

DIAZO-REACTIONS
IN THEIR RELATION TO BIOCHEMISTRY.

by

George Hunter, M.A., B.Sc., B.Sc. (Agric).

~~Assistant Professor of Pathological Chemistry,~~

~~University of Toronto.~~

Professor ~~Wass~~ of Biochemistry,

University of Alberta, Edmonton, Canada.

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List of Papers by the Writer and Collaborators.

By George Hunter:

1. The Estimation of Carnosine in Muscle Extract. Biochem.J.(1921) 15, 689.
2. The Estimation of Carnosine in Muscle Extract. Biochem.J.(1922) 16, 640.
- A Critical Study -
3. Note on Knoop's Test for Histidine. Biochem.J.(1922) 16, 637.
4. The Diazo-Reaction, with Special Reference to
Urine in Measles. Brit.Med.J.(1922) pg.751
5. Observations on the Distribution and Variation
of Carnosine in Cat Muscle. Biochem.J.(1924) 18, 408
6. The Diazo Reaction In Urine Biochem.J.(1925) 19, 25.
7. Carnosine of Muscle and Iminazole
Excretion in the Urine Biochem.J.(1925) 19, 34
8. Colour Standards for Use in the
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9. Uric Acid Determinations in Blood. J.Biol.Chem.(1925) 63, 17

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10. The Isolation from Blood of a Hitherto
Unknown Substance, and its Bearing on Present
Methods for the Estimation of Uric Acid. J.Biol.Chem.(1925) 65, 623

By George Hunter and B.A.Eagles.

11. Non-Protein Sulfur Compounds of Blood.

1. Sympectothion.

J.Biol.Chem.(1927) p.123

12. Non-Protein Sulfur Compounds of Blood.

2. Glutathione

J.Biol.Chem.(1927) p.133

13. Glutathione. A Critical Study.

J.Biol.Chem.(1927) p.147

14. On the Presence of Cystine in Liver

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15. The Colorimetric Estimation of Cystine
and Glutathione

J.Biol.Chem.(1927) p.177.

By George Hunter.

16. A New Test for Ergothioneine upon which
is Based a Method for its Estimation in
Simple Solution and in Blood-Filtrates.

Biochem.J.(1928) 22, 1-10.

By George Hunter and R.C.Montgomery.

17. On the Diazo Reaction in Uraemic Serum
and in Normal Urine.

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PREFACE

When studying methods for the estimation of carnosine in mammalian muscle I was led to the adoption of a colorimetric method based on the fact that this substance forms a highly coloured compound with diazotised sulphanilic acid. In a subsequent search for related substances in urine I succeeded in isolating histidine from the urine of a patient suffering from measles and at that time regarded it as responsible for the so-called Ehrlich diazo-reaction found in febrile conditions. About the same time I had the opportunity of studying what is generally called the van den Bergh reaction in the serum of patients with jaundice. Shortly after coming to Toronto I had occasion to test urine from patients with typhoid, military tuberculosis, and certain other diseases, and soon discovered that the true Ehrlich test is not attributable to the presence of histidine in urine. On perusal of the literature on Ehrlich's diazo-reaction it became apparent that there are several substances in urine which react with diazo-compounds, and react differently according to the conditions under which the tests are done. It was also recorded that pyrroles, pyrimidines, purines, glucose, aceto-acetic acid, and various other substances found in biological material, react with diazo-compounds forming

highly coloured substances.

The subject of diazo-reactions is thus one of some complexity. Much has been written on Ehrlich's diazo-reaction and in recent years on the van den Bergh reaction for bilirubin in blood serum. The Pauly test for glyoxalines has also been extensively used. However, knowledge of other diazo-reactions given by biological material has recently become more important for two main reasons. One of these is the increasing tendency towards the adoption of colorimetric methods in biochemical work, both for qualitative and quantitative purposes. Colorimetric methods are applicable to the detection or estimation of substances in amounts far beyond the range of gravimetric or volumetric methods, and with a modern colorimeter certain of them yield results of high quantitative accuracy. Diazo-compounds are amongst the most sensitive reagents we possess in biochemistry. Most of the reactions with which we deal are given quite satisfactorily by a hundredth part of a mg. in a volume of about 5 cc. Half a cubic centimeter of human blood serum, containing 0.1 mg. of bilirubin per 100 cc. suffices for the detection of this substance by the diazo-reagents. We thus employ less than one two millionth part of a gram of the substance for the test. The other reason is that we now

possess in the Koessler and Hanke diazo-reagent a means for the precise colorimetric determination of glyoxalines, phenols, and thioglyoxalines in simple solution; and the successful application of these methods to biological fluids depends to a large extent on our knowledge of other substances capable of reacting with diazo-compounds and possibly present in the material to be tested. With such knowledge, suitable fractionation methods may then be applied as a preliminary to the actual estimation of particular constituents.

It thus appeared to the writer that there was some need for a systematic treatment of the whole subject of diazo-reactions in their relationship to biochemistry. No such study exists in the English language and the only paper the writer has been able to discover with this object in view is that of Clemens in 1899; although of course, there are numerous theses and dissertations in German and French on the clinical aspects of Ehrlich's diazo-reaction.

The present study thus represents an attempt to classify diazo-reactions on a chemical basis. The substances with which we are concerned are divided into two large groups according as they couple with diazo-compounds in an acid or an alkaline medium, and are thus respectively treated in

Part I and Part II of the present study. Throughout the work, attention has been given primarily to the chemical aspect of the reactions. Azo-dyes, on account of their economic importance, have of course been extensively studied, but the vast literature on the subject appears to be of little help in our present problem, for the reason that aromatic amines and phenols constitute practically exclusively the commercial azo-dyes. Unfortunately for our purposes, aromatic amines do not appear to exist in biological material and phenols do not play a conspicuous part, at any rate in animal metabolism. Thus no attention has been given in the present study to aminoazo-compounds, and hydroxyazo-compounds have been but briefly dealt with. A systematic treatment of the commercial azo-dyes is given by Cain and Thorpe, [The Synthetic Dyestuffs, 1905, p.47]. On the other hand, the pure chemistry on azo-dyes from pyrroles, pyrimidines, purines, thiolglyoxalines, and other substances of much significance in the animal economy, has not been systematically undertaken, and information on such subjects sometimes does not exist, is widely scattered, and is frequently found in the most diverse subject-matter. The writer's treatment of this rather difficult literature must necessarily have many shortcomings, yet it may be adequate perhaps to the extent of

emphasising that at least in the field of biochemical diazo-reactions the aid of the organic chemist is urgently called for.

The objects of next importance have been the determination of the optimum conditions for coupling, the description and characterisation of the colours, ^{and} the sensitivity and ~~the~~ specificity of the reactions. It should be emphasised, however, that such means of differentiation are dependent on the performance of the tests under specified conditions. Apart from the reaction of the coupling medium, the amount of substance used for the tests is of the greatest importance. It may be noted as a general principle of colour tests, and of diazo colour tests in particular, that the colours are characteristic only when the reactive substance is present in amount just slightly greater than the minimum which gives a perceptible colour. Thus the maximum amount of the common glyoxalines may be set at rather less than 0.1 mg. where the total volume of test solution and reagents amounts to less than 10 cc. Over a short range just above the limit of sensitivity there is a linear proportionality between the concentration of substance present and the amount of colour produced. Under definite conditions to be specified later this holds true for bilirubin, phenols, glyoxalines, and thioglyoxalines, and probably for other substances which have not yet been

quantitatively studied. When, however, excessive amounts of glyoxalines, for example 2-3 mg. of histidine, are used under the same conditions, the colours cease to be proportional to the amount of substance present, the shade of colour is more yellow than that obtained under optimum conditions, and the time of colour development can not be judged. The same is true for other diazo-tests. So that without due regard to these considerations much useful information which is otherwise obtainable from diazo-tests, is lost.

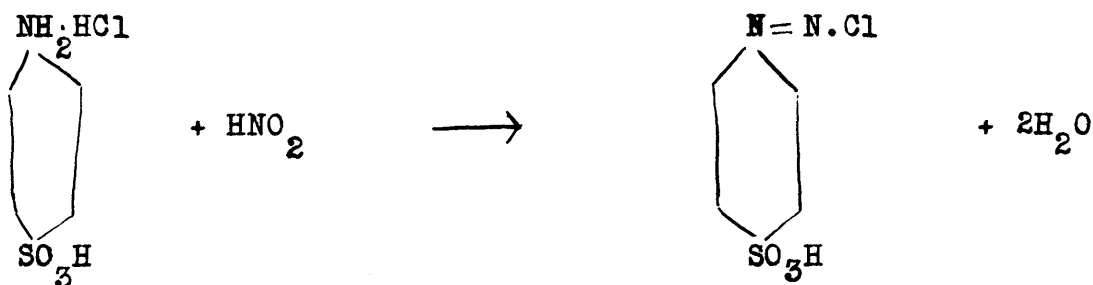
The diazo-reagent and technique used in performing the tests are also important, and the main features of different diazo-reagents recommended in the literature are examined.

Certain biochemical and physiological problems intimately associated with particular diazo-reactions have also been discussed.

Part III is a re-examination of the subject of ~~the~~ diazo-reactions of urine in the light of our study of the individual types of diazo-reaction.

Introduction.

Of the various reactions of diazo-compounds, such as the formation of phenols in hot aqueous solution, the formation of halogen derivatives by the Sandmeyer methods, and so on, we are concerned only with the property possessed by diazo-compounds of reacting, or "coupling", with other substances to form coloured compounds, or as synonymously used here, dyes. For the performance of a diazo-reaction as thus defined, it is first necessary to choose a suitable aromatic amine, from which by the action of nitrous acid, we prepare the diazo-compound. Sulphanilic acid — for such reasons as its ready availability in a high degree of purity, the ease with which it can be diazotised, the stability of its diazotised product at ordinary temperature, and the fact that the diazotised product does not readily combine with unchanged sulphanilic acid — has been generally adopted for biochemical tests. When, to a solution of sulphanilic acid in hydrochloric acid, the requisite amount of an aqueous solution of sodium nitrite is added we get as the product of diazotisation, p-sulphodiazobenzene chloride according to the following equation:



sulphanilic acid·HCl

p-sulphodiazobenzene chloride.

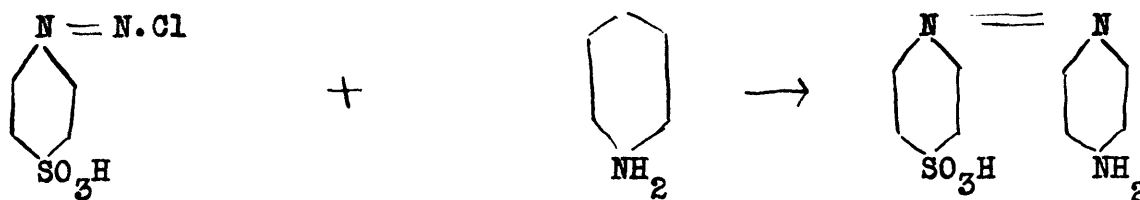
Such diazo-compounds, from their resemblance to ammonium compounds are also called diazonium salts, and diazobenzene chloride, $\text{Ph}\cdot\text{N}=\text{N}\cdot\text{Cl}$, may also be written as benzenediazonium chloride, $\text{PhN}\equiv\text{N}$; one N-atom

being pentavalent as in ammonium chloride, $\text{H} - \text{N} \begin{array}{l} \diagup \text{H} \\ \diagdown \text{H} \\ | \text{H} \\ | \text{Cl} \end{array}$

In weakly alkaline solution there exists the corresponding diazo-hydrate,

$\text{Ph}\cdot\text{N}=\text{N}\cdot\text{OH}$, or the diazonium hydroxide, $\text{Ph} - \text{N} \begin{array}{l} \equiv \text{N} \\ | \text{OH} \end{array}$.

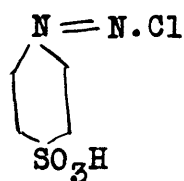
The coupling process, which occurs with other aromatic amines or with phenols as second component, may be illustrated thus:



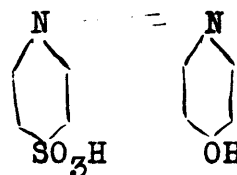
p-sulphodiazobenzene chloride

aniline

aminoazo-compound.



+



phenol

hydroxyazo-compound.

Diazo-compounds couple in an acid medium with aromatic amines, but with phenols generally only in a weakly alkaline medium.

The group $-N=N-$ between two carbon atoms, is called the azo-group, and the dyes containing it the azo-dyes. (In the diazo-compounds only one N of the group $-N=N-$ is attached to carbon.). More than one arylazo-group may couple with the second component: when two, the derivatives are called bisazo- and when three, trisazo-dyes.

We shall be mainly concerned with compounds in which the azo-group is coupled to a carbon-atom of the second component, in which case the compounds are C-azo-dyes. Coupling may however, be with a nitrogen atom, in which case the compounds are N-azo-dyes.

Azo-compounds in which the second component is an aliphatic compound are termed mixed azo-compounds.

Part I - Diazo-Reactions in an Acid Coupling Medium.

AZOPYRROLES.

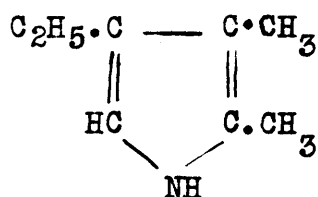
Derivatives of pyrrole will be described according to the usual numbering, positions 2 and 5 being sometimes designated as α and α' , and positions 3 and 4 as β and β' .

4. CH CH 3.

5. CH CH 2.

NH
1.

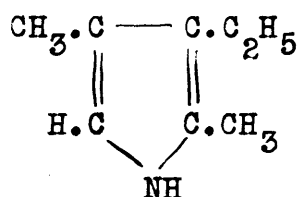
Of immediate interest to us are the reduction split products of haemin, which may be listed according to H. Fischer [1927] as follows:

Haemopyrrole Bases

2:3-dimethyl-4-ethylpyrrole

or

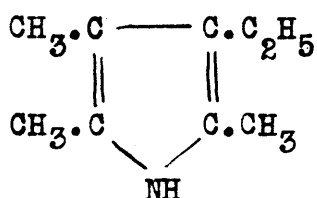
1. Haemopyrrole



2:4-dimethyl-3-ethylpyrrole

or

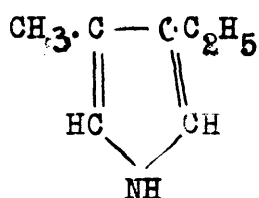
2. Cryptopyrrole



2:4 :5-trimethyl-3-ethylpyrrole

or

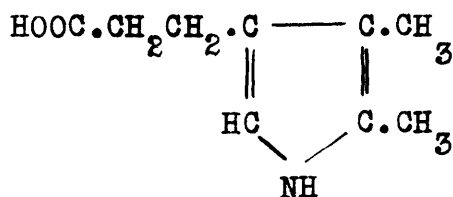
3. Phyllopyrrole



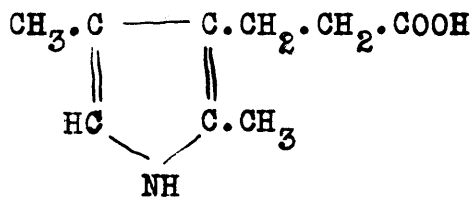
3:4-methylethylpyrrole

or

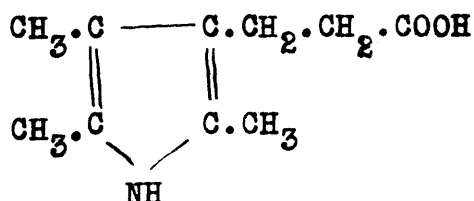
4. Opsopyrrole

Haemopyrrole Acids

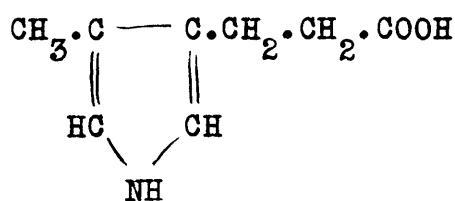
5. Haemopyrrole-carbonic acid



6. Cryptopyrrole-carbonic acid.



7. Phyllopyrrole-carbonic acid



8. Opsopyrrole-carbonic acid.

The behaviour of these pyrroles towards diazo-compounds will be described as we proceed in a systematic study of the conditions under which pyrrole and its simple substituted products couple with diazo-compounds. We shall thus consider in sequence pyrrole, mono-, di-, tri-, and tetra-C-substituted pyrroles, and finally N-substituted pyrroles. We shall then seek to state any valid generalisations on the formation of azopyrroles which may result from this study.

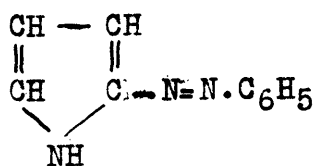
O. Fischer and Hepp [1886] first showed that pyrrole in alcoholic sodium acetate solution reacts with diazobenzene chloride to form a monoazo-derivative of the composition, $C_4H_4N.N_2.C_6H_5$. The citron-yellow crystals have basic properties and are easily soluble in hydrochloric acid giving a reddish-yellow solution. The base is recovered unchanged by the addition of ammonia or fixed alkali.

If, however, a molecular proportion of pyrrole in alcohol is left to act with two molecular proportions of diazo-compound in presence of sodium carbonate or sodium hydroxide a bisazo-dye of the composition $C_4H_3N(N_2C_6H_5)_2$ is formed. The crystalline product in this case consists of red-brown leaflets. It dissolves in concentrated hydrochloric acid

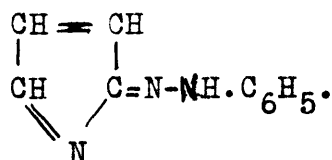
with a green-black colour, in concentrated sulphuric acid with a beautiful blue colour, in contrast to the monoazo-derivative which dissolves in the same medium with a yellow colour. Addition of water to the ^l sulphuric acid solution changes the colour to red. The solution in alcoholic sodium or potassium hydroxide is fuchsin-red.

Analogous toluene-, α -naphthylene-, and β -naphthyleneazo-derivatives were prepared by O. Fischer and Hepp. They conclude that the dyes formed are in all cases true C-azo-compounds and that there is no coupling through the imino-group. The bisazo-compounds are regarded as either α -: α' - or β -: β' -compounds, with the azo-groups arranged symmetrically to the N-atom.

That the benzeneazopyrrole of Fischer and Hepp is an α -derivative is confirmed by the finding of Plancher and Soncine [1901] that urea derivatives may be prepared from α -benzeneazopyrrole by treatment with phenylisocyanate, whereas such substances are not formed when the azo-group is in the β -position. It is thus supposed that α -benzeneazopyrrole exists in the tautomeric forms.



and



Monosubstituted Pyrroles.

O. Fischer and Hepp [1886] record that α -pyrrolecarboxylic acid forms a bisazo-compound with loss of CO_2 when coupled in alkaline solution, but that α -carbmethoxypyrrole under the same conditions forms a monoazo-compound. Khotinsky and Soloweitschik [1909] find that in acid solution with diazotised sulphanilic acid, α -phenylpyrrole forms an α' -monoazo compound.

I have been unable to find any record of the preparation of an azo-derivative of a mono- β -substituted pyrrole.

Disubstituted Pyrroles.

α : α' -dimethylpyrrole in slight ^{α} acid solution forms a monoazo-compound with diazobenzene chloride [Fischer and Hepp, 1886]. The analogous compound has been prepared from the same substance with diazobenzene-*p*-sulphonic acid by H Fischer and Bartholomäus [1911-'12]. These are yellow compounds unchanged in colour in contact with strong acids.

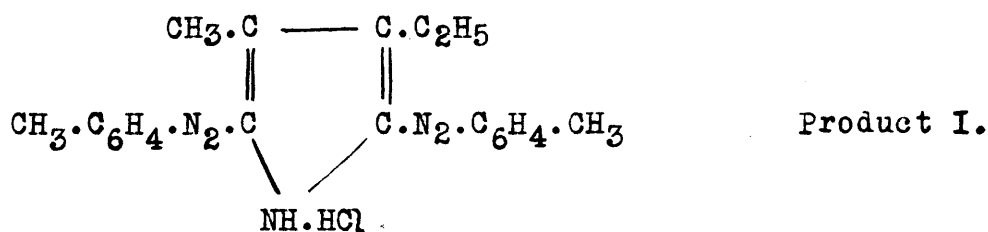
2:4 -dimethylpyrrole forms in acid solution a yellow-brown monoazo-derivative [H. Fischer and Bartholomäus, 1911-'12], and 2-methyl-3-carbethoxypyrrole a red-brown monoazo-derivative with diazobenzene-*p*-sulphonic acid [H. Fischer and Schubert, 1926]. Whether the azo-

group attaches itself to the α - or β -position in these cases has not been determined.

