe)	lemon-yellow	"
	1.84	red	"
	2.20 (2-Ac-4-Me)	orange	"
Hydroxy-pyruv-aldehyde	0-0.60(streak)	orange	1min
	0.70	orange	"
	0.93*(4-OHMe)	orange-red	"
	1.25*(4-Me)	red	"
	1.33(2-OHMe-4-Me)	red	"
Dihydroxy-acetone	0.47	red	1h.
	0.61	red	1h.
	0.93*(4-OHMe)	orange-red	1 min.
	1.25*(4-Me)	red	"
	1.33(2-OHMe-4-Me)	red	2H.

(cont.)

Compound	R _{Im}	Diazo Colour	Time of First Appearance
Glycer-aldehyde	0.22	red	32h.
	0.47	red	60h.
	0.60	red	60h.
	0.70	red	60h.
	0.93 (4-OHMe)	orange-red	1 min.
	0.99 (4-OHEt)	red	33h.
	1.25* (4-Me)	red	1min.
	1.33 (2-OHMe-4-Me)	red	17h.
Eryth-rose	0.32	red	66h.
	0.50	red	66h.
	0.78*	red-orange	1 min.
	1.00* (4-OHEt)	red	1 min.
	1.40	red	"
Lyxose	0.53	red	3h.
	0.78	red	15h.
	0.92	red	123h.
	0.99	red	35h.
	1.25 (4-Me)	red	15h.
	1.33	red	220 + H5 h.
Xylose	0.52	red	9h.
	0.77	red	4h.
	0.98	red	9h.
	1.25 (4-Me)	red	15h.
	1.33	red	220 + H1 h.
	1.37	red	220 + H5 h.
Ribose	0.52	red	3h.
	0.79	red	9h.
	0.98	red	9h.
	1.01	red	40h.
	1.26 (4-Me)	red	40h.
	1.30	red	220 + H5 h.
Arabinose	0.54	red	15h.
	0.78	red	9h.
	0.98	red	9h.
	1.27 (4-Me)	red	74h.
	1.33	red	220 + H1 h.
	1.47	yellow	220 + H1 h.

contin.

Compound	R _{Im}	Diazo Colour	Time of first Appearance
Glucose	0.16	red	9h.
	0.40	red	4h.
	0.61	orange	9h.
	0.73	orange	9h.
	0.90	orange-red	84h.
	0.99(4-OH _{Et})	red	220+H5 h.
	1.25*(4-Me)	red	84 h.
	1.35(2-OHMe-4-Me)	red	220 + H1 h.
Galactose	0.20	red	4h.
	0.41	red	10h.
	0.60	orange	2h.
	0.73	orange	3h.
	0.90	orange-red	88h.
	0.99(4-OH _{Et})	red	146h.
	1.24*(4-Me)	red	88h.
	1.32(2-OHMe-4-Me)	red	220 + H1 h.
Mannose	0.22	red	64h.
	0.38	red	64h.
	0.59	orange	220 + H1 h.
	0.66	orange	64h.
	0.91	red-orange	64h.
	0.99(4-OH _{Et})	red	220 + H4 h.
	1.24*(4-Me)	red	64h.
	1.35(2-OHMe-4-Me)	red	220 + H5 h.
Rhamnose	0.68	red	59h.
	0.81	red	220 + H3 h.
	0.97	red	59h.
	1.24(4-Me)	red	123h.
	1.46	red	220 + H3 h.
	1.65	red	220 + H8 h.
Fucose	0.82	red	220 + H1 H.
	0.97	red	220 + H1 h.
	1.25(4-Me)	red	220 + H1 h.
	1.46	red	220 + H1 h.
Fructose	0.22	red	2h.
	0.35	red	96h.
	0.46	red	2h.
	0.60	red	4h.
	0.76	red	34h.
	0.92(4-OHMe)	orange-red	10h.
	0.99(4-OH _{Et})	red	220 + H1 h.
	1.25*(4-Me)	red	26h.
	1.34(2-OHMe-4-Me)	red	220 + H3 h _{contin.}

Compound	R _{Im}	Diazo colour	Time of first Appearance
Sorbose	0.20	red	10h.
	0.33	red	10h.
	0.44	red	3h.
	0.60	red	3h.
	0.79	red	10h.
	0.99(4-OHEt)	red	48h.
	1.12	red	220 + H5 H.
	1.26(4-Me)	red	40h.
Tagatose	0.21	red	26h.
	0.32	red	220 + H1h.
	0.45	red	10h.
	0.60	red	26h.
	0.72	red	220 + H2 h.
	0.91(4-OHMe)	orange-red	26h.
	1.03	red	220 + H2 h.
	1.25(4-Me)	red	170h.
Lactose	0.11	red	23h.
	0.21	red	34h.
	0.40	red	50h.
	0.73*	red-orange	15h.
	0.98	red	220 + H7 h.
	1.13	red	220 + H7 h.
	1.27(4-Me)	red	220 + H7 h.
Maltose	0.11	red	16h.
	0.22	red	40h.
	0.41	red	40h.
	0.75*	red-orange	16h.
	1.00	red	220 + H7 h.
	1.28(4-Me)	red	220 + H7 h.
Cellobiose	0.11	red	16h.
	0.23	red	40h.
	0.41	red	40h.
	0.74*	red-orange	16h.
	1.28(4-Me)	red	220 + H4 h.

contin.

Compound	R _{Im}	Diazo Colour	Time of first Appearance
Melibiose	0.00	red	24h.
	0.14	red	27h.
	0.23	red	24h.
	0.35	red	40h.
	0.58	red	35h.
	0.71	red	220 + H2 h.
	0.91	red	48h.
	0.99	red	220 + H2 h.
	1.13	red	220 + H5 h.
	1.25*(4-Me)	red	27h.
	1.50	red	220 + H2 h.
Gentio- biose	0.00	red	48h.
	0.13	red	2h.
	0.25	red	48h.
	0.60	red	72h.
	0.98	red	83h.
	1.27*(4-Me)	red	29h.
Sucrose	1.25(4-Me)	faint pink	220 + H3 h.
Raffinose	1.27(4-Me)	pink	220 + H8 h.
Melezitose	1.24	red	220 + H4 h.
Amylose	-	-	-
Amylopectin	0.00	orange	220 + H24 h.
Starch	0.00-0.76	pink streak	220 + H5 h.
	1.27(4-Me)	red	220 + H8 h.
Deoxyglucose	1.00	red	96h.
	1.30	red	220 + H1 h.

contin.

Compound	R _{Im}	Diazo Colour	Time of first Appearance
Glucos-amine	0.00	red	99h.
	0.10	orange	3 min.
	0.18	orange	3 min.
	0.30	red	4h.
	0.40	yellow	15 min.
	1.03	red	220 + H1 h.
	1.25	red	220 + H1 h.
Galactos-amine	0.03	red	11h.
	0.16	red	11h.
	0.29	red	11h.
	0.40	yellow	1h.
	1.00	red	171 h.
	1.26	red	220 + H1 h.

Key: * Major Component

Im: imidazole
 4-Me: 4(5)-methylimidazole
 4-OHMe: 4(5)-hydroxymethylimidazole
 4-OHEt: 4(5)-(2-hydroxyethyl)imidazole
 2-OHMe: 2-hydroxymethylimidazole
 bis-Im: 2,2'-bis-imidazole
 2-OHMe-4-Me: 2-hydroxymethyl-4(5)-methylimidazole
 2-Ac-4-Me: 2-acetyl-4(5)-methylimidazole
 2,4-diMe: 2,4(5)-dimethylimidazole

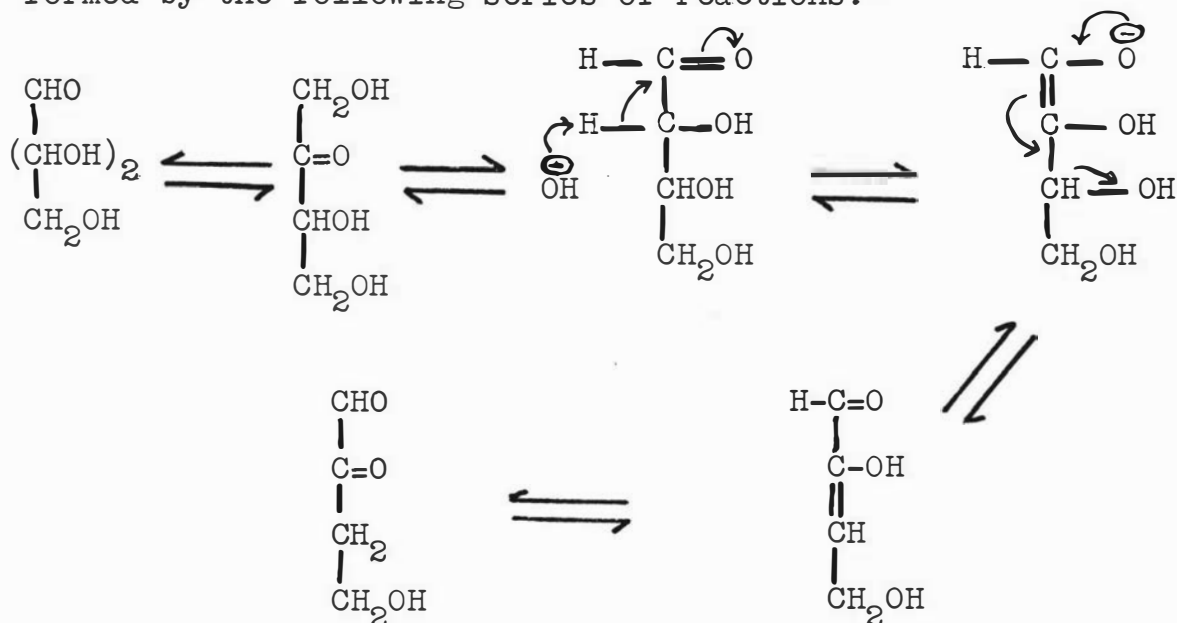
(all suspected present)

220 + H1 h. = 220 hours at room temperature +
 1 hour heated.

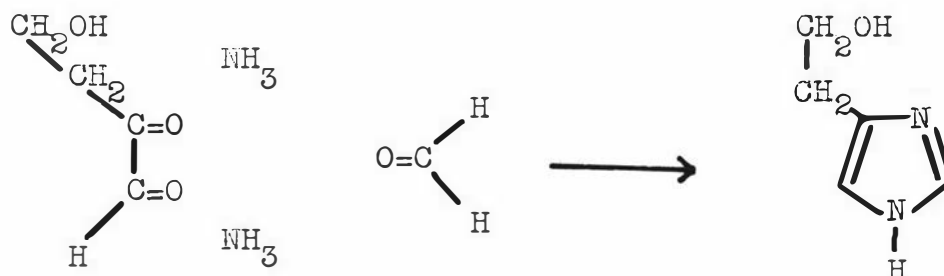
Glyoxal rapidly darkened with the evolution of heat, and within minutes glycosine appeared as crystals. Reaction occurred at such a rate that it was not possible to compare the rates of formation of the component imidazoles. Although the reaction of glycolaldehyde with ammonia was much less violent, it was possible to detect both imidazole and the major product, 2-hydroxymethylimidazole, within a minute of mixing. It seems evident that a portion of the glycolaldehyde is converted swiftly to glyoxal under the ammoniacal conditions. Pyruvaldehyde also reacted violently with aqueous ammonia with evolution of heat and rapid darkening. Within one minute 4(5)-methylimidazole, 2,4(5)-dimethylimidazole and 2-acetyl-4(5)-methylimidazole had been formed. A similar reaction rate was observed with hydroxypyruvaldehyde as the aqueous ammoniacal mixture became yellow and then black with the immediate (within one minute) formation of 4(5)-hydroxymethylimidazole, 4(5)-methylimidazole and 2-hydroxymethyl-4(5)-methylimidazole. Although dihydroxyacetone in ammoniacal solution produced a similar mixture of imidazoles, the rate of browning was very much slower (the solution was still only an amber colour after 220 hours at room temperature) and the 2-hydroxymethyl-4(5)-methylimidazole was not detected until two hours had elapsed. It is conceivable that different mechanisms are involved in the production of this latter compound, but it is often difficult to differentiate

2-hydroxymethyl-4(5)-methylimidazole from 4(5)-methylimidazole on paper chromatograms where the methyl-substituted compound is the major component. The time disparity noted between hydroxypyruvaldehyde and dihydroxyacetone for the formation of the disubstituted imidazole could be accounted for by this difficulty of detection. As might be expected, glyceraldehyde reacted with aqueous ammonia to give a mixture of imidazoles similar to that given by dihydroxyacetone, although dihydroxyacetone appeared to give a much higher concentration of 4(5)-hydroxymethylimidazole than did glyceraldehyde.

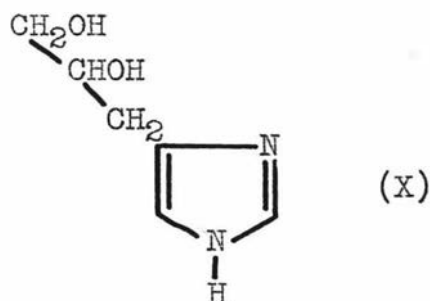
Erythrose in ammoniacal solution produced a number of imidazolic compounds, two of which chromatographically resembled the main products of reaction of 1,4-dihydroxybutan-2-one with ammonia. It seems likely that the compound with R_{Im} 1.00 was 4(5)-(2-hydroxyethyl)imidazole which could be formed by the following series of reactions:-



The deoxyosone could be formed by removal of the elements of water from erythrose, while a reverse aldolisation would account for the one-carbon fragment giving thus:-

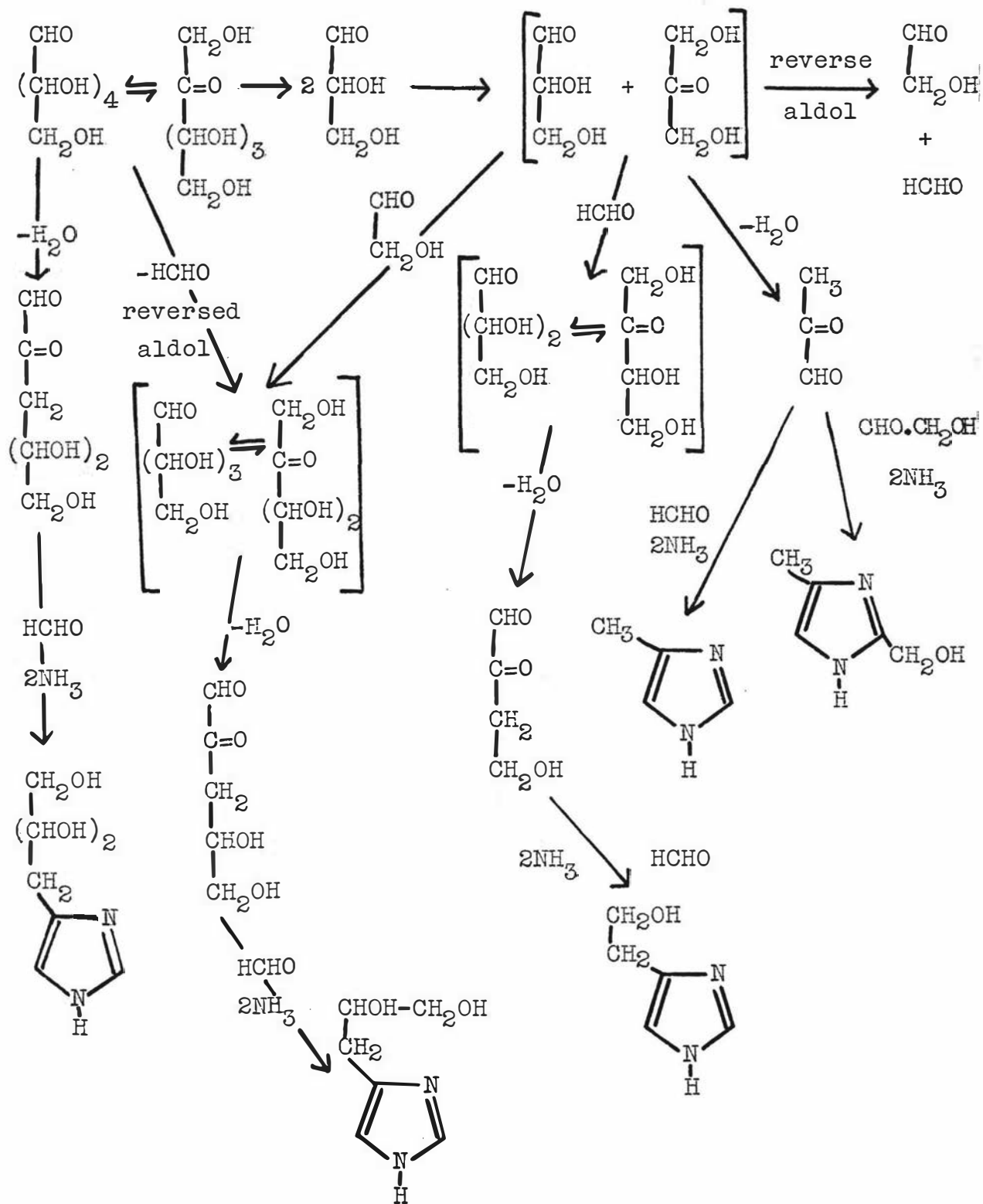


All of the aldopentose sugars gave similar patterns of imidazoles in which 4(5)-methylimidazole was a major constituent, although it seemed to be formed less rapidly in the cases of ribose and arabinose. The first imidazoles to appear from the reactions of the pentoses with ammonia were those with R_{Im} values of less than 1.00. The compounds with R_{Im} values in the vicinity of 0.50 and 0.80 were formed in all cases in less than 15 hours. Although not identified it seems possible that one of these compounds could have a structure represented by X if a deoxypentosone is formed in the same way as the deoxytetrosone mentioned earlier.



The aldohexose sugars, glucose, mannose and galactose, all gave products resembling 4(5)-(2-hydroxyethyl)imidazole, 4(5)-methylimidazole and 2-hydroxymethyl-4(5)-methylimidazole. However, although 4(5)-methylimidazole eventually became a major product, in no case was it detected earlier than after 64 hours of reaction at room temperature. A number of chromatographically-slower-moving imidazoles (presumably polyhydroxyalkylimidazoles) were formed as rapidly as 4(5)-methylimidazole from mannose-ammonia, and very much more quickly from glucose and galactose-ammonia. This result fails to confirm Komoto's⁴⁷ findings that for the glucose-ammonia system the order of formation was:- (i) 4(5)-methylimidazole (ii) 4(5)-(2',3',4'-trihydroxybutyl)imidazole, (iii) 2-hydroxymethyl-4(5)-methylimidazole, (iv) 4(5)-(2-hydroxyethyl)imidazole, (v) an unidentified imidazole and then (vi) 4(5)-(2',3'-dihydroxypropyl)imidazole for the reaction at 100°. When the glucose-ammonia reaction was carried out at room temperature during the present survey, no 4(5)-methylimidazole was evident until after 84 hours of reaction although at least four slower moving imidazoles were detected on chromatograms before this time. It seems probable that the compounds 4(5)-(2',3',4',-trihydroxybutyl)imidazole and 4(5)-(2',3'-dihydroxypropyl)imidazole are among the first formed. Such a result would indicate that the rate of deoxyhexosone and deoxypentosone formation in ammoniacal

solution is greater than the rate of formation of pyruvaldehyde. The following reaction scheme summarises the reactions undergone by a hexose sugar in ammoniacal solution:-



The fact that 2-hydroxymethyl-4(5)-methylimidazole was also slow to form supports the theory that pyruvaldehyde formation is slower than reverse aldolisation of the hexose to pentose and formaldehyde. A number of other reactions may contribute to the structures of the imidazolic products e.g. formation of tetrose and glycolaldehyde directly from hexose⁷¹; recombination of smaller fragments by aldol condensations; fission of 1,2-dicarbonyls.

The interaction of rhamnose (6-deoxymannose) with aqueous ammonia produced a number of imidazoles among which 4(5)-methylimidazole could be identified. Although Windaus and Ullrich⁹¹ obtained 2,4(5)-dimethylimidazole from a solution of rhamnose in ammoniacal zinc sulphate, it did not appear as a constituent of the present reaction mixture. Its presence may possibly have been masked by the presence of two unknown imidazoles with high R_{Im} values, and giving red dyes with the diazo spray reagent. Fucose (6-deoxygalactose) gave a similar mixture of imidazoles with aqueous ammonia but heating was required before their presence was noted.

The reaction mixtures produced by ketoses, fructose, sorbose, and tagatose with ammonia, closely resembled one another and those produced by the aldohexoses. This was to be expected since the Lobry de Bruyn and Alberda van Eckenstein

were formed. Lactose, maltose and cellobiose (all 1-4 linked) slowly gave a wine-red colour when treated with concentrated ammonia solution, and formed a number of imidazoles among which a compound giving an orange-red colour with the diazo spray at R_{Im} 0.74 was the major product. 4(5)-Methylimidazole was present as a minor component of the mixture. Melibiose and gentiobiose (1-6 linked) slowly assumed a yellow-brown colour in ammoniacal solution and gave a very different pattern of imidazoles from that produced by the 1-4 linked disaccharides. 4(5)-Methylimidazole was the major imidazolic product from the 1-6 linked disaccharides.

The non-reducing disaccharide, sucrose, proved very unreactive in ammoniacal solution and it was only after 220 hours at room temperature followed by heating for three hours at 75° that 4(5)-methylimidazole was detected in very low concentration, presumably after some hydrolysis of the disaccharide. Jezo³⁷ carried out a similar reaction with sucrose in the presence of a catalyst at 220° and under these conditions was able to detect a number of imidazoles (including 4(5)-methylimidazole) and pyrazines (See Tables 1 and 2, pages 25 and 28 of Introduction).

The non-reducing trisaccharides, raffinose and melezitose, and starch also formed traces of 4(5)-methylimidazole after long standing and heating. Amylose and amylopectin were unreactive under the same conditions.

2-Deoxy-D-glucose reacted with aqueous ammonia with the

slow formation of at least two imidazolic products, while glucosamine and galactosamine hydrochlorides formed mixtures containing a number of Pauly-positive components. It is probable that a complex mixture of heterocyclic compounds is formed in the glucosamine-ammonia mixture as Taha⁸² isolated a number of pyrazine derivatives from such a mixture.

IV. LINKAGE IDENTIFICATION IN HEXOSE DISACCHARIDES BY
FORMATION OF IMIDAZOLES.

Determination of the structure of a disaccharide involves, among other things, definition of the glycosidic link. This requires both definition of the configuration (α or β) of the link and of the carbon atoms of the two sugar units involved. With non-reducing disaccharides this latter problem is easy as both of the reducing carbons (aldose C_1 , ketose C_2) comprise the link. In the case of reducing disaccharides the link depends on which carbon of the reducing monosaccharide is involved. e.g. With aldohexoses this may be C_2 , C_3 , C_4 or C_6 (1-2, 1-3, 1-4 or 1-6 links) and with ketohexoses C_1 , C_3 , C_4 or C_6 (1-1, 1-3, 1-4 or 1-6). Of much greater rarity are links involving C_5 (1-5) of the ketopyranose or aldofuranose reducing unit. With oligosaccharides (2-10 monosaccharide units) of degree of polymerization greater than two the problem of defining the glycosidic links is complicated by the fact that the polymer may be homogeneous or heterogeneous with respect to the positions of the linkages.

It is not unusual, with the development of the various fractionation techniques depending on chromatography, to have only 20-50 mg. of an unknown disaccharide available for structural studies, and in many cases it is desirable

to be able to identify the disaccharide present in the hydrolysate when only 1-2 mg. has been isolated.

For this reason any suitable method for the determination of linkages in disaccharides should fulfil the requirements of high specificity of linkage differentiation, high sensitivity (requiring less than 1 mg. of sugar), ease of manipulation (preferably no quantitative determinations) and speed.

A number of methods exist for the determination of linkage in reducing di- and oligosaccharides.

Acid spray reagents have been used e.g. the failure of a reducing disaccharide to react with triphenyltetrazolium chloride indicates a glycosidic link on C-2 of a reducing aldose or C-1 of a reducing ketose². Acidic diphenylamine-aniline gives a deep blue colour with 1-4 linked reducing disaccharides⁷⁸. It is often possible to distinguish between 1-3, 1-4, and 1-2 or 1-6 glycosidic links of reducing oligosaccharides by a study of the paper-ionophoretic mobility of the corresponding alditols in molybdate buffer⁸. Micromethods involving periodate oxidation have been widely used³ for distinguishing between disaccharide linkages, but, although the determination may require as little as 1 mg. of sugar, the technique is time-consuming, requiring careful microdeterminations of such fragments as carbon dioxide, formaldehyde and formic acid. A study of any individual

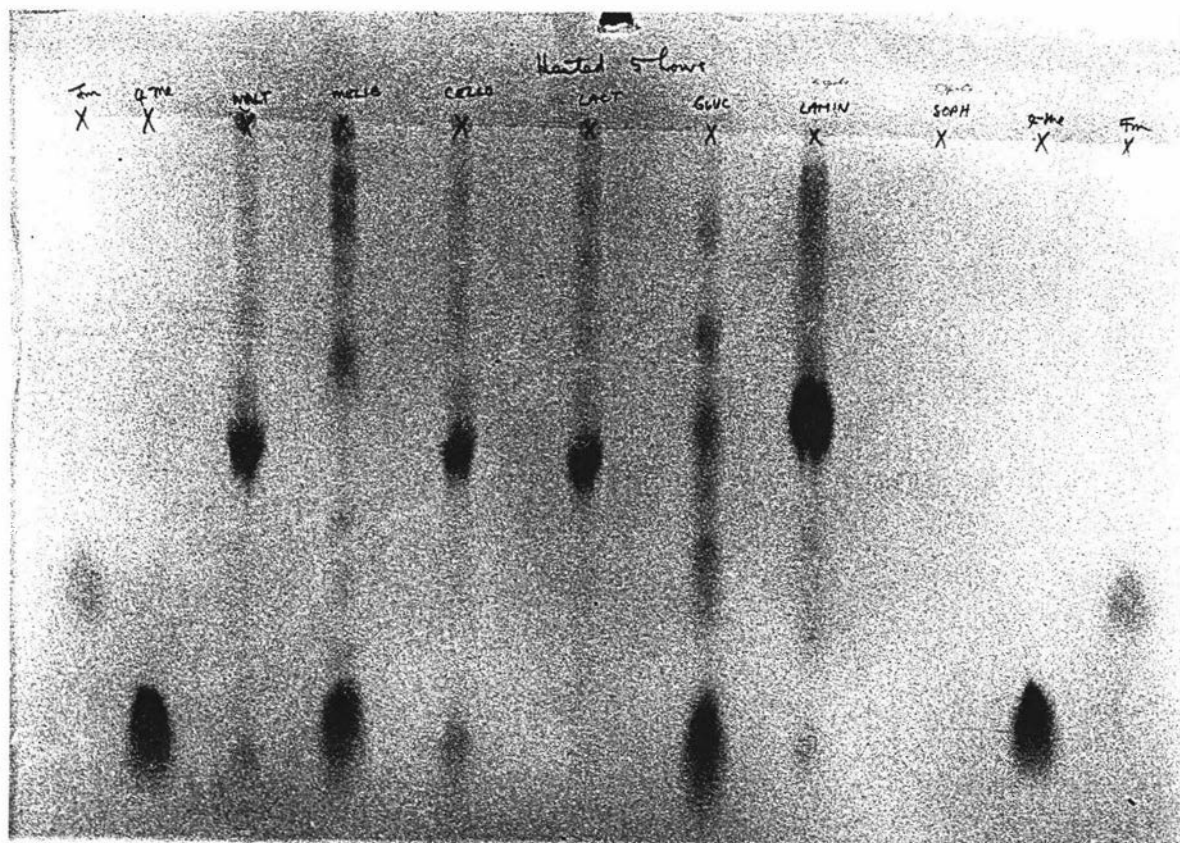
monosaccharide residues which survive periodate oxidations have also often proved useful in structural work. Similar methods exist for lead tetracetate.⁶⁹ Methylation analysis has been perhaps the most widely-used method for structural analysis of oligosaccharides, and, combined with gas-chromatographic analysis of the resulting methyl O-methylglycosides, is suitable for 0.5-2 mg. of sugar.⁶⁸ Enzymic methods can also be used to determine disaccharide linkages⁴ but the usefulness of this technique is rather dependent on the availability of highly-purified enzymes. A study of the saccharinic acids formed on alkaline degradation has been suggested by Whistler⁹² for the determination of linkage, but the procedure does not appear to have been used widely as an analytical method.

Most of the methods mentioned above do not fulfill all of the criteria laid down, so that there is room for the development of other methods. Particularly required are methods in which the main manipulation is paper chromatography and all four linkages (1-2, 1-3, 1-4 and 1-6) may be distinguished from one another.

Because of the strikingly different imidazole patterns produced by the action of ammonium hydroxide on 1-4 and 1-6 linked disaccharides during the previous survey, it was of interest to test 1-2 and 1-3 linked disaccharides under the same conditions. Accordingly, small (50 mg.)

samples of laminaribiose (3-O- β -D-glucosyl-D-glucose) and sophorose (2-O- β -D-glucosyl-D-glucose) were dissolved in aqueous ammonia and allowed to stand first at room temperature for 42 hours and then heated at 75° for five hours. Spots were placed on chromatograms along with those from control mixtures containing glucose, lactose, cellobiose, maltose, sophorose and melibiose after 42 hours and then hourly during the heating period. The optimum reaction time was found to be between four and five hours at the elevated temperature, as reaction at room temperature proved too slow. The imidazole patterns obtained are shown in Plate I.

PLATE I



If this method of identification of linkage-type was to be of real use in disaccharide studies, it would have to be readily adaptable to quantities of sugar of the order of 5 mg. or less. Various disaccharides, in one milligram quantities, were treated with ammonium hydroxide (0.1 ml.) in sealed capillary tubes at 110° for two hours. Paper chromatography of the resultant reaction products showed that there was a striking contrast in imidazole patterns between 1-1, 1-2, 1-3, 1-4, 1-5 and 1-6 linked reducing disaccharides, and that the microscale of the experiment was an advantage rather than a disadvantage, as the concentrations of the minor imidazolic products were decreased. In fact, as it was possible to detect as little as 0.3 µg. of imidazole with the sulphanilic acid spray reagent, care had to be taken to avoid overloading the chromatograms.

The standard procedure, outlined in the Experimental Section (page 224) was tested for the reducing hexose disaccharides sophorose (1-2 link), laminaribiose, turanose (1-3 link), lactose, maltose, cellobiose, lactulose, maltulose(1-4 link), melibiose, gentiobiose, isomaltose and isomaltulose (1-6 link). Glucosyl-1-1-fructose (1-1) and leucrose (1-5) gave somewhat similar patterns but both could be distinguished from the other compounds. The non-reducing disaccharides, sucrose, trehalose and melibitol gave no imidazoles under the standard conditions, while β -methylmaltoside, as might

be expected, gave traces of 4(5)-methylimidazole. Only traces of this compound were present among the reaction products of di- and trigalacturonic acids with ammonia, showing that the carboxyl group hinders the normal reaction. Xylobiose, a 1-4 linked pentose disaccharide, gave three spots of about equal intensity at R_{Im} values of 0.77, 0.92 and 1.30. The results obtained for a number of these compounds are summarised in Table 5, while Plates 2 and 3 show the chromatographic patterns obtained for the complete range of carbohydrates tested.

TABLE 5

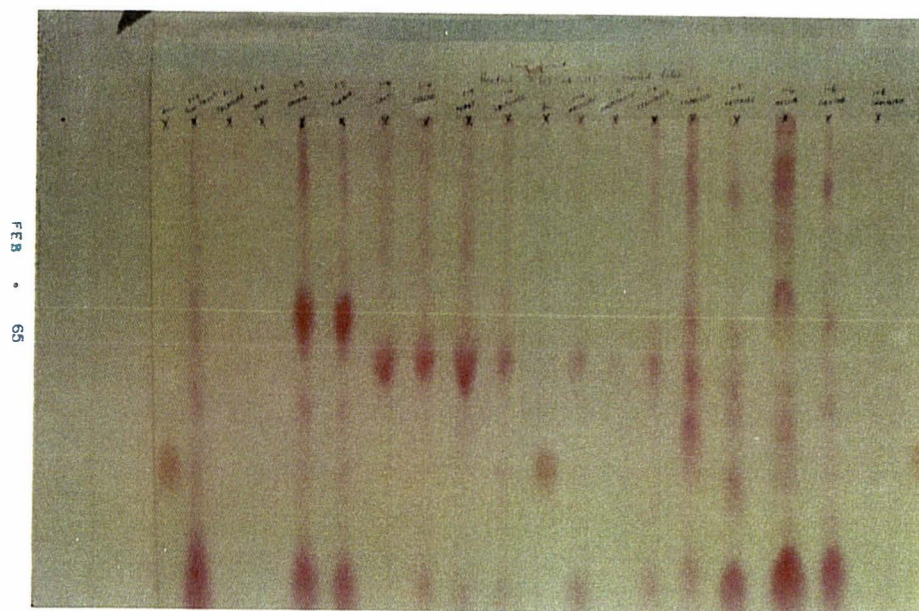
Linkage R_{Im} values of major imidazolic products from reducing Type hexose disaccharides with ammonia.

1-1	-	-	0.62(w)	-	0.81(w)	1.03(w)	1.29(s)
1-2	-	-	-	-	-	-	-
1-3	0.19(w)	-	0.62(s)	-	-	-	1.28(m)
1-4	0.16(w)	0.29(w)	-	0.74(s)	-	-	1.27(w)
1-5	-	0.26(m)	-	0.78(s)	-	1.03(m)	1.27(s)
1-6	0.20(w)	-	0.60(m)	-	0.83(w)	-	1.28(s)

s = strong intensity; m = medium; w = weak.

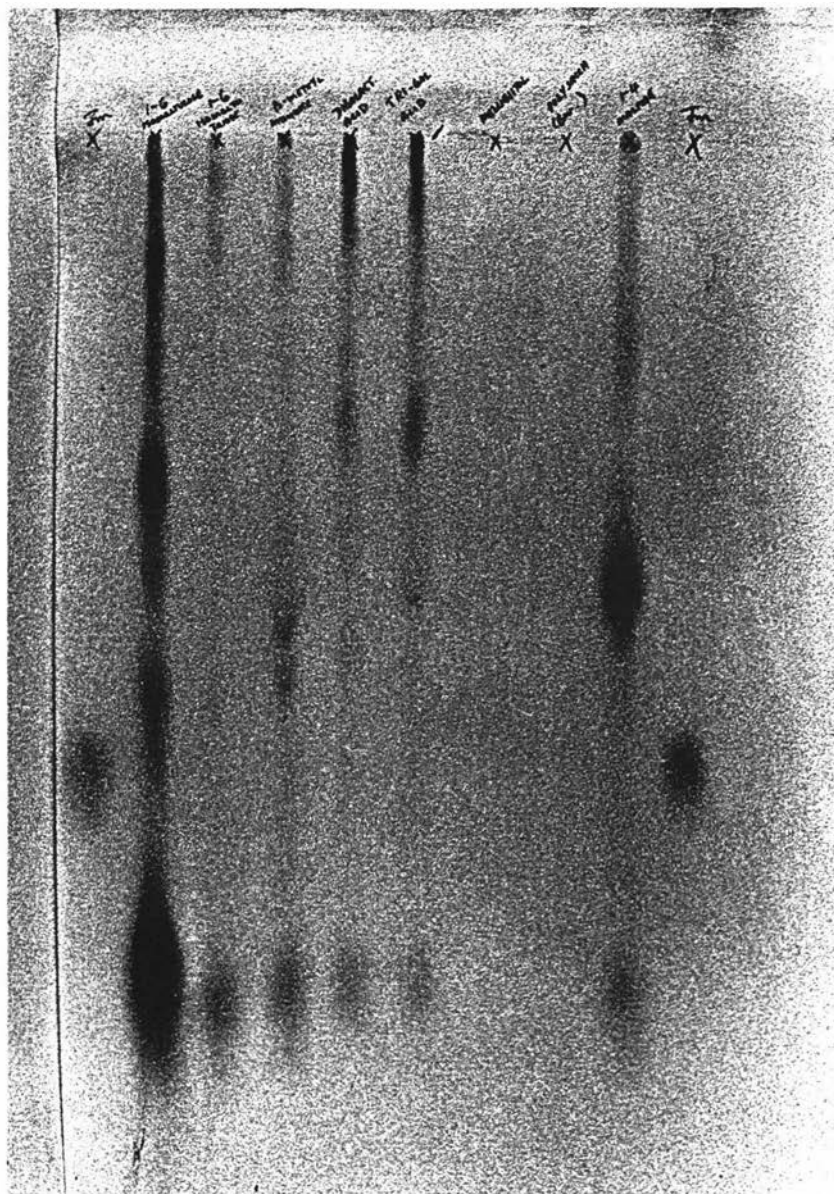
It was of interest to test the method for higher oligosaccharides and polysaccharides homogeneously linked with respect to their glycosidic linkages. With members of the malto-

PLATE 2



Imidazole patterns: L to R:- Imidazole(marker), glucosyl-1-1-fructose, trehalose, sophorose, laminaribiose, turanose, lactose, cellobiose, maltose, lactulose, imidazole, maltulose, maltotriose, cellotriose, xylobiose, leucrose, melibiose, gentiobiose, iso-maltose, imidazole.

PLATE 3



Imidazole patterns: L to R:- Imidazole (marker), isomaltulose, isomaltotriose, β-methylmaltoside, digalacturonic acid, trigalacturonic acid, melibiitol, unknown polysaccharide, maltose, imidazole.

dextrin series (degree of polymerisation 3-8) the same pattern of imidazoles was obtained as for maltose, but longer heating was required (8-9 hours at 110°). With similar heating in ammoniacal solution individual members of the isomaltodextrin series (d.p. 3-9) gave the typical series of imidazoles for a 1-6 linked sugar. The corresponding straight chain polysaccharides, amylose and dextran were also tested, but only the dextran gave a positive test, and then only on prolonged heating. Solubility appeared to be a limiting factor with the amylose.

Table 6 lists the reaction times of the separate homologous series. Celotriose, in ammoniacal solution, was found to give the characteristic imidazoles of a 1-4 linked sugar.

TABLE 6

Times of appearance of characteristic imidazole pattern.

Maltose Series		Isomaltose Series	
Degree of polymerisation.	Time (hrs)	Degree of polymerisation.	Time (hrs)
2	20	2	27
3	20+2(110°)	3	20+1(110°)
4	"	4	"
5	20+3(110°)	5	20+2(110°)
6	"	6	20+3(110°)
6+7	"	6+7	"
8	"	8+9	"
n(amylose)	-	n(dextran)	12(110°)

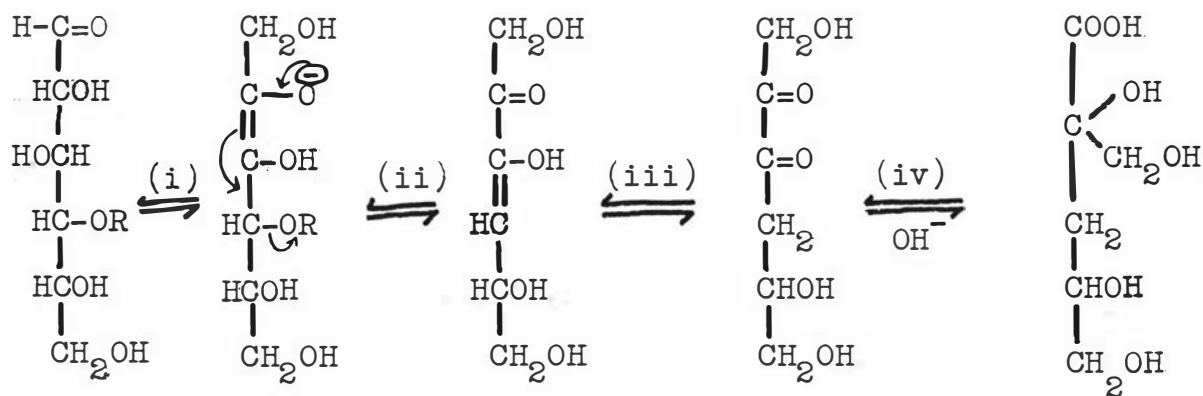
It may be concluded that the method developed for linkage determination in reducing hexose disaccharides (and for homologous series of homogeneously-linked oligosaccharides) satisfies the required criteria of speed, specificity, sensitivity and ease of manipulation.

There has been little mention in the literature of the effects of ammonia on oligosaccharides. Hough, Jones and Richards²⁹ reported that ammonium hydroxide converts lactose to a mixture of lactulose and galactose, and maltose to maltulose, mannose, fructose, glucose and another unidentified ketose disaccharide. Melibiose, under the same conditions, yields melibiulose, 6-O- α -D-galactopyranosyl- β -D-mannose,⁹⁶ D-galactose, D-tagatose and various heterocyclic products.⁵⁰ These results show that aqueous ammonia causes both epimerisation and fragmentation of disaccharides.

It might be expected that the action of ammonium hydroxide on reducing oligosaccharides should parallel, initially, the action of alkali, which causes epimerisation and also results, in some cases, in a stepwise degradation from the reducing end of the molecule. The acetal links of non-reducing glycosides are generally stable to alkalis as is evidenced by the non-reducing properties of trehalose, sucrose³² and many alkyl glycosides,²⁰ and therefore alkaline hydrolysis is only known to occur under drastic conditions e.g. 10% aqueous sodium hydroxide at 170° for some hours.⁴⁰

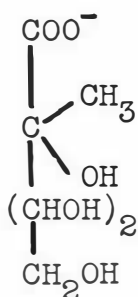
Among the reaction products of alkalis on carbohydrates are the various saccharinic acids, mixtures of which are formed from unsubstituted hexoses. When the sugars exist in a substituted state, however, the type of saccharinic acid formed depends on the position of substitution. A number of mechanisms have been proposed to account for the formation of these acids. The most widely-accepted appears to be that suggested by Isbell³⁴ who postulated the following steps:-

- (i) formation and ionisation of an enediol
- (ii) β -elimination of an hydroxyl or alkoxy group
- (iii) rearrangement to an α -dicarbonyl intermediate, and
- (iv) a benzilic acid type of rearrangement to the saccharinic acid. e.g. formation of a glucoiso-saccharinic acid from a 4-O-substituted hexose.

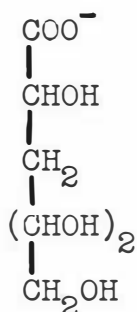


Ordinarily 1-O-substituted sugars are stable in oxygen-free alkaline solutions, but when substitution occurs on

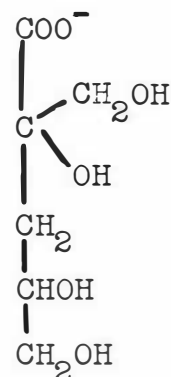
the C-1 hydroxyl group of fructose (e.g. glucosyl-1-1-fructose), high yields of lactic acid and some saccharinates (I) are formed.⁴⁰ 2-O-Substitution does not lead to saccharinates because formation of the required carbonyl group, β to the substituted hydroxyl group, is hindered.⁹⁵ When substitution occurs on the 3-OH, the effect of alkali produces metasaccharinates (II) e.g. laminaridose (3-O- β -D-glucopyranosyl-D-glucose) and turanose (3-O- α -D-glucopyranosyl-D-fructose) are degraded at equal rates to metasaccharinic acids.¹⁰ Alkaline degradation of 4-O-substituted sugars produces isosaccharinates (III). In general,



(I)



(II)

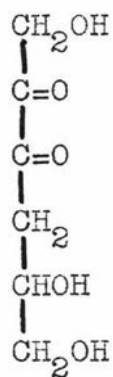


(III)

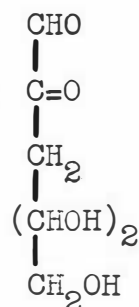
ketoses are degraded faster than aldoses e.g. maltulose faster than maltose.⁹³ Sugars substituted in the 6-position cannot eliminate an alkoxy anion directly because of their inability to enolise to form a carbonyl group at C-4. However, substitution of the C-6 hydroxyl group results in more pronounced attack on the C-4 hydroxyl group by alkali.

Ionisation of this C-4 hydroxyl group causes a reverse aldol-ization reaction, giving dihydroxyacetone and a 3-O-substituted glyceraldehyde. From the dihydroxyacetone, via pyruv-aldehyde, lactic acid is formed.⁸⁰

Each of the above reactions requires the formation of a dicarbonyl intermediate. A number of these have been synthesised and were found to give the expected saccharinic acid on alkali treatment e.g. Whistler and BeMiller⁹⁴ isolated the dicarbonyl intermediate (IV) involved in the formation of isosaccharinic acids, while Anet¹ has prepared the corresponding dicarbonyl (V) for metasaccharinic acids.



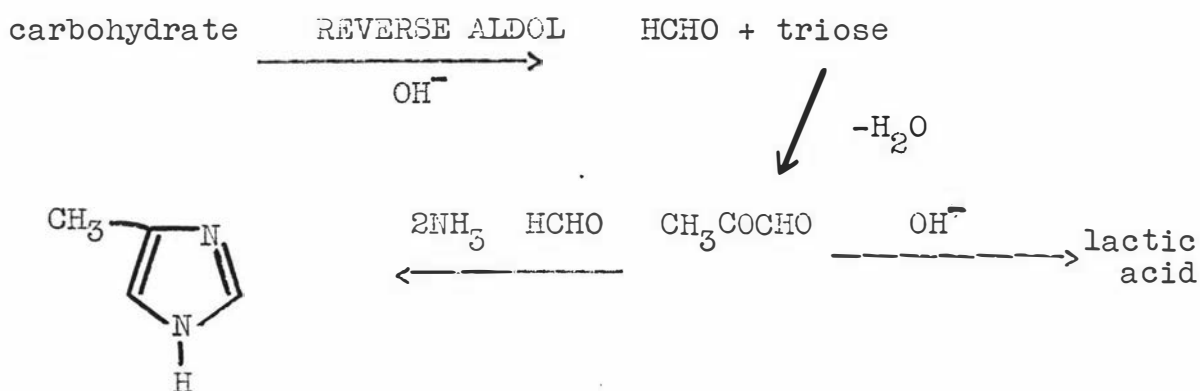
(IV)



(V)

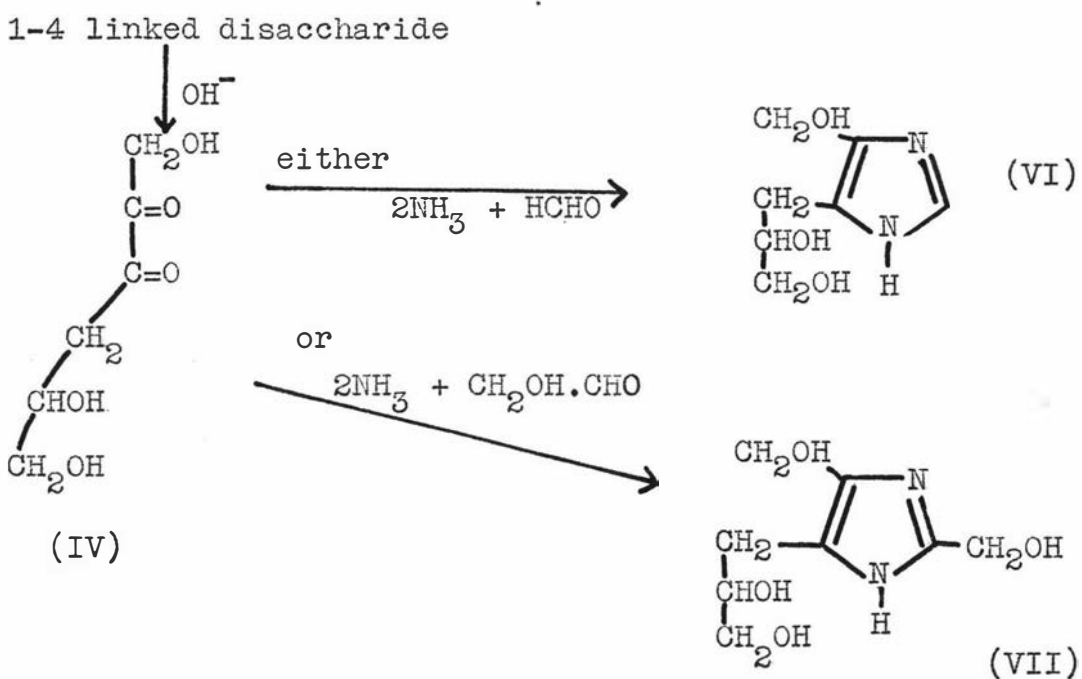
It is suggested in this thesis that saccharinic acid formation in ammoniacal solution stops at this dicarbonyl intermediate stage, when, under the influence of ammonia, imidazoles are formed. This would account for the different patterns of imidazoles produced from differently linked

oligosaccharides. The presence of 4(5)-methylimidazole in the reaction products of all of the reducing disaccharides can be attributed to a certain amount of reverse aldolization of the carbohydrates forming small quantities of formaldehyde and pyruvaldehyde.⁵⁹ In non-ammoniacal alkaline conditions the pyruvaldehyde would be converted to lactic acid⁴⁰ but with ammonia, acid formation would be suppressed by the conversion of pyruvaldehyde to 4(5)-methylimidazole.