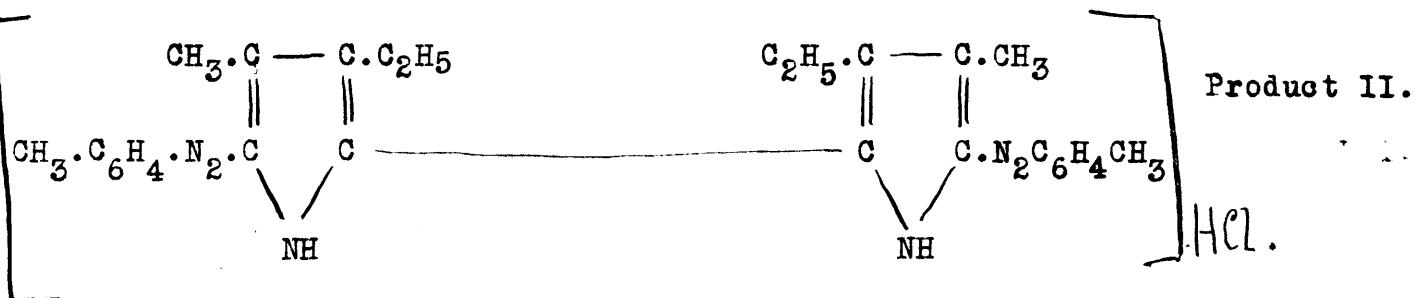
The behaviour of 3:4-methylethylpyrrole, or opsopyrrole, with diazo-compounds is of particular interest in relation to the study of the action of diazo-compounds on bilirubin. The literature on the azo-derivatives of opsopyrrole is, however, too incomplete to permit as yet of definite conclusions as to whether this substance forms mono- or bisazo-derivatives, or both. The evidence appears sufficiently strong, as may be seen in the following discussion, to warrant that it forms some compound other than a simple α -monoazo-dye.

In 1904-05, before the complex nature of pyrrole oil from chlorophyll or haemin was understood, Marchlewski and his pupils [Goldmann and Marchlewski, 1904-05; Goldmann, Hetper, and Marchlewski, 1905] isolated from the oil prepared from haemin, crystals with a definite m.p., soluble in chloroform with a blue-violet, in hydrochloric acid with a blue, and in potassium hydroxide with a red colour. The substance was regarded as a bisazo-derivative of "haemopyrrole". It was suggested [Buraczewski and Marchlewski, 1906] that the dye thus prepared from pyrrole oil was the same as that obtained from 3:4-

methyl-n-propylpyrrole, but it was later shown that two azo-derivatives were obtainable from synthetic 3:4 methyl-ethylpyrrole, identical with the two main azo-products isolated from pyrrole oil [Grabowski and Marchlewski, 1912; 1914]. The two ~~diaz~~^{azo-}toluene derivatives are thus formulated by Marchlewski [1922]:



and



The ~~diaz~~^{azo-}benzene derivative corresponding to Product I. formed red crystals m.p. 233°, belonging to the mono- or triclinic system.

The spectrum of the base showed two bands, $\lambda = 553-530.5$ and $\lambda = 519-495$, whilst the hydrochloride showed only one band situated on the green thallium line.

The ~~diaz~~^{azo-}benzene derivative corresponding to Product II. formulated

as a conjugated monoazo-dye, though the manner of linkage of the pyrrole nuclei is regarded by Marchlewski as not clear, forms ruby crystals m.p. 268° . The chloroform solution is blue-violet, the hydrochloric acid solution clear blue, and the neutral or alkaline solution red. The chloroform solution shows two poorly defined bands in the region $\lambda = 571.5 - 511$, besides a strong absorption in the violet and ultraviolet regions.

H.Fischer and Walach [1926] have found that 3:4-dimethyl-pyrrole couples in acid solution with diazobenzene-p-sulphonic acid to form a blue azo-dye, which was not further investigated owing to scarcity of material. H.Fischer and Treibs [1926] mention that under the same conditions opsopyrrole forms a blue azo-dye and that opsopyrrole-carbonic acid gives, along with a brown intermediate product, a blue azo-dye. The blue solution shows a broad spectral band at $\lambda = 645-650$. With sodium hydroxide the colour of the blue solution is changed to red.

These findings with $\beta:\beta'$ -dimethylpyrrole, opsopyrrole, and opsopyrrole carbonic acid are important, inconclusive as they are, in that they give to the work of Marchlewski and his pupils a significance which perhaps it has not hitherto enjoyed.* From the work of O.Fischer

and Hepp and of H.Fischer it seemed unlikely that isazopyrroles are ever formed by coupling in acid solution. Pyrrole itself forms a monoazo-product under these conditions and the mono-alkylated pyrroles so far investigated have been found to yield monoazo-products, as do also the other than $\beta:\beta'$ -disubstituted pyrroles. We are not yet certain, of course, that these yield bisazo-derivatives but the evidence to date is in favour of this assumption. It is found that the monoazo-pyrroles, formed at least with diazobenzene chloride or diazobenzene-sulphonic acid, are apparently without exception yellow or orange-coloured substances, which, unlike the azo-derivative of opsopyrrole, are not turned blue in colour with acids. The mention by H.Fischer and Trieb of the formation of a brown intermediate product in the coupling of opsopyrrole-carbonic acid suggests that two azo-derivatives of opsopyrrole are formed in acid solution; and Marchlewski's evidence on this point is not reasonably in doubt. The red colour of the neutral or alkaline solution and the blue colour of the acid solution are, according to O.Fischer and Hepp, characteristics of bisazopyrroles. As we shall see, this colour change is also akin to that

obtained with bisazo-bilirubin.

Note to Page 17.

I have studied carefully the various papers of Marchlewski on azopyrroles as he was the first observer in this particular field; and as noted by Küster [1922,1] , he showed by means of the azo-derivatives the complex nature of pyrrole oil and demonstrated the identity of the products prepared from chlorophyll and from haemin; but of his work we can only agree with Küster and with H.Fischer and Bartholomäus [1911-'12] that the findings are not clear. After it was found by Küster [1908] that pyrrole oil had at least two constituents Marchlewski [1908; Marchlewski and Retinger, 1908] reported the isolation of no less than five azo-derivatives from the crude pyrrole oil, four of which were described with respect to crystal form, m.p., colour, and the property of forming complex metallic salts. Several structural formulae were suggested for certain of these compounds by Marchlewski [1909; Marchlewski and Robel, 1910] but these were later modified. It is stated [Marchlewski, 1912] that "haemopyrrole" - presumably opsopyrrole - forms a mono-azo derivative but in excess of diazo-compound this soon changes to the bisazo-compound. Marchlewski,

[1922] later confines his claims to the preparation of two azo-pyrroles from 3:4-methylethylpyrrole and regards the other azo-compounds prepared from pyrrole oil as probably mixtures. The conjugated monoazo-dye, called product II throughout Marchleswki's papers, has apparently a deeper colour than the bisazo-dye, product I. The difficulty in interpreting the findings of Marchleswki is greatly increased by the frequent errata; e.g. product II is first given [Marchleswki and Retinger, 1908] as $C_{23}H_{27}N_5O_2$ and later [Marchlewski, 1922] as $C_{23}H_{33}N_6Cl$ formulated as $(C_6H_5N_2 \cdot C_8H_{11}N \cdot C_8H_{11}N \cdot C_6H_5) \cdot HCl$.

Trisubstituted Pyrroles.

H.Fischer and Bartholomäus [1911-'12] prepared mono-C-azo-compounds from the following substituted pyrroles by treatment of their alcoholic solutions with 1 per cent. aqueous diazobenzene-p-sulphonic acid in a medium approximately 0.05 normal acid: 2:4-dimethyl-3-acetylpyrrole; 2:5-dimethylpyrrole-3-carbonic acid, and 2:5-dimethyl-3-carbethoxy-pyrrole. Other azo-derivatives described are those from 2:4:5-trimethylpyrrole [H. Fischer and Bartholomäus, 1912,3] ; 2:4-dimethylpyrrole-5-propionic acid [H. Fischer and Rose, 1914] ; and the diazotised benzidine derivatives of 2:4-dimethyl-3-carbethoxypyrrole and 2:4-dimethyl-3-acetylpyrrole [H. Fischer and Schneller, 1923]. From such findings H.Fischer and Bartholomäus [1912,1] showed that trialkylated α -azo-dyes do not change appreciably in colour with acids, whilst trialkylated β -azo-dyes at the same dilution turn red with acids. They [H.Fischer and Bartholomäus, 1912,2] describe the α - and β -derivatives in more detail as follows: the distinction holds good only for trialkylated azopyrroles made with diazobenzene-p-sulphonic acid.

α -azotrialkylated pyrroles are unstable and change on exposure to air and light. They are difficult to recrystallise. They do not change

appreciably in colour, as contrasted with the β -azo-derivatives, in contact with acids. They are further characterised by the following test. When a sodium hydroxide solution of an α -azo-derivative is poured on filter paper and touched with a drop of diazotised nitraniline, the margins of the drop become purple-red and the whole surface covered by it becomes blue after a short time. This reaction has been obtained with other diazo-compounds but it then appears more slowly and is not so marked. β -azo-trialkylated pyrroles are on the other hand stable and easily recrystallized. They change to red with acids and do not give the reaction above described with diazotised nitraniline.

Küster [1922,1] has confirmed these observations on the distinction of α -and β -derivatives. Thus he found that the dye formed with diazobenzene-p-sulphonic acid from 2:4-dimethyl-3-carbethoxypyrrole behaved as an α -derivative and that from 2:4-dimethyl-5-acetylpyrrole as a β -derivative.

The azo-derivative of haemopyrrole has been described by H.Fischer and Bartholomäus [1911-'12] as an orange-yellow substance, $C_8H_{12}N.N.C_6H_4.SO_3H$, hardly affected by contact with acid. It behaves as an α -azo-trialkylated pyrrole [H.Fischer and Bartholomäus, 1912,2] .

The formation of this dye was mentioned by Willstätter and Asahina [1911] but it was not further investigated by them.

Cryptopyrrole likewise gives a beautiful crystalline α -azo-dye of the same empirical composition [H. Fischer and Bartholomäus, 1912, 4] .
Haemopyrrole-carbonic acid, formerly called phonopyrrole-carbonic acid, gives a yellow-red to orange dye of the α -series; yet its distillation product is not, as expected, haemopyrrole, which as we have seen gives an α -azo-dye, but, through a shift of the ethyl-group from the β -to α -position, is 2-ethyl-4:5-dimethylpyrrole, since it forms an azo-derivative of the β -series [H. Fischer and Bartholomäus, 1912, 2] .

Benary and Silbermann 1913. formed from 3-hydroxy-4-carbethoxy-5-methylpyrrole the 2-benzenazo-derivative, which they observed gives a red colour with ferric chloride, and thus retains the OH-group and is an azo-compound. H. Fischer and Herrmann [1922] also prepared from this compound derivatives with diazotised p-dichloroaniline, p-nitraniline, and sulphanilic acid. These all form yellow crystals.

Tetrasubstituted Pyrroles.

Phyllopyrrole and Phyllopyrrole-carbonic acid do not react with diazo-compounds. [Willstätter and Asahina, 1911 ; H. Fischer and Bartholomäus,

1912,3] . A free CH-group in the pyrrole ring is necessary for coupling, and the only tetra-C-substituted products which will form azo-compounds are those containing a carboxyl group, which is eliminated in the process of coupling. H. Fischer and Rothweiler [1923] ^{ou} find that 2:4-dimethyl-3-acetyl_{pyrrole}-5-carbonic acid couples with diazobenzene-sulphonic acid in alkaline solution with the formation of a yellow colour and evolution of CO₂, which was conspicuous when the coupling mixture was acidified. The substance is identical with that previously obtained from 2:4-dimethyl-3-acetylpyrrole by H. Fischer and Bartholomäus [1911-'12] . CO₂ is eliminated even in acetic acid solution when diazotised p-nitraniline is used as coupling agent. The corresponding ester does not couple.

N-Substituted Pyrroles.

Khotinsky and Soloweitschik [1909] showed that 1-phenylpyrrole couples in glacial acetic acid with diazotised sulphanilic acid to form 1-phenyl-2-p-sulphobenzeazopyrrole. This is a stable compound forming a golden yellow sodium salt. The corresponding diazotised benzidine derivative is unstable.

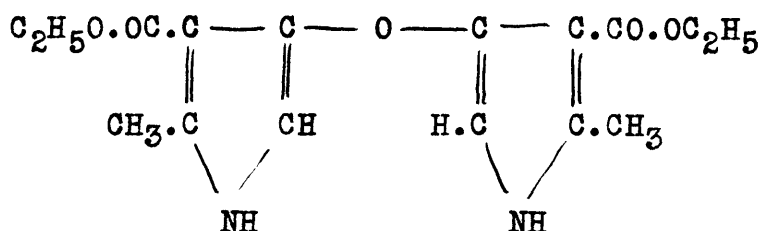
From the finding H. Fischer and Bartholomäus 1911-'12. that 1-phenyl-2:5-dimethylpyrrole-3-carbonic acid (still containing a free

CH-group) did not couple with diazobenzene-p-sulphonic acid it was thought that a free imino-H in the pyrrole ring was a necessary condition for coupling, but they later showed [H. Fischer and Bartholomäus, 1912.5] that 1:2:5-trimethylpyrrole and 1-ethyl-2:5-dimethylpyrrole couple smoothly to form monoazo-dyes of the composition $C_{13}H_{15}O_3N_3S$ and $C_{14}H_{17}O_3N_3S$ respectively, and that also 1-phenyl-2:5-dimethylpyrrole readily couples to give a red colour, though the product was not isolated.

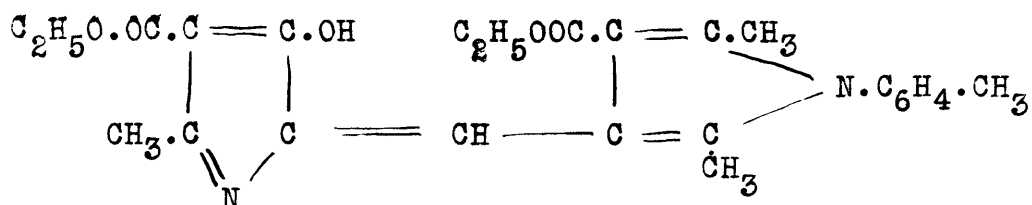
Reichstein [1927] has described azo-derivatives prepared from N-methylpyrrole, N-isoamylpyrrole and N-butylpyrrole.

Conjugated Pyrroles.

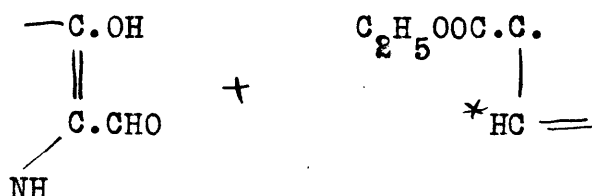
It is of interest to note that the compound:



couples easily with diazobenzene-p-sulphonic acid to form a beautiful crystalline azo-dye [H. Fischer and Loy, 1923]. The same workers have shown that the compound:



couples with diazobenzene-p-sulphonic acid as a result of hydrolysis and release of a free CH- group.



These will be considered in relation to the formation of azo-compounds by bilirubin.

ADDENDUM

The writer's experimental observations have been confined to the behaviour of pyrrole with Ehrlich's diazo-reagent (see p. 55) .

From an alcoholic solution of pyrrole (Kahlbaum) containing 2 mg. per cc., 1 cc. portions were put in three tubes, A, B and C. To A was added a little powdered sodium acetate and to B a little dry sodium carbonate. 0.5 cc. of the diazo-reagent was then added to each tube, A and B being shaken to dissolve the solid substances. In A there was the immediate appearance of a yellow colour which remained unchanged for several hours. In B the colour developed from yellow to orange

and after several hours to a dirty red. In C₂ⁿ *to which the reagent alone was added,* the colour developed from yellow to orange, to red, and after several hours to a deep permanganate colour seen better after dilution with water.

In tubes A and B the conditions for the reaction correspond to those adopted by O. Fischer and Hepp, (except that there is an excess of diazo compound in both cases), and the yellow and red colours obtained undoubtedly correspond to the mono- and bisazo- compounds described by these workers. The development of a deep purple colour in tube C was unexpected. The colour is so much more blue and more intense than that obtained in tube B that one is tempted to hazard the opinion that pyrrole *in a strongly acid medium* ~~under these conditions~~ couples with more than two azo-groups.

It is also of interest to note the behaviour of the above solutions towards strong acids and alkalies. By the addition of a large excess of conc. sulphuric acid to A there is little change in colour; to B, especially at the orange stage, a marked deepening of colour to red. This corresponds with the findings of Fischer and Hepp when using aqueous or alcoholic solutions of the mono- and bisazo-compounds. It may be noted that the changes in colour of the bisazo-

compound to a dirty green with conc. hydrochloric acid and to a blue with conc. sulphuric acid are obtainable only with dry crystalline material. The colour in tube C was little affected by strong acid or alkali. The behaviour of these compounds in aqueous alcoholic solution towards strong acids and alkalies thus differs markedly from the behaviour of azobilirubin towards the same reagents.

It was also considered desirable to determine the sensitivity of the reaction using 1 cc. of pyrrole solution and 0.5 cc. of diazo-reagent, as in tube C above. Thus it was found that with 0.2 mg. of pyrrole, the purplish red colour developed very slowly at room temperature, but quite quickly on the application of heat. With 0.02 mg. of pyrrole only an orange-yellow colour was obtained, even on heating, and various modifications of the test failed to improve the colour. With 0.002 mg. of pyrrole, and even with half this amount, a perceptible yellow colour is obtained. The test is thus sensitive to a dilution of over 1 in a million.

CONCLUSIONS

When coupled in acid solution with diazo-compounds pyrrole forms monoazo-derivatives and in alkaline solution bisazo- derivatives. The latter are more highly coloured than the monoazo-derivatives, and their solutions become red in contact with acid, whereas solutions of the monoazo-derivatives remain practically unchanged with acid. The α -position is occupied by the azo-group in preference to the β -position, when both are open. ^{no β} It is suggested that pyrrole combines under certain conditions in acid solution with more than one azo- group.

A free CH- group in the pyrrole ring is a prerequisite for the formation of an azo-derivative although a tetra-C-substituted pyrrole may couple with diazo-compounds under certain conditions provided one of the substituents is a COOH- group. CO_2 is removed from such a radical most readily by coupling in alkaline solution, but also by certain diazo-compounds in acid solution.

There is yet doubt as to whether β -monosubstituted or $\beta:\beta'$ -disubstituted pyrroles form mono- or bisazo-derivatives, or both, when coupled in acid solution. α -monosubstituted and $\alpha:\alpha'$ -disubstituted

pyrroles coupled under the same conditions form monoazo-derivatives.

α -azo- trialkylated pyrroles are distinguishable from β -azo-tri-alkylated pyrroles in their behaviour towards acids, and towards diazotised p -nitraniline.

N-alkyl- or aryl-substituted pyrroles form azo- compounds provided there is a free CH- group in the pyrrole ring. In this respect the pyrrole ring differs from the glyoxaline ring to be discussed later. There is no evidence that N-azopyrroles are ever formed.

THE ACTION OF DIAZO-COMPOUNDS ON BILIRUBIN

Ehrlich. [1883,1] found that a dark colour appeared on the addition of diazotised sulphanilic acid to urine containing bile pigments and later in the same year he [Ehrlich, 1883,2] determined that bilirubin was the reactive substance present.

Chemical

The first crystalline product of the reaction of bilirubin and a diazo-compound was prepared by Pröscher [1900]. To a chloroform-alcohol solution of bilirubin, strongly acidified with hydrochloric acid, an alcoholic solution of diazotised amino-acetophenone was added until no further blue colour developed. Excess of water, strongly acidified with hydrochloric acid, was then added to the reaction mixture. The chloroform layer carried out all the azo-dye as a blue solution. On washing this with water, the colour changed to red, with the removal of the acid. The dry chloroform solution on evaporation yielded microscopic prismatic needles. The analysis yielded the empirical formula $C_{24}H_{25}N_4O_4$, regarded by Pröscher as a compound of 1 molecule of bilirubin with 1 molecule of diazoacetophenone. Now

that it is agreed [H.Fischer, 1915; Küster 1917] that the empirical formula for bilirubin is $C_{33}H_{36}N_4O_6$ instead of the Staedeler formula $C_{16}H_{18}N_2O_3$ or $C_{32}H_{36}N_4O_6$ we have for acetophenonemonoazo-bilirubin the formula $C_{33}H_{35}N_4O_6$ ($C_8H_7N_2O$) and for acetophenonebisazo-bilirubin, $C_{33}H_{34}N_4O_6$ ($C_8H_7N_2O$)₂,^{and} it is clear that Pröscher's product was the bisazo-compound and not the monoazo. Orndorff and Teeple [1905] have described both acetophenonemonoazo- and acetophenonebisazo-bilirubin, and found the bisazo-compound to agree in analysis and properties with the compound described by Pröscher.

H.Fischer and Barrenscheen [1921] have confirmed the findings of Orndorff and Teeple, that mono- and bisazo-compounds are always formed together whatever changes are made in the conditions of coupling. With an excess of diazo-compound some monoazo-derivative is formed and with one equivalent of diazo-compound some bisazo-derivative is formed. Moreover the reaction is never quite complete as some uncoupled bilirubin was always recoverable. The reaction of the coupling medium was altered from strongly acid to 0.1 normal sodium hydroxide with apparently the same findings.

Mono- and bisazo- derivatives of bilirubin have been prepared by the use of acetophenonediazonium chloride [Pröscher, 1900; Orndorff and Teeple, 1905], tribromobenzenediazonium acid sulphate [Orndorff and Teeple, 1905] and benzenediazonium chloride [H. Fischer and Barrenscheen 1921] . The last workers also describe the crystalline barium bisazo-compound prepared *with* diazobenzene-p-sulphonic acid.

Acetophenonemonoazo-bilirubin has not been obtained in crystalline form. It is nearly insoluble in chloroform and carbon disulphide. Its alcoholic solution is red, and turns bluish green with alkalies. In concentrated hydrochloric acid it is deep purple. Its solutions in acids or alkalies show no absorption band.

Tribromobenzenemonoazo-bilirubin has not been obtained in crystalline form. It is insoluble in ethyl acetate and carbon disulphide and nearly insoluble in chloroform. In acids and alkalies its solutions are predominantly red and show no absorption band. It is insoluble in potassium carbonate.

Benzenemonoazo-bilirubin crystallises in small red-brown prisms,

insoluble in water, dilute acids and strong alkalies. It is easily soluble in ammonia with a brown-red colour.

Acetophenonebisazo-bilirubin^{is} obtained from chloroform solution in microscopic prismatic needles with a red lustre in reflected light. It is slightly soluble in carbon disulphide and easily soluble in alcohol. It dissolves in alkalies forming a clear blue solution which gradually changes to green then finally becomes colourless. Neutral and acetic acid solutions are red and hydrochloric and sulphuric acid solutions are purple or blue depending on the amount of acid and pigment present. Pröscher notes the similarity of the behaviour of safranine and suggests that the red solution is the mono-acid, and the blue the diacid-solution of the dye, (see p 88). The spectral hands recorded by Pröscher (from Formánek's observations) agree very closely with those found by Orndorff and Teeple. The positions of maximum absorption are given:-

	Formánek.	Orndorff and Teeple
In alcohol, λ =	$\mu\mu$ 524.9	$\mu\mu$ 522
In alcoholic hydrochloric acid, λ	561.4 - 525.8	2 bands with light space between at 539
In alcoholic potassium hydroxide, λ =	643.1	639

Tribromobenzenebisazo-bilirubin crystallises from glacial acetic acid in large well formed rosettes, almost black in colour with a bronzy lustre. It is soluble in carbon disulphide, ethylacetate, chloroform, and alcohol, but dissolves in alkalies and in acids with bluish-purple colours. Neutral or ammoniacal solutions are red and show no absorption band, but in alcoholic potassium hydroxide solution there is a well defined band in the region $\lambda = 606-536\mu$ and in alcoholic hydrochloric acid solution a similar band at $\lambda = 573.5-495.5\mu$.

Benzenebisazo-bilirubin forms monoclinic dark red crystals with a metallic lustre. They are insoluble in water, dilute acids, or strong alkalies, but soluble in hydrochloric acid with a blue-violet colour, and in acetic acid and dilute sodium hydroxide with a red-violet colour. The substance is easily soluble in ammonia with a beautiful violet colour.

p-sulphobenzenebisazo-bilirubin. A crystalline barium salt described by Fischer and Barrenscheen [1921] agreed in analysis more closely with this compound than with the corresponding monoazo-product. The free base, presumably contaminated with the monoazo-

dye is described by these workers as having a green fluorescence, is easily soluble in water, amyl alcohol, and pyridine, and insoluble in dilute acetic acid and ethyl acetate. Its solution in pyridine absorbs from the green.

CONCLUSIONS TO CHEMICAL

We may thus conclude that bilirubin forms with diazo-compounds in an acid coupling medium both monoazo- and bisazo- dyes. The latter are the more readily prepared in crystalline condition and form the greater part of the mixed reaction product.

The method of coupling of bilirubin with diazo-compounds still remains obscure. The formulations of the bilirubin molecule suggested by Küster and ^{by} Fischer have no free methene groups, necessary in a pyrrole which may form an azo-derivative. These formulations are partly based on the fact [Küster, 1915] that bilirubin gives a negative test with Ehrlich's aldehyde reagent (p-dimethylaminobenzaldehyde), but it may be questioned if such a negative test is stronger evidence for the absence of, than a positive diazo-test is for the presence of a free methene group.

The possibility of coupling through an unsaturated side-chain appears to be excluded, from the fact that mesobilirubin with saturated side-chains forms a bisazo-compound similar to that from bilirubin (*see under urobilinogen*^{p 87}). It is possible that a free methene group may be exposed in the manner outlined at the end of the chapter on pyrroles, but there can be no complete splitting of the bilirubin molecule as evidenced by the composition and molecular weights of the azo-products. The apparently similar behaviour of opsopyrrole and bilirubin towards diazo-compounds is suggestive but awaits further investigation. There is no reason to believe that bilirubin forms imino-azo-compounds.

PHYSIOLOGICAL

There is no record of Ehrlich's having tested blood serum but he

notes [Ehrlich, 1886] that by means of the diazo-test bilirubin was

found to be present in all pleuritic exudates tested. Upon this he

has the comment, interesting in the light of modern knowledge, "Es

erklärt sich der Bilirubingehalt, den ich auf diese Weise nachweisen

konnte, wohl am Ungezwungensten in der Weise, dass sich eben in

jedem entzündlichen Exsudate rothe Blutkörperchen befinden und in

ihm fort und fort unter Bildung von Bilirubin zerstört werden. (The

bilirubin content which I demonstrated in this way, ^{is} explained ~~itself~~

in the most natural way in that red blood cells are present in every

inflammatory exudate and are broken down in it continually with the

formation of bilirubin). As noted by McNee [1922-'23], Minkowski and

Naunyn in 1886 ~~by their findings on removing the livers from geese,~~

brought to prominence the view that bilirubin formation is exclusively

a hepatic function. It is interesting that the study of the action

of bilirubin on the diazo-reagents in the hands of van den Bergh

should have been one of the main factors in the shaping of the modern

view that the liver is not the sole seat of bilirubin formation.

Indeed, there is no significant contribution on the physiological aspects of the bilirubin diazo-test from Ehrlich's paper till the advent of van den Bergh's methods in 1913, with the exception of Pröscher's paper in 1901. Pröscher, [1901], who had previously prepared a crystalline azo-derivative of bilirubin [Pröscher, 1900] pointed to the sharpness and beauty of the test but observed that despite this, it had been little used for clinical purposes. Pröscher confined his attention mainly to the test in urine, and merely mentioned that bilirubin may be tested for in serum by precipitation of the proteins by alcohol, and addition of diazo-solution to the filtrate. To van den Bergh is due the credit of first using the test as a means of estimating bilirubin in blood and other body fluids. Van den Bergh and Snapper [1913] first published a method for the quantitative estimation of bilirubin by which they established the earlier claims of Gilbert that bilirubin is present in normal human serum in small amount, and in relatively great amount in the serum of the horse. In the next two years van den Bergh and his associates published a number of papers in the "Nederl. Tijdschr. voor Geneesk" mainly on the

question of extra-hepatic bilirubin formation. In 1916 appeared the findings which have subsequently proved of greatest importance from a clinical aspect, and which have earned for the test the name of the "van den Bergh reaction".

Van den Bergh and Muller [1916], when testing bile, inadvertently omitted to add alcohol, and found to their surprise that the test thus succeeded just as well as in the presence of alcohol. Yet a neutral sodium bilirubinate solution would not couple in the absence of alcohol. On testing horse serum, ^{and} the serum from patients with pernicious anaemia and certain types of haemolytic jaundice, it was found that a colour was not given by the diazo-reagent alone; whereas, under the same conditions the serum from patients with obstructive jaundice gave an immediate colour just as had been found for bile. The test as obtained with bile or with serum from obstructive jaundice was thus termed a direct, and that given only in the presence of alcohol an indirect diazo-reaction. All sera, of course, which contain bilirubin give an indirect reaction, and determinations of bilirubin are performed on protein-free alcoholic extracts. Van den Bergh and Muller suggested that haemolytic jaundice

could thus be differentiated clinically from obstructive jaundice and postulated the existence of two types of bilirubin. At this point three main differences were evident. (1) Difference in coupling with diazo-compounds. (2) Difference in adsorption by protein precipitates: when "obstructive sera" were precipitated by alcohol much of the bilirubin was precipitated with it, whereas this was not so for "haemolytic sera". (3) Different speed of oxidation: serum from obstructive jaundice rapidly lost its bilirubin on exposure to air, but that ~~now~~^{from} haemolytic jaundice was very stable. We shall return to these later, as they are important in any attempt to explain the direct and indirect van den Bergh reactions. Van den Bergh and Muller further showed that coupling is promoted by the presence of such bile acids as glycocholic, dehydrocholic and cholic acids, but did not regard the presence of glycocholic acid in bile as providing a satisfactory explanation of its behaviour. After bile is heated for a short time it ceases to give a direct reaction but the indirect remains. No satisfactory explanation for the difference of behaviour was, however, obtained. They suggest that in haemolytic sera the bili-

rubin is bound in such a manner to protein that the combination must be broken by the addition of alcohol before coupling takes place. In his monograph "Der Gallenfarbstoff im Blute" [1918] van den Bergh reviews his work up to this date.

The chief biochemical interest in the extensive clinical literature which has grown up since that date lies in the problem of the existence of two types of bilirubin. The evidence is too strong to admit of any doubt that there are two such types. Two sera containing the same amount of bilirubin may react differently. The distinction was first brought to light by the findings of van den Bergh and Muller above described but in reality it has ^{α} longer history. It has long been a moot point whether bilirubin prepared from gall stones is the same substance as haematoidin recognized by histologists, and it has only recently been proved that these substances are identical in chemical composition and crystallographic form, [Fischer and Reindel, 1923]. Body fluids such as bile and serum from obstructive jaundice, which have been regarded as containing bilirubin, give the so-called direct test of van den Bergh; whereas, body fluids such as exudates,

transudates, and haemorrhagic fluids, ~~the common sites of~~
~~haemateidin crystals~~, do not give the direct test, but couple
 after treatment with alcohol. Also, presumably without a know-
 ledge of the van den Bergh distinction, Hoover and Blankenhorn
 [1916] showed that "obstructive bilirubin" easily dialyses where-
 as "haemolytic bilirubin" does not.

We may thus sum up the evidence upon which the existence
 of two types of bilirubin depends. We may call these the direct
 and delayed types, using "delayed" in reference to all reactions
 which are regarded as non-direct, rather than in the more restricted
 clinical sense. (The use of indirect in contra-distinction to direct
 is confusing in that both types of bilirubin, as they exist in the
 body fluids, couple in presence of alcohol; that is, give an indirect
 test).

Direct bilirubin, as it occurs in bile or in the serum of patients
with obstructive jaundice,

- (a) rapidly couples with diazo-compounds in an acid medium.
- (b) is changed to the delayed type after heating for a short time

[van den Bergh, 1918; Lepehne, 1920], or allowing to stand in an

oxygen-free atmosphere [Newman, 1928].

- (c) ^{when} ~~allowing to remain~~ exposed to an atmosphere containing oxygen, [van den Bergh, 1918], or by the use of very mild oxidising agents [Andrewes, 1924], ^{suffers} ~~there is~~ a relatively rapid oxidation, or change of the pigment in such a way that it no longer couples under any conditions with diazo-compounds.
- (d) dialyses through a collodion membrane [Hoover and Blankenhorn, 1916; Blankenhorn, 1917; Brulé, Garban, and Weissmann, 1922]; corresponding ^{to} ~~with~~ the general finding of bilirubinuria in obstructive jaundice, with a kidney threshold in man of 3-4 units of bilirubin [van den Bergh, 1918].
- (e) is not extractable with chloroform [Grunenberg, 1922; Andrewes, 1924; Roberts, 1928].
- (f) is readily adsorbed by the protein precipitated from the fluid by alcohol, [van den Bergh, 1918; Thannhauser and Andersen, 1921].
- (g) is not readily prepared in crystalline form.

Delayed bilirubin, as it occurs in haemorrhagic exudates and in the serum of patients with haemolytic jaundice.

- (a) couples slowly or not at all with diazo-compounds. After treatment of the fluid with alcohol it reacts as direct bilirubin.
- (b) may be converted to the direct type on addition of alkali in the proper amount [Collinson and Fowweather, 1916; Newman, 1928], although this is questioned [Roberts, 1928].
- (c) is less readily oxidised [van den Bergh, 1918; Andrewes, 1924].
- (d) does not dialyse through a collodion membrane [Refs. as under (d), above]; corresponding ^{to} with the general absence of bilirubinuria in haemolytic jaundice, and a much higher kidney threshold of excretion.
- (e) is extractable with chloroform [Refs. as under (e), above].
- (f) is not adsorbed by the protein precipitated from the fluid by alcohol, [Refs. as under (f), above].

(g) is readily prepared. Van den Bergh [1918] has described a simple method by which bilirubin may be prepared in crystalline form from as little as 10 cc. of blood serum. Fischer and Reindel [1923] confirmed these findings.

There have been various attempts to account for these two types of bilirubin. Van den Bergh [1918] suggested that the delayed type is present in the serum in combination with protein, and ^{that} preliminary treatment with alcohol is necessary to effect disruption of the union before it will couple with diazo-compounds. The experimental evidence available on the subject is against this view, [Andrewes, 1924]. Feigl and Querner [1919] suggested combination with a lipoid, and Rosenthal and Holzer [1921], from their general findings of hypercholesterolaemia in obstructive jaundice and hypocholesterolaemia in haemolytic jaundice, attributed the direct reaction to the accelerating action of cholesterol. Van den Bergh and Muller [1916] noted the accelerating action of bile salts on the coupling, as later did Thannhauser and Andersen [1921]; but in vitro experiments of the former workers indicated that the presence of bile salts did not account for the direct reaction, and Andrewes [1924]

has advanced some evidence against ^{the} lipoid hypothesis. E. Adler and Strauss [1922] attribute the direct and delayed reactions to the hydrated or dehydrated state of the proteins in association with the bilirubin in the serum, and instance the enhancing effect on the colour of such substances as ammonium sulphate and caffeine-sodium salicylate. The hypothesis of Collinson and Fowweather [1926], that direct bilirubin is present in the serum probably as an ammonium salt, whereas delayed bilirubin is present as the free acid in a colloidal condition, is certainly one of the most suggestive that has been advanced, especially if kept to the more general terms of an alkali salt. Of course bilirubin is a dibasic acid and may theoretically occur as such, or as the mono- or dialkali salts. At the hydrogen-ion concentration of blood it is most probably there as a monoalkali salt and the assumption that the direct type is a monoalkali salt, for example sodium hydrogen bilirubinate, fits practically all of the findings. The assumption of the presence of colloidal bilirubin to account for the delayed type, fits equally ^{well} its solubility in chloroform, inability to dialyse, and greater stability towards oxidising agents. We shall

examine these hypotheses in more detail.

In certain endeavours to explain the direct and indirect tests it has been stated that "pure bilirubin" gives a direct test in alcohol and a delayed test in water. Bilirubin is, as a matter of fact, quite insoluble in water and an aqueous suspension shows no sign of coupling even after standing for twelve hours in presence of the diazo compound. An alcoholic suspension of bilirubin, with the reagent added, shows merely a trace of azo-bilirubin after standing twelve hours. I have likewise been unable to obtain the semblance of a direct test by adding varying amounts of sodium hydroxide-still maintaining the coupling medium acid. If, however, a very small quantity of bilirubin is just dissolved in sodium hydroxide and a few drops of 6 per cent. aqueous disodium hydrogen phosphate added, then mixing with the diazo-reagent is followed by immediate coupling in a definitely acid medium. Under these conditions it is possible to duplicate with pure bilirubin the direct reaction obtainable in serum. Similar buffering systems are present in serum and in all likelihood the direct van den Bergh reaction is attributable to the presence in the serum of sodium hydrogen bilirubinate. This

is in accordance with the hypothesis of Collinson and Powweather

It is less easy to devise conditions to obtain a delayed reaction with pure bilirubin. Consideration of this problem has led the writer to believe that the behaviour of delayed bilirubin can not be fully explained on the hypothesis that it is present in the serum simply as colloidal bilirubin. Colloidal bilirubin is presumably quite insoluble in alcohol, and the addition of alcohol to serum would not bring it into solution. In short, we must suppose that the delayed type of bilirubin is in solution in the serum, and even more securely in solution than the direct type; as there is some absorption of the latter on the addition of alcohol. We must regard the addition of alcohol to serum, not as a means for obtaining bilirubin in solution, but as a means for the precipitation of protein without precipitating the bilirubin. We may regard a chloroform solution of bilirubin as analogous to a "delayed serum". Coupling is almost negligible when the diazo-reagent is added to a chloroform solution of bilirubin (a delayed reaction), but after the addition of alcohol, we obtain an "indirect reaction". This would appear a somewhat remote analogy,

yet, when we find that for example, 0.1 cc. of a chloroform solution of bilirubin to which has been added 9.9 cc. of alcohol, then 0.5 cc. of diazo-reagent, reacts quantitatively ^(see p. 64) ~~in next section~~, whereas the same amount of bilirubin suspended in 10 cc. of alcohol gives no colour with the reagent, the conditions then approach what we might expect to find physiologically. The bilirubin solvent—it may be a lipoid or sterol—need be present in relatively small amount to retain the bilirubin in solution on the addition of alcohol. The solvent containing the bilirubin is probably insoluble in acid aqueous solution but soluble in alcohol. The action of alcohol in hastening coupling in the delayed serum, I should thus regard, not as one of breaking any complex of bilirubin with protein or other material, but ^{as} attributable to the mutual solvent action of alcohol for water and bilirubin solvent, and ^{sh} ~~w~~ould regard a chloroform solution of bilirubin as providing a crude model of a delayed serum.

On this hypothesis the bilirubin of the delayed serum is regarded as free bilirubin, or at any rate not combined with base as is most probably the case with direct bilirubin. It may, of

course, be in combination with its solvent, or some other serum constituent so that it resists salt formation and oxidation.

It would not be dialysable but extractable with chloroform. Such a hypothesis to account for the delayed type of bilirubin would appear to the writer to fit the findings better than the assumption of the presence of colloidal bilirubin.

We have the further consideration of the relationship of haematoidin to the delayed type of bilirubin. As previously observed, Fischer and Reindel [1923] have proved the chemical identity of haematoidin with bilirubin. Thannhauser [1922] reported that haematoidin did not couple with diazo-compounds but Fischer and Reindel found that in presence of alcohol it couples very slowly with benzenediazonium chloride to form a pigment similar to that from bilirubin, but on treatment with chloroform and alkali the coupled products show a characteristic difference. If benzene-azo-bilirubin in chloroform is shaken with alkali all the dye, of a bluish-violet colour, passes into the alkaline aqueous layer. But if benzene-azo-haematoidin is treated similarly, the colour changes to yellow and the chloroform layer retains a

weak yellow colour. The yellow alkaline extract is no longer changed to blue with acid. It is remarkable that this behaviour is found only in freshly prepared haematoidin. The dye formed from haematoidin 2-3 days old behaves exactly as the bilirubin azo-dye. Fischer and Reindel note that these observations must be taken with reserve. They have been unable to elucidate this behaviour but suggest that a primary combination is formed analogous to a diazoamino-compound, which secondarily goes to an azo-dye.

The behaviour of fresh haematoidin with diazo-compounds recalls the finding of Küster [1912] that freshly prepared bilirubin combines with four atoms of silver but older preparations combine with only two atoms of silver.

In this connection it is also to be remembered that the structure of bilirubin still remains in several respects obscure. Küster [1917] has suggested five possible formulae for the substance. Besides containing two carboxyl groups it contains two hydroxyl groups and probably occurs in both keto and enol modifications. Küster notes that none of the formulae suggested admits of the formation of either a mono- or bisazo- compound. Fischer

and Barrenscheen [1921] likewise note that the study of the azo-compounds of bilirubin is probably of much importance in the elucidation of ^{its} ~~the~~ structure of ~~bilirubin~~.

CONCLUSIONS TO PHYSIOLOGICAL

An attempt has been made to determine the probable nature of the two ~~kinds~~ of diazo-reaction for bilirubin obtainable in ~~normal or~~ icteric sera. The so-called direct reaction is most probably due to the presence of sodium hydrogen bilirubinate, and the so-called delayed reaction to the presence of free bilirubin in an undetermined solvent which ~~is~~ soluble in alcohol.

THE DETERMINATION OF BILIRUBIN BY THE DIAZO-REAGENTS

Van den Bergh [1918] made a standard solution by dissolving 5 mg. of bilirubin (Schuchardt) in 100 cc. of chloroform. One cc. of this solution was evaporated on the water bath and the residue taken up in 10 cc. of a solution containing 3 gm. NaCl and 0.6 gm. NaHCO_3 in a litre of 70 per cent. alcohol. To this were added 2.5 cc. of the diazo-reagent made as follows:

Solution A. 1 gm. sulphanilic acid, 15 cc. conc. hydrochloric acid, and water to one litre.

Solution B. 0.5 per cent. sodium nitrite.

The diazo-reagent is made by taking 25 cc. of Solution A and adding 0.75 cc. of Solution B.

[Note. For complete diazotisation 25 cc. of Solution A. requires 2.0 cc. of Solution B. The diazo-reagent here described is substantially the same as that used by Ehrlich [1883,2; 1886]. About two-thirds of the sulphanilic acid used remains as such, and under certain conditions some coupling occurs with the diazotised substance ^{giving} to ~~give~~ a brownish-red colour. Such a partially diazotised reagent ensures the absence of nitrous acid,

in the presence of which bilirubin is quickly changed in such a manner that it no longer couples. This action of nitrous acid may be explained on the assumption of the formation of the oxime of a pyrrole group [Fischer and Rose, 1912], or by an oxidising action similar to that displayed by nitrous acid in the Gmelin test].