The discovery that 4(5)-methylimidazole was the major imidazolic product of the reaction between aqueous ammonia and 1-6 linked disaccharides (which form mainly lactic acid with alkali) would appear to support the hypothesis that ammonia reacts with the intermediate dicarbonyl compound. This explanation also accounts for the finding that 4(5)-methylimidazole is the major imidazolic product of the interaction of glucosyl-1-1-fructose (which yields mainly lactic acid with alkali) with aqueous ammonia.

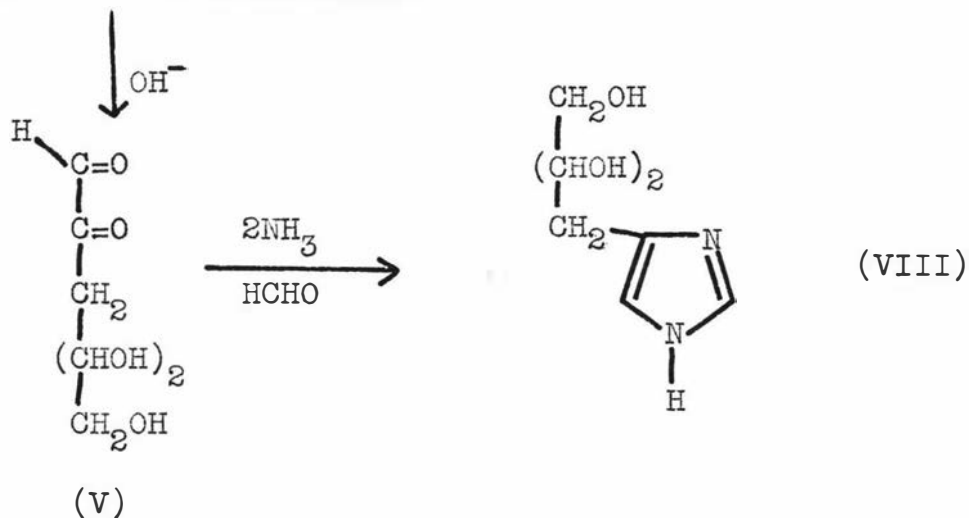
The imidazoles formed in highest concentration from the 1-3 and 1-4 linked disaccharides gave, with the diazo reagent, orange-red dyes which have been noted to be often characteristic of imidazoles with an hydroxyalkyl side chain. These compounds also moved more slowly on chromatograms than 4(5)-hydroxymethylimidazole, indicating an appreciably more complex substitution pattern. If, as is now suggested, the main imidazoles formed are derived from the respective dicarbonyl intermediates (i.e. IV from 4-O-substituted- and V from 3-O-substituted disaccharides) then their structures could be as follows:-



As compound VII is trisubstituted it would not give a colour with the diazotised sulphanilic acid, and thus the

compound VI would be the only one detected on chromatograms.

1-3 linked disaccharide



Compound VIII, 4(5)-(2',3',4'-trihydroxybutyl)imidazole, has been isolated previously by Komoto⁴⁴ as a minor product from the interaction of glucose and ammonia, and therefore it would be of interest to compare his product with the major imidazole from ammoniacal solutions of turanose and laminaribiose. Unfortunately Komoto's chromatographic data refer to an unidentifiable chromatography paper and thus it is not possible to compare R_f values at this time, although both compounds appear to move slowly. Elucidation of the structures of the imidazoles formed from the different disaccharides may best be attacked through the interaction of the dicarbonyl intermediates (which have been synthesised) with aqueous ammonia.

The action of ammonium hydroxide on the homologous maltodextrin and isomaltodextrin series was similar to its action on the parent disaccharides, but the reaction proceeded more slowly as the degradation probably occurred in a step-wise fashion from the reducing end of the chain.

Future Projects Arising from the Results of this Thesis.

A number of problems arising from this thesis and other work remain unsolved, and would appear to merit further study:

- (i) Is the compound giving a blue colour with the diazo spray in the glyoxal-ammonia mixture an amino-substituted imidazole?
- (ii) Why was it possible to isolate imidazole from the glycolaldehyde-ammonia mixture and yet not possible to detect "glycosine"?
- (iii) What are the identities of the large number of imidazoles of low R_f values present in ammoniacal solutions of glyceraldehyde and dihydroxyacetone?
- (iv) What is the main source of formaldehyde for 4(5)-methylimidazole formation from hexoses? Is it from reverse aldolizations of hexose (or pentose, tetrose, triose) or from ammoniacal "fission" of pyruvaldehyde?
- (v) What is the mechanism of formation of 4(5)-hydroxymethylimidazole from glyceraldehyde and ammonia?
- (vi) What are the mechanisms of formation of 4(5)-methylimidazole and 2-hydroxymethyl-4(5)-methylimidazole from hydroxypyruvaldehyde in ammoniacal solution?
- (vii) Are the dicarbonyl intermediates involved in formation of the various saccharinic acids also responsible for the imidazolic patterns formed by the various reducing disaccharides? It would be interesting to compare the imidazoles formed when these dicarbonyls react with ammonia with those imidazoles formed by the parent disaccharides.

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EXPERIMENTAL

EXPERIMENTAL

Analyses were carried out by Dr. A.D. Campbell and staff, Chemistry Department, University of Otago. Infrared spectra were determined on a Perkin-Elmer 421 infrared spectrophotometer by Mr. S.F. Downes, University of Otago, and on a Perkin-Elmer Infracord locally. Ultraviolet and visible spectroscopy was carried out using a Beckman DU Spectrophotometer. Proton magnetic resonance spectra were determined on a Varian DP 60 spectrometer with the assistance of Dr. R. Golding and Dr. B. Thomas, of Dominion Chemical Laboratories, Lower Hutt. Chemical shifts are given in c./sec. from tetramethylsilane as internal reference. Most evaporations were carried out at low temperature under vacuum. All melting points were determined on a Gallenkamp electrical melting point apparatus.

Ia. SYNTHESIS OF IMIDAZOLES.

IMIDAZOLE: Imidazole was prepared from dinitrotartaric acid, ammonia and formaldehyde by the method of Maquenne.²⁵ It had m.p. 90°.

2-METHYLIMIDAZOLE: 2-Methylimidazole-4,5-dicarboxylic acid (5 g.) was prepared by the method of Fargher and Pyman.⁹ The dicarboxylic acid was decarboxylated by distillation from soda-lime and the resultant distillate of 2-methylimidazole (2 g.) was recrystallised from diethyl ether m.p. 142° (Found: C, 58.8; H, 7.4. Calc. for C₄H₆N₂: C, 58.4; H, 7.4%). Fargher and Pyman⁹ report m.p. 142-3. The picrate crystallised from water m.p. 213° in agreement with published figures.

4(5)-METHYLIMIDAZOLE: 4(5)-Methylimidazole picrate (8.5 g.; m.p. 160°), prepared from glucose, ammonia and copper acetate by the method of Koessler and Hanke,¹⁸ was dissolved in a mixture of hot water (50 ml.) and dilute sulphuric acid (0.2N; 20 ml.) and then boiled (20 min.) and cooled. Picric acid was removed by extraction with benzene, the aqueous residue made strongly alkaline with sodium carbonate, and evaporated to dryness. Extraction of the residue with ether followed by distillation (120-5°; 0.02 mm.) yielded 4(5)-methylimidazole (2.2 g.) as a syrup. The base was purified by partition chromatography on a cellulose column using n-butanol half-saturated with water containing

0.25% acetic acid as eluant. The product was evaporated to dryness in vacuo and allowed to stand at -10° (approx. 2 days) when hygroscopic crystals were obtained m.p. 55° . (Found: N, 34.4. Calc. for $C_4H_6N_2$: N, 34.1%).

2,4(5)-DIMETHYLIMIDAZOLE: This compound was prepared by two methods.

(i) The picrate m.p. 142° was isolated from the product of interaction of pyruvaldehyde, ammonia solution and acetaldehyde held at 100° during 12 hours.

(ii) A mixture of α,β -bis-(benzoylamino)- α -propene (2 g.) (obtained from the interaction at 0° of 4(5)-methylimidazole and benzoyl chloride in 10% sodium hydroxide solution) and acetic anhydride (8 g.) in a sealed tube were heated at 180° for 6 hours. The product was washed out with water, evaporated in vacuo, made alkaline with potassium carbonate and extracted with ether. The ethereal solution formed a picrate m.p. 142° . Windaus and Langenbeck⁴⁴ give 2,4(5)-dimethylimidazole picrate m.p. 142° . (Found: N, 21.4. Calc. for $C_{11}H_{11}N_5O_7$: N, 21.5%).

4,5-DIMETHYLIMIDAZOLE: (i) This compound was prepared, as described by Castle and Seese,³ by the lithium aluminium hydride reduction of methyl imidazole-4,5-dicarboxylate. The excess lithium aluminium hydride was destroyed by the careful addition of water and the ether layer evaporated.

The aqueous layer was filtered and extracted continuously with ether for a week. 4,5-Dimethylimidazole was isolated as a picrate m.p. 190°. Cowgill⁴ gives m.p. 191-192°.

(ii) The same dimethylimidazole was prepared at the same time as 2,4,5-trimethylimidazole by the method of Fargher and Pyman.⁸ To a mixture of diacetyl (8.6 g.) and formaldehyde (50 ml. of 40%) was added ammonia solution (80 ml. of 25%) with cooling. After 12 hours the ammonia was removed, the product made strongly alkaline with potassium carbonate and extracted with ether. The resultant oil (after removal of the ether) was dissolved in water and a saturated aqueous solution of picric acid added. By fractional crystallisation 4,5-dimethylimidazole picrate (4.8 g.) m.p. 191°, and 2,4,5-trimethylimidazole (2.0 g.) m.p. 163° were obtained.

2-ETHYL-4(5)-METHYLIMIDAZOLE: A modification of the method of Windaus and Langenbeck⁴⁴ was used for the preparation of this compound. The product of interaction of α,β -bis-(benzoylamino)- α -propene with propionic anhydride gave a mixture of products. Thin layer chromatography on alumina using toluene/ethylacetate/25% ammonia (1:3:0.1) showed compounds at R_{Im} values of 1.00 and 1.91, giving red and lemon-yellow colours respectively with the diazo spray reagent. Separation of these products on sheets of Whatman No. 3MM paper in n-

butanol/glacial acetic acid/water (4:1:1) showed that the faster-moving compound was 2-ethyl-4(5)-methylimidazole.

The picrate crystallised from water m.p. 131°. (Found: C, 42.7; H, 4.3; Calc. for $C_{12}H_{13}N_5O_7$: C, 42.5; H, 3.9%).

2-HYDROXYMETHYLIMIDAZOLE: 2-Hydroxymethylimidazole was prepared by a modification of the method of Jones.¹⁶ N-Benzylamino acetal (30 g.) b_{10} 168° was prepared from chloroacetal (50 g.) and benzylamine (107 g.). To a mixture of this compound with sodium thiocyanate (14 g.) in 50% ethanol (70 ml.) was added with stirring concentrated hydrochloric acid (15 ml.). The mixture was heated in an open beaker on a steam bath until a violet solid remained (5 hours). The product was dissolved in 50% sodium hydroxide solution, treated with charcoal, filtered and acidified with concentrated hydrochloric acid to yield 1-benzyl-2-mercaptoimidazole (28 g.). A sample recrystallised from ethyl acetate had m.p. 144-5° (Lit.¹⁶ gives m.p. 144-5°). A stirred solution of concentrated nitric acid (35 ml.) in water (85 ml.) was heated to 45° and a small portion (1 g.) of 1-benzyl-2-mercaptoimidazole was added to initiate the reaction. Further portions of the mercaptoimidazole were added while the temperature was maintained between 40-50° by means of an ice bath. The reaction product was made alkaline with 12N sodium hydroxide, extracted with chloroform, the chloroform evaporated and the residue distilled (b_{13} 166-7°)

to give 1-benzylimidazole (19 g.) m.p. 71-2°. A picrate derivative crystallised from water m.p. 75-6°.

When Jones' method was followed for the next stage of the reaction a mixture of products was obtained. The modified method involved treatment of 1-benzylimidazole (19 g.) with 40% formaldehyde solution (30 ml.) in a sealed tube at 140° for 8 hours. After cooling the faintly yellow product was washed from the tube with methanol and repeatedly evaporated in vacuo from methanol until the odour of formaldehyde could no longer be detected. The syrup (12.2 g.) was dissolved in absolute ethanol (60 ml.), treated with 12N hydrochloric acid (20 ml.) and evaporated to dryness in vacuo. The white crystalline residue was repeatedly re-crystallised from ethanol/dry ether until the hydrochloride had m.p. 158° (10 g.). (Found: C, 59.2; H, 6.1; N, 12.3. Calc. for $C_{11}H_{13}N_2OCl$: C, 58.8; H, 5.8; N, 12.3%).

A quantity of 1-benzyl-2-hydroxymethylimidazole hydrochloride (5 g.) was suspended in water (100 ml.) and made alkaline with barium carbonate. After boiling, the hot solution was filtered and, on cooling, white plates (2.5 g.) separated from the filtrate m.p. 56°. (Found: C, 69.6; H, 6.4; N, 15.0. $C_{11}H_{12}N_2O$ requires: C, 70.2; H, 6.4; N, 14.9%). A further quantity of the free base (1 g.) of 1-benzyl-2-hydroxymethylimidazole was extracted from

the barium carbonate residue with chloroform. Comparison of the infrared spectra of this compound and 1-benzylimidazole showed a strong hydroxyl band at 3200 cm^{-1} in the spectrum of the former, absent in the latter. (See Fig. 3. page 65). A picrate prepared from the free base had melting point (from water) 135° . Jones¹⁶ gives 1-benzyl-2-hydroxymethylimidazole picrate m.p. $132-3^\circ$.

1-Benzyl-2-hydroxymethylimidazole (0.8 g.) in liquid ammonia (50 ml.) was treated with small pieces of sodium metal until the solution retained a deep blue colour. The colour was then discharged with ammonium chloride, the ammonia evaporated, and the residue extracted with hot absolute ethanol (50 ml.). The ethanolic solution was evaporated to 10 ml., cooled, filtered, and then evaporated under reduced pressure to a syrup (0.4 g.) which was purified by separation on sheets of Whatman No. 3MM paper in n-butanol/acetic acid/water (4:1:1). The resultant syrupy 2-hydroxymethylimidazole (0.3 g.) gave an orange colour with alkaline diazotised sulphanilic acid and travelled to R_{Im} 1.12 on No. 3MM paper in the solvent mentioned above. A portion (0.2 g.) of the free base was taken up in water (10 ml.), a saturated aqueous solution of picric acid added and the solution evaporated to a small volume. After chilling overnight, yellow-orange crystals separated (0.1 g.) m.p. 148° . Recrystallisation from absolute ethanol raised

the melting point to 152°. (Found: C, 36.8; H, 2.9; N, 21.5. Calc. for $C_{10}H_9N_5O_8$: C, 36.7; H, 2.8; N, 21.4%). Jones¹⁶ gives 2-hydroxymethylimidazole picrate m.p. 152-3°.

A further portion of the syrupy free base (0.2 g.) was extracted with hot ethanol, filtered, evaporated in vacuo to a syrup and separated in turn on a column containing alumina using chloroform/methanol (1:1) and then again on a cellulose column using n-butanol half-saturated with water and containing 0.25% acetic acid. Evaporation of the fractions containing 2-hydroxymethylimidazole gave a pale yellow syrup which crystallised rapidly on cooling. The white crystals (0.15 g.) were collected, washed with ether, and then recrystallised from methanol/ether (1:3) giving large white plates m.p. 110° raised to 112° on further recrystallisation. (Found: C, 49.1; H, 6.4; N, 28.9. $C_4H_6N_2O$ requires: C, 49.0; H, 6.2; N, 28.9%). An infrared spectrum (nujol) showed the presence of a broad hydroxyl band with ν_{\max} 3200 cm^{-1} .

4(5)-HYDROXYMETHYLIMIDAZOLE: 4(5)-Hydroxymethylimidazole picrate (10 g.), prepared by the method of Totter and Darby,⁴¹ was boiled with sulphuric acid (0.2N; 20 ml.) and the picric acid extracted with benzene (5 x 30 ml.). Excess barium carbonate was added to the aqueous solution and, after filtration, more barium carbonate was added to the filtrate, the solution was again boiled (10 min.) and then evaporated to

dryness in vacuo. An ethanol extract of the residue gave white crystals which, on recrystallisation from absolute ethanol/dry ether, gave 4(5)-hydroxymethylimidazole (1.6 g) m.p. 92°. (Found: N, 28.3; Calc. for $C_4H_6N_2O$: N, 28.5%). Jones and McLaughlin¹⁷ report 4(5)-hydroxymethylimidazole m.p. 91-2°.

2-HYDROXYMETHYL-4(5)-METHYLIMIDAZOLE: A mixture of pyruvaldehyde (0.1 g.) with glycolaldehyde (0.08 g.) in 4.7% ammonia solution (12 ml.) was heated on a steam bath for 48 hours. The product was evaporated in vacuo and the residue extracted with dry acetone which was then concentrated to a small volume. 2-Hydroxymethyl-4(5)-methylimidazole was isolated from this solution as a picrate which was recrystallised from 95% ethanol/ether as the sesquihydrate m.p. 83°. (Found: N, 19.0. Calc. for $C_{11}H_{11}N_5O_8 \cdot 1\frac{1}{2}H_2O$; N, 19.0%). Dehydration of this picrate in vacuo at 60° for two days raised the melting point to 143°. Komoto²⁰ reports 2-hydroxymethyl-4(5)-methylimidazole picrate m.p. 143-5°.

4(5)-HYDROXYMETHYL-2-METHYLIMIDAZOLE: To a solution of 4(5)-hydroxymethylimidazole (2.25 g.) in 10% sodium hydroxide solution (50 ml.) at 0° was added benzoyl chloride (4 g.). The mixture was maintained at 0° for four hours, extracted with benzene (5 x 30 ml.), washed with water, and the benzene evaporated. The white solid residue after evaporation

of the benzene was extracted with hot absolute ethanol and then ether was added to the extract in order to precipitate the 1,2-dibenzamido-3-hydroxy-1-propene as silky white crystals m.p. 168-9°. The product (0.37 g.) was treated in a sealed tube at 180° for six hours with acetic anhydride (1.6 ml.). The evaporated product was treated with potassium carbonate, evaporated to dryness, and then extracted with acetone. From this extract was obtained a syrup (0.05 g.) which yielded a picrate m.p. 174-8°. Insufficient material was obtained for analysis, but the infrared spectrum differed from that of 2-hydroxymethyl-4(5)-methylimidazole picrate. Mackay and Shepherd²³ prepared 4(5)-hydroxymethyl-2-methylimidazole by the interaction of dihydroxyacetone, acetaldehyde and ammoniacal cupric carbonate and give a melting point for the picrate of 172-3°.

4(5)-(2'-HYDROXYETHYL)IMIDAZOLE: (i) To prepare this compound an adaptation of the method of Erlenmeyer et al.⁵¹ was used. A solution of histamine dihydrochloride (3 g.) in water (30 ml.) was treated with dilute hydrochloric acid (2 ml. of 4N) and then with a slight excess (5.5 ml.) of 10% barium nitrite. After 10 minutes the mixture was warmed on the water bath (15 min.) and after cooling was made alkaline with barium carbonate. The solution was filtered, evaporated to dryness and the residue extracted with hot, dry acetone and then evaporated again to give a yellow oil. To this was added a solution of picric acid in ethanol/water

(1:1), the mixture was concentrated almost to dryness, and then frozen when crystals of 4(5)-(2-hydroxyethyl)imidazole picrate (0.01 g.) separated m.p. 142° . The free base travelled to R_{Im} 0.99 on Whatman No. 3MM paper and No. 1 paper when n-butanol/acetic acid/water (4:1:1) was used as solvent, and to R_{Im} 0.94 on Whatman No. 1 paper in acetone/chloroform/water/ammonia (30:5:4:0.2). It gave a red-orange colour with diazotised sulphanilic acid.

(ii) A further sample of the compound was prepared by a method essentially the same as that of Turner.⁴³ A 50% solution containing 1,4-dihydroxybutan-2-one was prepared using the method of Reppe.³⁶ To 100 ml. of this solution was added 40% formaldehyde (120 ml.) and the whole was added at $70-80^{\circ}$ to a mixture of cupric sulphate (250 g.) in water (1 l.) and 25% ammonia solution (900 ml.). After the reaction had proceeded for two hours at $70-80^{\circ}$, the solution was cooled, the copper complex filtered, suspended in water, acidified with acetic acid and then decomposed with a stream of hydrogen sulphide. The copper sulphide was removed by centrifuging and the aqueous solution concentrated to dryness and then distilled to give 4(5)-(2-hydroxyethyl)-imidazole b₁ $170-5^{\circ}$ (1 g.). The compound gave a picrate from a very concentrated aqueous solution m.p. 143° in agreement with the published value.⁴³

4(5)-D-ARABOTETRAHYDROXYBUTYLIMIDAZOLE: (i) This compound was isolated from an aerated mixture of fructose, copper hydroxide and ammonium hydroxide using the method of Parrod.³¹ The picrate had m.p. 113° and the free base m.p. 164° which is in agreement with published data.³¹

(ii) The same compound was prepared from a mixture of glucosone,²⁶ formaldehyde, ammonium hydroxide and copper hydroxide.

IMIDAZOLE-4(5)-ALDEHYDE (4(5)-FORMYLIMIDAZOLE): Imidazole-4(5)-aldehyde was prepared by the oxidation of 4(5)-hydroxymethylimidazole.³⁴ To 4(5)-hydroxymethylimidazole (1 g.) was added concentrated nitric acid (1.1 ml.) and the mixture was then digested on a water bath in a covered beaker.

When no further brown fumes were evolved the mixture was evaporated to dryness at 100° and made alkaline with warm, concentrated sodium carbonate solution. On cooling, 0.2 g. of the aldehyde crystallised m.p. 170°. The aldehyde yielded a picrate which crystallised from water m.p. 194°, and a 2,4-dinitrophenylhydrazone from ethanol m.p. 198°. Lit.³⁴ reports free base m.p. 173-4°; picrate m.p. 195-6°; 2,4-dinitrophenylhydrazone m.p. 199-200°.