As the bilirubin solution in chloroform was found to be unstable, van den Bergh devised the following standard:

0.1508 gm. ammonium iron alum dissolved in 50 cc. conc.

hydrochloric acid with water to 100 cc.

10 cc. of this solution with 25 cc. conc. hydrochloric

acid and water to 250 cc.

The colour standard was obtained by taking 3 cc. of this solution along with 3 cc. of a 10 per cent. ammonium thiocyanate solution and shaking out the iron thiocyanate with 12 cc. of ether. The ether solution was found to match the azo-bilirubin solution described above.

Another artificial standard has since been described by van den Berg and Muller [1927] which consists of 2.0 gm. anhydrous

cobalt sulphate dissolved in water to 100 cc. This is also stated to match the azo-bilirubin solution.

Fischer and Barrenscheen [1921] have criticised the

van den Bergh method of estimation in certain important respects.

On repeating van den Bergh's described procedure for the preparation

of the azo-bilirubin standard they found that the residue after

evaporation of the chloroform did not dissolve completely in the

sodium chloride, sodium bicarbonate, and alcohol mixture; that

more colour to the extent of 66 per cent. was obtained by adding

the diazo-reagent to a chloroform alcohol mixture than was given

by the solution obtainable by the van den Bergh procedure. They

also found that full colour production was not reached in the time

given by van den Bergh for coupling, and that complete coupling

was not attained even in 10 minutes, as evidenced by their finding,

that on addition of water and chloroform to the test solution, free

bilirubin was still extractable. They thus conclude that, useful

as the method may be for clinical purposes, (yet it) gives no measure

of the absolute amount of bilirubin in ^{simple} ~~pure~~ solution or in plasma.

Van den Bergh and Muller [1927] have subsequently ad-

vised that the chloroform standard should be evaporated, not to dryness, but so that the bilirubin remains dissolved in 0.1 to 0.2 cc. of chloroform. Apart from this concession the objections of Fischer and Barrenscheen have so far remained unanswered, and as they are of fundamental importance in the determination of bilirubin by the diazo-reagents, they obviously call for a re-examination of the subject. This I have attempted as follows:

Ten mgs. of bilirubin were transferred to a dry 100 cc. glass stoppered flask and about 90 cc. of pure chloroform added. After about a day, with occasional shaking, the substance completely dissolved. Chloroform was then added to the 100 cc. mark and the contents of the flask thoroughly mixed. Fischer and Barrenscheen found such a solution to be stable for over a year and I can attest that it is stable at least for several months.

[Note. The bilirubin used here was prepared by the Eastman Kodak Company, ^{Rochester, N. Y.} who kindly provided me with a description of its method of preparation. The substance was a microcrystalline bright orange powder, completely soluble in chloroform, and leaving no ash on ignition. There is no doubt that the material was of a high degree of purity.]

0.5 cc. of this solution was transferred to a small glass basin and evaporated to dryness in a water bath. The addition of 10 cc. of the van den Bergh bicarbonate-alcohol mixture dissolved only a trace of the residue, and subsequent addition of 2.5 cc. of the diazo-reagent gave only a perceptible pink colour. This amply confirms the statement of Fischer and Barrenscheen that bilirubin is practically insoluble in alcohol and that the addition of sodium chloride and sodium bicarbonate in the concentration mentioned, does not appreciably affect the question.

To 0.5 cc. of the bilirubin solution I next added 9.5 cc. of 96 per cent. alcohol followed by 2.5 cc. of diazo-reagent. A pink colour soon appeared which changed to a deeper reddish-purple after five to ten minutes. On comparing this in a test-tube side by side with the artificial standard of ethereal iron thiocyanate it was found that the colours were of almost the same intensity, as was also the standard made with cobalt sulphate, although all the solutions were of a different hue. The finding of Fischer and Barrenscheen is thus confirmed, yet at the same time van den Bergh

must have treated his standard chloroform solution in such a way that it was all obtained in solution.

Van den Bergh regards the method as providing an estimation rather than a quantitative determination of bilirubin in serum, and if, as stated by Fischer and Barrenscheen, the coupling is not complete, we can hardly hope for a satisfactory means of measuring the amount of bilirubin by the diazo method. ~~It appeared to the writer that the question had not been adequately examined and~~ ^{Nevertheless,} it was decided to test accurately the relation of the amount of colour developed to the amount of bilirubin in the test solution. In this connection, however, certain preliminary observations should be recorded:

When coupling takes place under the conditions described above, or in presence of the negligible amount of sodium bicarbonate recommended by van den Bergh, the colour developed is appreciably more blue than the iron thiocyanate solution, and very markedly more blue than the cobalt sulphate solution. Matching colours under these circumstances is open to a relatively large error.

The speed of coupling is markedly influenced by the

reaction of the medium. Under the above described conditions increase of colour over a period of 5-10 minutes is quite appreciable without recourse to a colorimeter. With more acid present the reaction is even more retarded and is probably not complete. The solution may, however, be buffered to a weakly acid reaction, under which conditions the coupling is apparently practically immediate and as will be shown complete. The azo-bilirubin colours are less stable in weakly acid solution than in ^{more} stronger ^{ly} acid solution.

The presence of relatively large proportions of chloroform in the coupling medium also retards coupling. Thus if to 0.5 cc. of the chloroform solution of bilirubin, 2 cc. of chloroform and 7.5 cc. of 96 per cent alcohol ^{are} ~~be~~ added, followed by 2.5 cc. of Ehrlich's diazo-reagent, (under which conditions we have still a homogenous solution), then less colour develops, and on the addition of water at the end of five minutes uncoupled bilirubin is found to be present in the chloroform layer.

These findings appear to account for the statement of Fischer and Barrenscheen that the coupling is not complete.

After various attempts to modify the conditions of coupling so that the reaction was likely to be rapid and complete the following method was adopted.

A solution of 6 gm. of $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ in 100 cc. is, on the basis of one equivalent of sodium, about one-sixth normal alkali. The diazo-reagent is about one-sixth normal acid due to hydrochloric acid. On mixing an equal volume of the diazo-reagent with the disodium hydrogen phosphate solution, we obtain a weakly acid solution buffered by NaH_2PO_4 . Under these conditions a faint reddish colour becomes perceptible after about ten minutes in the diazo-solution, due probably to the coupling with ~~undiazotised~~ sulphanilic acid in the solution. For this reason the diazo-solution is made immediately before using for each pair of tests in the following procedure. For example, to 4 cc. of the Ehrlich diazo-reagent, 4 cc. of the disodium hydrogen phosphate solution are added, from which 2.5 cc. were used in each test.

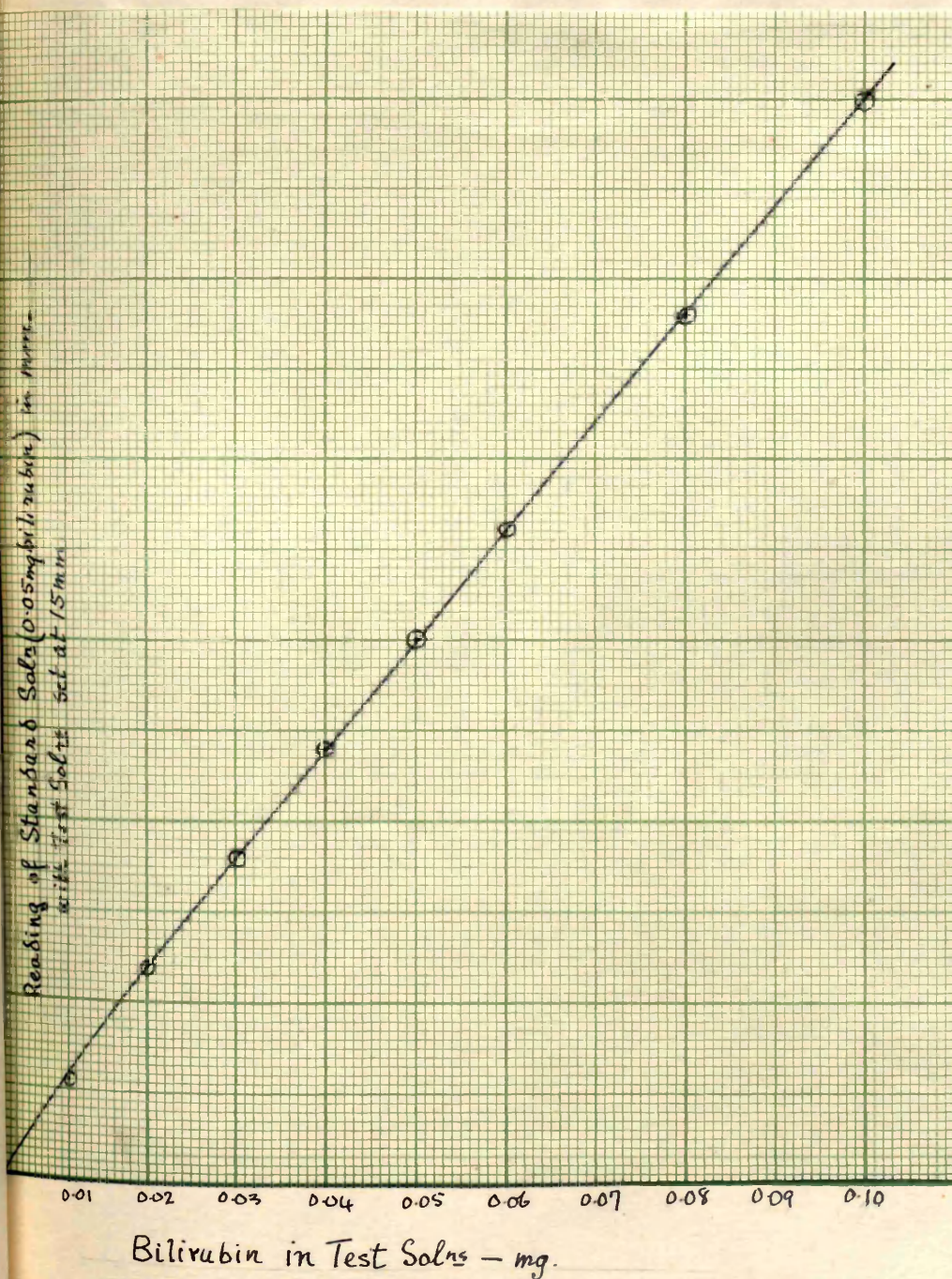
[Note. The concentration of diazobenzene-p-sulphonic acid is here of course only half that used in the Van den Bergh procedure, yet there is still present more than a thousand times the theoretical

requirement for 0.1 mg. bilirubin].

The method was tested over the range of 0.1 to 0.01 mg. of bilirubin, that is, using 1.0 to 0.1 cc. of the standard bilirubin solution in chloroform. 96 per cent. alcohol was added to make each volume 10 cc. and to this 2.5 cc. of the phosphate buffered diazo-reagent were added. The colour produced from each was read in a Duboscq colorimeter against a standard containing 0.05 mg. of bilirubin. To eliminate any error that might arise from fading, the standard was remade with each solution to be measured. To obtain a direct proportionality between the amount of bilirubin present and the amount of colour produced, each solution, which we ^{shall} ~~will~~ term the test solution, is placed in the left-hand cup of the colorimeter with the plunger set at a constant depth of 15 mm., and the standard solution is placed in the right-hand cup, and the depth of colour altered to match.

The solutions were in each case mixed and the colours produced in test-tubes. Suitable amounts of the fluids were then transferred to dry colorimeter cups and readings made without delay. The results are given in the following graph, from which it is seen

that there is a strict linear proportionality between the amount of bilirubin present and the amount of colour produced from it by the diazo-reagent.



The addition of water to the azo-bilirubin solutions ~~until~~ the separation of the chloroform, failed to reveal the presence in the chloroform layer of the slightest trace of bilirubin. The evidence is thus altogether in favour of the conclusion that, under the above described conditions, bilirubin couples quantitatively with diazo-benzene-p-sulphonic acid. The reagents in themselves have no colour and, as the colour produced is strictly proportional to the amount of bilirubin present, this diazo-reaction obviously supplies a method of the highest accuracy for the determination of bilirubin in simple solution. The colour obtained under these conditions from pure bilirubin matches the artificial cobalt sulphate solution much more closely than that obtained by the method of van den Bergh. The colour hues are, however, not sufficiently near to each other to admit of precise standardisation, but from a number of readings it was found that when the azo-bilirubin solution containing 0.05 mg. of bilirubin in 12.5 cc. was set at a depth of 15 mm., the cobalt sulphate solution read about 14.2 mm.; indicating that 1.9 gm. of anhydrous cobalt sulphate in 100 cc. provides a more accurate standard than 2.0 gm. in 100 cc. The amount given by McNee and Keefer [1925], that is 2.161 gm. in 100 cc.

is definitely too high.

[Note: Some care is necessary in drying $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$, the usual commercial salt. When heated for six hours in an electric oven at about 150° , the resultant apparently dry powder still contained a relatively large amount of water. When heated in a muffle furnace beyond a very dull-red heat there is ~~apparently~~ a slow decomposition of the sulphate and the production of a black insoluble oxide. The dry powder dissolves slowly in cold and easily in warm water. The standard solution is finally filtered from a trace of insoluble material].

The colour of the azo-bilirubin solution produced by the buffered diazo-^{reagent} also closely resembles in hue that obtained from serum. Indeed the conditions of coupling here may be regarded as very similar to those obtaining in an extract from serum. Such a standard solution may be used for the determination of bilirubin in serum, but there is much to be said for the use of a stable artificial standard such as is provided by the cobalt sulphate solution.

The colour values obtained by using the chloroform solution containing 10 mg. ^{bilirubin} in 100 cc. were checked by using another

~~chloroform solution containing 10 mg. in 100 cc. were checked by~~
~~using another~~ chloroform solution containing 25 mg. per 100 cc.

Determination of Bilirubin in Serum

The procedure of van den Bergh and Muller [1927] is as follows:

To a centrifuge-tube add 0.5 cc. of serum and 1.0 cc. of 96 per cent. alcohol. Centrifuge, and remove 1.0 cc. of the supernatant fluid

to a test-tube. To this add 0.5 cc. of 96 per cent. alcohol and 0.25 cc. of diazo-reagent. The colour is matched with that prepared from

a 1:200,000 solution of bilirubin, or with the artificial standards mentioned above. The amount of bilirubin present in the serum is

calculated by van den Bergh as follows: On the addition of 1 cc.

of 96 per cent. alcohol to 0.5 cc. of serum, the resultant volume is

found to be $\frac{10}{7}$; ^{and the solution thus $\frac{20}{7}$} the volume occupied by protein precipitate being

neglected. By the addition of 0.5 cc. of alcohol and 0.25 cc. of

diazo-reagent to 1 cc. of the supernatant fluid there is a further

dilution of $\frac{7}{4}$, so that the total dilution of the serum is $\frac{20}{7} \times \frac{7}{4} = 5$.

That is, the value found by comparison with the standard solution

must be multiplied by 5. If, for example, in the colorimeter a

given depth of test solution matches the same depth of standard solu-

tion, there will be present in the serum a concentration of

$$5 \times \frac{1}{200,000} \text{ of bilirubin.}$$

If the test solution had only one-fifth the colour of the standard then the concentration would be

$$5 \times 0.2 \times \frac{1}{200,000}.$$

For convenience, van den Bergh used for such an expression as this, simply the product of 5×0.2 , it being understood that it is further multiplied by $\frac{1}{200,000}$.

The method of expression is, despite this simplification, obviously a clumsy one, as a bilirubin value of, let us suppose 10, is meaningless in itself, and in its more complete form of 1 in 20,000 it still conveys to us little, as it is an unusual form of expression of the concentration of a blood constituent. The general adoption of McNee's translation of the term "Bilirubinwert" by "bilirubin unit" is even more unfortunate, as the term "unit" appears to have been adopted in physiology, especially since the standardisation of insulin, for purposes of biological assay. It thus connotes the lack of quantitative accuracy. In the writer's opinion the simplest ~~process~~ ^{way} is to express the concentration of bilirubin in the serum in the manner in which it has become the custom to express the concentration of other non-protein blood constituents, that is, as mg. per 100 cc. Had

the expression originally been in this form it is unlikely that a gross inaccuracy (of 20 per cent.) in the calculations of van den Bergh, could have hitherto escaped the notice of the various workers who have used his method. The error lies in the fact that van den Bergh describes his standard as one of 1 in 200,000, whereas it is actually one of 1 in 250,000 of bilirubin; the standard solution, containing 0.05 mg. of bilirubin in the form of azo-bilirubin, in a volume of 12.5 cc., ^(see p. 55) ~~and~~ not 10 cc. This means, caeteris paribus, that all the values in the literature to date are 20 per cent. too high.

The calculation is simply performed as follows: Since 12.5 cc. of standard solution contain 0.05 mg. of bilirubin then 100 cc. of standard contain 0.4 mg. of bilirubin. If the total dilution of the blood serum is taken as 1 in 5, and a given depth of azo-colour from the serum matches the same depth of colour from the standard, then 100 cc. of serum will contain 5×0.4 mg. or 2.0 mg. of bilirubin. For a normal human blood serum at this dilution the colour would amount to about $\frac{1}{10}$ of that of the standard; that is, one ^w should have $5 \times \frac{1}{10} \times 0.4$ mg. = 0.2 mg. of bilirubin per

100 cc. In general terms the total dilution of the blood serum
 \times ratio of the readings \times 0.4 = mg. of bilirubin per 100 cc. of
fluid under investigation.

Various modifications of the van den Bergh method for the determination of bilirubin in serum have been suggested. That which has received most attention is the modification of Thannhauser and Andersen [1921]. This was introduced to overcome the adsorption of bilirubin frequently encountered in sera from patients with obstructive jaundice. In this method 0.5 cc. of diazo-reagent is added to 1 cc. of serum, followed by 2.5 cc. of 96 per cent. alcohol and 1.0 cc. of saturated ammonium sulphate. The object of the method is to allow the formation of azo-bilirubin before protein precipitation, and thus eliminate adsorption. The main purpose of the incorporation of ammonium sulphate appears to be ^{to} complete protein precipitation.

From 1 cc. of serum the total volume is thus 5.0 cc., so that the dilution appears to be 1 in 5. It is, however, taken as 1 in 4 for the reason that only 0.2-0.3 cc. of the ammonium sulphate goes into solution, and the volume occupied by protein precipitate

may be taken as compensating approximately for this amount in excess of a 1 in 4 dilution.

Thannhauser and Andersen also used a stronger diazo-reagent, and converted the reddish azo-colour into a blue by the addition of conc. hydrochloric acid before reading. But these changes are of doubtful value and have not been generally adopted.

The main arguments for the adoption of this modification are that it simplifies the technique, and enables the worker to test at the same time whether the serum gives a "direct" reaction. In an acid medium the serum proteins do not affect the activity of ~~the~~ diazotised sulphanilic acid, and they provide a greater buffering effect on the coupling medium, thus leading to greater similarity in colour hue than is obtained under the van den Bergh conditions. It may also be accepted that the ^{Thannhauser and Andersen method} reduces adsorption, although in the writer's experience ~~they~~^{it} do not eliminate it.

When the icteric serum contains a relatively large amount of bilirubin, amounts smaller than 1 cc. of serum must be taken for the test, if quantitative accuracy is desired. Apart from the question of adsorption, it is perhaps unsafe to have in serum test solutions

a concentration of bilirubin greater than the maximum of the range in which I have shown that the reaction is strictly quantitative, that is, 0.1 mg. of bilirubin in a volume of 12.5 cc.

Using the Thannhauser and Andersen method in which 1 cc. of serum is diluted to approximately 4.0 cc., this means that not more than about 0.03 mg. of bilirubin should be present in the test portion of serum. In other words, if the serum has more than 3.0 mg. of bilirubin per 100 cc., an amount less than 1 cc. should be used to bring the concentration within the approved range.

In his monograph, van den Bergh had already made it clear that in bilirubin-rich sera dilution with water before the addition of alcohol leads to higher bilirubin values. This he attributed to decreased loss from adsorption, but the limit of concentration at which bilirubin actually in solution will react quantitatively with the diazo-reagent is probably also exceeded in such sera. The question of whether the van den Bergh procedure, with proper attention to dilution, gives a lower value than the Thannhauser and Andersen method, under the same conditions, does not appear to have been investigated. But certainly, accurate results can not

be obtained by either method without due regard to dilution.

The incorporation of ammonium sulphate appears to the writer to be of doubtful value. The additional protein precipitated by it is negligible and ~~its removal~~ of no apparent advantage to the determination. The presence of indoxyl in sera from uraemic patients is not detectable by the diazo method in the presence of ammonium sulphate (~~we~~ under indoxyl^{p94}). The procedure for routine work might well be simplified by adding in place of ammonium sulphate 0.3 cc. additional alcohol, thus: 1 cc. of serum + 0.5 cc. of diazo-reagent + 2.8 cc. of 96 per cent. alcohol; giving a dilution of approximately 1 in 4.

The quantitative recovery of bilirubin added to serum is shown by the following experiment. To two centrifuge-tubes add 0.1 cc. of chloroform containing 0.01 mg. of bilirubin. To one tube add 1.0 cc. of ^aserum practically free from bilirubin, along with 0.5 cc. of diazo-reagent and 2.8 cc. of 96 per cent. alcohol. Centrifuge. To the other add 3.8 cc. of 96 per cent. alcohol and 0.5 cc. of buffered diazo-reagent. The amount of colour developed in each case was found to be the same.