On acidification of the alkaline residue (after filtration of the aldehyde) and allowing the mixture to stand imidazole-4(5)-carboxylic acid separated (0.13 g.) m.p. 282°.

IMIDAZOLE-2-ALDEHYDE (2-FORMYLIMIDAZOLE): Concentrated nitric acid (0.5 ml.) was added to 2-hydroxymethylimidazole (0.5 g.) and the mixture digested at 100° in a covered beaker. When no further fumes were evolved, the cover was removed and the mixture was allowed to evaporate to dryness. The residue was made alkaline with warm concentrated sodium carbonate solution but no aldehyde separated on cooling. Chromatography of the product on Whatman No. 3MM paper in n-butanol/acetic acid/water (4:1:1) indicated that little of the alcohol had been oxidized, but there was present a small amount of a compound giving a pale orange-brown colour with the diazo reagent at R_{Im} 0.53. The mixture was evaporated to dryness, extracted with hot absolute ethanol (5x 20 ml.), filtered and evaporated in vacuo. To the resultant syrup (mainly 2-hydroxymethylimidazole; 0.35 g.) was added 10% sulphuric acid (10 ml.) mixed with chromium trioxide (0.4 g.). The solution was heated for an hour on the steam bath, after which the product was treated with excess aqueous sodium carbonate and evaporated to dryness in vacuo. After two extractions with hot absolute ethanol the resultant yellow solution gave a brown syrup (0.08 g.) containing some solid material. It did not prove possible to obtain a crystalline derivative from the product, but an infrared spectrum (nujol) gave a broad band $1600-90\text{ cm}^{-1}$, similar to that obtained by Turner⁴³ for imidazole-4(5)-aldehyde ($1654-96\text{ cm}^{-1}$). The compound gave an orange-brown dye of low

intensity at R_{Im} 0.53 in n-butanol/acetic acid/water (4:1:1) (0.57 on Whatman No. 1 paper) and R_{Im} 0.78 in ethyl acetate/acetic acid/-n-butanol/water (1:1:1:1) on Whatman No. 3MM paper.

2-ACETYL-4(5)-METHYLIMIDAZOLE: The nitrosoester of ethyl acetoacetate¹ was prepared by treatment of the latter with nitrous acid at -10° to yield a product (16 g.) which had m.p. 58° after recrystallisation from toluene/petroleum ether. (Found: C, 44.9; H, 6.0; N, 8.7. Calc. for $C_6H_9NO_4$: C, 45.3; H, 5.7; N, 8.8%).

The nitrosoester (16 g.) and stannous chloride (45 g.) were dissolved with shaking and cooling in concentrated hydrochloric acid (75 ml.). Tin (10 g.; granular) was added and the mixture was warmed for 10 minutes at 100° . The solution was diluted with 1.5 l. of water and "detinned" with hydrogen sulphide, the stannous sulphide being removed by centrifugation. The supernatant was completely dehydrated in vacuo at 45° giving a syrupy residue which slowly crystallised as colourless crystals. Recrystallisation from ethanol/ether gave the aminoester hydrochlorid \hat{e} (22.4 g.), m.p. 95° .

Reaction of the above aminoester in aqueous solution with a solution of potassium thiocyanate (21 g. in 100 ml. of water and 20 ml. concentrated hydrochloric acid) on the water bath for an hour gave (after concentration) yellow crystals

of ethyl 2-mercapto-4(5)-methylimidazole-5(4)-carboxylate (19 g.) m.p. 229° (darkens 175°). (Found: C, 45.2; H, 5.6. Calc. for $C_7H_{10}NSO_2$: C, 45.5; H, 6.1%). Ochiai and Ikuma²⁸ report m.p. 229°.

The thiol group was removed by warming the mercaptoimidazole (18.8 g.) with concentrated nitric acid. Concentration of the solution gave ethyl 4(5)-methylimidazole-5(4)-carboxylate nitrate (12 g.) m.p. 167°. The **nitrate** was decomposed with a concentrated solution of sodium hydroxide, filtered, and the product recrystallised from 40 ml. of ethanol (3.2 g.) m.p. 205°. (Found: C, 54.6; H, 6.8; N, 18.2. Calc. for $C_7H_{10}N_2O_2$: C, 54.5; H, 6.5; N, 18.2%).

The ester (3.0 g.) was dissolved in chloroform (90 ml.), cooled in ice and then treated at 0° with bromine (3 ml.) in chloroform (30 ml.). Concentration of the product yielded ethyl 2-bromo-4(5)-methylimidazole-5(4)-carboxylate (1.3 g.) m.p. 151°. (Found: C, 35.9; H, 4.0; N, 12.0. Calc. for $C_7H_9N_2O_2Br$: C, 36.0; H, 3.9; N, 12.0%). Pyman and Timmis³⁵ give m.p. 153°.

The ethyl ester (1.3 g.) was hydrolysed by heating in 20% hydrochloric acid (15 ml.) at 100° for 3 hours. The pH was adjusted to 4 with sodium carbonate, and, on cooling, the solution yielded crystals (1 g.) m.p. 232° (darkens 220°). Pyman and Timmis³⁵ report 2-bromo-4(5)-methylimidazole-5(4)-carboxylic acid m.p. 234°.

Decarboxylation was carried out for $5\frac{1}{2}$ hours in water at 150° in a sealed tube. The compound failed to crystallise but when treated with a concentrated aqueous solution of picric acid gave a picrate m.p. 171° . Pyman and Timmis³⁵ report 2-bromo-4(5)-methylimidazole picrate m.p. $172-3^\circ$. The solution from the tube was evaporated to dryness in vacuo to yield the free base (6.2 g.) which gave a yellow colour with diazotised sulphanilic acid and moved to R_{Im} 1.90 in chloroform/pyridine(2:1) on aluminium oxide G thin layer chromatoplates.

The bromo-compound (0.2 g.) in dry ether (10 ml.) was added to a flask (fitted with a stirrer and reflux condenser with drying tube) containing magnesium turnings (6.6 g.) and a crystal of iodine in anhydrous ether (32 ml.). The mixture was heated under reflux for $\frac{1}{2}$ hour and then anhydrous methyl cyanide (0.15 g.) was added in dry ether during 10 minutes. The mixture was then heated for a further four hours, at the end of which time ammonium chloride (0.2 g.) in water (2 ml.) was added to the cooled solution. The ether was evaporated and the mixture heated for an hour to ensure hydrolysis of the ketimine. Extraction with chloroform gave a product (0.02 g.) which appeared to be mainly 2-bromo-4(5)-methylimidazole. Thin layer chromatography as before indicated imidazolic products at R_{Im} values 0.46 (faint red); 0.90 (strong, yellow - probably unchanged

bromo-compound) and 0.97 (yellow). The fastest moving compound also resembled 2-acetyl-4(5)-methylimidazole with TLC on alumina in toluene/ethylacetate/25% ammonia. (1:3:0.1)

IMIDAZOLE-4(5)-FORMAMIDE: This compound was prepared by treatment of ethyl imidazole-4(5)-carboxylate (2 g.) in a sealed tube for $3\frac{1}{2}$ hours at 150° with 20 ml. of concentrated ammonia solution.² The amide (1.1 g.) precipitated from the pale yellow solution as white crystals m.p. 215° . The picrate separated from water m.p. 228° in agreement with the figure obtained by Balaban.²

Ib. CHROMATOGRAPHY OF IMIDAZOLES:

(i) Paper Chromatography.-

Paper chromatography of imidazoles was carried out at room temperature by the descending method using both large and small sheets of Whatman Nos. 1 and 3MM papers. The R_{Im} values listed in Table C1 (page 45) were obtained on approximately 9" square sheets of chromatography paper developed in glass battery jars. The imidazolic compounds were applied to the sheets of paper using a small platinum loop which was flamed before and after use. A fresh batch of solvent was prepared for each chromatogram. After development the air-dried chromatograms were sprayed with one of the following spray reagents.

Sulphanilic Acid (Pauly reagent).³⁹

Sulphanilic acid (9 g. in 90 ml. conc. HCl and 900 ml.
of water) 1 volume
Sodium nitrite (5% in water) 1 volume
Sodium carbonate anhydrous (10% in water) 2 volumes
added in the above order.

Iodine reagent.³⁸

Iodine (1 g.) dissolved in carbon tetrachloride (100 ml.).

(ii) Cellulose Thin-layer Chromatography.-

R_f and R_{Im} values were determined on 20 X 20 cm. glass plates using Macherey Nagel MN-Cellulose powder (300 g.;

15% in water) spread to a thickness of 250 μ using a Desaga apparatus. The plates were dried at room temperature for 30 minutes and then at 105° for 10 minutes. Quantities (1-5 μ g.) of imidazolic compound were applied to the chromatoplate from a glass capillary and the plates developed to a distance of 10 cm. at 20°. After air-drying, the plates were sprayed with diazotised sulphanilic acid to locate the imidazoles. See Table C2 (page 47).

(iii) Alumina and Silica Gel Thin-layer Chromatography.-

Silica gel G and aluminium oxide G were applied to 20 x 20 cm. glass plates to a thickness of 250 μ by the method of Stahl.^{40a} After 15 minutes at room temperature the silica plates were activated in an oven at 110° for an hour while the alumina chromatoplates were allowed to stand in the air overnight.

The base line was fixed at 3 cm. from the edge of the plate and the compounds applied in ethanol or diethyl ether solution from a micropipette. Each chromatoplate was developed to a height of 12.5 cm. at 20°. After air-drying, the plates were sprayed with Pauly reagent. Five different solvent systems were employed as the mobile phase and are reported in Table C3 along with R_f and R_{Im} values. (page 48)

Two-dimensional chromatography was employed for **very** complex mixtures. It was found that documentation was best achieved by colour photography. A tracing taken from a black and white photograph of a two-dimensional chromatoplate is included on page 50.

II. THE SEPARATION AND DETERMINATION OF IMIDAZOLES BY COMBINED PAPER-CHROMATOGRAPHIC AND SPECTROPHOTOMETRIC TECHNIQUES.

(1) Preparation of Reagents.

Aqueous solutions of 4(5)-methylimidazole, 4(5)-hydroxymethylimidazole, 4(5)-(2-hydroxyethyl)imidazole, imidazole, 2-methylimidazole, 2-hydroxymethyl-4(5)-methylimidazole, L-histidine hydrochloride and histamine dihydrochloride were made up to contain 100 µg./ ml. and stored at approximately 3°. The imidazoles were tested for purity by chromatography, analysis and titration with standard hydrochloric acid.

AR Sulphanilic acid (2.25 g.) was dissolved in 23 ml. of concentrated hydrochloric acid (specific gravity 1.19) and made up to 250 ml. with distilled water.

Recrystallised p-toluidine (1.35 g.) was dissolved in 23 ml. of concentrated hydrochloric acid (s.g. 1.19) and 25 ml. of absolute ethanol (AR) and made up to 250 ml. with distilled water.

Recrystallised p-bromoaniline (2.15 g.) was dissolved in 23 ml. of concentrated hydrochloric acid (s.g. 1.19) and absolute ethanol (AR) (75 ml.) and made up to 250 ml. with distilled water.

AR 90% Sodium nitrite (12.50 g.) was made up to 250 ml. with distilled water.

AR Anhydrous Sodium carbonate (2.75 g.) was made up to 250 ml. with distilled water.

Diazotised Sulphanilic Acid Reagent: Aliquots (0.75 ml.) of each of the solutions of sulphanilic acid and sodium nitrite were mixed (5 min.) at 0° in a 25 ml. volumetric flask. A further 3 ml. of sodium nitrite were added and the mixture allowed to stand (5 min.). Cold distilled water was added to make the volume 25 ml. and the mixture allowed to stand in an ice bath for at least 20 minutes. A fresh batch of this reagent was made up each day.

p-Bromoaniline Spray Reagent: Stock (0.9%) p-bromoaniline containing 10% v/v concentrated hydrochloric acid (1 volume) was mixed (30 sec.) with 5% sodium nitrite solution (1 volume) and then 10% sodium carbonate (2 volumes) was added and the mixture shaken (1 min.).

(2) Standard Procedure for Estimation of Imidazoles.

(a) Chromatographic Separation.-

A known volume (0.1-1.0 ml.) of a solution containing imidazoles is applied as a narrow band (2-3 mm.) to Whatman No. 3MM chromatography paper and marker spots of the same solution are placed on either side. The paper is irrigated as a descending chromatogram with a solvent chosen from Table E1, removed from the tank and dried at room temperature. The marker strips are then sprayed with Pauly diazo reagent³⁹ to reveal the positions of the imidazoles.

TABLE E1

R_{Im} VALUES OF IMIDAZOLES ON WHATMAN NO. 3MM PAPER

COMPOUND	SOLVENT I	SOLVENT II	SOLVENT III	DIAZO COLOUR
Imidazole	1.00	1.00	1.00	orange
4(5)-Methylimidazole	1.28	1.10	1.41	red
4(5)-Hydroxymethylimidazole	0.93	0.59	0.83	red-orange
2-Methylimidazole	1.30	1.08	1.34	yellow
L-Histidine. HCl	0.03	0.05	0.23	red
Histamine. di HCl	0.10	0.68	0.22	red

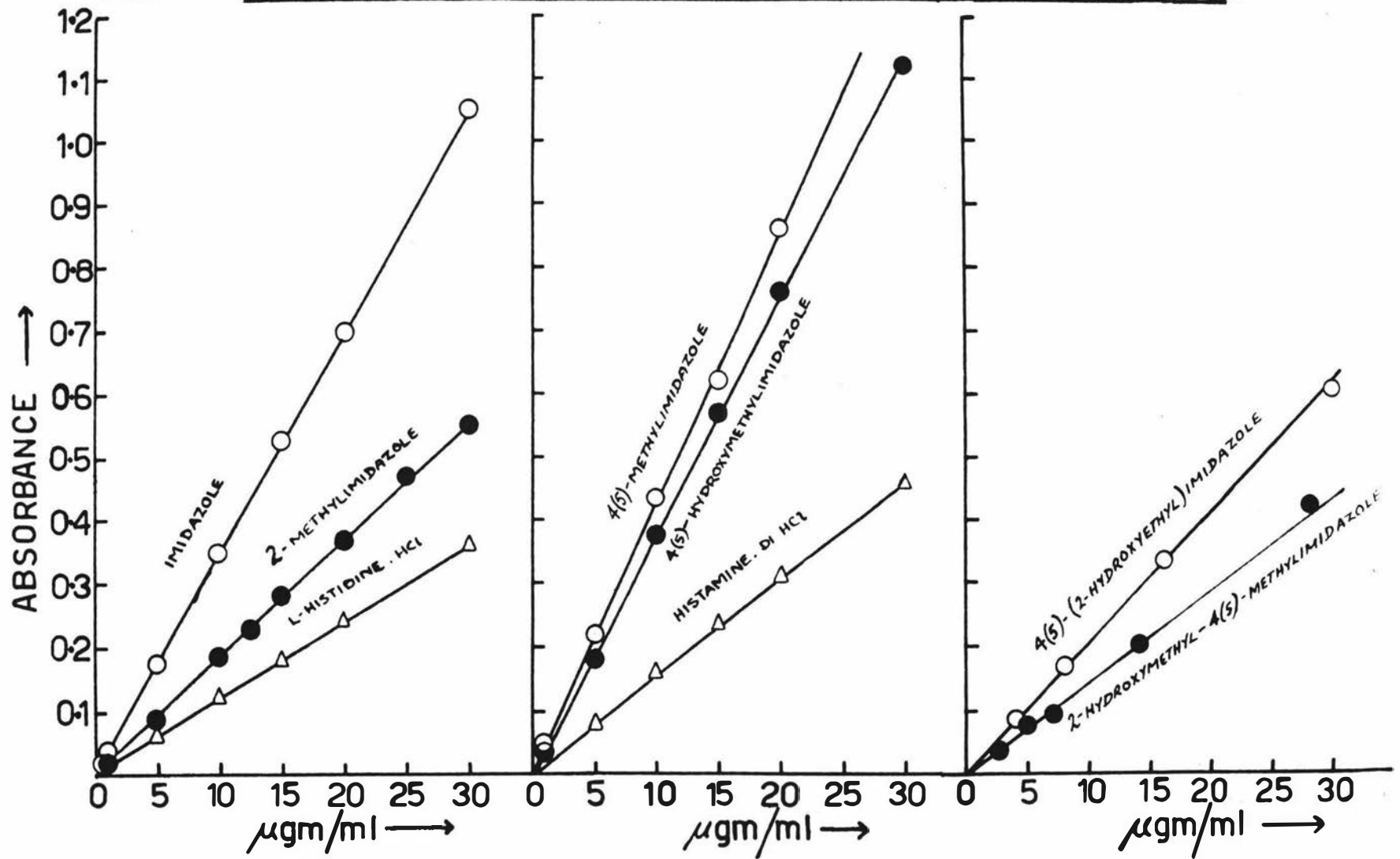
R_{Im} refers to the ratio (distance travelled by compound: distance travelled by imidazole).

Solvent I : n- butanol:acetic acid:water (4:1:1)

Solvent II : acetone:chloroform:water:28% ammonia (30:5:4:0.2)

Solvent III : ethylacetate: acetic acid: water (3:1:3) - top layer.

FIG. 1 STANDARD CURVES FOR ANALYSIS OF IMIDAZOLES.



By reference to the markers the areas of paper containing the component imidazoles are cut out from the main body of the chromatogram. The imidazoles are eluted from the excised strips by irrigation with distilled water in a chromatographic tank and the eluates are each made up to a known volume with distilled water. Most imidazolic compounds may be eluted into about 3-10 ml.

In a complex mixture of imidazoles it may not be possible to achieve complete separation by the use of one particular solvent system. In such a case it may be necessary to concentrate those eluates containing mixtures and carry out a further chromatographic separation in another solvent.

(b) Spectrophotometric Determination.-

Diazotised sulphanilic acid reagent (0.8 ml.) is mixed (30 sec.) in a stoppered test tube with the sodium carbonate solution (2 ml.). An aliquot (0.4 ml.) of one of the eluted imidazole solutions is added, the mixture shaken, and then transferred to a Corex spectrophotometer cell. The absorbance of the dye produced is determined at 480 m μ on a Beckman Model DU Quartz Spectrophotometer at a time specified in Table E2, using a reagent blank in which distilled water (0.4 ml.) replaces the imidazole solution. The concentration of the eluted imidazole solution is determined by reference to a standard curve (See Fig. 1)

TABLE E2

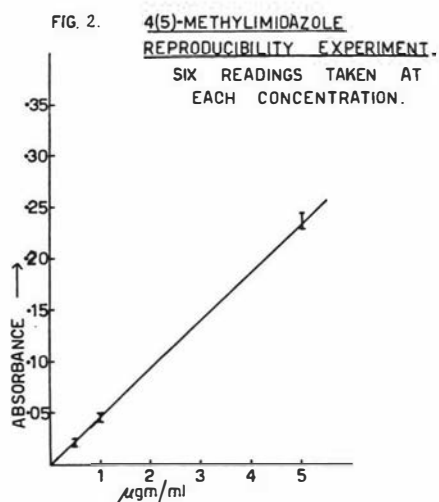
OPTIMUM READING TIMES, WAVELENGTHS OF MAXIMA AND ABSORBANCES AT λ MAX AND λ 480.

Compound	Optimum time for reading (minutes)	Wavelength of Maximum (λ_{max}) $m\mu$	Absorbance at λ_{max} for 10 μ g./ml. of Imidazolic Compound	Absorbance at 480 $m\mu$ for 10 μ g./ml. of Imidazolic Compound
Imidazole	24	480	.350	.350
4(5)-Methylimidazole	16	500	.470	.435
4(5)-Hydroxymethylimidazole	14	485	.382	.371
2-Methylimidazole	38	480	.191	.191
L-Histidine.HCl	6-7	490	.135	.128
Histamine.di HCl	5	500	.179	.170
4(5)-(2-Hydroxyethyl)imidazole	4	495	.210	.205
2-Hydroxyethyl-4(5)-methylimidazole	12	500	.163	.142

prepared from a series of suitable dilutions of stock imidazole solution.

Variation of absorbance with concentration:

Standard curves, determined from readings taken at 480 m μ and optimum times, were found to be linear within the range of concentrations studied (See Fig. 1). A series of six separate determinations was made for imidazole and 4(5)-methylimidazole at concentrations of 0.5, 1.0 and 5.0 $\mu\text{g./ml.}$ The results obtained demonstrated a good order of reproducibility even at the lower concentrations (See Table E3 and Fig. 2). The method estimated 5-30 $\mu\text{g./ml.}$ (2-12 $\mu\text{g.}$) with a 3% error and 0.5-1.0 $\mu\text{g./ml.}$ (0.2-4.0 $\mu\text{g.}$) with a 6-8% error.



Variation of absorbance with time:

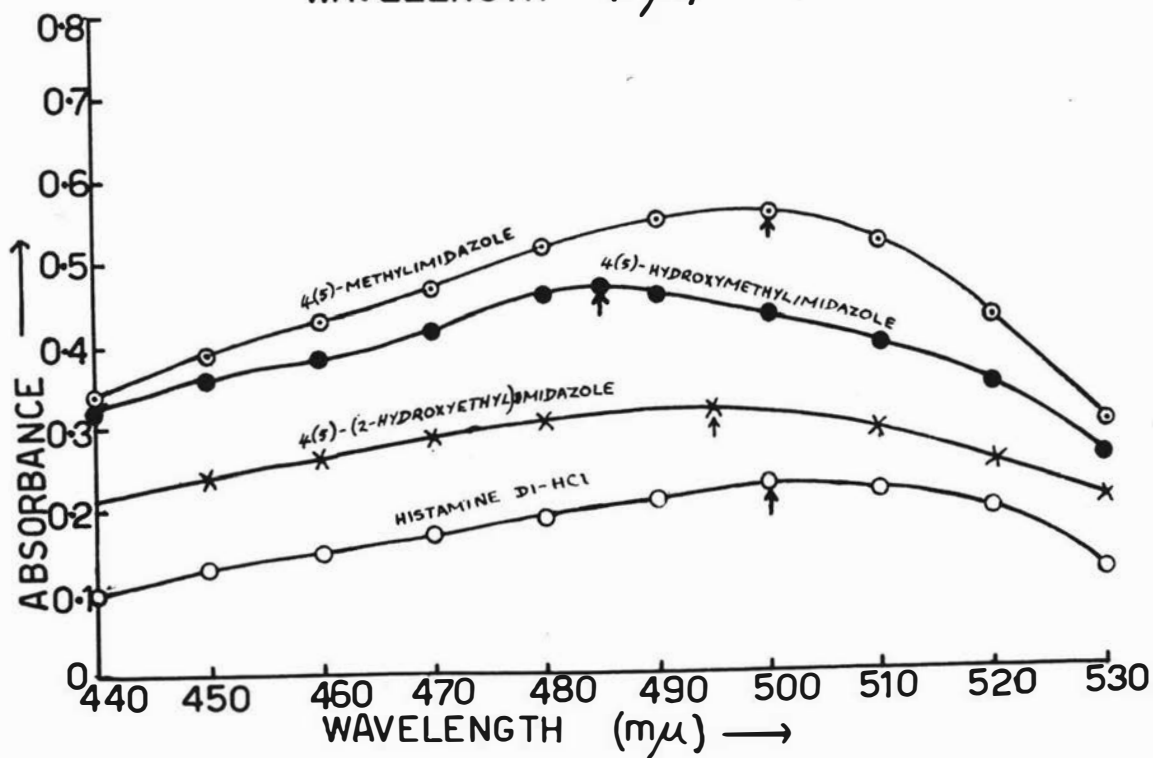
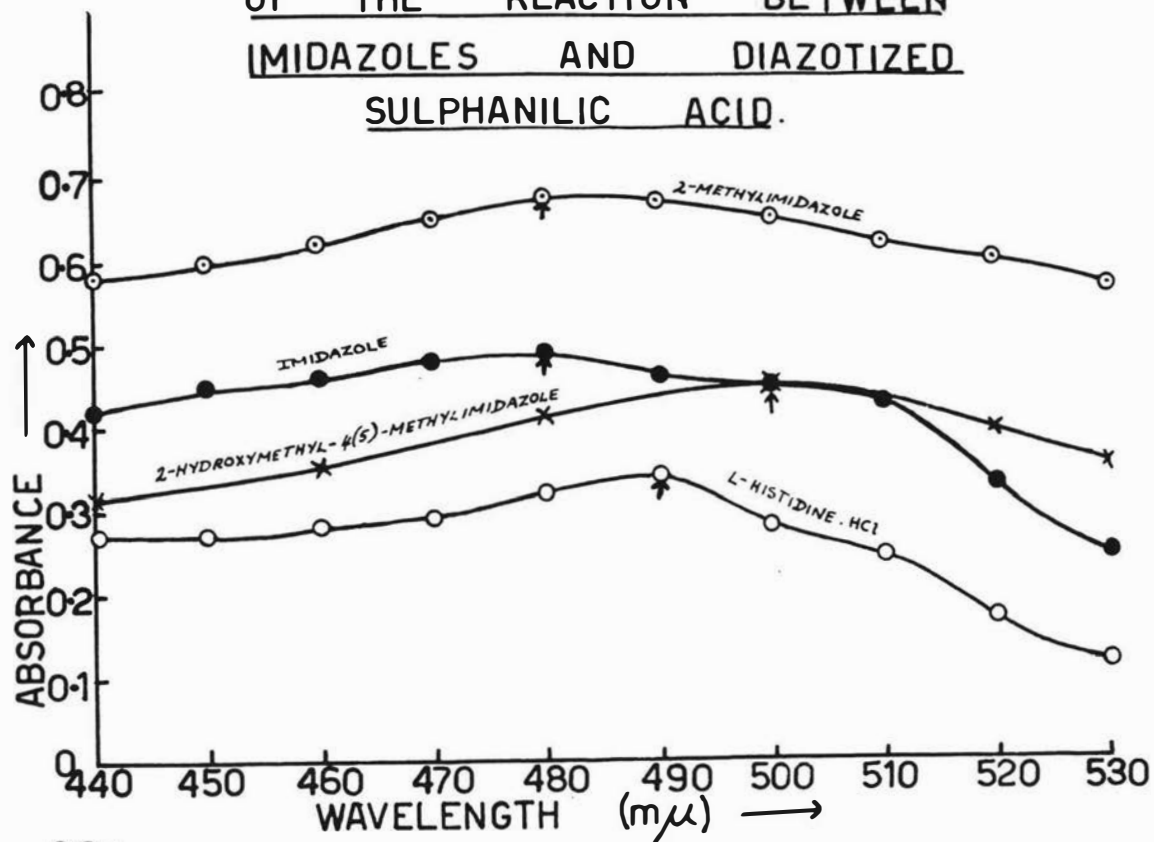
Plots of absorbance against time at 480 m μ showed that in most cases the colour intensity rose to a maximum in 2-8 minutes, remained near that value for 1-10 minutes and then decreased at a rate depending on the particular imida-

TABLE E3

REPLICATE DETERMINATIONS OF ABSORBANCE AT LOW CONCENTRATIONS.

Concentration μg./ml.	Absorbance of Dye With Imidazole, Readings at 480mμ - 24 minutes			Absorbance of Dye with 4(5)-Methylimidazole. Readings at 480mμ - 16 minutes		
	0.5	1.0	5.0	0.5	1.0	5.0
1	.022	.040	.190	.021	.045	.220
2	.018	.046	.198	.018	.042	.215
3	.018	.039	.190	.019	.045	.220
4	.022	.040	.200	.024	.045	.225
5	.020	.043	.193	.019	.041	.209
6	.021	.045	.190	.022	.044	.220
Average Absorbance	.020	.042	.194	.021	.044	.218
Standard error	±.002	±.003	±.004	±.002	±.002	±.005

FIG 3 ABSORPTION SPECTRA OF PRODUCTS
OF THE REACTION BETWEEN
IMIDAZOLES AND DIAZOTIZED
SULPHANILIC ACID.



zole and on its concentration. The colour developed by the weaker solutions was more stable and for concentrations much above 50 $\mu\text{g./ml.}$ there were deviations from Beer's Law. From the plots of absorbance against time an optimum time for reading was established for each of the imidazoles studied (See Table E2). The time chosen did not necessarily correspond to that of the maximum colour intensity but was so selected as to allow readings to be taken when the colour was either constant or changing relatively slowly.

Variation of absorbance with wavelength:

The absorption spectrum of the dye formed with each imidazole was plotted between 420 and 520 $\text{m}\mu$ (See Fig. 3) at the optimum time and the maxima were as tabulated in Table E2. As the peaks of the absorption spectra proved to be rather flat it was decided to standardise all absorbance readings at 480 $\text{m}\mu$ to simplify the procedure. Comparative readings of absorbance at the wavelength of the maximum and at 480 $\text{m}\mu$ are also shown in Table E2.

Effect of temperature:

Although most workers have carried out the reaction between diazotised aniline derivatives and imidazoles at 0° , it was found²⁴ that reproducible results could be obtained by carrying out the reaction at room temperature ($15-20^\circ$). This eliminates the use of an ice bath.

Recovery of Imidazoles from Paper Chromatograms:

(i) Paper chromatography of single imidazoles.- 100 $\mu\text{g.}$

quantities of each of six imidazoles, applied as narrow (2-3 mm.) bands to Whatman No. 3MM chromatography paper and developed overnight as descending chromatograms in n-butanol/acetic acid/water (4:1:1), were eluted with water and determined spectrophotometrically. Almost quantitative recovery was achieved in all experiments. (See Table E4).

(ii) Paper Chromatographic Separation of Mixtures of Imidazoles.- Although, as has been mentioned previously, it was not possible to separate adequately 2-methylimidazole and 4(5)-methylimidazole on Whatman No. 3MM paper, mixtures of three and five different imidazoles were readily separated and quantitatively eluted using the standard procedure. Mixtures consisting of L-histidine hydrochloride, histamine dihydrochloride, 4(5)-hydroxymethylimidazole, imidazole and either 2-methylimidazole or 4(5)-methylimidazole applied to Whatman No. 3MM paper were first developed for 36 hours in n-butanol/acetic acid/water(4:1:1). All but histidine and histamine were clearly separated (See Table E1) and were cut out and eluted. As histidine and histamine moved very slowly in this solvent there remained sufficient length of chromatogram after removal of the other compounds to allow further separation of these two in acetone/chloroform/water/28% ammonia(30:5:4:0.2). Recoveries are tabulated in Table E4.

TABLE E4

RECOVERIES OBTAINED FOR 100 μ g. QUANTITIES OF IMIDAZOLES SEPARATED ON AND
ELUTED FROM WHATMAN NO 3MM PAPER

	Imidazole	4(5)-Methyl- imidazole	4(5)-Hydroxy- methylimid- azole	2-Methyl- imidazole	L_Histid- ine HCl	Histamine Di-HCl
μ g. of Imidazole recovered when run separately	99,98,99	96,95,100	98,98,102	98,100,98	98,95,98	102,100,99
μ g. of Imidazolic compound recovered from sep- aration of mixt- ures of imidazoles	1.	101	101		97	
	2.		101	102	102	
	3. 99	99				94
	4. 96	102	98		96	97
	5. 100		100	95	103	101
Average % Recovery	98.6	98.8	99.7	99.0	98.4	98.9

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FIG 4 ABSORPTION SPECTRA OF PRODUCTS
OF THE REACTION BETWEEN
IMIDAZOLES (30µgm/ml of iso-butanol)
AND DIAZO-*p*-TOLUIDINE.

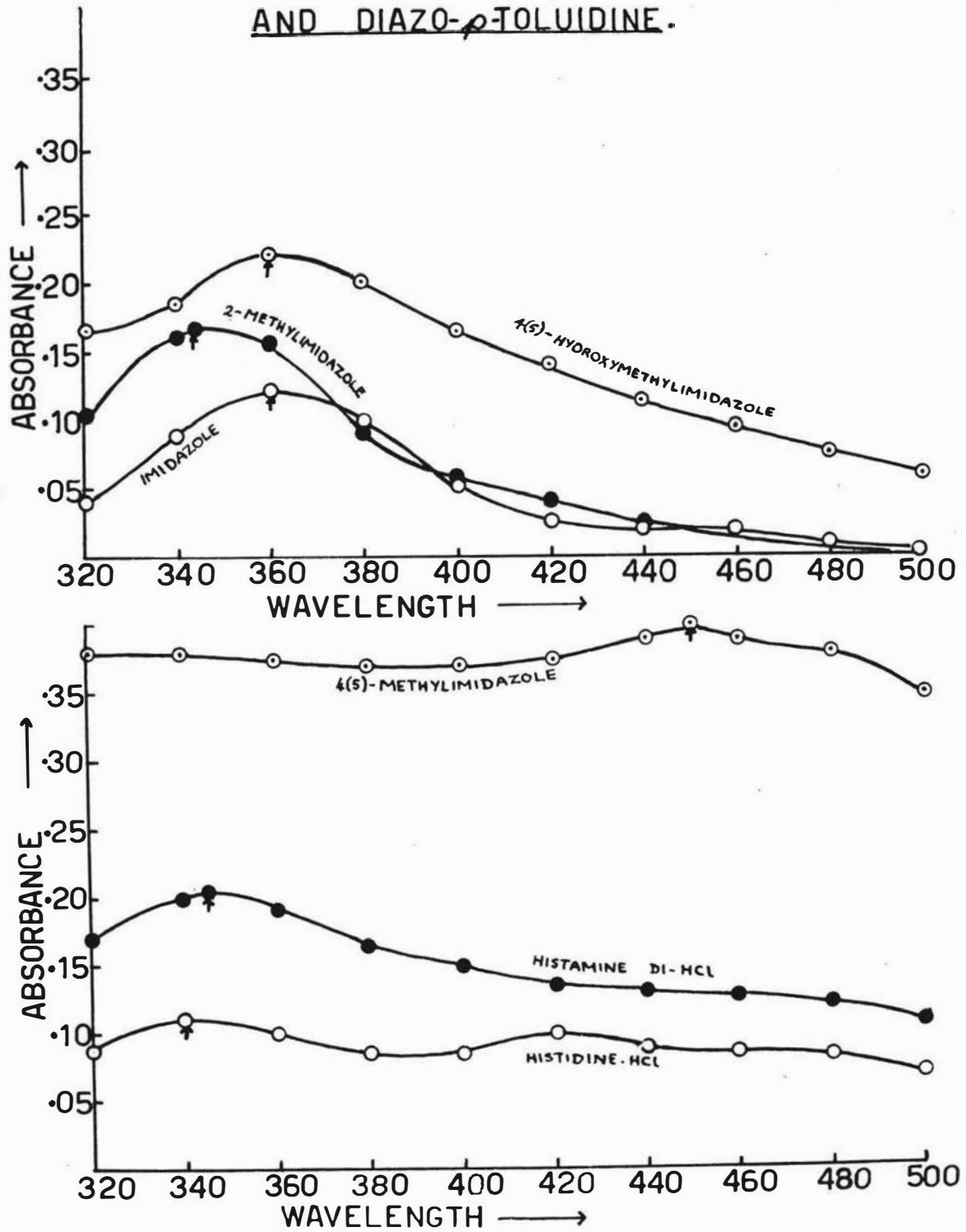
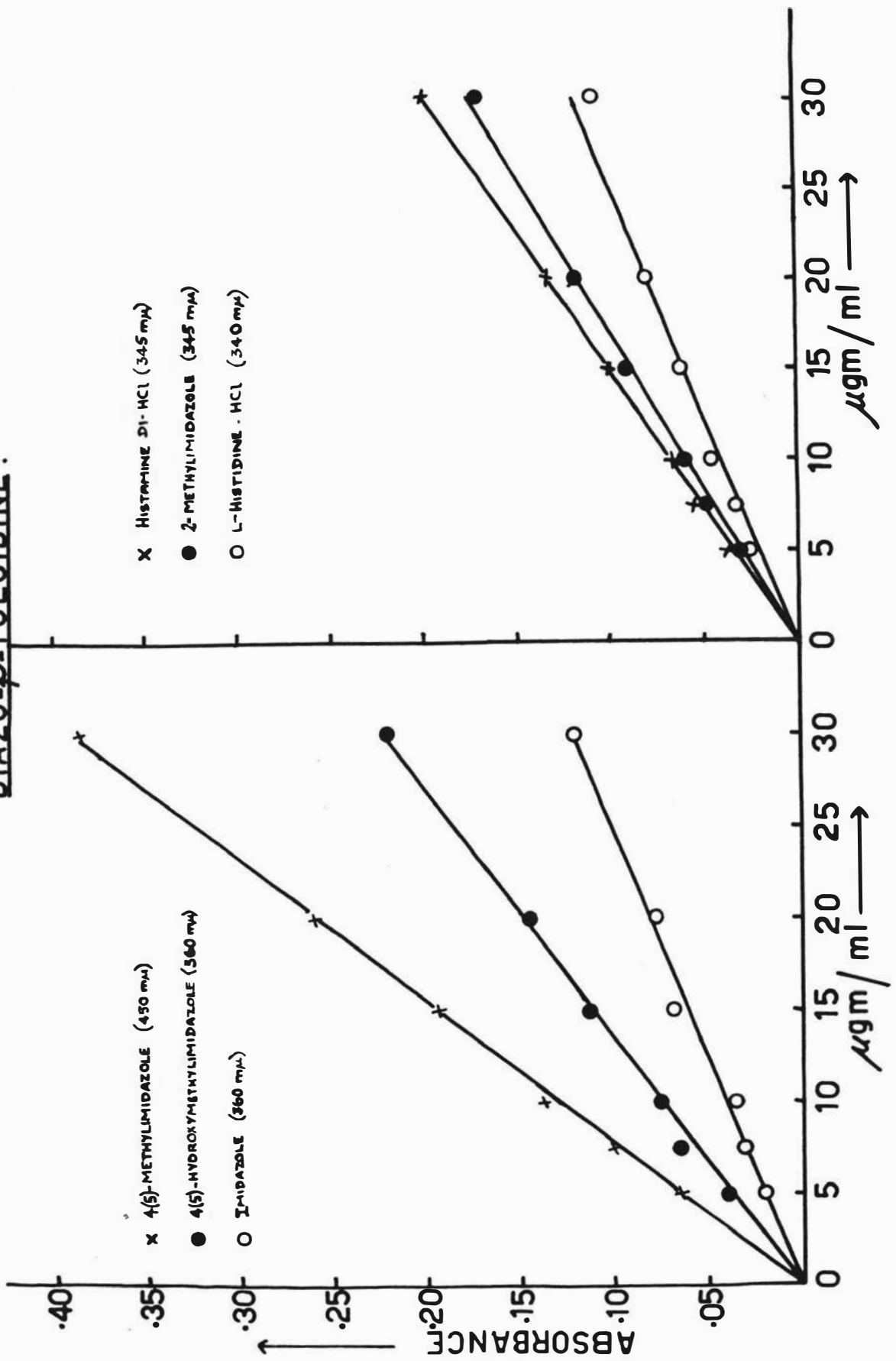


FIG. 5. STANDARD CURVES FOR IMIDAZOLES WITH DIAZO-*p*-TOLUIDINE.



(3) Use of Diazotised p-Toluidine in Imidazole Estimation.

Diazo p-toluidine reagent was made up as for diazotised sulphanilic acid. Sodium carbonate solution (10%, 2 ml.) was added to 0.8 ml. of this reagent in a stoppered test tube and mixed (30 sec.). The imidazole solution (0.4 ml.) was added to the tube, the mixture shaken and then allowed to stand (10 min.) for the colour to develop. Iso-butyl alcohol (5 ml. portions) was added and the colour extracted into the organic layer by vigorous shaking. Complete separation of the layers was achieved by centrifugation at 25,000 r.p.m. (5 min.) and the colour of the iso-butyl alcohol layer was measured against a reagent blank. As this blank developed quite an intense yellow colour on standing it was found advisable to take readings within a short time of centrifuging.

The wavelength of the maximum was determined for each imidazole (See Fig. 4) and standard curves were plotted. (See Fig. 5). As the wavelengths of the maxima in iso-butyl alcohol were in some instances approaching the ultra-violet it was necessary to use silica cells for all measurements.

(4) Use of Diazotised p-Bromoaniline in Imidazole Estimation.

(a) Preparation of Standard Curves.- Determination of absorption maxima and standard curves was carried out as for p-toluidine (See Fig. 6).

FIG. 6 STANDARD CURVES FOR IMIDAZOLES WITH DIAZO-*p*-BROMOANILINE.

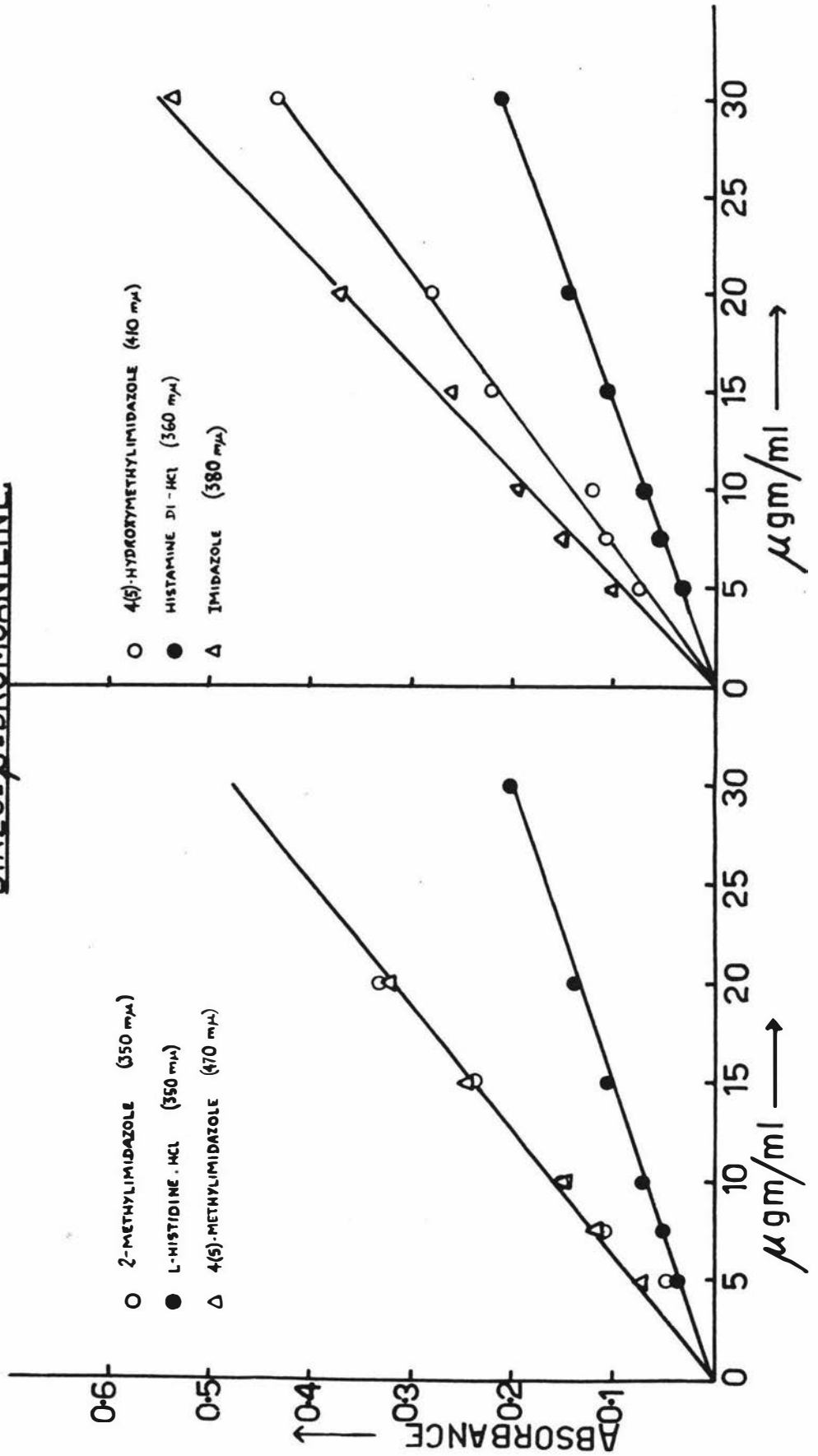
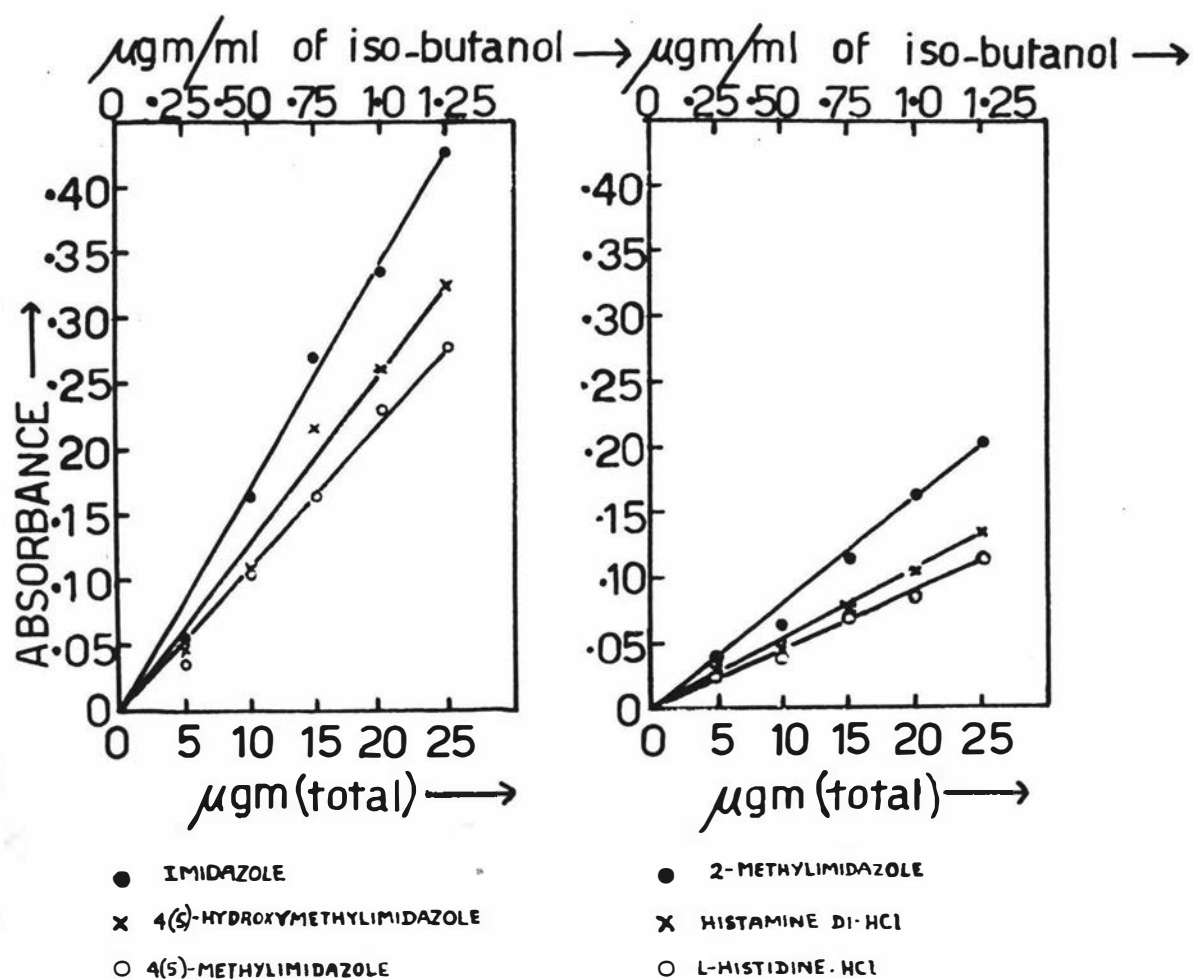


FIG. 7 STANDARD CURVES FOR IMIDAZOLES
SPRAYED WITH DIAZO-*p*-BROMO-ANILINE
IN ALKALI AND ELUTED FROM
WHATMAN N° 3MM PAPER WITH
ISO-BUTANOL.