The precision with which bilirubin may be estimated in serum and similar body fluids rests then with ^{the} degree of accuracy desired by the worker rather than with the principles of the method. The routine method as outlined above is relatively accurate despite the very small amounts of substance measured. A low normal bilirubin value for human blood of say 0.1 mg. per 100 cc. is, of course, open to a relatively high percentage error,--perhaps 50 per cent--but when we consider that such a value may be obtained from 0.5 cc. of blood serum, or with 0.0005 mg. of the substance to be measured, ~~at the present stage of analytical chemistry,~~ we can hardly expect much better. When the amount present is greater than 0.5 mg. per 100 cc. the degree of accuracy then depends mainly on two factors, the measurement of the dilution, and the presence of pigments other than bilirubin in the serum. The main factors affecting the accuracy of the dilution figure are variability in the amount of protein in the serum, and change of volume on mixing alcohol and water. Interfering pigments could be compensated for by the use of such a colorimeter as that described by Bürker, [1927], but for almost all practical purposes the error from pigment interference is negligible.

Testing for Bilirubin in Urine by the Diazo-Method.

Ehrlich, [1883,1] observed that when normal urine is treated with an equal volume of diazo-reagent little change in colour takes place on mixing. When bile pigments are present in marked amount, however, an intense dark colouring results. On heating, the colour may change to a red-violet, the addition of a large amount of acetic acid sometimes bringing about the same colour change. From such a test solution ~~when~~ strongly acidified with hydrochloric acid, saturated with sodium chloride, and allowed to stand for a few days, a precipitate separates, which may be filtered, washed, and found to give colour changes similar to the azo-derivative prepared from bilirubin, [Ehrlich 1883,2]. Ehrlich further showed that the test was highly specific for bilirubin. Other bile pigments, as biliverdin, bilifuscin, biliprasin, bilihumin, and urobilin, failed to react with diazobenzene-sulphonic acid.

[Note: It has been stated by Davies and Dodds [1927] that biliverdin, perhaps the best defined of the above substances, gives an immediate colour in the presence of alcohol with the diazo-reagent, and Roberts [1928] records a similar observation. Such a finding is of

course contrary to a mass of previous evidence, and an examination of the above papers ^{suggests} ~~makes it appear probable~~ to the writer that the biliverdin preparations there used were not free from bilirubin. Tests by the writer with biliverdin, prepared by the Eastman Kodak Company, proved to be entirely negative. Dastre and Floresco [1899] state that biliprasin reacts like bilirubin, while Küster [1914] notes that dehydroxybilirubin, a non-crystalline oxidation product of bilirubin gives a colour with diazo-compounds. The possible presence of bilirubin in both products would account for the findings].

Pröscher [1901] notes that the detection of bilirubin by the diazo-reaction in icteric urine offers some difficulty. If to such urine hydrochloric acid and alcohol ~~are~~ added, followed by the diazo-reagent, a brownish-red colour results instead of a blue. There is no characteristic colour change on the addition of alkali. Pröscher supposes that in icteric urine another substance is present which gives the brown colour with the diazo-reagent, and he considers that this substance is not present in normal urine, as added bilirubin is detectable in the latter. Pröscher finally recommends saturation

of the urine with ammonium sulphate, dissolving the filtered precipitate in acid 96 per cent. alcohol, and adding the diazo-reagent. Under these conditions a blue colour, showing a characteristic behaviour with strong alkali, was obtained. The test, however, has never been adopted.

As the diazo-reagent has proved so valuable in the detection and estimation of bilirubin in other media it appeared worth while to re-examine the possibilities of obtaining a satisfactory test by this means, especially as existing clinical tests for bilirubin in urine are not very favourably regarded. I was soon able to confirm the earlier observations that in icteric urines containing large amounts of bilirubin the diazo-reagent gives rise to badly defined colours, and the addition of acids, or alcohol does not appreciably improve the colours. Contrary to the finding of Pröscher, I was unable to obtain a satisfactory test with normal urine to which a suitable solution of pure bilirubin had been added, and so would not explain the failure of the test by supposing that the interfering substance, (or substances), is excreted only in jaundice. It was apparent from such consider-

ations that some preliminary separation of the bilirubin is necessary before a satisfactory diazo-test can be carried out in urine.

It has long been known that full saturation of urine with ammonium sulphate precipitates bilirubin [Méhu, 1876]. The bilirubin ~~thus~~ obtained from the precipitate dissolves in slightly acid alcohol. The addition of the diazo-reagent to this alcoholic filtrate often gives a fairly satisfactory colour, although it is frequently changed or obscured by the presence of other pigments, especially urobilin; for full saturation with ammonium sulphate precipitates the greater part of ^{the} pigment in normal or pathological urines, besides a part at least of the uric acid. This method has the further disadvantage that the precipitate can not be centrifuged out on account of the high specific gravity of the mother-liquor.

The separation of bilirubin by calcium or barium salts in alkaline solution similarly leads to very impure and bulky precipitates, and its extraction with chloroform is uncertain and unspecific. For our present purposes the best method available for the precipitation of bilirubin from urine is that employed by

Nakayama [1902]. This procedure depends on the precipitation of bilirubin by the addition of a solution of barium chloride to the weakly acid urine. Cole [1919] adds a small amount of magnesium sulphate to favour the precipitation. Little or no pigment is discharged from normal slightly acid urine on the addition of barium chloride, and the centrifuged precipitate is nearly white. If a small amount of bilirubin in alkaline phosphate solution is added to such a urine followed by barium chloride, the centrifuged precipitate is perceptibly yellow in colour. If the supernatant fluid is poured cleanly off, and the precipitate stirred with a glass rod in presence of diazo-reagent and alcohol, the presence of bilirubin is indicated by the development of a pink colour.

In certain highly pigmented pathological urines which contain relatively large amounts of urobilin and unknown pigments, there is some coloration of the barium precipitate even in the absence of bilirubin. Nevertheless, under these conditions there is relatively little of the total urinary pigment precipitated, and the supernatant fluid gives practically as strong a test for urobilin as the native urine. There may however be sufficient pigment present in the

precipitate to vitiate to some extent the subsequent diazo-test. This difficulty, however, can be overcome in all urines so far examined by the writer, by extracting the barium precipitate with a little alcohol weakly acidified with phosphoric acid, adding about two volumes of water to the alcoholic extract, a few drops of ammonium sulphate solution, and finally reprecipitating with barium chloride. The resultant barium precipitate then gives a characteristic diazo-reaction if bilirubin is present in the original urine.

Pathological urines are so variable in composition that it is impossible to give directions covering all types for such a test as the present. Ten cc. of urine may be regarded as a maximum amount to use when only traces of bilirubin are present, and as little as 1 cc., diluted to 5 cc. with water, is sufficient in many icteric urines. In most cases the test is satisfactorily performed as follows:

To 5 cc. of urine in a 15 cc. centrifuge-tube, 2 cc. of 10 per cent. barium chloride are added, and the contents mixed and centrifuged. The supernatant fluid is poured off, the precipitate washed with a few cc. of water, again centrifuged, and the liquid poured off. 0.5 cc. of diazo-reagent is now added and the precipitate

stirred with a glass rod. Two cc. of 96 per cent. alcohol are then added and the contents mixed. As the mixture at this stage is almost free from buffering substances coupling is accelerated by the addition of 0.3 cc. of 6 per cent $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ solution (see p. 62). ~~previously described.~~ The colour thus obtained in the presence of bilirubin is of the same hue as that obtained ^{with} ~~by~~ the diazo-reagent from icteric serum.

Urine generally contains enough free sulphate for the precipitation of bilirubin by this method. The reaction of the urine need not be altered before addition of the barium chloride unless it is alkaline, in which case it should be made weakly acid with acetic acid.

In highly pigmented urines the barium precipitate is obtained as above. It is then stirred with 2 cc. of 96 per cent. alcohol to which has been added one drop of 10 per cent. phosphoric acid. The contents are centrifuged and the supernatant fluid transferred to another tube, along with 4 cc. of water. 2 drops of 10 per cent. ammonium sulphate are added, followed by 2 cc. of 10 per cent. barium chloride, and the bilirubin thus reprecipitated. The centrifuged precipitate is then treated with diazo-reagent, alcohol,

and sodium phosphate, as above. There appears to be very little loss in the process of reprecipitation.

It would appear to the writer that this test should prove useful for the detection of bilirubin in urine, especially when other tests yield inconclusive results. It is at least as sensitive as any other test devised (the Huppert-Cole test having been tried in parallel with it) and may be regarded as entirely specific for bilirubin, as indole and indoxyl (~~sup. 89~~) are not precipitated by barium chloride in acid solution. In certain bilirubin-free urines a slight brownish colour has been obtained on treatment of the barium precipitate with the diazo-reagent. For this reason the colour obtained should be tested with strong acid and alkali towards which the brown colour is inert. If the colour is due to azobilirubin, acid changes it to a purplish-blue, and alkali to a bright green colour. By this means positive proof can be obtained of the presence or absence of bilirubin in urine.

It would appear that with little change in technique the test could readily be adapted to the quantitative estimation of bilirubin in urine.

Summary to the Qualitative and Quantitative Determination
of Bilirubin by means of the Diazo-Reagent.

The basic principles of the method for the estimation of bilirubin by the diazo-reagent have been re-examined, and details given for the precise determination of bilirubin in pure solution or in blood serum.

The possibilities of detecting bilirubin in icteric urine by means of the diazo-reagent have been reinvestigated, and a satisfactory qualitative test, which might form the basis of a quantitative method, has been described.

Urobilinogen

Ehrlich [1883; 1884] called attention to a second diazo-reaction obtainable in acid urine, which, in contradistinction to the primäre Verdunklung given by bilirubin, he describes as the primäre Vergilbung or primary Eigelb reaction. When the urine is mixed with an equal volume of diazo-reagent an orange colour develops. When made alkaline with ammonia the colour is changed to sulphur-yellow, but the latter change is frequently masked by the presence of other pigments. He observed that under certain conditions, "with a certain excess of diazobenzene-sulphonic acid", the orange solution can be changed to a dye which in strong acid solution is a fluorescent blue-violet colour, in weak acid green, in weak alkali yellow and in strong alkali red. The substance giving the test was found to be extractable from urine by ether, is frequently found in diarrhoeal stools, and most frequently in the urine in cases described by Gerhardt as "urobilin icterus". From these findings he concluded [Ehrlich, 1886] that the substance giving the reaction is urobilinogen.

No further attention was given to the subject until Thomas [1907] showed that this reaction paralleled the Ehrlich p-dimethylamino-benzaldehyde reaction in urine, although it was found to be less

sensitive than the latter test.

Fischer [1911] , by sodium amalgam reduction of bilirubin, prepared a substance which he called hemibilirubin which is stated to form a dye with diazo-salts. This changes quickly in air to a red orange pigment. Hemibilirubin was shown by Fischer and Meyer-Betz [1911] to be the same substance as urobilinogen prepared by them in crystalline form from urine. Fischer [1912] states that urobilinogen is the substance giving the Ehrlich primary Eigels reaction in urine.

The colour-changes with acids and bases of the azo_urobilinogen described by Ehrlich have not again been recorded. It would appear probable that certain of these were obtained from ether extracts. I have been able partially to confirm Ehrlich's findings and with more suitable material it is probable that they may (be yet) completely confirmed. In a dark room 300-400 cc. of normal urine are passed into a beaker, made alkaline with a solution of sodium bicarbonate, and shaken with 20-30 cc. of pure chloroform in a separatory funnel. The chloroform emulsion is separated, centrifuged, and filtered through a dry paper, so that it is obtained quite free from droplets of water. The colourless chloroform solution is then evaporated to dryness at a low temperature,

and the residue taken up in 1-2 cc. of alcohol. The whole procedure is conducted with as little exposure to light as possible, but a pinkish brown colour is always present in the residue. A few drops of the alcoholic solution added to a little Ehrlich's aldehyde solution gives a bright red colour, even without heat.

If to 0.5 cc. of the alcoholic solution a few drops of Ehrlich's diazo-reagent ^{are} ~~is~~ added an orange colour develops immediately. This orange colour is quickly changed to a clear yellow on the addition of a few drops of ammonia, and more slowly to a dark red if a layer of strong sodium hydroxide is run below the solution. The red solution shows two strong spectral bands, one in the position of the urobilin band, and probably due to urobilin in the original solution, and a second poorly defined band of maximum darkness about $\lambda = 590\mu\mu$. This behaviour with weak and strong alkalies is in accordance with Ehrlich's findings.

With regard to the fact that both bilirubin and urobilinogen form azo-dyes it is interesting to note that an intermediate reduction product, mesobilirubin does likewise. The relationship of the three substances is shown; they differ by 4 hydrogen atoms -

$C_{33}H_{36}N_4O_6$ - Bilirubin.

$C_{33}H_{40}N_4O_6$ - Mesobilirubin.

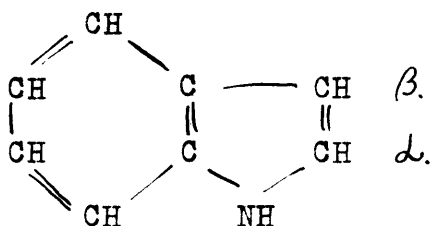
$C_{33}H_{44}N_4O_6$ - Mesobilirubinogen, Hemibilirubin, or
Urobilinogen.

As an explanation ~~for~~^{of} the formation of az^obilirubin it was suggested by Fischer and Barrenscheen [1921] that an azo-group might attach itself to an unsaturated side-chain in the bilirubin molecule, but the finding of Fischer and Niemann [1924] that mesobilirubin, which has saturated side-chains, couples with diazobenzene-p-sulphonic acid or with diazobenzene chloride to form reddish-violet dyes precludes this possibility. With diazobenzene chloride mesobilirubin forms shining leaflets m-p. 192° , of the composition $C_{33}H_{38}N_4O_6 \cdot (N_2 \cdot C_6H_5 \cdot HCl)_2$. Mesobilirubin-dimethylester forms a similar dye of analogous composition. m.p. 178° . The dye from mesobilirubin is more soluble in chloroform than the corresponding dye from bilirubin. In the case of mesobilirubin coupling is also complete. The colour changes of the mesobilirubin dye resemble those obtained with the bisazo-dye from bilirubin. The neutral or slightly alkaline solution is red, the strongly alkaline blue-violet, and the strongly acid blue-violet. The mesobilirubin dye changes colour more rapidly near the neutral point than does the bilirubin dye. The shade of colour in acid solution is

related to the dissociation of the acid used. Thus in glacial acetic acid ($K = 0.0018$) the dye dissolves with a violet colour, but with 90 per cent. acetic acid a reddish shade appears, and in dilute acetic acid the colour is purple-red. In oxalic acid ($K = 10.0$) even in great dilution the colour is still blue. The same is true for great dilutions of mineral acid. Fischer and Niemann thus conclude that the dye is dissociated in the red modification but undissociated in the blue. A similar explanation is perhaps more applicable to the colour changes of azo-bilirubin than that suggested by Pröscher (see p. 34) .

INDOLE, SKATOLE, AND INDOXYL.

Having considered the action of diazo-compounds on pyrroles it is in order to consider indole which from a structural aspect may be regarded as a fusion of the two cyclic structures benzene and pyrrole, and thus systematically called benzopyrrole, represented as



the substituents in the pyrrole nucleus being denoted as in α - or β -positions, as shown.

β -indole - α - aminopropionic acid, or tryptophane, by the action of intestinal bacteria gives rise to indole and a number of derivatives amongst which indole-propionic acid, indole-acetic acid (the chromogen of "urorosein"), β -methylinole or skatole and β -hydroxyindole or indoxyl have been found in the intestinal contents, in the faeces, or in the urine. Indole and skatole are normal constituents of human faeces, and indoxyl of human urine. Indoxyl occurs in the urine as "urinary indican", as a salt of indoxyl-sulphuric acid or indoxyl-glycuronic acid, and in certain plants as "indican" in the form of a

glucoside. Although all of these substances have a free methene group in the β -position, yet they do not all react with diazo-compounds; and in those that do there are notable differences in behaviour.

Of the substances mentioned above there appears to be no record of the behaviour of indole-propionic acid and indole-acetic acid towards diazo-compounds. It would be of interest to know their behaviour as the mother substance tryptophane does not couple with diazo compounds, whereas skatole does. The action of diazo-compounds on indole, skatole, and indoxyl may be considered at greater length.

Indole.

Benzeneazoindole was prepared by Pieroni [1921]. Madelung and Wilhelmi [1924] prepared benzeneazoindole by the gradual addition of a methyl alcohol solution of indole to a solution of benzenediazonium chloride made alkaline with sodium-carbonate. The substance, $C_{14}H_{11}N_3$, was obtained as yellow-brown crystals m.p. 133-134°.

Pauly and Gundermann [1908] found that indole couples readily with diazo-compounds in alkaline solution. There are a number of other records [e.g. Monfet, 1903; Herzfeld, 1922] that indole gives coloured solutions with the diazo-compounds, but little attention has

been given towards determining the best conditions for coupling. My own observations on the test are as follows:

20 mg. of indole (Kahlbaum)^{were} ~~was~~ dissolved in 10 cc. of 96 per cent. alcohol. One cc. of this solution was made to 10 cc. with water, and latest portions, containing 0.2 mg. of indole were used for the following tests. along with 0.5 cc. of Ehrlich's diazo-reagent,

- A. 1 cc. of test solution + 0.5 cc. of diazo-reagent → brown colour.
- B. " " " " + sodium acetate → clear yellow colour.
- C. 1 cc. of test solution + 0.5 cc. of diazo-reagent + 1 drop conc hydrochloric acid → deep purple colour.
- D. 1 cc. of test solution + 0.5 cc. of diazo-reagent + 0.1 cc. of 2.5 normal sodium hydroxide → yellow colour with orange tinge.
- E. 1 cc. of test solution + 0.5 cc. of diazo-reagent + Na_2CO_3 → yellow colour.

The best conditions for coupling thus appeared to be in presence of strong acid. The test under these conditions was repeated with 1 cc. of test solution containing 0.02 mg. of indole. A beautiful clear red solution is thus obtained, the reaction being promoted by heating for a short time in a boiling water bath. Under the same conditions 0.002 mg. of indole gives merely a trace of colour, but

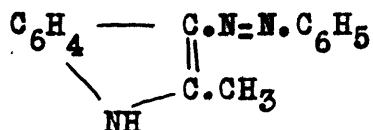
of the same purplish-red hue. The test is thus sensitive to a dilution of about 1 in 750,000, and compares favourably in delicacy with the best tests we possess for indole.

The purplish-red azo-colour in acid solution shows a general spectral absorption between the D and E lines. On adding a large excess of strong hydrochloric acid the colour remains predominantly red though acquiring a little more blue. On the addition of sodium hydroxide the azoindole rapidly changes to an unstable bluish-green colour.

It would not appear that the azoindole thus prepared by coupling in strong acid solution is the same substance as that described by Madelung and Wilhelmi [1924] by coupling in alkaline solution. The similarity in the behaviour of indole and pyrrole with respect to the reaction of the coupling medium is striking (see p. 27).

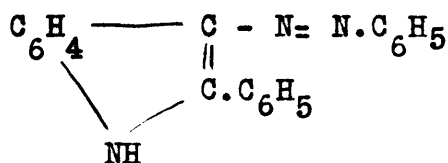
Skatole

It was observed by Emil Fischer [1886] that Δ -methylindole couples easily with diazobenzene chloride in presence of sodium acetate to form yellow needles of the constitution:



[Wagner, 1887].

Under the same conditions N-methylindole coupled^s fairly readily, but skatole reacts very slowly and gives rise to differently constituted products. Plancher and Soncini [1903] record similar observations with α -methyl- and with α -phenylindole. The latter does not react with phenylisocyanate, and thus has the azo-group in the β -position, (see p. 13):



The same workers observed that indole and skatole scarcely react with diazobenzene chloride in the presence of sodium acetate.

Pauly and Gundermann [1908] observed that diazobenzene-sulphonic

Pauly and Gundermann [1908] observed that diazobenzene-sulphonic

acid, in the presence of sodium hydroxide, gives coloured solutions

with indole and α -methylindole, but not with skatole, α : β -dimethylindole, nor with tryptophane.

My own observations with skatole are as follows:

A solution of skatole (Kahlbaum) containing 0.2 mg. per cc. was made as for indole and the following tests performed.

A. 1 cc. of test solution + 0.5 cc. of diazo-reagent \rightarrow yellow colour,

less marked than for indole.

- B. 1 cc. of test solution + 0.5 cc. of diazo-reagent + sodium acetate → more orange than for indole.
- C. 1 cc. of test solution + 0.5 cc. of diazo-reagent + 1 drop conc. hydrochloric acid → a buff colour, less intense than the corresponding indole colour.
- D. 1 cc. of test solution + 0.5 cc. of diazo-reagent + 0.1 cc. of 2.5 normal sodium hydroxide → no colour - confirming Pauly and Gundermann.
- E. 1 cc. of test solution + 0.5 cc. of diazo reagent + Na_2CO_3 → very pale yellow like the corresponding indole solution.

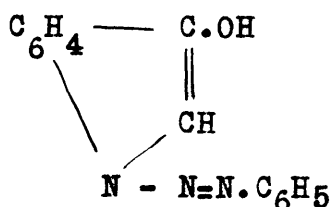
The addition of sodium hydroxide to the buff solution obtained under C was followed by the appearance of a yellowish-green colour.

Tryptophane gives no colour under any conditions with the diazo-reagent.

Indoxyl

By the action of diazobenzene chloride on an aqueous dilute hydrochloric acid solution of indoxyl, Baeyer 1883 prepared a compound of the composition $\text{C}_{14}\text{H}_{11}\text{N}_3\text{O}$, consisting of orange coloured prisms, m.p. 236°. In very dilute solutions he obtained a yellowish red colour, and recommended the reaction as a means of testing for small amounts of indoxyl. He regarded the compound as being of the probable

constitution



Heumann and Bachofen [1893] reprepared the same substance as well as p-sulphobenzeneazoindoxyl. They observed that the addition of sodium hydroxide to solutions of the buff azo-dye gives rise to a dark purple-red colour. The questionable constitution attributed by Baeyer to azoindoxyl does not appear to have been re-examined.

From a biochemical and medical aspect attention has in recent years been redirected to this test for indoxyl. When investigating the hypobilirubinaemia indicated by the van den Bergh test (v.Bilirubin) in uraemic sera, Andrews² [1924] found that a buff colour developed, and that this was changed by the addition of strong alkali to a bright red colour. The buff colour was found to be slow to develop at room temperature but Hewitt [1925] showed that the colour developed quickly on the application of heat. According to Hewitt the colour was not produced in presence of acetic acid. The writer with Montgomery [1927] showed that the inhibiting action of acetic acid is overcome by the addition of strong acid, from which observation the technique of the

test was improved according to the following excerpt from our paper; (No 9.)

"The test is performed by precipitating and centrifuging the serum or plasma with 2 volumes of 95 per cent alcohol, taking 1 cc. of the supernatant fluid in a small test tube, adding 0.4 cc. of the diazo reagent and 0.1 cc. of concentrated hydrochloric acid. The tube is heated over a flame, or in a water bath at about $80^{\circ}\text{C}.$, for a minimum of two minutes. If the test is positive the buff colour will then have fully developed. The tube is cooled and 1.0 cc. of 2.5 normal sodium hydroxide is added and the tube quickly inverted. The intensity of the red colour thus produced accords with the buff colour obtained in acid solution. As the buff colour is very stable it is the more suited for comparative tests. At any rate it can be preserved until it is convenient to add alkali to a series of tubes at the one time.

As originally pointed out by Andrewes the secondary red colour is produced only by the addition of strong alkali. Nor will this colour develop with strong alkali in the presence of a large amount of ammonia. So that ammonium sulphate can not be used in this test in association with alcohol to precipitate the proteins from the serum as recommended by Thannhauser and Andersen for the van den Bergh test.

As is true for the diazo reaction with bilirubin there must be no excess of nitrous acid in the diazo reagent used for the present test."

Hunter and Montgomery further showed that the test was obtainable with normal human urine.

Finally, Harrison and Bromfield [1927] showed conclusively that the so-called Andrewes' diazo-test is due to indoxyl. It is given by potassium indoxyl sulphate and by N-acetylindoxyl.

This very delicate test for indoxyl might well be made the basis of a method for the determination of "urinary indican" in urine or in pathological blood serum.

Part II- Diazo-reactions in an Alkaline Coupling Medium.

Introduction and Reagents.

As we have seen in Part I the only type of substance which reacts in an acid medium to give dyes with diazo-compounds is one containing the pyrrole nucleus. As aromatic amines, which form dyes under the same conditions, do not exist in biological material, diazo-reactions in acid solution are ~~thus~~ highly specific for pyrrole derivatives. In an alkaline coupling medium, however, various substances of different structure, such as phenols, iminazoles, thioliminazoles, pyrimidines, and purines, form dyes with diazo-compounds. Certain aliphatic substances, all apparently containing a tautomeric hydrogen, such as acetoacetic acid and acetaldehyde, give under certain conditions intense colours with diazo-compounds. Lastly may be mentioned the unknown substance called by Weiss "urochromogen" which is responsible for the true Ehrlich diazo-reaction of urine in certain febrile conditions.

From these typical substances we get in most cases typical diazo-reactions. The type of diazo-reaction is determined by such factors as the shade of colour, the speed of colour development, the stability of

the colour, its behaviour towards acids and alkalies, and other factors which will be mentioned under the different "types". It is our purpose here merely to outline and indicate methods for the differentiation of different types of diazo-reaction .

At the present point it is, however, necessary to discuss shortly the most suitable diazo-reagents to use under different circumstances. We have the choice of three main diazo-reagents now in use for the carrying out of so-called diazo-reactions. They are as follows:

(1) The Ehrlich diazo reagent is described on page 55.

(2) The Pauly reagent, consisting of solid diazobenzene-p-sulphonic acid, is prepared according to Pauly [1904], as follows:

2 gm. of powdered sulphanilic acid are shaken with 3 cc. of water and 2 cc. of conc. hydrochloric acid. 1 gm. of potassium nitrite in 1 to 2 cc. of water is gradually added within one minute to the cooled mixture. Most of the sulphanilic acid quickly dissolves and there appears at once in its stead a thick white crystalline precipitate of diazobenzene-sulphonic acid. After a few minutes this is filtered by suction and washed with a little water.

(3) The Koessler and Hanke reagent is made from two stock solutions

[Koessler and Hanke, 1919], as follows:

(a) Stock sulphanilic acid - 4.5 gm. of sulphanilic acid are dissolved in 45 cc. of 37 per cent. hydrochloric acid (sp.gr. 1.19) in a 500 cc. flask and water added to the mark.

(b) Stock sodium nitrite - 25 gm. of 90 per cent. sodium nitrite are dissolved in water and diluted to 500 cc.

The reagent is made from these as follows: 1.5 cc. each of the stock sulphanilic acid and sodium nitrite solutions are measured into a 50 cc. volumetric flask. The flask is then immersed in an ice bath for 5 minutes. Then 6 cc. more of the stock nitrite solution are added and the well mixed solution left in the ice bath for five minutes. Cold distilled water is then added to the mark and the flask returned to the ice bath where it is kept. The reagent should not be used for at least 15 minutes after diluting with water. It is stable for at least 24 hours.

It has been previously mentioned that there is an excess of sulphanilic acid in the Ehrlich reagent in order to ensure the absence of free nitrous acid; as in the presence of nitrous acid pyrrole compounds are changed in such a way that they no longer react with the diazonium compound. As we shall also see ~~the presence of nitrous acid: a marked effect~~ ^{decomposes} ~~on the azo-compound formed with urochromogen.~~ ^{(see p. 153).} ~~a character which has been~~

~~used to advantage by the writer in characterising the urochromogen type of diazo-reaction.~~ With phenols, iminazoles, thioliminazoles, pyrimidines, ^{and urochromogen} and purines, ~~however~~, the presence of excess of nitrite in alkaline solution has no effect on the reactions.

It is thus necessary to use a reagent free from nitrous acid ^{in an acid coupling medium,} when testing, pyrrole derivatives including bilirubin and certain substances related to it; *care must also be taken when working with a reagent containing free nitrous acid, that it is made alkaline before bringing in contact with urochromogen, substances capable of decamination, or substances otherwise affected by free nitrous acid.*

The chief disadvantage of Ehrlich's reagent lies in the fact that it gives a colour on the addition of alkalies, due to coupling with the unchanged sulphanilic acid which it contains. This is a serious disadvantage ~~in a colour test~~ and practically precludes its application as a *reagent for accurate work in neutral or alkaline coupling media.*

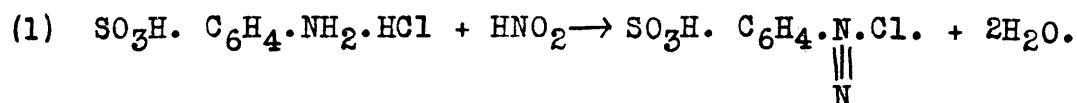
The use of solid p-diazobenzene-sulphonic acid in presence of sodium carbonate was recommended by Pauly [1904] as a delicate test for histidine, and it has since been widely adopted as a general test for the presence of the glyoxaline ring. Like the Ehrlich reagent, however, the substance prepared according to Pauly's directions quickly gives a yellow colour, which progressively intensifies in the presence

of sodium acetate or sodium carbonate, indicating the presence of unchanged sulphanilic acid. It is thus frequently difficult to determine, without recourse to controls, whether the colour produced is due to the reagents in themselves or to the presence of the substance under investigation. At any rate the final shade of the colour is altered by the underlying yellow or orange colour arising from the reagents. Pauly recommends the use of freshly prepared substance, although some workers use the dried powder which is fairly stable in the absence of light. The latter method is, however, not unattended by danger as the dried product has been known to explode violently.

The Koessler and Hanke reagent was devised for the colorimetric estimation of iminazoles and phenols. The sulphanilic acid used in its preparation is completely diazotised and the hydrochloric acid ~~originally present~~ completely converted into sodium chloride by the large excess of sodium nitrite added. If we examine its composition it appears difficult on theoretical grounds to justify the large amount of sodium nitrite used - some seventy times more than the theoretical requirement for complete diazotisation, and more than three times the amount necessary to neutralise the hydrochloric acid in the sulphanilic acid solution.

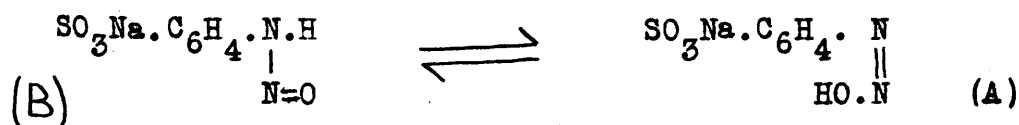
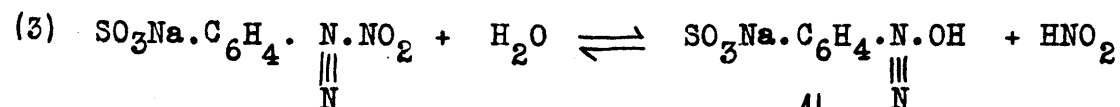
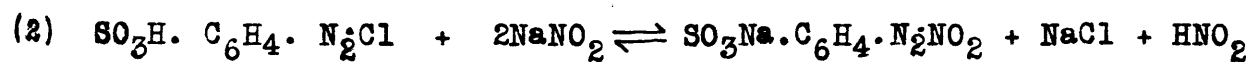
50 cc. of the reagent contain approximately 0.0135 gm. of sulphanilic acid (diazotised), 0.08 gm. of sodium chloride, and 0.27 gm. of sodium nitrite, with some free nitrous acid.

The main reaction in the first stage of the preparation is formulated as:



The nitrous acid used here, however, amounts to about fourteen times the requirement for the above equation and some neutralisation of the free hydrochloric acid must also occur.

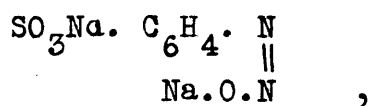
The addition of the final 6 cc. of sodium nitrite solution leads to changes formulated by Koessler and Hanke as follows:



One or both of (A) and (B) are regarded as the active coupling agents.

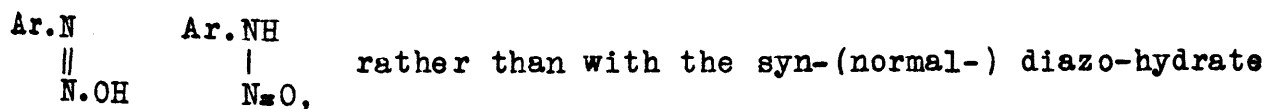
The addition of alkali to the reagent neutralises the excess of nitrous acid and displaces the equilibrium represented in equation (3) towards the right, leading to an increase in the concentration of the active

coupling compounds (A) and (B). With strong alkali such as sodium hydroxide there would be the formation of the disodium salt:



but with weak alkali any disodium salt formed would be rapidly hydrolysed by water back to (A), since the normal diazo-hydrate (A) is a weak acid.

The above explanation by Koessler and Hanke of the underlying principles of their reagent is for the most part acceptable. The assumption that the nitrosamine isomer (B) is an active coupling constituent is perhaps open to question as the nitrosamines are coloured (yellow) compounds and couple very slowly with phenols. They are further generally regarded as in equilibrium with the anti-(iso-) diazo-hydrate (which also couples slowly) represented as:



(A) as represented by Koessler and Hanke. (see Sidgwick, The Organic Chemistry of Nitrogen, 1910 p. 270). The yellow colour which appears in old reagents is perhaps partly due to nitrosamine formation.

We may thus regard the active coupling constituent of the Koessler and Hanke reagent as the syn-(or normal-) diazo-hydrate (A), which indeed

is generally regarded as the active coupling constituent of any diazo-reagent in an alkaline medium. The essential features of the reagent are that it contains no undiazotised sulphanilic acid, and thus gives no colour with sodium acetate or with sodium carbonate, and secondly, it contains no strong acid. A reagent embodying these features should be obtainable with about one third the amount of sodium nitrite employed by Koessler and Hanke, but as it is, the reagent is eminently suited for the purposes for which it was devised and it is doubtful if the labour entailed in testing new modifications would justify itself.

The Koessler and Hanke reagent can be used for all purposes for which the Pauly reagent has been previously used, and in the writer's opinion the latter can now be advantageously dispensed with.

Outline of different types of diazo-reaction in alkaline solution
(to be later discussed at length) with special reference to the
writer's own work.

1. The Phenol-type.

Chemical.

Discussion of (a) the "laws" regulating the formation of
hydroxyazo-compounds.

(b) the intermediate products in the formation
of hydroxyazo-compounds.

(c) the structural formulations of the o- and p-
series of hydroxyazo-compounds.

(d) factors governing the speed of formation of
hydroxyazo-compounds. [Goldschmidt and Merz, 1897].

Colorimetric detection and estimation of phenols by means of diazo-
reagents.

The methods of Hanke and Koessler [1922.1].

From 0.01 to 0.03 mg. of the phenol is used in the estimation. The
p-hydroxyazo-compounds are predominantly yellow; the o-hydroxyazo-
compounds red. Under the conditions of the determination the time of

colour development is in most cases fast, compared with other compounds to be dealt with.

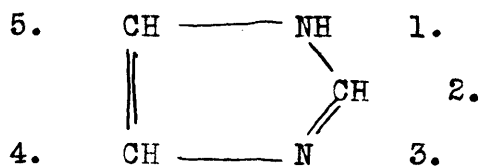
The artificial colour standards made from Congo red and methyl orange by Hanke and Koessler require revision in the light of the writer's findings [Hunter, 1925], (see Paper.No.7).

Physiological.

- (a) The fractionation of different types of phenol in biological or bacteriological material according to Hanke and Koessler [1922,2].
- (b) Phenols in urine and blood, and the degree to which they affect diazo-reactions in such fluids.

2. The Iminazole or Glyoxaline Type.

The iminazole or glyoxaline ring is represented and numbered as follows:



the iminic-hydrogen being attached to either N-atom. The chief iminazole in biological material is the amino acid histidine (α -amino ^{$-\beta$} -4(or-5)-iminazole-propionic acid), and the various other iminazoles may be regarded as derivatives of it. Thus in vertebrate muscle carnosine, or alanylhistidine, frequently occurs in relatively high concentration, and in invertebrate muscle a related iminazole derivative, called avertebrin, has recently been described by Kutscher and Ackermann [1926]. By the action of certain bacteria on histidine we may also obtain β -4(or-5)-iminazole-propionic acid, histamine, and urocanic acid.

Trimethylhistidine is obtainable from certain mushrooms (see Barger, The Simpler Natural Bases, 1914).

Chemistry.

Among the chief workers in the history of the chemistry of the azo-iminazoles are Rung and Behrend [1892], Burian [1904], Pauly [1904],

~~Congress 1900~~, and above all Pyman and his associates. We owe to the latter indeed a relatively complete knowledge of the laws of coupling in the iminazole ring, and much miscellaneous information on the behaviour of individual iminazoles is to be found in *his* numerous papers in the Journal of the Chemical Society from 1910 onwards. Fargher and Pyman [1919], in reviewing the position, thus conclude "--that glyoxalines, in order to be capable of coupling, must contain a free imino-group and also a hydrogen atom, or some other displaceable group, such as a carboxyl group, in one of the 2-, 4-, or 5- positions, and that the arylazoglyoxalines hitherto prepared are C-azo-compounds."

Colorimetric detection and estimation of iminazoles by means of diazo reagents.

Diazobenzene-p-sulphonic acid dissolved in sodium carbonate as recommended by Pauly has been the most widely used reagent both in chemical and biochemical work for the detection of iminazoles.

Koessler and Hanke [1919,1], by the use of their reagent already described (see p. 99), devised a method which they showed to be accurate for the estimation of histidine, histamine, iminazole-propionic acid,

iminazole-acetic acid, and methyliminazole. They found that as a group colour production is slower than with phenols. In all cases the colour is predominantly red though influenced by the groups in the side-chain. Both amino- and carboxyl- groups tend to yellow colours.

They further showed that the intensity or amount of colour produced by the iminazole ring depends on the side chain, as evidenced by the different "molecular-colour-values" obtained. [The molecular-colour-value is the calculated length of the column in mm. of a certain standard (in this case a mixture of methyl orange and Congo red) which is necessary to match the colour produced by one molecular weight of the iminazole; e.g. 0.01 mg. of histidine gives a reading of 7.4 mm; whence the molecular-colour-value of histidine (molecular weight = 155) is:

$$155 \times 7.4 \times 10^5 = 114.7 \text{ million mm.}]$$

The following values have been determined:

	Weight, mol.	Molecular-colour- value Million mm.	Determined by
Histidine	155.0	114.7	Hunter [1925,2].
Histidine monohydrochloride	209.5	113.1	Hunter [1925,2].
Histidine ^{hydro} dichloride	228.0	114.0	Koessler and Hanke [1919,1].
Histamine	184.0	138.0	" "

	Weight, mol.	Molecular-colour- Value Million mm.	Determined by
Iminazole-propionic acid	140.	191.0	Koessler and Hanke [1919,1].
Iminazole-acetic acid (as hydrochloride)	162.5	182.0	" "
Methyliminazole	82.0	201.0	" "
Carnosine	226.0	140.1	Hunter [1925,2].
Carnosine nitrate	289.0	141.6	" "
Urocanic acid $\cdot 2H_2O$ 2	174.	109.0	Hunter [1925,1].

The differences thus found in molecular-colour-value are suggestive from a chemical aspect. It is remarkable that methyliminazole should give nearly twice the amount of colour given by urocanic acid or histidine. As observed by Koessler and Hanke both the shade of colour and the intensity or amount of colour are influenced by the side-chain. All of the iminazoles above examined are similar to the extent that the side-chain is always in the 4-(or 5-) position and they differ only in the constituents of the side-chain. It thus appears reasonable to assume that the molecular-colour-values are entirely dependent on the constituents of the side-chain. The chemical composition of the azo-iminazole in solution has not been determined. According to Pyman's findings it is.

most probably a C-azo-compound. It may be a mono- or bisazo-compound. but, judging from the strict proportionality between the amount of iminazole used for the test and the intensity of colour produced, it is unlikely that it is a mixture.

The diazo-method for the estimation of carnosine in simple solution has been examined and found to be satisfactory by the writer, as shown in Paper, No.1.

Physiological.

- (a) Application of the diazo-method for the determination of histamine in culture media, with methods for the fractionation of different iminazoles [Koessler and Hanke, 1919, 2 ; 1919, 3 ; Hanke and Koessler, 1922].
- (b) The estimation of histidine in protein by the diazo-method [Hanke and Koessler, 1920].
- (c) The estimation of carnosine in muscle by the diazo method is critically examined by the writer in Paper, No.2.

Certain questions on the accuracy of the method have been treated in the writer's Papers Nos. 4 and 6.

The accuracy of the writer's methods and carnosine standards have been recently confirmed by Kuen [1927].

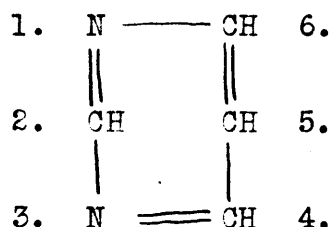
The necessity for the use of pure dyes as artificial standards in colorimetric work in relation to diazo-methods has been pointed out in the writer's Paper No.7.

(d) The determination of iminazoles in urine by the diazo-method.

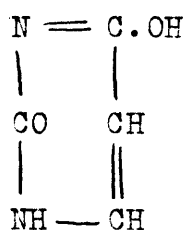
[Koessler and Hanke, 1924; Hunter, 1925, 1] . The writer's Paper No. 6 may be referred to.

3. Pyrimidine Diazo-Reactions.

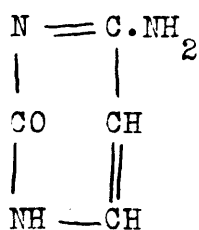
Pyrimidine is numbered thus:



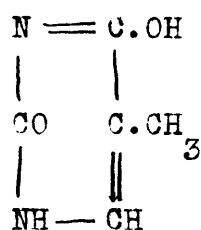
It is represented in the nucleic acids by uracil, cytosine, and thymine, which may be written as:



uracil



cytosine



thymine.

though also existing in other tautomeric forms.