(b) Adaptation to the Estimation of Imidazoles on Paper Chromatograms.- Varying amounts of imidazoles (5-25 μ g.) were spotted on Whatman No. 3MM paper and sprayed with the diazo-p-bromoaniline reagent. The colours were allowed to develop (10 min.), and the areas of paper cut out (along with a blank), macerated with water (5 ml.) in a stoppered test tube, shaken vigorously with iso-butyl alcohol (5 ml.), transferred to a centrifuge (a little celite was added to carry down the paper) and spun (25,000 r.p.m.; 5 min.). The liquid was decanted and the paper extracted again (three times) with 5 ml. portions of iso-butyl alcohol until all of the colour had been extracted. The combined extracts were again centrifuged and the absorbance of the iso-butanol layer measured (See Fig. 7).

III. THE REACTION OF α -DICARBONYL AND α -HYDROXYCARBONYL
COMPOUNDS WITH AQUEOUS AMMONIA.

(i) The Reaction of Formaldehyde with Aqueous Ammonia.

A solution of 40% formaldehyde (100 ml.) was treated, with cooling, with 25% ammonia solution (150 ml.) and stored in a stoppered flask for six weeks at room temperature. A similar mixture containing, in addition to the above components, glycolaldehyde (0.1 mg.) was stored under identical conditions. Although the mixtures became slightly yellow it was not possible to detect imidazolic components by paper chromatography. Aliquots from each mixture heated for 12 hours at 100° failed to produce imidazoles.

(ii) The Reaction of Glyoxal with Ammonia.

A 30% solution of glyoxal in water (400 ml.) was added slowly with cooling to concentrated ammonia solution (600 ml.). The mixture rapidly darkened with the separation of some solid material. Paper chromatography on Whatman No.1 paper in n-butanol/acetic acid/water (4:1:1) and spraying with diazotised sulphanic acid indicated imidazolic compounds in high concentration at R_{Im} values 1.00 (orange) and 1.64 (orange). Further imidazoles were present in low concentration at R_{Im} values 0.00(brown), 0.40 (red), 0.53 (orange-brown), 0.82 (yellow-orange), 1.21 (purple) and 1.31 (orange-red). After standing six weeks at room temperature, the mixture was filtered and a grey-black crystalline material (11.25 g.) collected. Nitrogen was bubbled through the filtrate to remove the ammonia, and then the solution was evaporated in vacuo to 500 ml. The black solution was extracted continuously with ether for two weeks, the ether extract was dried over anhydrous sodium sulphate, filtered, and then evaporated to dryness giving 32.05 g. of product. Paper and thin layer chromatography on alumina followed by spraying with the diazo reagent indicated that those compounds of R_f values 0.30 and greater had been extracted. Examination of an alumina thin-layer plate (developed in chloroform/pyridine (3:1)) under ultra-violet light indicated a number of fluorescent spots at R_f values: 0.00,

0.07, 0.14, 0.29, 0.38, 0.55 and 0.70.

Examination of solid material ("Glycosine"):- Sublimation of a portion at 250°/20 mm. gave white crystals m.p. (sublimes) 300-20°. (Found: C, 54.12; H, 5.97; N, 41.85%. There was a residue). Paper chromatography on Whatman No. 1 paper in n-butanol/acetic acid/water (4:1:1) gave an orange spot with the diazo spray at R_{Im} 1.65. A sample (c.a. 5 g.) of the solid was boiled with 95% ethanol and charcoal for 30 minutes, then filtered and allowed to cool, when cream-white needles separated, sublimation point circa 320°. The compound was recrystallised from 95% ethanol. (Found: C, 53.73; H, 4.85; N, 41.32. Calc. for $C_6H_6N_4$: C, 53.72; H, 4.51; N, 41.77%). The compound gave a green colour with bromine water and an orange colour with diazotised sulphanilic acid, thus resembling the "glycosine" isolated by Lehmstedt²¹. The infrared spectrum closely resembled that of imidazole while a proton magnetic resonance spectrum obtained in glacial acetic acid gave a single peak at 45¹c./sec. This confirmed the formulation of glycosine as 2,2'-bis-imidazole with four equivalent C-H protons. A picrate separated from glacial acetic acid m.p. 290°. (Found: N, 23.50, Calc. for $C_{18}H_{12}N_{10}O_{14}$: N, 23.66%). Lehmstedt²¹ gives picrate m.p. > 270°.

Examination of ether extract (imidazole):- A portion (5 g.) of the brown gum was distilled at 2.5 mm. pressure. A

crystalline material 1.2 g. was collected m.p. 65-75°. Paper and thin-layer chromatography showed that the solid consisted mainly of a compound giving an orange colour with the imidazole spray and moving to R_{Im} 1.00 in a number of solvents. A sample recrystallized from absolute ethanol/ether had m.p. 85°. A mixed melting point determination with an authentic sample of imidazole had m.p. 83°. Hofmann¹⁰ gives imidazole m.p. 90°. The picrate separated from ethereal solution as pale-yellow needles m.p. 205-6°. Recrystallization raised the melting point to 210°. Parrod²⁹ gives imidazole picrate m.p. 208-12°. (Found: C, 36.8; H, 2.7; N, 23.2. Calc. for $C_9H_7N_5O_7$: C, 36.4; H, 2.4; N, 23.6%).

Attempts to separate further imidazolic compounds using adsorption and partition chromatography proved unsuccessful owing to the high concentration of imidazole in both the ether extract and aqueous residue.

Tentative identification of 2-Formylimidazole:- Paper and thin-layer chromatography in a number of solvents showed that the compound from the glyoxal-ammonia with R_{Im} 0.53 (Whatman No. 3MM paper; n-butanol/acetic acid/water (4:1:1)) was chromatographically identical to (and gave the same colour reaction as) the oxidation product of 2-hydroxymethylimidazole (see page 161).

Further minor fractions from the mixture were not investigated.

(iii) The Reaction of Glycolaldehyde with Ammonia.

Concentrated ammonia solution (15 ml.) was added to a solution of glycolaldehyde (1.8 g.) in water (5 ml.) and the mixture was stored at room temperature in a stoppered flask. The solution slowly assumed a pale yellow colour. After three days paper chromatography on Whatman No.1 paper in n-butanol/acetic acid/water(4:1:1) showed the presence of imidazoles at R_{Im} values 1.00 and 1.12, both giving an orange colour with diazotized sulphanilic acid. After eight weeks a paper-chromatographic study showed imidazoles at R_{Im} values: 0.00-0.40 (pink streak), 1.00 (orange) and 1.12 (orange). The solution was then evaporated in vacuo to give a brown gum (1.85 g.) which was dissolved in water and aluminium oxide (5 g.) added. The mixture was evaporated to dryness in vacuo and the resultant mixture of alumina with the brown material adsorbed on to it was placed on top of an alumina column. The column was developed in turn with chloroform (250 ml.), chloroform/methanol(1:1; 250 ml.), chloroform/methanol/ammonia (1:3:0.01; 250 ml.), methanol/ammonia (1:0.01; 250 ml.), and methanol/water (1:1; 250 ml.). Fractions (10 ml.) were collected and examined individually on paper chromatograms using the diazo reagent as a spray to indicate the presence of imidazoles.

Fraction 1. - Imidazole: A number of tubes of the chloro-

form/methanol elution contained a compound travelling to the same position as imidazole on paper and thin-layer chromatograms. Evaporation in vacuo gave a syrup (0.25 g.) which had an infrared spectrum (film) identical to that of imidazole. (See Fig. 2. Facing page 64.) The syrup was taken up in ether (10 ml.) and a concentrated solution of picric acid in ether was added. Yellow crystals (0.3 g.) separated immediately m.p. 204°. Recrystallization from ethanol/ether raised the melting point to 210°. (Found: C, 36.7; H, 2.6; N, 23.5. Calc. for $C_9H_7N_5O_7$: C, 36.4; H, 2.4; N, 23.6%). Parrod²⁹ reports imidazole picrate m.p. 208-12°.

Fraction 2 - 2-Hydroxymethylimidazole: Passage of chloroform/methanol/ammonia (1:3:0.01) and methanol/ammonia (1:0.01) through the alumina column gave a series of fractions containing the imidazolic compound with R_{Im} value 1.12. These fractions were evaporated to a syrup (0.1 g.) the infrared spectrum (film) of which had features in common with that of 2-hydroxymethylimidazole (synthesised by the method described on page 153). The isolated compound was chromatographically identical to 2-hydroxymethylimidazole on Whatman No. 1 paper in n-butanol/acetic acid/water (4:1:1). A portion (0.04 g.) of the syrup was dissolved in water (10 ml.), boiled with charcoal and filtered through filter-aid. To the filtrate was added a saturated aqueous

solution of picric acid after which the solution was concentrated and cooled. A small amount (0.01 g.) of imidazole picrate separated m.p. 206° and was removed by filtration. To the filtrate was added picric acid (0.025 g.) and the solution boiled and then chilled. Yellow crystals (0.02 g.) separated m.p. 149°. (A mixed melting point determination with 2-hydroxymethylimidazole picrate gave m.p. 148°). Recrystallization from water raised the melting point to 151°. (Found: C, 37.1; H, 2.3; N, 21.6. Calc. for $C_{10}H_9N_5O_8$ C, 36.7; H, 2.8; N, 21.4%). Jones¹⁶ reports 2-hydroxymethylimidazole picrate m.p. 151-2°.

The remainder of the syrup (0.05 g.) was treated in glacial acetic acid (2ml.) with red phosphorus (0.05 g.) and hydriodic acid (0.05 g.). The mixture was heated at 180° for five hours in a sealed tube. After cooling, the product was filtered, treated with concentrated aqueous sodium hydroxide until alkaline, and then evaporated to dryness. The residue was extracted with hot absolute ethanol giving a solution which contained a number of imidazolic products. Paper chromatography showed that the major of these products gave a lemon-yellow colour with the diazo reagent and had the same R_{Im} value as 2-methylimidazole.

(iv) The Reaction of DL-Glyceraldehyde with Aqueous Ammonia.

(a) Initial Qualitative Examination.

A mixture of DL-glyceraldehyde (5 g.) in water (100 ml.) and 25% ammonia solution (25 ml.) was stored in a sterilised, stoppered flask at 37°. The solution gradually darkened until after three weeks it was almost black. The absorbance of the mixture was measured at intervals during the reaction period and the results are shown in Fig. 4, page 69. Chromatography of the mixture on Whatman No. 1 chromatography paper in n-butanol/acetic acid/water (4:1:1) gave the following results with a series of reagents:-

TABLE E5

R_f values and colours with various reagents.

1. <u>AgNO₃</u> ⁴⁰ :	0.18(brown); 0.41(brown);	0.24(brown); 0.51(brown);	0.34(brown); 0.62(brown).
2. <u>p-Anisidine</u> ¹² <u>HCl</u> :	0.15(brown);	0.19(brown);	0.24(pink).
3. <u>U.V.</u> :	0.34;	0.43;	0.62.
4. <u>Diazotised</u> ³⁹ <u>Sulphanilic</u> <u>Acid</u> :	0.00(orange); 0.40(orange); 0.72(yellow).	0.25(red); 0.43(red);	0.34(red); 0.58(red);
5. <u>Ninhydrin</u> :	0-0.1(yellow); 0.32(yellow); 0.59(green).	0.15(violet); 0.33(violet);	0.26(violet); 0.40(yellow);
6. <u>Urea</u> ¹² <u>Phosphate</u> :	0.15(blue).		
7. <u>Anisidine</u> ³⁸ <u>Reagent</u> :	0-0.5(pink); 0.44(red);	0.26(red); 0.60(orange-red).	0.33(orange);
8. <u>I₂/CCl₄</u> ³⁸ :	0.00(brown); 0.40(brown);	0.25(yellow); 0.44(brown);	0.34(brown); 0.58(brown).

(b) Large-scale Experiment.

DL-Glyceraldehyde (45 g.) in distilled water (900 ml.) and 25% ammonia solution (225 ml.) was left in a sterilised, stoppered vessel at 37°. After eight weeks the ammonia was removed in vacuo and the residue dissolved in 250 ml. of water, passed through a column of Amberlite IR-120 ion-exchange resin in the hydrogen form. The material on the column was washed with water and the eluates giving a positive Molisch reaction were combined and evaporated in vacuo to a syrup (6.9 g.).

(i) Qualitative Investigation of Fractions Giving a Positive Molisch Reaction:-

Paper chromatography showed the presence of a triose (yellow-brown), pentoses (pink), and hexoses (brown) when the papers were sprayed with p-anisidine hydrochloride.¹²

Spraying with urea phosphate¹² indicated the presence of a ketotriose and a ketohexose. The syrup was fractionated by partition chromatography on a cellulose column using n-butanol half-saturated with water as the mobile phase. Portions of effluent (5 ml.) were collected, examined on paper chromatograms, and combined into five main fractions. The carbohydrates were identified by chromatography in a number of solvents. (See Table 1, page 71).

(ii) Identification of Basic Compounds:

The basic compounds were eluted from the ion-exchange column with 4N HCl and the eluate evaporated repeatedly

from water in vacuo until all traces of HCl had been removed. In all later experiments it was found more convenient to displace the basic compounds from the ion-exchange resin with 4N ammonia solution. The resulting dark coloured syrup (43.5 g.) was found to contain Pauly-positive³² compounds with R_{Im} values: 2.22, 1.37, 1.35, 1.30, 0.99, 0.91, 0.82, 0.55, 0.41, 0.21, 0.07, 0.00 in n-butanol/acetic acid/water (4:1:1) on Whatman No. 3MM paper. The individual imidazolic compounds were separated from a portion of the syrup (10 g.) on cellulose columns with n-butanol half-saturated with water and containing 0.25% acetic acid (4 ml./l.). On the basis of a chromatographic survey the eluates were combined into a number of fractions which were evaporated in vacuo.

(1) Unknown compound at R_{Im} 2.22: A small quantity (5 mg.) of a syrup giving a yellow colour with diazotised sulphanilic acid was dissolved in water and treated with saturated aqueous picric acid. Yellow needles were obtained (5 mg.) m.p. 200°. Recrystallisation from water gave 2 mg. of a compound m.p. 203°.

(2) 2-Hydroxymethyl-4(5)-methylimidazole: A fraction (0.2 g.) which gave a red colour with Pauly reagent at R_{Im} 1.37 was taken up in absolute ethanol, treated with charcoal, filtered, and evaporated to 20 ml. To 5 ml. of this solution a saturated ethanolic solution of picric acid was added, the

mixture concentrated and allowed to stand when yellow crystals (40 mg.) separated; m.p. 78-80°. On recrystallisation from ethanol/ether the melting point was raised to 83°. (Found: N, 19.4. Calc. for $C_{11}H_{11}N_5O_8 \cdot 1\frac{1}{2}H_2O$: N, 19.0%). After drying the picrate in vacuo at 60° for 2 days the melting point was raised to 142° with loss of water of crystallisation. Komoto¹⁹ reports 2-hydroxymethyl-4(5)-methylimidazole picrate m.p. 143-5°.

For comparison 2-hydroxymethyl-4(5)-methylimidazole picrate and 4(5)-hydroxymethyl-2-methylimidazole picrate were prepared using the methods described on page 157.

A mixed melting point determination with 2-hydroxymethyl-4(5)-methylimidazole picrate showed no depression with the product from the glyceraldehyde/ammonia mixture. The paraffin mull infrared spectra of the picrate of the isolated compound and the two synthetic isomeric hydroxymethyl-methylimidazole picrates are shown in Fig. 5, page 73. It may be seen that the isolated compound is identical to 2-hydroxymethyl-4(5)-methylimidazole. Chromatographic evidence obtained in several solvent systems confirms this conclusion.

Reduction of the isolated 2-hydroxymethyl-4(5)-methylimidazole (0.03 g.) with red phosphorus (0.03 g.) and hydriodic acid (0.03 g.) in glacial acetic acid (2 ml.) in a sealed tube at 160° for 3 hours gave a compound chromatographically

identical to 2,4(5)-dimethylimidazole. The latter compound was prepared for comparison by the reaction of α,β -bis-(benzoylamino)- α -propene with acetic anhydride in a sealed tube at 130° for 3 hours. See page 151.

(3) Unknown Compound at R_{Im} 1.35: A fraction obtained as a syrup (0.2 g.) was taken up in absolute ethanol (10 ml.), boiled (charcoal), filtered and evaporated to dryness. Paper chromatography showed that the fraction contained two compounds, one of which appeared to be 2-hydroxymethyl-4(5)-methylimidazole. A 5% aqueous solution (1 ml.) formed a picrate m.p. 65°, which on two recrystallisations from ethanol/ether was raised to 84°. (Found: C, 36.9; H, 4.8; N, 19.6%). This corresponds to a free base of formula $C_6H_{16}N_2O$ but the picrate could have been an impure sample of that of Fraction (2); ($C_{11}H_{11}N_5O_8 \cdot 1\frac{1}{2}H_2O$ requires: C, 35.9; H, 3.8; N, 19.0%). The remainder (0.15 g.) of the fraction was separated in n-butanol/acetic acid/water (4:1:1) on sheets of Whatman No. 3MM paper giving 0.06 g. of a syrup which gave a red colour with diazotised sulphanilic acid and had the following R_{Im} values on Whatman No. 1 paper:-
n-butanol/acetic acid/water (4:1:1); $R_{Im} = 1.54$
ethyl acetate/acetic acid/n-butanol/water (1:1:1:1); $R_{Im} = 1.06$
ethanol/diethyl ether/water/28% ammonia (4:5:1:0.1); $R_{Im} = 1.03$
The compound formed a chloroplatinate m.p. >220° (decomp.).
Periodate oxidation showed that 0.42 moles of formic acid

were produced.

An n.m.r. spectrum run in deuterium oxide allowed the following assignments to be made: s 1H 435 c./sec. 5(4)-H; s 2H 275 c./sec. CH₂OH attached to an aromatic ring. A number of other small peaks were unable to be assigned. The sample appeared to be impure.

(4) 4(5)-Methylimidazole: A further fraction (1.3 g.) giving an intense red colour with diazotised sulphanilic acid at R_{Im} 1.30 in n-butanol/acetic acid/water (4:1:1) on Whatman No. 3MM paper was dissolved in water, treated with charcoal, filtered and concentrated. This compound chromatographically resembled 4(5)-methylimidazole. A portion of the filtrate was converted to a picrate (Found: C, 38.7; H, 3.4; N, 22.2. Calc. for $C_{10}H_9N_5O_7$: C, 38.6; H, 2.9; N, 22.5%) m.p. 163°, undepressed on admixture with an authentic sample of 4(5)-methylimidazole picrate prepared by the method of Koessler and Hanke.¹⁸ (See page 150). Further derivatives prepared from this fraction were: oxalate, m.p. 206°, chloroaurate, m.p. 200°, nitrate, m.p. 109° and chloroplatinate, m.p. 215-20°. Analysis of the last named derivative gave: (Found: Cl, 37.0; Pt, 33.2. $(C_4H_6N_2)_2H_2PtCl_6$ requires: Cl, 37.1; Pt, 34.0%).

To 0.2 g. of the original syrup in 0.4 g. of 10% NaOH at 0° was added 0.04 g. of benzoyl chloride and the mixture allowed to stand at 0° for 3 hours. The resulting

solution was extracted with benzene, the benzene evaporated, the residue taken up in boiling absolute ethanol and allowed to crystallise at 0°, yielding white needles (5 mg.)

(Found: N, 9.7. Calc. for $C_{17}H_{16}N_2O_2$: N, 10.0%) m.p. 140° undepressed in a mixed melting point determination with α,β -bis(benzoylamino)- α -propene.

(5) 4(5)-(2-Hydroxyethyl)imidazole: A small quantity (10 mg.) of an imidazole running to R_{Im} 0.99 in n-butanol/acetic acid/water (4:1:1) on Whatman No. 3MM paper was purified by chromatography on sheets of Whatman No. 3MM paper in the above solvent. The areas of paper containing the compound were located by spraying marker strips with the diazo reagent, cut out and then the imidazolic compound was eluted with water. A saturated aqueous solution of picric acid was added to the combined eluates and the solution boiled, cooled, the excess picric acid removed by ether-extraction, and the residue evaporated almost to dryness. After two days at -10° yellow crystals (3 mg.) separated m.p. 130°. Recrystallisation from acetone (dry) after treatment with charcoal gave a picrate (1 mg.) m.p. 143°. A mixed melting point determination with a sample of 4(5)-(2-hydroxyethyl)imidazole picrate prepared from histamine by the method of Erlenmeyer, Waldi and Sorkin⁶ gave m.p. 142°. The isolated imidazole travelled to the same position as 4(5)-(2-hydroxyethyl)imidazole with paper chromatography in n-butanol/acetic acid/water

(4:1:1) and acetone/chloroform/water/28% ammonia (30:5:4:0.2).

(6) 4(5)-Hydroxymethylimidazole: A fraction containing an imidazole with an R_{Im} value of 0.91 in n-butanol/acetic acid/water (4:1:1) on Whatman No. 3MM paper, and giving an orange-red dye with diazotised sulphanilic acid spray, was evaporated in vacuo and the residue (0.1 g.) taken up in 10 ml. of water. To 3 ml. of this solution was added a saturated aqueous solution of picric acid. Yellow needles (40 mg.) separated immediately and on recrystallisation from hot water had m.p. 206°. (Found: C, 36.5; H, 3.1; N, 21.0. Calc. for $C_{10}H_9N_5O_8$: C, 36.7; H, 2.8; N, 21.4%), A mixed melting point with a specimen of 4(5)-hydroxymethylimidazole prepared by the method of Totter and Darby⁴¹ (see page 156) was undepressed.

An n.m.r. spectrum was obtained for the free base in D_2O . See Fig. 6 page 80. The following assignments were made: -s 1H 424 c./sec. 5(4)-H; s 1H 472 c./sec. 2-H; s 2H 273 c./sec. 4(5)-CH₂OH. As the compound was not soluble in $CDCl_3$ it was not possible to obtain proof of NH or CH₂OH.

A small quantity (50 mg.) of the isolated syrup was reduced with red phosphorus (0.05 g.) and hydriodic acid (0.05 g.) in glacial acetic acid (2.5 ml.) in a sealed tube at 160° for 3 hours. Paper chromatography of the product showed the presence of 4(5)-methylimidazole.

A further sample (0.3 g.) of 4(5)-hydroxymethylimidazole isolated from a glyceraldehyde/ammonia mixture was dissolved in 10% sodium hydroxide solution (6 ml.) and benzoyl chloride (0.5 g.) was added. The mixture was kept at 0° during 3 hours. The product was extracted with benzene (3 x 10 ml.), washed with dilute sodium bicarbonate and then with water. The benzene extract was evaporated to dryness, the residue taken up in hot ethanol and allowed to crystallise giving silky white crystals of 1-hydroxymethyl-1,2-dibenzoylamino-ethylene m.p. 168-9°.

(7) Unknown Compound at R_{Im} 0.55: This fraction (0.1 g.) was obtained as a dark-brown syrup which gave a red colour with diazotised sulphanilic acid. Treatment of an aqueous solution with saturated aqueous picric acid gave chunky red crystals m.p. 284-5°. The melting point was not raised on recrystallisation from water. Analysis of the picrate and the free base gave apparently contradictory results.

Picrate: (Found: C, 29.4; H, 2.85; N, 21.8%).

Free base: (Found: C, 45.0; H, 8.5; N, 9.8%).

An n.m.r. spectrum in D₂O showed the absence of aromatic protons. It did not prove possible to assign the signals to any specific groups although the integral showed that there were about 8 non-exchangeable protons present. Three of these (87 c./sec.) were possibly a methyl group in an environment Ar-C-CH₃.