The behaviour of pyrimides ⁱⁿ with diazo-compounds has been most closely studied by Johnson and Clapp [1908-'09]. Uracil, cytosine, and thymine, were all found to give colours when mixed with diazobenzene-sulphonic acid in presence of excess of sodium hydroxide. Of these thymine gives the most intense colour. From an examination of various derivatives of uracil, cytosine, and thymine, it was found that no reaction occurs if the iminic hydrogen in position -3 is replaced by a methyl group.

6-hydroxy-and 6-aminopyrimidine do not couple, so it is suggested that the grouping-CO-NH must be present in the 1:2 or 2:3-positions of the pyrimidine ring to enable coupling.

That the coupling does not occur in the 4- or 5- positions is shown by the findings of Evans [1893] that 2-oxy-4:6-dimethylpyrimidine gives a red compound, of Pauly [1904] that 4-methyluracil gives an intense colour and of Johnson and Clapp that 4:5-dimethyluracil gives a red colour.

Johnson and Clapp made an unsuccessful attempt to isolate the reaction product of thymine and diazobenzene-sulphonic acid. The nature of the linkage thus remains obscure although the available evidence appears to favour the view that coupling takes place in position-3; in which case the reaction products would be N-azo-compounds.

Steudel [1904] first observed that thymine couples with diazo-compounds in presence of sodium hydroxide.

The method of testing recommended by Johnson and Clapp is:

5- 10 mg. of the pyrimidine and an equal weight of diazobenzene-sulphonic acid prepared according to Pauly [1904] are mixed together with a glass rod in a dry watch glass. A drop of 10 per cent. sodium hydroxide is then allowed to flow on the mixture. The red colour develops immediately under these conditions.

The writer has repeated the tests of Johnson and Clapp. with uracil, cytosine, and thymine, and finds their behaviour as described by these workers.

[Note: I am indebted to Prof. Treat B. Johnson of Yale for a gift of synthetic thymine] .

It should be noted, however, that the colours are obtained only with relatively large amounts of the pyrimides ⁱⁿ in question. The sensitivity of the tests can be raised slightly by the application of heat to the solutions, but it is unnecessary to emphasise this in view of the much more delicate test for thymine which has been discovered by the writer, and which will now be described.

A Delicate Diazo-Reaction for Thymine, which is not given
by Uracil or Cytosine.

Reagents Required:

- (1) The Koessler and Hanke diazo-reagent described on page 99.
- (2) 1.1 per cent sodium carbonate solution.
- (3) 3.0 normal sodium hydroxide solution.
- (4) 20 per cent. hydroxylamine hydrochloride in water.
- (5) Solution of thymine. Here used 11.4 mg. of thymine in 25 cc. of water.

The test is performed as follows:

To 2.5 cc. of sodium carbonate solution add 1 cc. of the diazo-reagent. At the end of one minute add 0.5 cc. of the thymine solution. Allow to stand for 5 minutes, after which period a faint yellow colour is present. Add 1 cc. of 3 normal sodium hydroxide and let stand ^{for} 1 minute. Then add 1 drop of the hydroxylamine solution and mix quickly. An intense red colour rapidly develops. It is stable for several hours.

The sensitivity of the test was determined by using decreasing amounts of thymine. It was found that a marked colour is still obtainable with less than 0.01 mg. of thymine present in the test solution.

The test is not given by as much as 0.5 mg. of uracil or cytosine, although yellow colours are produced by these in contact with the reagent and sodium carbonate.

It is necessary that coupling take place in sodium carbonate solution, as no colour is obtained at the above dilutions with excess of sodium hydroxide even on the addition of hydroxylamine.

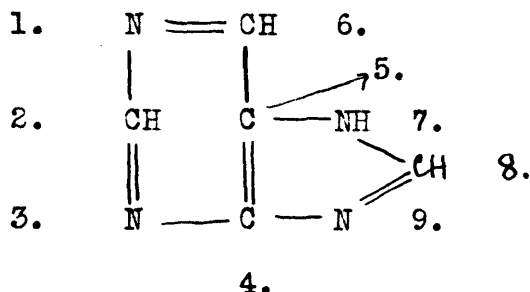
The test would appear to be of value in distinguishing thymine from certain other pyrimidines. It is at least 100 times more delicate than any other test which has been described for thymine. It is, however, not

specific, as it is given by those substances which will be dealt with under the "Keto-enol" type of diazo-reaction. Further relationships of the test will also be considered there. (see p. 143).

4. Purine Diazo-Reactions.

Chemistry.

The purines have the basic structure and numbering as follows:



The purine skeleton may thus be regarded as a fusion of the pyrimidine and iminazole rings at the 4- and 5- positions, and indeed Burian [1904,1,2] regarded the reaction products of iminazoles and of purines with diazo-compounds as of the same type. Following Rung and Behrend, ~~1892~~ Burian regarded the azo-iminazoles as coupled through the iminic-hydrogen in the 1-position of the iminazole nucleus, and thus as N-azo-compounds; and on the evidence that purines substituted in the 7-position (corresponding to the 1-position in the iminazole ring) do not couple with diazo-compounds, he concluded that purines which do react with diazo-compounds couple in the 7-position. Hans Fischer [1909], however, showed that the p-dichlorodiazobenzene derivatives of theophylline, of xanthine, and of guanine, are all reduceable to the corresponding 8-aminopurines,

and thus proved that purines couple in the 8-position. The method has been patented by Kalle and Co. [1911] for the manufacture of 8-aminopurines. As we have indicated, Pyman and his associates have shown that iminazoles couple only in the 2-, 4-, or 5- positions, and as only the 8-position (the 2-position in iminazoles) is available in purines the analogous behaviour of iminazoles and of purines towards diazo-compounds still remains. It is further of interest to observe that Burian's negative findings with 7-methyl-purines is paralleled by Pyman's finding that the iminic-hydrogen in this position in the iminazole ring is necessary in the coupling process.

The position assigned to the azo-group in purines by Burian was called in question by Steudel [1904] on the ground that, since thymine was found to react with diazo-compounds, the coupling of purines might take place through the pyrimidine ring. Pauly [1904], as we have mentioned (p. 115), confirmed Steudel's findings. Burian [1904, 1], however, pointed out that substitution in the pyrimidine ring did not affect the power of purines to react with diazo-compounds. Thus theopylline (1:3-dimethyl-2:6-[&]oxypurine) couples readily, but theobromine (3:7-dimethyl-2:6-[&]oxypurine) and caffeine (1:3:7-trimethyl-2:6-[&]oxypurine).

both with methyl groups in the 7-position of the purine ring, do not couple.

Burian also found that uric acid (2:6:8-trioxypurine) does not couple, although the 7-position is open.

It would thus appear that a purine in order to be capable of coupling with diazo-compounds must have both the 7- and 8-positions open.

Burian prepared crystalline p-sulphobenzeneazo-derivates from xanthine, theophylline, guanine, hypoxanthine, and adenine [1904, 2; 1907]. Xanthine, theophylline, and guanine, couple even in great excess of sodium hydroxide to form orange coloured products. Hypoxanthine and adenine form yellow azo-derivatives, and adenine does not couple in an excess of strong alkali.

The position in which purines couple with diazo-compounds has been of interest in determining the point of attachment of the purine to phosphorus in the nucleic acid molecule, as nucleic acid itself does not couple with diazo-compounds.

Colour-tests and Physiological.

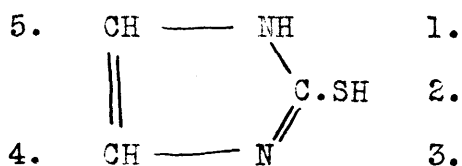
The writer [Hunter 1922] has briefly dealt with the behaviour of guanine, adenine, xanthine, and hypoxanthine, towards the Koessler and Hanke

reagents (see Paper No 2). It may further be noted that the conditions (if they exist) for the quantitative coupling of small amounts of purines has not yet been determined. Further work may improve the present unsatisfactory colour tests obtainable with purines and diazo-compounds.

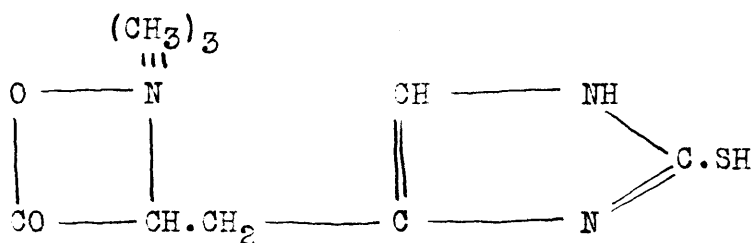
It is of interest to note that uric acid, which does not couple with diazotised sulphanilic acid, reacts to give a bluish-green colour with diazotised p-nitraniline in presence of sodium hydroxide, and in the absence of air. The writer has confirmed this test, first described by Riegler [1897].

5. The Diazo-Reaction of Thioliminazoles (Thiolglyoxalines)

Thioliminazole is represented as:

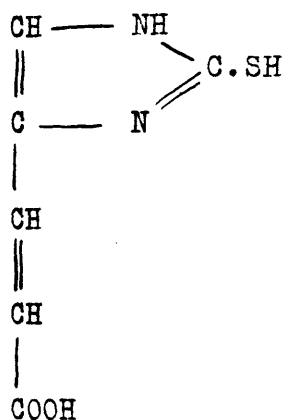


The only known derivative in biological material is ergothioneine,

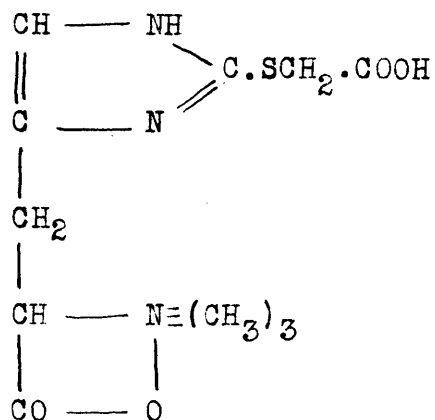


for which the writer discovered the peculiar type of diazo-reaction described in ~~the following~~ Paper No.8.

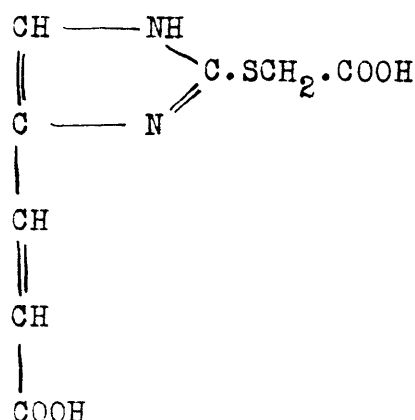
subsequent to the publication of ~~the foregoing~~ ^{No. 8} Paper, the writer has had the opportunity of testing the additional thioliminazole derivatives listed below.



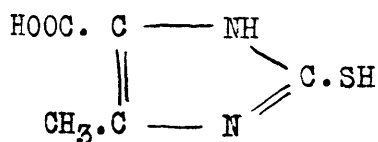
(I)



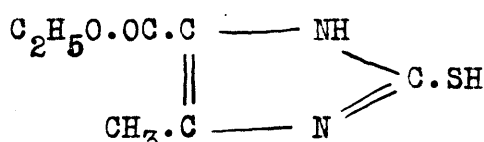
(II)



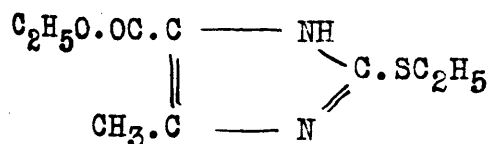
(III)



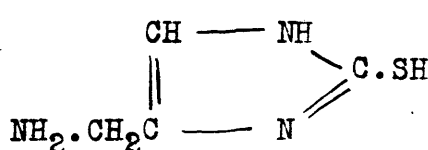
(IV)



(V)



(VI)



(VII)

Pauly's test and the Koessler and Hanke test for iminazoles and the writer's test for ergothioneine were applied to each of the above substances. Of these only (VI) shows no colour with any of the tests

while (V), in relatively high concentration (about 1 mg.), gives only a faint yellow colour with the Pauly and Koessler and Hanke tests. Three of the substances, (I), (IV), and (VII), give the writer's test for ergothioneine. The colour obtained with substance (I) is practically identical with that described for ergothioneine. The colours obtained from substances (IV) and (VII) match each other very closely and are of a pinkish-orange hue, rather similar to ^adilute solution of sodium alizarine sulphonate at the neutral point; the colours are much less blue than in the case of ergothioneine and substance (I).

With the Koessler and Hanke test substances (I), (IV), and (VII), give like ergothioneine, clear yellow colours with no sign of red after standing for 5 minutes or more. In the case of substances (IV) and (VII) coupling is more rapid than with ergothioneine. With substances (II) and (III), where the thiol-hydrogen is replaced by acetic acid, the writer's test is negative. Substance (III) gives a clear pink iminazole test with the Koessler and Hanke reagents, although, rather surprisingly, substance (II) gives a very yellow colour. This may be accounted for by the presence of trimethylamine in the side-chain.

The Pauly test gives results parallel with the Koessler and Hanke test,

but in the former, the yellow colours are overlaid with a certain amount of red from the reagents, and distinction is more difficult.

We may thus conclude that the writer's test for ergothioneine is a general one for the thio^oiminazole ring provided it contains a free sulphhydryl-group in the 2-position and has at least one of the 4- or 5-positions open, or containing a displaceable carboxyl-group. A free iminic-hydrogen may also be necessary. It is also clear that the presence of a thiol-group in the 2-position alters the reaction of the glyoxaline ring with diazo-compounds in such a way that it can no longer be identified by the glyoxaline type of diazo-reaction. That the thiol-hydrogen plays a part in the interference is indicated by the finding of a characteristic glyoxaline diazo-test with substance (III) but a negative test (a yellow, not a red colour) with substance (I).

Acknowledgments. I am much indebted to Dr.Blythe A.Eagles for small samples of the substances (I), (II) and (III), prepared by him in Prof. Treat B.Johnson's laboratory at Yale; to Dr.Harold King of London for specimens of the substances (IV), (V) and (VI); and to Professor F.L. Pyman for substance (VII).

Dr.Eagles has also informed me that he has applied my test to

thiohydantoin, thiotyrosine, and thiophenol, with negative results.

This is valuable contributory evidence to the high specificity for the thioliminazole ring, claimed for the test.

Physiological.

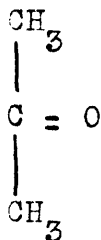
Certain physiological aspects of the test have been dealt with in the attached reprint.

The test has found perhaps its most interesting application in the hands of Eagles and Vars [1928], in an investigation of the origin of ergothioneine in blood. Their preliminary communication indicates that the occurrence of ergothioneine in blood is fortuitous and dependent on the diet. Their investigations suggest that there is present a thioliminazole nucleus in protein of plant origin but not in protein of animal origin, as there is present in the hydrolysate of the former a constituent which gives the thioliminazole diazo-test.

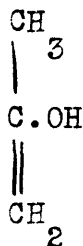
6. Keto-enol Types of Diazo-Reaction.

(1) Keto-enolic Tautomers.

Substances giving this type of diazo-reaction are capable of existing in at least two tautomeric forms. We may take as an example acetone which may be regarded as an equilibrium mixture of the keto and enol forms: ~~We may take an an example acetone which may be regarded as an equilibrium mixture of the keto and enol forms.~~



Keto form

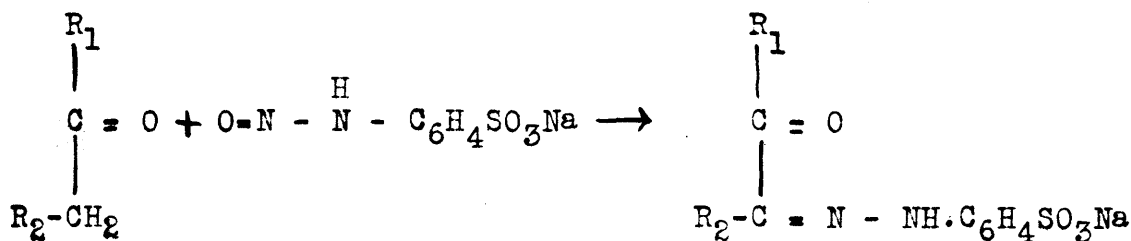


Enol form

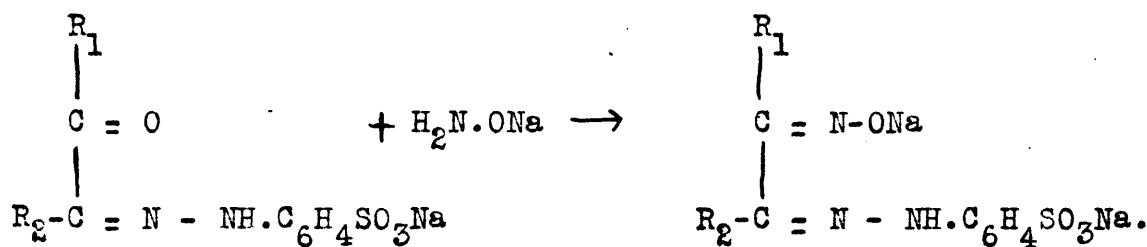
A suitable method for obtaining the test with acetone is described by Hanke and Koessler [1922,1] . . .

To a test-tube add 5 cc. of 1.1 per cent. sodium carbonate and 2 cc. of their diazo-reagent. After 1 minute add 1 cc. of an aqueous solution containing about 0.5 mg. of acetone and allow the reaction to proceed for 5 minutes. Then add 2 cc. of 3 normal sodium hydroxide and after 1 minute 0.10 cc. of a 20 per cent. aqueous solution of hydroxylamine hydrochloride and mix. A purplish colour immediately develops. If the

reaction with the diazo-reagent and sodium carbonate is allowed to proceed for 15 minutes a much greater final colour is obtained. Conditions for full colour development have not been determined. Hanke and Koessler have suggested the following formulation of the reactions taking place:



Pale Yellow



Red

The final red product they regard as a hydrazine^{ox} derivative. We shall return to a discussion of the probable reactions in the test.

The test for acetone, acetaldehyde, and acetoacetic acid, described by Hanke and Koessler would not appear to be a new one, although hydroxylamine may not have been hitherto used for the production of the final colour. The history of the test is of some interest.

Shortly after the publication of Ehrlich's first paper on the diazo-

reaction of the urine in typhoid fever [Ehrlich 1882] Penzoldt [1883,1;1893,2] and Petri [1883] independently found that under certain conditions the urine of diabetic patients gives a marked diazo-reaction. Both authors traced this reaction to the presence of glucose.

The finding incidentally led to some misunderstanding with regard to the specificity of "Ehrlich's diazo-reaction", but as we shall see the results of Penzoldt and Petri were obtained by departing from the conditions of the test laid down by Ehrlich (see p. 144).

Penzoldt used for the test crystalline diazobenzene-sulphonic acid dissolved in 60 parts of water, along with potassium hydroxide as alkali. He further noted that a slight excess of nitrous acid was not harmful to his test. The test for glucose was performed as follows:

2 cc. of glucose solution (containing more than 0.1 per cent) are made strongly alkaline with potassium hydroxide, and 2 cc. of the diazo-reagent, previously made slightly alkaline, added. In presence of a large amount of sugar a purplish colour develops after 15 minutes, but with small amounts of glucose only after an hour.

Penzoldt and Emil Fischer [1883] showed that the test is a general one for aldehydes, for which reason it has been frequently referred to in

subsequent literature as ^{the} ~~Penzoldt~~ ^{Fischer} aldehyde test. Easily oxidisable aldehydes were found to behave as glucose but a colour ~~is~~ obtained from aromatic aldehydes only in the presence of sodium amalgam. By the use of sodium amalgam a much greater colour ~~is~~ also obtained from glucose and acetaldehyde.

Positive tests were obtained by Penzoldt and Fischer with acetaldehyde, acetone, ethylacetoacetate, valerian aldehyde, furfural, glyoxal, and benzaldehyde. The same test was also found to be given by phenol, resorcinol, and pyrocatechol, if care was taken to bring them in contact with the diazo-compound in presence of excess of alkali so that the known dyes are not formed.

Petri [1883; 1883-'84; 1884] added to 5 cc. of urine 2 cc. of 30 per cent. potassium hydroxide and 5 cc. of a diazo-reagent, ~~made~~ with approximately the theoretical amount of potassium nitrite. He found that colour development ~~is~~ hastened by warming, that the colour ~~is~~ given only with fixed alkalies and not with ammonium hydroxide, and that reduction with zinc in the absence of oxygen may replace reduction with sodium amalgam. He made the further interesting observation that over-reduction with zinc produces a colourless solution, which on exposure to

oxygen rapidly becomes coloured again. Petri later used, as did Penzoldt, solid diazobenzene-sulphonic acid and found that peptones and proteins by the use of zinc give the same fuchsin-red colour as obtained from aldehydes.

It appears probable that the glucose test is not given by glucose as such, but by certain substances of the type of acetaldehyde produced by relatively long contact with the strong alkali. Of course, it is possible on the hypothesis of Nef [1910], of the existence of 2 : 3 or 3 : 4 dienols in the hexose molecule in contact with the strong alkali, that the reaction is given by glucose with the carbon chain intact.