(8) Further Fractions: A number of minor fractions were not further investigated. The structure of the compound appearing at R_{Im} 0.21 (in n-butanol/acetic acid/water (4:1:1) on Whatman No. 3MM paper) was not elucidated as purification proved difficult. In its chromatographic behaviour it resembled 4(5)-D-arabotetrahydroxybutylimidazole.³⁰ Because of its low R_{Im} value and relatively compact orange-red spot with Pauly reagent it is unlikely to be a carboxyl-substituted imidazole, which generally give low-intensity yellow spots with tailing. The compound is probably an imidazole with a polyhydroxyalkyl side chain.

(iii) Rate of Formation of Imidazolic Compounds.

Aliquots (0.1 ml.) of the original glyceraldehyde/ammonia mixture were taken regularly during the period of reaction and separated by quantitative paper chromatography on Whatman No. 3MM paper, the component imidazoles eluted with water, and the quantities determined spectrophotometrically using the method described in Section III of the Experimental, p. 168. From the results obtained, the rates of formation were found for the imidazoles which have been identified. (See Fig. 7, page 83).

(iv) Order of Formation of Imidazolic Compounds.

In order to slow down the reaction rate to determine more accurately the order of formation of the individual imidazoles, a mixture of 0.1 g. of DL-glyceraldehyde in 2 ml.

of water and 0.5 ml. of 28% ammonia solution was left at 20° in a stoppered tube. A chromatographic survey at suitable intervals over a period of 3 days showed the order of formation to be: 4(5)-methylimidazole and 4(5)-hydroxymethylimidazole (almost immediately); 2-hydroxymethyl-4(5)-methylimidazole (17 hr.); unknown compound at R_{Im} 0.21 (32 hr.); 4(5)-(2-hydroxyethyl)imidazole (33 hr.), and a number of unidentified imidazoles. The diazotised sulphuric acid spray allowed a lower level of detection for the imidazolic compounds of less than 0.5 μ g.

(v) The Interaction Of Pyruvaldehyde With Ammonia.

(1) Initial Experiments.

Pyruvaldehyde.- Pyruvaldehyde was prepared by the method of Riley,³⁷ A mixture of selenium dioxide (400 g.) and acetone (2 l.) was heated under reflux for 4 hours, the yellow liquid produced was decanted, the residue washed with acetone and the product and washings fractionally distilled to give pyruvaldehyde (10 g.) b_{50} 54-70°. The compound was purified by distillation from anhydrous calcium chloride and yielded a bisphenylhydrazone m.p. 145°. Thin-layer chromatography of the 2,4-dinitrophenylhydrazone confirmed the purity.

Qualitative Survey.- A mixture of pyruvaldehyde (5 g.) in water (20 ml.) and 25% ammonia solution (30 ml.) was stored in a stoppered flask for 5 days at room temperature.

Paper chromatography on Whatman No. 1 paper in n-butanol/ acetic acid/water (4:1:1) and use of the diazotised sulphanic acid spray indicated imidazolic compounds at R_{Im} 1.31 (red), 1.58 (lemon-yellow), 1.87 (red), and 2.27 (orange-yellow). Thin-layer chromatography on aluminium oxide G chromatoplates using a number of solvent systems gave the following results:

TABLE E6

Solvent System	R _{Im} Value and Diazo Colour						
Toluene/ethyl acetate/ammonia (1:3:0.1)	2.55 (orange),	2.00 (red),	1.45 (yellow),	1.09 (red)			
Chloroform/pyridine (2:1)	1.97 "	2.06 "	1.33 "	1.04 "			
Toluene/95% ethanol (1:1)	1.13 "	1.11 "	1.02 "	1.03 "			

(2) Large Scale Experiment.

For this experiment pyruvaldehyde (25% in water) , obtained from L. Light and Co., was purified by the method of Lento et al.²² Thin-layer chromatography of the 2,4-dinitrophenylhydrazone on an alumina plate in ethyl acetate/toluene (1:1) showed a single spot corresponding to the mono-2,4-dinitrophenylhydrazone. Gas chromatography on a 4½ foot column of 20% Pluronic F68 supported on 30-80 mesh ASTM firebrick C22 (73°; A = 32; speed = 1200; Hi Fi Gas Chromatogram) showed that the pyruvaldehyde contained no lower carbonyls in detectable amounts.

Ammonia solution (Analar; 25%, 140 ml.) was added carefully with cooling to a 25% aqueous solution of pyruvaldehyde (95 ml.) and the mixture was stored at 19° in a stoppered flask for 5 weeks. The solution rapidly became dark-brown in colour and within 24 hours chromatography

showed the presence of four imidazolic compounds. At the end of the reaction period the ammonia and water were evaporated in vacuo and the resultant dark-brown oil (30.6 g.) added to a column containing Amberlite IRC-50 resin (H-form; 400 g.) and washed with distilled water (5 l.). On concentration, the eluate and washings gave a neutral fraction (5.3 g.).

Neutral fraction from ion-exchange resin.- Spots were examined on paper chromatograms in n-butanol/acetic acid/water (4:1:1). No aldoses or ketoses could be detected using the p-anisidine hydrochloride spray and the urea phosphate spray. The aniline-xylose spray gave a faint red spot running to the same position as lactic acid in the above solvent and in ethyl acetate/acetic acid/water (14:3:3).

Basic fraction from ion-exchange resin.- The basic material was eluted from the resin with 4N ammonium hydroxide and concentrated to yield a product (20.2 g.) again containing a large proportion of brown material. Thin-layer chromatography showed four compounds giving dyes with Pauly reagent (See Table E6). A quantity (1 g.) of the brown material was separated on a cellulose column using n-butanol half-saturated with water and containing 0.25% glacial acetic acid as mobile phase. Three main fractions were collected (on the basis of a chromatographic survey of eluates) and evaporated to dryness.

Fraction 1. 2-Acetyl-4(5)-methylimidazole: The syrup (0.04 g.) was purified by extraction with chloroform and then by passage in the same solvent through a column containing alumina. Evaporation of the chloroform gave white crystals (0.03 g.) m.p. 109°. (Found: C, 57.9; H, 6.7; N, 22.75. $C_6H_8N_2O$ requires: C, 58.05; H, 6.5; N, 22.6%). A paraffin mull infrared spectrum gave ν_{max} 3160 (aromatic CH), 1678 (aryl C:O), 1397, 1370 (methyl ketone CH) and 1170 cm^{-1} (aryl ketone). See Fig. 8, page 87. An ultraviolet spectrum in absolute ethanol had λ_{max} 293 μ . Addition of a saturated aqueous solution of picric acid to 0.01 g. of the compound dissolved in 1 ml. of water gave yellow needles m.p. 152°, raised to 157° on recrystallisation from water. (Found: N, 20.2. $C_{12}H_{11}N_5O_8$ requires: N, 19.85%). The compound formed a 2,4-dinitrophenylhydrazone m.p. 275° (decomp.), and gave a positive iodoform reaction.

The n.m.r. spectrum was determined giving:- 790 c./sec. s 1H NH ; 424 c./sec. s 1H C4(5)- H ; 160 c./sec. s 3H $COCH_3$; 142 c./sec. s 3H C4(5)- CH_3 . For comparison similar spectra were determined for 4(5)-methylimidazole and 2,4,5-trimethylimidazole (See Fig. 9, p. 88).

Reduction of the isolated 2-acetyl-4(5)-methylimidazole.

A mixture of 2-acetyl-4(5)-methylimidazole (0.04 g.) and potassium hydroxide (0.03 g.) dissolved in hydrazine hydrate (50%; 0.1 ml.) and diethylene glycol (1 ml.) was refluxed

for $1\frac{1}{2}$ hours. The water was then drained from the condenser and the temperature allowed to rise to 180° for 6 hours. The product was extracted with ether and was found to be chromatographically identical to 2-ethyl-4(5)-methylimidazole which was obtained pure by adsorption of a sample obtained from L. Light and Co. on an alumina column, and chromatography in chloroform/pyridine (3:1).

The compound obtained from L. Light and Co. appeared to consist of two imidazolic compounds. One of these gave a lemon-yellow colour with diazotised sulphanilic acid spray reagent at R_{Im} 1.91 on an alumina thin-layer chromatoplate in toluene/ethyl acetate/25% ammonia (1:3:0.1). The picrate (m.p. 131°) obtained for this compound showed it to be 2-ethyl-4(5)-methylimidazole. Lit.⁴⁴ gives m.p. 131° . (Found: C, 42.3; H, 4.3; N, 20.2. Calc. for $C_{12}H_{13}N_5O_7$: C, 42.5; H, 3.9; N, 20.6%).

An attempted Clemmensen reduction of 2-acetyl-4(5)-methylimidazole (13.2 mg.) with zinc amalgam (60 mg.) in water (2.5 ml.), concentrated hydrochloric acid (0.5 ml.) and toluene (1 ml.) heated under reflux for 30 hours, with the addition of 0.5 ml. portions of concentrated hydrochloric acid at six-hourly intervals, gave an unidentified imidazolic product.

An improved and more rapid isolation of 2-acetyl-4(5)-methylimidazole from pyruvaldehyde/ammonia mixture involved

a preliminary chloroform extraction of the basic fraction followed by distillation at 140-80°/25mm. Final purification was again achieved by chromatography in chloroform on alumina.

Fraction 2.- This fraction (0.07 g.) contained two imidazolic compounds, the major of which was chromatographically identical to 2-acetyl-4(5)-methylimidazole. A further separation on alumina resulted in the isolation of a yellow-brown syrup (0.02 g.) which gave a red colour with alkaline diazotised sulphanilic acid at R_{Im} 2.00 on an alumina chromatoplate in toluene/ethyl acetate/ammonia (1:3:0.1). A picrate formed from this substance blackened at 140° and finally melted with decomposition at 180-210°. An infrared spectrum of the syrup had ν_{max} 3100 (broad) (OH or aromatic CH?), 2900 (strong) (C-CH₃?), 2950 (weak), 2850 (weak) (CH₂?), and 1720 cm.⁻¹ (weak) (C=O?). Proton magnetic resonance spectra in deuteriochloroform and D₂O showed one exchangeable proton at 800 c./sec. The CDCl₃ n.m.r. spectrum gave:- 800 c./sec. s 1H NH; 398 c./sec. s 1H C4(5)-H; 252 c./sec. 2H split; 208 c./sec. 1 H split; 132.8 c./sec. s 3H 4(5)-CH₃; 106.5 c./sec. 3H split; 78.7 and 73.8 c./sec. 3H split. Spin decoupling showed that the doublet could be condensed to a single peak but it was not possible to detect any change in the other signals.

Fraction 3. 4(5)-Methylimidazole and 2,4(5)-Dimethylimidazole: The final fraction (0.56 g.) contained the imidazoles with R_{Im} values 1.45 and 1.09 in toluene/ethyl acetate/ammonia (1:3:0.1) on alumina thin-layer chromatoplates. There appeared to be a quantity of brown polymeric material present as well. The imidazoles were distilled at 120-200°/2mm. and then separated on alumina using chloroform/pyridine (4:1) as irrigant.

2,4(5)-Dimethylimidazole.- The faster moving compound (0.091 g.) was chromatographically identical to 2,4(5)-dimethylimidazole giving, with diazotised sulphanilic acid spray, the yellow colour often characteristic of 2-substituted imidazoles.¹³ It crystallised from chloroform as white needles m.p. 88°, (Lit.⁴⁴ m.p. 92°) and readily formed a picrate, m.p. and mixed m.p. 142°. (Found: N, 21.3. Calc. for $C_{11}H_{11}N_5O_7$: N, 21.5%). The infrared spectrum was identical to that of a sample of 2,4(5)-dimethylimidazole picrate prepared by the method of Windaus and Langenbeck.⁴⁴ (See page 151) The infrared spectra are shown in Fig. 10, page 91.

4(5)-Methylimidazole.- The remaining compound (0.077 g.) gave a red colour with Pauly reagent at R_{Im} 1.09 and appeared to be identical to 4(5)-methylimidazole. The syrup obtained from the alumina column failed to crystallise but formed a picrate m.p. 159°, undepressed on admixture with 4(5)-methylimidazole picrate¹⁸ (See page 150). (Found: C, 38.9;

H, 3.0; N, 22.5. Calc. for $C_{10}H_9N_5O_7$: C, 38.6; H, 2.9; N, 22.5%). An infrared spectrum was identical to a similar spectrum determined for 4(5)-methylimidazole picrate.

(See Fig. 11, p.92)

(3) Comparison of Yields of 4(5)-Methylimidazole under Different Reaction Conditions:

The following reaction mixtures were stored at room temperature:

- (a) Pyruvaldehyde (10 ml. of 25% soln.) and 25% ammonia soln. (15 ml.) in a stoppered flask;
- (b) As (a) but with air continuously bubbled through the solution;
- (c) As (a) but with nitrogen continuously bubbled through the solution;
- (d) Pyruvaldehyde (10 ml. of 25% soln.), ammonium acetate (9.25 g.) and glacial acetic acid to make 25 ml.

After a week 0.05 ml. aliquots were taken, the 5(4)-methylimidazole separated by chromatography on Whatman No. 3MM paper using n-butanol/acetic acid/water (4:1:1), and determined spectrophotometrically using the method described in Section III of the Experimental. It was not possible to estimate 2,4(5)-dimethylimidazole by this method because an azo dye of very low colour intensity was formed and even slight contamination with 4(5)-methylimidazole gave erroneous values. The results are shown in Table 2, page 92. Paper

and thin-layer chromatography showed that all of the mixtures (a) to (d) contained the same component imidazoles.

(4) Quantitative Gravimetric Separation of 4(5)-Methylimidazole and 2,4(5)-Dimethylimidazole.

Aliquots (1.196g.; 1.761 g.) of the bases from the pyruvaldehyde/ammonia mixture were adsorbed on a column packed with aluminium oxide (Analar; 150 g.). Irrigation with chloroform removed the fast-moving imidazoles (0.078g.; 0.112 g.). Further passage of chloroform/pyridine (3:1) and collection of 10 ml. fractions allowed separation of 2,4(5)-dimethylimidazole and 4(5)-methylimidazole. Finally, irrigation with pyridine removed the remaining 4(5)-methylimidazole from the adsorbent. The appropriate fractions were combined, evaporated, dried in vacuo and weighed giving 2,4(5)-dimethylimidazole (0.3335 g.: 0.555 g.) and 4(5)-methylimidazole (0.312 g.; 0.458 g.). These results gave molar ratios for 2,4(5)-dimethylimidazole: 4(5)-methylimidazole of 1:1.09 and 1:0.97.

Attempted Identification of Formaldehyde and Acetaldehyde in the Pyruvaldehyde/Ammonia Mixture. A mixture of pyruvaldehyde and ammonia in the same concentration as in previous experiments was treated continuously for a week with a slow stream of nitrogen which was previously passed through 25% ammonia solution. The issuing gases were passed through sintered discs into a series of chilled 2,4-dinitrophenyl-

hydrazine solutions (2 g. of 2,4-dinitrophenylhydrazine per litre of 2N HCl solution). Thin-layer chromatography in toluene/ethyl acetate (1:1) on silica gel G and aluminium oxide G chromatoplates indicated that the 2,4-dinitrophenylhydrazones of acetaldehyde and formaldehyde appeared to be present in low concentration. Separation of the mixture of 2,4-dinitrophenylhydrazones on a celite column in hexane saturated with nitromethane using the method of Day et al.⁵ followed by ultraviolet and visible spectroscopy of the fractions in chloroform solution showed that one fraction had a very small peak at λ_{\max} 345 m μ (corresponds to λ_{\max} 344 m μ for formaldehyde 2,4-dinitrophenylhydrazone¹⁵). A further fraction had λ_{\max} 355 m μ corresponding to λ_{\max} 355 m μ for acetaldehyde 2,4-dinitrophenylhydrazone¹⁵. In alkaline solution there was a maximum at 430 m μ for both fractions. Formaldehyde and acetaldehyde 2,4-dinitrophenylhydrazones have λ_{\max} 430 m μ in alkaline solution.¹⁵

(vi) The Reaction of Hydroxypyruvaldehyde with Aqueous Ammonia.

Synthesis of Hydroxypyruvaldehyde: The trimer alcoholate of hydroxypyruvaldehyde was prepared by a method that was essentially that of Evans et al.⁷ Anhydrous cupric acetate (2.25 moles; 412.9 g.) in the form of a fine powder was stirred into a solution containing one mole (90 g.) of dihydroxyacetone dissolved in 10 moles (180 ml.) of water. After 7 days at room temperature the excess copper was carefully precipitated by the addition of 225 ml. of 10% aqueous oxalic acid. The solution was centrifuged and the supernatant reduced by evaporation at 35° to a small volume to which successive portions of ethanol were added and the whole reduced to dryness at 35°. The residue was repeatedly evaporated from ethanol until no trace of acid remained, and the compound was then precipitated with dry ether from ethanol as a flocculent white mass. The solid was centrifuged and the mother liquors worked up with more ether to give a total yield of hydroxy-pyruvaldehyde trimer alcoholate of 55.4 g. The compound was dried in a vacuum oven at 70° for 10 hours and gave a pale-yellow amorphous solid m.p. (decomp.) 160°. Evans et al.⁷ report m.p. 155-160°. (Found: C, 42.42; H, 5.78. Calc. for $(C_3H_4O_3)_3C_2H_5OH$: C, 42.47; H, 5.85%). The hydroxypyruvaldehyde turned Schiff's reagent pink slowly on standing and yielded an

oxime (hydroxymethylglyoxime) m.p. 130°. (Lit.⁷ reports m.p. 134-5°). With phenylhydrazine it formed phenylglycerosazone m.p. 132°. (Lit.⁷ gives m.p. 132°), and with *o*-phenylenediamine it formed the quinoxaline derivative as yellow crystals m.p. 240°. (Evans et al.⁷ report m.p. 250-1°; Norish and Griffiths²⁷ report m.p. 165°).

Hydroxypyruvaldehyde alcoholate (55.4 g.) was dissolved in distilled water (300 ml.) and warmed to 60° for 30 minutes to depolymerise the compound. To this solution was added, with cooling, 25% ammonia solution (500 ml.).

The resulting mixture rapidly darkened and chromatography indicated the rapid formation of imidazoles.

Paper chromatographic survey: Paper chromatography on Whatman No. 1 paper in n-butanol/acetic acid/water (4:1:1) revealed imidazolic spots at R_{Im} values 0.00 (red), 0.25 (pink), 0.37 (pink), 0.91 (orange-red), 1.20 (orange-weak), 1.30 (red) and 1.38 (red). The compounds at 0.91, 1.30 and 1.38 were present in highest concentrations, the others being present only in trace amounts. From a paper- and thin-layer chromatographic survey the compound at R_{Im} 0.91 was found to resemble 4(5)-hydroxymethylimidazole, that at 1.30 resembled 4(5)-methylimidazole and that at 1.37 was similar in behaviour to 2-hydroxymethyl-4(5)-methylimidazole.

Separation of Components: After standing at room temperature for six weeks the mixture was evaporated in vacuo to yield

a dark-coloured, oily syrup (49.5 g.), which was dissolved in water (500 ml.) and passed through a column containing Amberlite IRC-50 ion-exchange resin (H-form) and then washed with water (2 l.). The basic material was then eluted from the resin with 4N ammonium hydroxide solution (1.7 l.) and the solution, which contained all of the imidazolic components from the original mixture, was evaporated to a syrup (31.4 g.).

Examination of water eluate: On evaporation to dryness, a dark-brown residue (15.5 g.) remained. This proved to contain no aldose, ketose or imidazole components and was not further investigated.

Examination of ammonia eluate: The basic fraction was extracted with hot, dry acetone (10 x 100 ml.) and on evaporation yielded a syrup (1.4 g.) which appeared to contain only those imidazoles with R_{Im} values 0.91 and greater.

Extraction with hot ethanol was found to be less selective in that all of the imidazolic compounds were extracted.

Separation of acetone extract: The syrup (1.4 g.) was mixed with cellulose powder, placed on top of a cellulose column and partitioned using n-butanol half saturated with water and containing 1% acetic acid. Fractions of 10 ml. were collected, examined on paper chromatograms and the appropriate fractions combined and evaporated.

(a) 4(5)-Methylimidazole

A fraction (0.4 g.), when examined on Whatman No. 1 paper in n-butanol/acetic acid/water (4:1:1) and sprayed with the diazo reagent, showed imidazoles at R_{Im} 1.30 (red), 1.38 (red), and a very weak component giving an orange spot at 2.00. The fraction was re-separated on an alumina column eluting in turn with chloroform, chloroform/pyridine (2:1) and methanol. The chloroform fractions contained the fast-moving imidazole and a trace of the compound R_{Im} 1.30. The chloroform/pyridine fractions were evaporated to a syrup (0.2 g.) travelling to the same position on chromatograms as 4(5)-methylimidazole (R_{Im} 1.30). A picrate was prepared which crystallized from water m.p. 158° raised to 162° on recrystallization, and undepressed on admixture with an authentic sample of 4(5)-methylimidazole picrate (Found: C, 38.7; H, 3.3; N, 22.5. Calc. for $C_{10}H_9N_5O_7$: C, 38.6; H, 2.9; N, 22.5%). The infrared spectrum (nujol) of the picrate was identical to that of 4(5)-methylimidazole picrate.

(b) 2-Hydroxymethyl-4(5)-methylimidazole

A further chromatographically-homogeneous fraction (0.05 g.) from the methanol eluate appeared to contain the imidazole with R_{Im} 1.38. Half of this fraction was taken up in 95% ethanol and an ethanolic solution of picric acid was added. The mixture was evaporated to a small volume and diethyl ether added. After standing for two days,

yellow plates separated (2 mg.) m.p. 83°. Komoto¹⁹ reports 2-hydroxymethyl-4(5)-methylimidazole picrate sesquihydrate m.p. 83°. The remainder of the fraction (0.025 g.) was treated at 160° for three hours in glacial acetic acid (2 ml.) with red phosphorus (0.025 g.) and hydriodic acid (0.025 g.). The resultant solution was continuously evaporated from water in vacuo until no further acid remained. The residue was extracted with absolute ethanol, filtered and spotted on chromatograms. The ethanol extract was found to contain a compound giving a lemon-yellow colour with the diazo spray reagent and moving to the same position on chromatograms and thin-layer chromatoplates as a sample of 2,4(5)-dimethylimidazole isolated from the pyruvaldehyde/ammonia mixture (see page 204).

(c) 4(5)-Hydroxymethylimidazole

A further fraction (0.8 g.) from the cellulose column was separated on an alumina column, eluting in turn with chloroform/methanol (1:1; 250 ml.), methanol (250 ml.) and methanol/water (1:1; 250 ml.). The methanol and methanol/water eluates gave an orange-red colour with the diazo spray and contained the compound with R_{Im} 0.91. The appropriate fractions were evaporated to a syrup (0.6 g.) which was dissolved in water (30 ml.), treated with picric acid, boiled and then allowed to cool. Yellow needles separated (0.25 g.) m.p. 197°, raised to 200° on recrystall-

ization from water (Found: C, 37.1; H, 3.0; N, 21.4. Calc. for $C_{10}H_9N_5O_8$: C, 36.7; H, 2.8; N, 21.4%), undepressed on admixture with 4(5)-hydroxymethylimidazole picrate. The infrared spectra of the picrate and that of 4(5)-hydroxymethylimidazole picrate were similar.

Estimation of Imidazole Concentrations.- Comparison with dihydroxyacetone/ammonia.

Two solutions of (a) dihydroxyacetone (0.9 g.; 0.01 mole) and (b) hydroxypyruvaldehyde trimer alcoholate (1.127 g. contains 0.01 mole of hydroxypyruvaldehyde) were prepared by dissolving each of (a) and (b) in concentrated aqueous ammonia (12.5 ml.) with cooling. The mixtures were allowed to stand in stoppered flasks for four weeks. Thin-layer chromatograms on alumina using toluene/ethanol (1:1), chloroform/pyridine (2:1) and toluene/ethyl acetate/ammonia (1:3:0.1) showed that both mixtures contained a similar mixture of imidazoles, although the hydroxypyruvaldehyde/ammonia mixture darkened at a much faster rate.

After four weeks, duplicate aliquots (0.1 ml.) of each solution were separated on Whatman No. 3MM paper and 4(5)-methyl- and 4(5)-hydroxymethylimidazoles estimated colorimetrically as described on page 170. The results are shown in Table 3 on page 99.

(vii) The Reaction of Diacetyl with Aqueous Ammonia.

A solution of diacetyl (40 g.) in absolute ethanol (200 ml.) was treated with dry ammonia for 2 hours. The ethanol was evaporated in vacuo to give a viscous yellow product (48.3 g.). Paper chromatography followed by spraying with diazotised sulphanic acid and ninhydrin showed no spots. A portion of the syrup (2 g.) was added to a column containing Dowex 50 resin and washed with distilled water (1 l.). Hydrochloric acid (1 l.; 2N) was then passed through the resin and the effluent evaporated to dryness giving a white crystalline product (1.58 g.). Recrystallization from ethanol gave 2,4,5-trimethylimidazole hydrochloride (1.0 g.) m.p. 314°, (decomp.). Lit.¹¹ m.p. 316°. The hydrochloride (1.0 g.) was neutralized with 10% sodium carbonate, extracted with ether, the ether washed, dried and evaporated in vacuo to give 2,4,5-trimethylimidazole as a syrup (0.5 g.). A portion in aqueous solution was treated with saturated aqueous picric acid, heated and cooled when 2,4,5-trimethylimidazole picrate crystallized m.p. 162°. Lit.¹¹ m.p. 163°. (Found: N, 20.53; Calc. for $C_{12}H_{13}N_5O$ N, 20.64%). The remainder of the impure 2,4,5-trimethylimidazole was distilled at 160°/2 mm. to give white crystals m.p. 130°. (Found: C, 65.0; H, 9.2; N, 25.0. Calc. for $C_6H_{10}N_2$: C, 65.4; H, 9.2; N, 25.4%). Lit.³³ gives m.p. 132.5-133°. An n.m.r. spectrum was determined in deuterium oxide. See Fig. 9, page 88. The following assignments were made: 119 c./sec.

s 6H and 134 c./sec. s 3H.

A sample of the original syrup (1 g.) from diacetyl/ ammonia was dissolved in absolute ethanol (10 ml.) and treated at 100° for 4 hours with 20 ml. of 5% potassium permanganate. Paper chromatography followed by use of a 5% ferrous sulphate spray indicated that no pyrazines were present.

(viii) The Reaction of Acetoin with Aqueous Ammonia.

A mixture of acetoin (10 g.) dissolved in water (10 ml.) and 25% ammonia solution (100 ml.) was stored in a stoppered flask. After a week an aliquot (25 ml.) of the mixture was evaporated in vacuo to yield a syrup (3.3 g.). Formation of a picrate as with the diacetyl-ammonia mixture afforded a product m.p. 157°. Recrystallization from water gave 2,4,5-trimethylimidazole picrate m.p. 162°. (Found: N, 21.2; Calc. for $C_{12}H_{13}N_5O_6$: N, 21.6%). The hydrochloride melted at 312°.

(ix) The Reaction of 1,4-Dihydroxy-2-butanone with Ammonia.
Hydration of 2-Butyne-1,4-diol.

The method followed was essentially that of Reppe.³⁶ A solution of mercuric sulphate (5 g.) dissolved in concentrated sulphuric acid (2 ml.) was added to a stirred aqueous solution of 2-butyne-1,4-diol (100 g. in 1 l. H₂O). The temperature of the solution was maintained at 15-20° for 6 hours, and then at 20-30° for the same period. After settling, the sludge (mainly mercury) was removed and the aqueous solution heated at 40° for 60 hours to complete the addition of water to the hydroxymethylvinyl ketone. The resulting solution was carefully neutralized to pH 4.5 with calcium carbonate, treated with charcoal, filtered and the filtrate then concentrated (at 50-60° and 60-70 mm. pressure) to about 200 ml. Any attempt to further concentrate this solution and distil the ketone resulted in a considerable degree of polymerization and isolation of a product which was mainly 2-butyne-1,4-diol. The aqueous solution contained a low concentration of 1,4-dihydroxy-2-butanone as was demonstrated by isolation of a 2,4-dinitrophenylhydrazone m.p. 109°. (Jadot and Mullers¹⁴ give 1,4-dihydroxy-2-butanone 2,4-dinitrophenylhydrazone m.p. 109°.).

Reaction of 1,4-dihydroxy-2-butanone with aqueous ammonia.

The impure solution of 1,4-dihydroxy-2-butanone (200 ml.) was added with cooling to 25% ammonia solution (500 ml.)

and allowed to stand in a stoppered flask. A mixture of 2-butyne-1,4-diol and ammonia in the same comparative concentrations was prepared as a control.

Paper chromatography on Whatman No. 1 paper using n-butanol/acetic acid/water (4:1:1) as solvent, and spraying the air-dried chromatogram with diazotized sulphanilic acid showed that there were no imidazolic compounds in the 2-butyne-1,4-diol/ammonia mixture. The dihydroxybutanone/ammonia mixture showed imidazolic compounds forming red colours with the diazo reagent at R_{Im} values: 0.00 (weak); 0.73 (weak); 0.99 and 2.10.

After four weeks the ammoniacal solution was evaporated in vacuo and the residue dissolved in water. The solution was passed through a column containing Amberlite IRC-50 ion-exchange resin (H-form) which was then washed with distilled water (5 l.). These washings were found to contain no imidazolic material.

The basic compounds were removed from the resin with 4N ammonium hydroxide (2 l.). The eluate was filtered and evaporated in vacuo to a black syrup (20.4 g.). Chromatography showed the presence in this syrup of all of the imidazoles from the original mixture. Ethanol extraction of the syrup and evaporation of the extract gave a further brown syrup (16.2 g.) again containing the same component imidazoles.

A quantity (2g.) of the syrup was separated by cellulose column chromatography using n-butanol half saturated with water and containing 0.25% acetic acid. Fractions (10 ml.) were collected using an automatic fraction collector and examined by paper chromatography. The appropriate fractions were combined and evaporated in vacuo.

Fraction 1. Examination of compound with R_{Im} 2.10:

Fraction 1 was evaporated to a dark-brown syrup (0.4 g.) which was taken up in absolute ethanol, boiled with charcoal, filtered and evaporated to a syrup (0.23 g.). This was dissolved in chloroform/pyridine (1:2) and passed through an alumina column. The eluate gave a yellow-brown syrup (0.1 g.) which was soluble in chloroform and gave a red colour with diazotised sulphanilic acid. Both infrared and n.m.r. spectra indicated a highly impure sample. It was not possible to obtain a crystalline derivative of this compound.

Fraction 2. Examination of compound with R_{Im} 0.99:

A further fraction from the cellulose column (0.35 g.) was purified by alumina column chromatography using chloroform/methanol (1:1) as eluant, and then again by passage through a cellulose column as before. The resultant syrup (0.12 g.) contained an imidazolic compound chromatographically resembling 4(5)-(2-hydroxyethyl)imidazole prepared by the action of nitrous acid on histamine (See page 158). When a sol-

ution of the syrup in ethanol/ether (1:1) was treated with a saturated aqueous solution of picric acid and allowed to evaporate in the air, a small quantity (c.a. 3 mg.) of a picrate m.p. 134-8° was obtained. Recrystallization failed to raise the melting point. Komoto¹⁹ gives 4(5)-(2-hydroxyethyl)imidazole picrate m.p. 144°. The infrared spectrum of the syrup (film) showed few features other than a broad band at 3400 cm.⁻¹

The other minor fractions which appeared to contain imidazoles were not further investigated.

(x) The Reaction of L-Arabinose with Aqueous Ammonia.

A solution of L-arabinose (125 g.) in water (1 l.) was treated with 25% ammonia solution (500 ml.) and stored in a stoppered flask for six weeks. Chromatography on Whatman No. 1 paper in n-butanol/acetic acid/water (4:1:1) showed that the mixture contained a very complex pattern of imidazoles (R_{Im} 0.00-2.70). The dark brown aqueous solution was continuously extracted with chloroform for three weeks. The chloroform extracts were combined, dried with anhydrous sodium sulphate, and then evaporated to a syrup (10.2 g.) which contained Pauly-positive compounds with R_{Im} values 1.19 (pink), 1.26 (red), 1.41 (yellow) and 1.46 (orange). A portion (1.5 g.) of the syrup was fractionated on a cellulose column using n-butanol half-saturated with water and containing 0.25% acetic acid. The effluent from the column was examined by paper chromatography and the appropriate fractions combined. The fraction containing the compound giving a red colour with diazotised sulphanilic acid at R_{Im} 1.26 was evaporated to a syrup (0.2 g.) in vacuo, boiled with charcoal, filtered, treated with concentrated aqueous picric acid, concentrated and cooled giving chunky orange crystals (10 mg.) m.p. 157°. (raised to 159° on recrystallization, undepressed on admixture with 4(5)-methylimidazole picrate).

(xi) Reactions of Carbohydrates and a Number of Their Alkaline Degradation Products with Aqueous Ammonia.

(a) Study of the Imidazoles Formed and their Orders of Formation.- Duplicate samples of glyoxal, glycolaldehyde, dihydroxyacetone, glyceraldehyde, pyruvaldehyde, hydroxy-pyruvaldehyde, D-erythrose, L-arabinose, D-ribose, D-xylose, D-lyxose, D-mannose, D-glucose, D-galactose, D-fructose, L-sorbose, D-tagatose, L-rhamnose (6-desoxy-L-mannose), L-fucose (6-desoxy-L-galactose), 2-desoxy-D-glucose, D-glucosamine hydrochloride, galactosamine hydrochloride, sucrose, maltose, lactose, D-cellobiose, gentiobiose and D-melibiose (1 mole) were treated at room temperature with concentrated ammonium hydroxide (3 moles). Raffinose, D-melezitose, amylose (from potato starch), amylopectin (from potato starch) and starch (soluble) were similarly treated in the ratio of 0.9 g. of carbohydrate to 1.5 ml. of 25% ammonia solution. The reaction mixtures were cooled where necessary, and then stored in tightly-stoppered glass containers. Using a platinum loop (which was cleaned by flaming with a bunsen burner) spots of roughly equal size were applied to sheets of Whatman No. 3MM chromatography paper at one minute intervals for ten minutes, hourly for four hours, and then six-hourly for about 212 hours. After the first hour of reaction the duplicate mixture was employed for all further samples. After the period at room temperature the mixtures were heated

at 75° for six hours, and then at 110° for eighteen hours. Samples were taken hourly for the first twelve hours of this heating period, and then finally at the end of the 24 hours. Marker spots consisting of imidazole, 4(5)-methylimidazole, 4(5)-hydroxymethylimidazole, 2-hydroxymethyl-4(5)-methylimidazole, 4(5)-(2-hydroxyethyl)imidazole and 2,4(5)-dimethylimidazole were run concurrently on the appropriate sheets of chromatography paper. After development overnight in n-butanol/glacial acetic acid/water (4:1:1) the chromatograms were allowed to dry in the air before being sprayed with alkaline diazotised sulphanic acid to locate the imidazolic compounds.

Results are listed in Table 4 on page 112.

(b) A Study of the Imidazole Patterns Obtained from the Aqueous Ammoniacal Solutions of Reducing Disaccharides: Maltose, lactose, melibiose, cellobiose, laminaribiose and sophorose (70 mg. quantities) in stoppered vials were separately treated with ammonia solution (0.6 ml.). The latter two disaccharides were obtained from Dr. R. Bailey, Plant Chemistry Division, D.S.I.R., Palmerston North. Samples of each mixture were chromatographed, as in the previous section, at the end of the 42 hour period. Imidazole, 4(5)-methylimidazole and a glucose/ammonia mixture were run concurrently as markers. Reaction was then continued at 75° for a further six hours, samples being taken hourly. The results obtained from the sprayed chromatograms are shown in Plate I,

IV DEVELOPMENT OF A MICROTECHNIQUE FOR LINKAGE DETERMINATION
IN HEXOSE DISACCHARIDES BY IMIDAZOLE FORMATION.

Small quantities (ca. 1 mg.) of sophorose, laminaribiose (from the partial hydrolysis of laminarin), turanose, lactose, maltose, cellobiose, lactulose, maltulose, melibiose, gentiobiose, isomaltose, isomaltulose, glucosyl-1-1-fructose, trehalose, sucrose, leucrose, melibiitol, β -methylmaltoside, xylobiose, di- and trigalacturonic acids were treated with aqueous ammonia (0.1 ml.) in a sealed container at 110°. Samples were taken hourly and chromatographed as described on page 223, showing that the optimum time of imidazole formation was 3 hours. The R_{Im} values of the imidazoles produced from most of these mixtures are summarised on page 132, Table 5. See also Plates 2 and 3, pages 133 and 133A.

The standardised procedure for linkage-determination was formulated as follows:- To the carbohydrate (0.1-1 mg.) in a glass capillary tube is added 25% ammonium hydroxide (ca. 0.1 ml.), the tube is sealed and heated (3 hours) at 110°. A small spot of the brown reaction product (1-2 μ l) is developed overnight on Whatman No. 3MM paper using n-butanol/glacial acetic acid/water (4:1:1) as solvent. The thoroughly air-dried chromatogram is then sprayed with the diazo reagent to locate the positions of the imidazoles. Ammoniacal solutions of known disaccharides should be run concurrently as markers.

Application of the technique to linkage determination in homogeneously linked oligosaccharides.

A time study was carried out, as in the previous section, on heated ammoniacal solutions of celotriose, the maltodextrin series* (up to degree of polymerization = 8) and the isomaltodextrin series** (up to D.P. = 9) (All of these oligosaccharides were kindly donated by Dr. R.W. Bailey, Plant Chemistry Division, D.S.I.R., Palmerston North). The corresponding relatively straight chain polysaccharides, amylose (maltodextrin series) and dextran (isomaltose series) were similarly tested, but under more prolonged periods of heating (up to 24 hours). The results obtained are listed in Table 6, page 134.

* obtained from the partial hydrolysis of amylose

** obtained from the partial hydrolysis of dextran.

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APPENDIX

SPECTROSCOPY OF IMIDAZOLES.

(a) Ultraviolet Absorption Spectra.-

Although imidazoethiones and imidazoles in which the ring is conjugated with a carbonyl group show selective absorption in the ultraviolet region, simple imidazoles fail to exhibit such selective absorption,^{2,5} except for some absorption attributed to cyclic excitation in the range 200-2 μ .⁷

(b) Infrared Spectra.-

A number of workers^{1,4,6,9,13} have studied the infrared spectra of imidazoles. Their results indicate very marked association through hydrogen bonding. Garfinkel and Edsall⁴ have made a detailed study of the infrared spectra of imidazole, the imidazolium ion, 4(5)-methylimidazole and histidine, but were unable to assign all of the peaks.

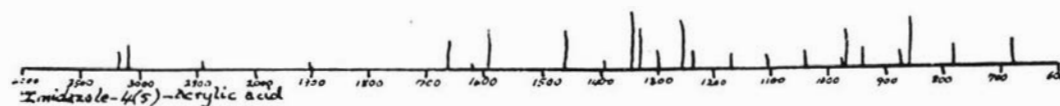
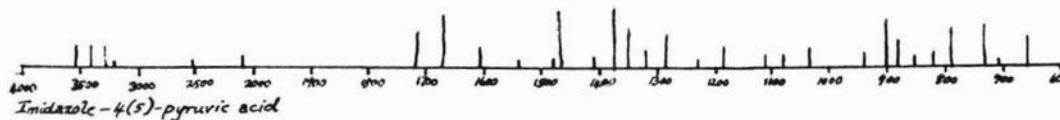
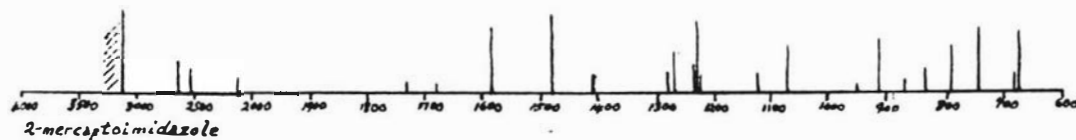
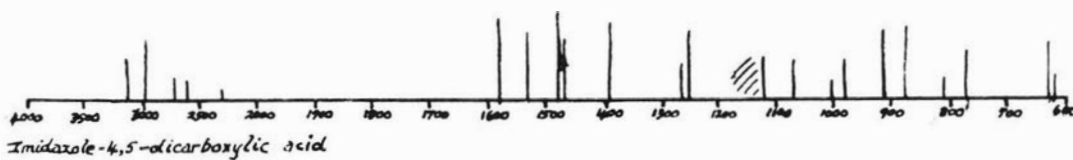
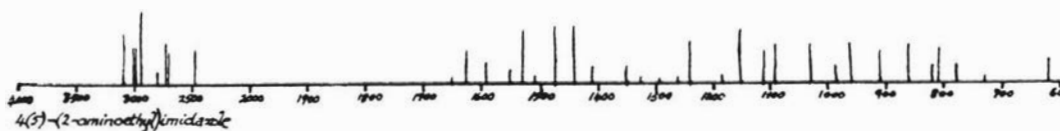
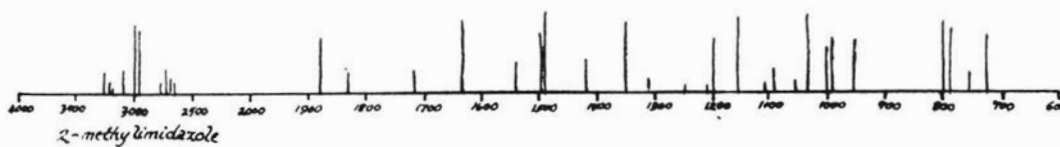
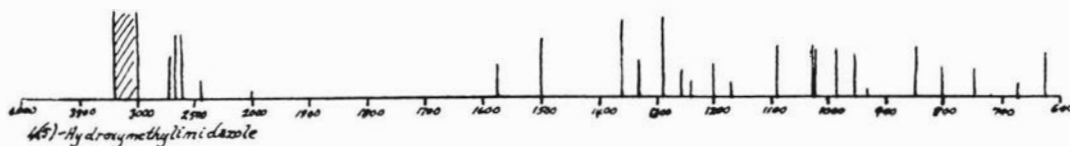
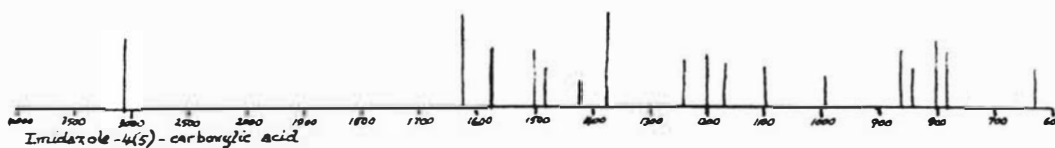
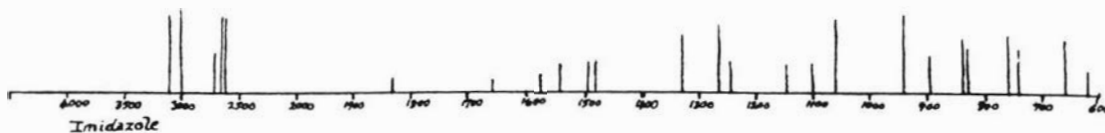
During the present project, infrared spectroscopy was used mainly as a "finger-printing" tool, although it occasionally proved useful in structural work. A collection of line spectra obtained for a range of imidazoles is included for reference purposes.

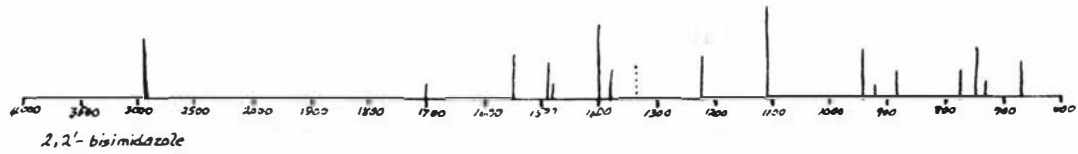
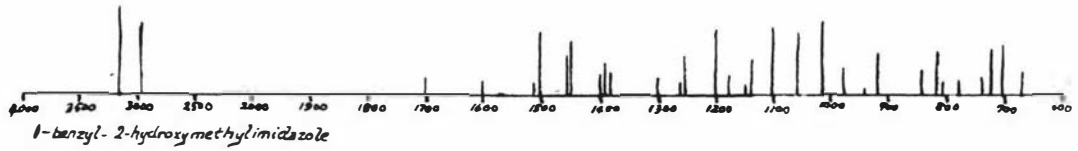
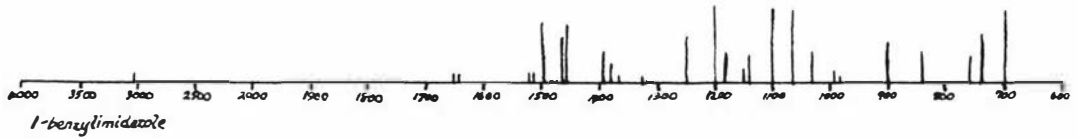
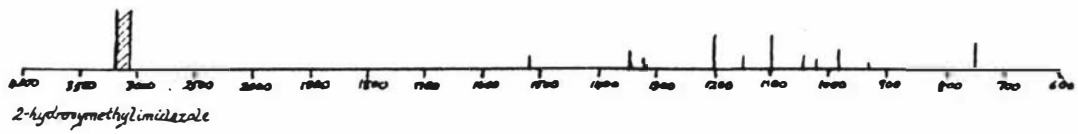
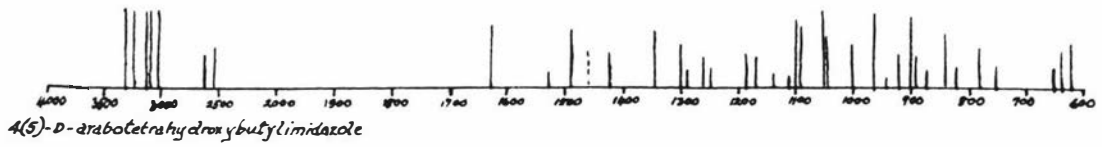
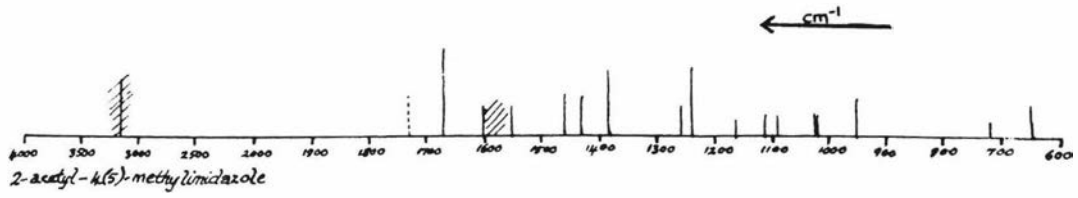
(c) Proton Magnetic Resonance Spectra.-

During this research project extensive use was made of nuclear magnetic resonance spectroscopy. As yet the literature dealing with this topic is not extensive, only the

I.R. LINE SPECTRA OF IMIDAZOLES.

← cm⁻¹





simpler substituted imidazoles having been studied.^{3,8,10,11,12,1} Solubility of the compounds poses some problems as most common imidazoles are rather insoluble in non-polar solvents, and therefore it was often not possible to compare spectra in deuteriochloroform with those in deuterium oxide. Reddy and his co-workers¹¹ have overcome this difficulty to some extent by converting imidazoles to N-acetyl derivatives which are readily soluble in mixtures of deuteriochloroform and dimethylsulphoxide.

The n.m.r. spectrum of the non-exchanging protons of imidazole consists of two peaks in the ratio 1:2 with chemical shifts of 462 c./sec. and 427 c./sec. respectively from tetramethylsilane. The low field peak can be assigned to the 2-H and the high field peak to the equivalent 4- and 5-H's.¹¹ A number of proton magnetic resonance spectra of imidazoles have been included in the text of this thesis.