It would thus appear that the tests described by Penzoldt and Fischer and by Petri for aldehydes, are fundamentally the same as that described by Hanke and Koessler for acetone, acetaldehyde, and acetoacetic acid; and that the hydroxylamine of the last workers serves the same purpose as sodium amalgam or zinc. A reducing agent is necessary for all the tests, but in the case of such substances as acetaldehyde a red colour develops without the addition of another reducing agent, as uncoupled acetaldehyde in solution itself serves as a reducing agent.

The writer has made certain investigations on this type of diazo-

reaction and confirmed the main findings of Penzoldt and of Petri, as well as those of Hanke and Koessler. It would appear that the best conditions for the test with such a substance as acetone lie between those adopted by Penzoldt and by Hanke and Koessler. It is true that coupling does take place, especially with the aid of heat, in the presence of a large excess of strong alkali; but it is at best very slow and incomplete owing to the fact presumably that there is little active coupling diazo-compound present under such conditions. On the other hand in a dilute sodium carbonate solution of acetone there is probably very little of the enolic tautomer, which according to Dimroth and Hartmann [1908] is the only form capable of coupling, present. The greater colour produced by acetone by prolonging the time of coupling in presence of dilute sodium carbonate, as found by Hanke and Koessler, or by the use of 10 per cent. sodium carbonate instead of 1.1 per cent. and keeping the coupling time 5 minutes, as found by the writer, is readily explainable on this hypothesis. A high concentration of weak alkali thus provides the best conditions for coupling as it does not greatly diminish the active diazo-compound and at the same time tends to increase the proportion of enolic form present.

The great variations in the extent of coupling of different substances of this type under such specific conditions as those adopted by Hanke and Koessler is also in accordance with the above explanation; the proportion of enolic form present under definite conditions being very variable for different substances.

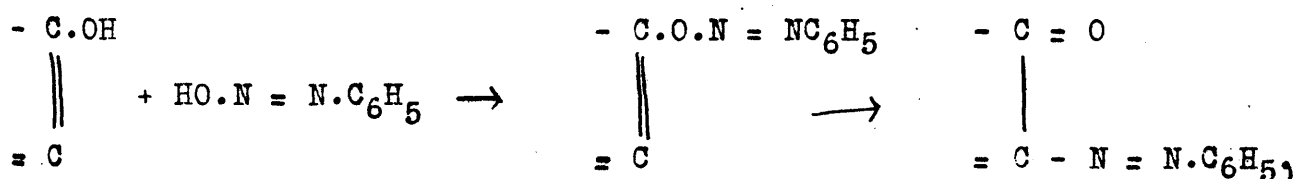
I have further found that many reducing agents may replace hydroxylamine in the production of the final colour. The addition, instead of hydroxylamine, of zinc, aluminium, and tin, or a drop of very dilute stannous chloride, is quickly followed by the appearance of the red colour. In the case of stannous chloride the colour intensification with acetaldehyde is practically as great as that obtained with hydroxylamine. With the former, however, the colour is unstable. On allowing the test-tube to stand, the colour rather rapidly fades except on the top layer. By shaking the contents it quickly returns, apparently to its full extent. The process may be repeated many times. We have thus present an autoxidisable substance, coloured in the presence of oxygen, and colourless in its absence.

Besides metallic reducing agents we may use acetaldehyde and even glycerol. The action of the latter is surprising, and may be due to

certain aldehyde impurities in the commercial product used by the writer.

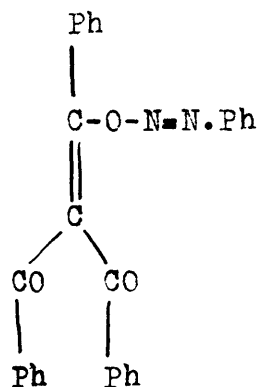
It is thus apparent that, although hydroxylamine may have an other than purely reducing function in the Hanke and Koessler test, yet it is not a necessary constituent in the general type of reaction under consideration. The Hanke and Koessler formulation above given is thus inadequate as an explanation of the mechanism of the reactions concerned.

The problem leads us into the difficult field of the chemistry of the mixed azo-compounds, and the most suggestive work here appears to be, from our point of view, that of Dimroth, Dimroth and Hartmann [1908] consider that the most probable reaction which takes place with an enol and diazo-compound is:

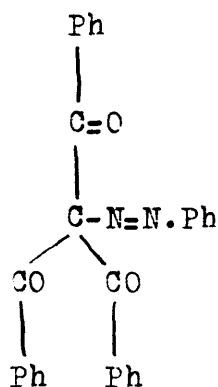


that is, the formation in the first place of an O-azo-compound followed by the migration of the benzeneazo-radicle to a carbon atom, with the simultaneous disappearance of the double bond. They found that the enolic form of tribenzoylmethane couples to give a yellow O-azo-compound (I), which by heat is transformed to a red C-azo-compound (II), which by further heat is transformed to a colourless hydrazone (III), through the

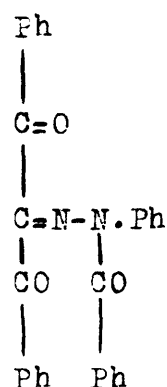
migration of a benzoyl group.



(I) yellow



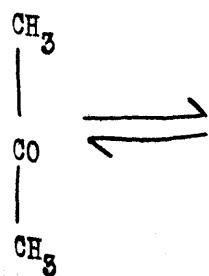
(II) red



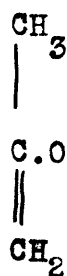
(III) colourless

By reduction of the red compound (II) there is produced a leuco-compound which is readily reoxidised with atmospheric oxygen or oxidising agents to the red substance. It may be observed that the hydrazone-form, and not the azo-form, is regarded by Dimroth as the stable one in such mixed azo-compounds.

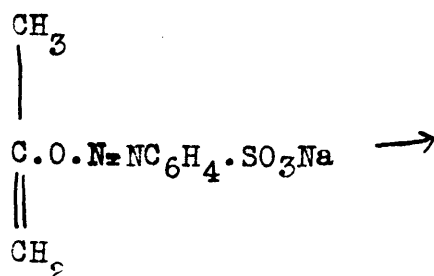
Partly on this analogy the following scheme is suggested for the reactions taking place when using acetone and diazobenzene-p-sodium-sulphonate.



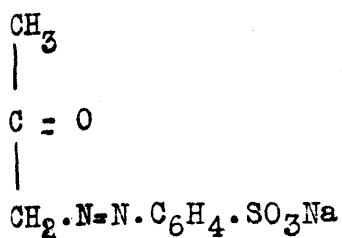
(A)



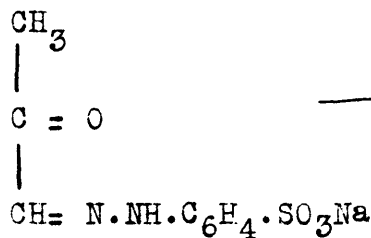
(B)



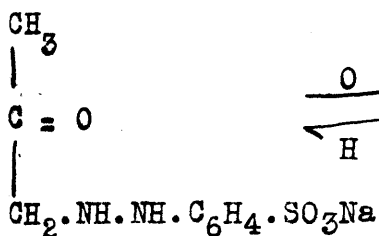
(C)



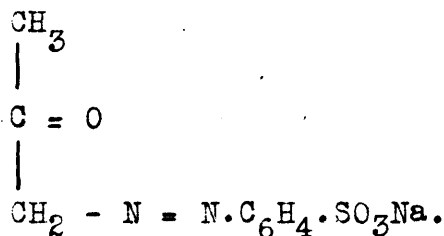
(D)



(E)



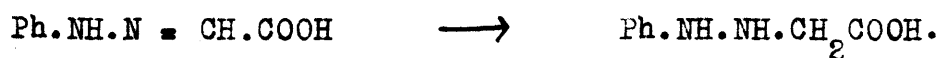
(F)



(G)

Acetone (A) in the presence of fairly concentrated sodium carbonate will tend to form the sodium salt of the enolic tautomer (B), which in presence of the diazo-compound first forms an O-azo-compound (C) or according to Auwers [1908] an O-diazonium salt. Then follows a migration of the arylazo-group to the carbon atom with the formation of a true azo-compound (D), which, however, is unstable in the sodium carbonate medium and changes to the hydrazone (E). This may be regarded as the end point of the reaction in the sodium carbonate coupling medium. With acetaldehyde the colour changes during the first stage of the reaction are very marked and appear to correspond with the formation of products

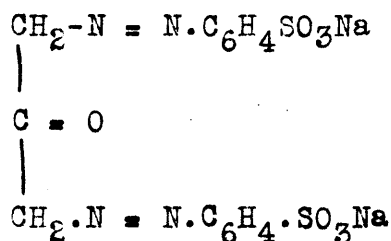
corresponding to (C), (D), and (E). The colour is first yellow corresponding to C, then a marked red colour appears due probably to the presence of the azo compound (D), and finally the colour rapidly fades to a pale yellow corresponding to the formation of the hydrazone (E). The addition of sodium hydroxide after 5 minutes has been allowed for coupling in the sodium carbonate medium, is generally followed by the production of a slight red colour. This may be accounted for by the tendency of hydrazones to revert to azo-compounds in the presence of strong alkali; the reverse process occurring with strong acid. (see Sidgwick, 1910). The strong alkali itself, however, produces little change in the absence of a reducing agent. In the presence of a reducing agent, ~~however~~ it seems probable that the hydrazone is reduced to the hydrazido compound (F). Sidgwick [1910] notes that by careful reduction in alkaline solution the following reaction takes place:



As hydrazo-compounds of the type $\text{Ph.NH} - \text{NH.Aryl}$ are the leuco-compounds of the corresponding azo-dyes, it appears probable that the hydrazido-compound (F) is the substance produced by reduction and that this is changed by atmospheric oxygen to the azo-compound (G).

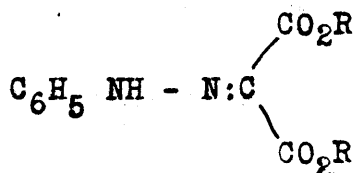
The disappearance of colour on standing and its appearance on exposure to atmospheric oxygen are thus represented by the equilibrium between (F) and (G).

It would also appear possible that in the case of acetone, after the migration of the benzeneazo-group to an end carbon atom, that an ethylene linkage may appear at the other end of the molecule, and by a repetition of the process the following bisazo compound form: .

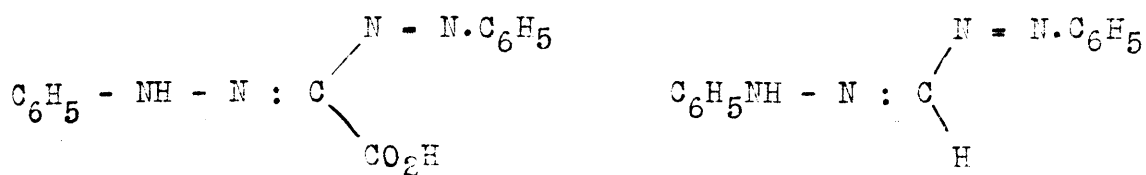


The carboxyl-group in presence of hydroxylamine may still form an oxime as suggested by Hanke and Koessler, and by so doing increase the stability of the colour.

There is also the possibility of formazyl formation in certain of the compounds giving this type of reaction. Thus malonic ester gives with diazobenzene in alkaline solution a phenylhydrazone of malonic ester :



but if malonic acid is used formazylcarboxylic acid and formazyl result:

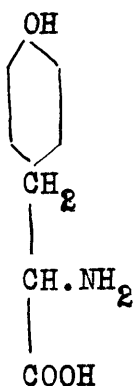


These are usually dark red crystalline substances giving on reduction colourless hydrazones. (see Cain 1920) .

The subject is thus a very complex one and it would appear that the course of the reaction can only be determined by the isolation and analysis of the final red products from each substance concerned. For although these diazo-reactions appear to follow a general type acetoacetic acid, for example, and its simple substitution products show a different behaviour with diazo-compounds, [See Cain, 1920].

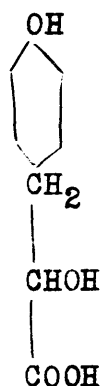
(2) Tyrosine and Tyramine.

Hanke and Koessler [1922, 1] discovered that tyrosine and tyramine give in a very beautiful fashion, and indeed quantitatively, the keto-enol type of diazo-reaction. Para-hydroxyphenylpropionic acid and p-hydroxyphenyllactic acid behave as normal phenols, the presence of NH_2 in the side-chain giving rise to this remarkable difference in behaviour.



Tyrosine

(positive test)



p-hydroxyphenyllactic acid

(negative test)

The writer has tested p-nitrophenol and p-aminophenol but with negative results. He has also found that tyrosine gives the Penzoldt aldehyde test but only in the presence of a metallic reducing agent. When the red colour obtained in the Penzoldt test with glucose is discharged by shaking in the presence of oxygen, it is recoverable by the addition of hydroxylamine as well as by metallic reducers. The use of stannous chloride in place of hydroxylamine hydrochloride in the Hanke and Koessler method gives the characteristic intensification of colour. As with the keto-enolic substances, the colour fades rather rapidly except on the surface layer and is quickly regenerated by shaking. As before the process can be repeated many times.

The parallel behaviour of tyrosine with the keto-enolic tautomers thus leaves little doubt that the red compound produced in both cases

is of the same type, and, in the case of tyrosine is explainable by a set of reactions similar to that outlined for acetone. The initial formation of an O-azo compound is in conformity with the conceptions of Chattaway and Hill [1922] of the mechanism of the coupling of phenols, but in the case of tyrosine, through the influence of the NH_2 - group in the side-chain, the migration of the arylazo group is in some way affected, or the hydrazone structure is the more stable form as in the mixed azo-compounds. *No aromatic derivative of tyrosine has yet been described.*

As Hanke and Koessler have shown, their test for tyrosine is a highly sensitive one, a distinct colour being obtained by 0.005 mg. of tyrosine in a volume of 10 cc. The writer has also found the test to be a very useful one, as interference from keto-enolic substances can as a rule be readily eliminated by distillation or other means. Glucose may be said to give a negative test, though in very high concentration a slight intensification is obtained: (indicating that the Penzoldt test is given by decomposition products of glucose). Hanke and Koessler [1922, 2] have also described methods for the separation of tyrosine from other phenols. The test also provides a means of detecting tyrosine in presence of iminazoles, a point which has been shortly dealt with by the writer, ~~Hunter~~, 1922, (see paper ^{Na2} ~~inserted pg~~). .

(3) Thymine.

The test for thymine has already been described. Here again, the final colour behaves with stannous chloride as does that from acetone or tyrosine, and the colour produced by hydroxylamine is very stable.

The fact that uracil and cytosine do not give the test is perhaps no more remarkable than that p-hydroxyphenylpropionic acid and similar substances without an amino-group in the side-chain, do not behave as tyrosine. The methyl-group in the 5-position of thymine has an effect on the reaction apparently analogous to that of the amino-group in the side-chain of tyrosine. In the case of ~~the~~ thymine we must also suppose that the labile hydrogen migrates between a carbon and a nitrogen atom.

7. Ehrlich's Diazo-Reaction, or the Diazo-Reaction of Urochromogen.

This is the oldest of the diazo-reactions with which biochemistry is concerned, but as yet the substance responsible for it has not been isolated. There is an extensive clinical literature on the test, which has been reviewed up to 1909 by Huber who quotes 261 references including some 30 theses or dissertations on the subject. Our main interest in the reaction will be confined to its characterisation, and differentiation from the diazo-reactions previously studied; and to a consideration of the probable nature of the substance giving the reaction.

The reaction was first described by Ehrlich [1882]. Further descriptions of the test and its application followed shortly after [Ehrlich, 1883, 1; 1883, 2; 1884]. In his first paper Ehrlich used a saturated solution of sulphanilic acid in nitric acid (30-50 cc. of conc. nitric acid in 500 cc.). To this he added "a few granules" of sodium nitrite, ^{noting} ~~and stated~~ that the reagent contained no free nitrous acid. He also recommended ammonium hydroxide or potassium hydroxide as alkali. After the publication of the papers by Penzoldt and by Petri (see pg. 130) Ehrlich [1883, 2] recommended the use of a saturated solution of sulphanilic acid containing 50 cc. of conc. hydrochloric acid per litre, stating at the same time that

1 gm. of sulphanilic acid per litre suffices. The reagent was then made by adding 5 cc. of 0.5 per cent sodium nitrite solution to 250 cc. of the sulphanilic acid solution. And, as Penzoldt and Petri had shown that glucose gives a colour with the reagent in contact with potassium hydroxide, Ehrlich definitely stated that ammonium hydroxide was the preferable alkali for his test, although it gives a yellow colour with the reagents alone. Later [Ehrlich, 1884] he stated that a reagent containing 15 cc. of hydrochloric acid per litre (that recommended for the bilirubin diazo-reaction, see pg. 55) gives the characteristic colour test but not the green precipitate (see below) obtained with the more strongly acid reagent.

The test is thus fully described by Ehrlich [1883, 1]: To a test-tube one-third filled with urine an equal volume of diazo-reagent is added followed by excess of ammonium hydroxide. A positive test is indicated by the appearance of an intense scarlet or carmine colour, especially marked in the foam obtained by vigorous shaking. When allowed to sediment for 12 to 24 hours the upper strata of the alkaline earthy precipitate are coloured green, or, when the urine contains relatively large amounts of aromatic hydroxy acids, violet. The green dye is very stable and difficultly soluble. Its colour is changed to red with acids and is recoverable by again making alkaline.

Ehrlich applied the test to most of the common urinary constituents, but could find no substance which behaved like this constituent of urine from febrile conditions. He observed that it is a reducing substance and is very readily oxidised, especially, in alkaline solution. The addition of a few drops of potassium permanganate, calcium oxychloride or other oxidising agents, quickly destroyed^s the substance giving the diazo-reaction. With respect to oxidising agents he observed its resemblance to pyrogallol.

Ehrlich and Brieger [Ehrlich, 1884] attempted to isolate the substance. They found that it is not precipitated by neutral lead acetate but that it is precipitated by basic lead acetate. The latter precipitate was well washed and freed from lead by H_2S . The filtrate from lead sulphide was then evaporated, mixed with alcohol, and treated with oxalic acid, to remove urea. The filtrate was again evaporated and treated with calcium carbonate. The calcium salt they found to be soluble in alcohol and insoluble in ether. They record that the calcium or zinc salts give an exquisite colour with the diazo-test, but found them to be too unstable to permit of further progress in the purification of the substance.

During the next thirty years, which saw the growth of the large clinical

literature already referred to, it is no overstatement to say that practically nothing was added to our knowledge of the nature of the substance giving the reaction. Nor would it appear that any of the various modifications suggested in this period for performing the test, has any advantage over the original technique described by Ehrlich. The period is adequately covered by Huber's monograph [1909].

The first significant advance after Ehrlich's studies, was made by Moritz Weiss in a series of papers dating from 1906. The main thesis of Weiss is, however, included in a series of papers on the pigment analysis of urine published since 1920, to which papers we may for the present confine our attention, [Weiss, 1920,1; 1920,2; 1922; 1923,1; 1923,2].

The main findings of Weiss are that the substance giving Ehrlich's diazo reaction:

- (a) is not precipitated by full saturation with $(\text{NH}_4)_2\text{SO}_4$, and it thus occurs in the urochrome fraction of Garrod. Like urochrome it is extractable from the ammonium sulphate solution with absolute alcohol.
- (b) It is not precipitated by neutral lead acetate but by excess of lead acetate in presence of a definite excess of sodium hydroxide. By this

means it is separable from various other urinary pigments, which are precipitated by neutral lead acetate, and from iminazole derivatives, which are not precipitated by neutral or basic lead acetate.

(c) ~~It~~ is oxidised by potassium permanganate to a yellow substance regarded by Weiss as identical with urochrome, and ultimately oxidisable to a melanine, termed by ^{him} ~~Weiss~~ uromelanine.

Weiss [1920,2] summaries the properties of the substance which he thus calls urochromogen:

It has acid properties, as it forms salts soluble in water and alcohol, but insoluble in ether. It gives a strong xanthoproteic acid test, and its solutions, when heated with 25 per cent. sulphuric acid and potassium bichromate have the odour of phenacetaldehyde; on the basis of which tests it is considered to be a phenylalanine derivative possessing, on the evidence of its positive diazo-test, at least one hydroxyl-group. It gives a negative Millon's test and a negative glyoxylic acid test, so that tyrosine and tryptophane derivatives are eliminated. As it gives a greenish colour with ferric chloride in neutral solution, it is considered probable that the side-chain is in the o-position to the hydroxyl-group. On the evidence of the positive permanganate test, its

oxidation in presence of alkali, and its power to reduce ammoniacal silver solutions, it is considered that other atomic groups are probably present.

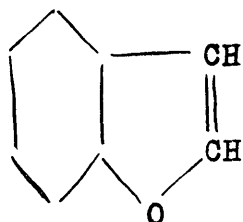
As Weiss [1922] found lead-blackening sulphur present in uromelanine prepared from purified fractions of urochromogen, he considers, in his last paper of this series [Weiss 1923, 2], that urochromogen is probably a derivative of phenylcysteine.

Urochromogen is regarded by Weiss as a normal constituent of urine.

The findings of Weiss rest for the main part on the use of Ehrlich's reagent and technique. For the estimation of urochromogen he advised the use of sodium hydroxide as alkali [Weiss 1923, 2].

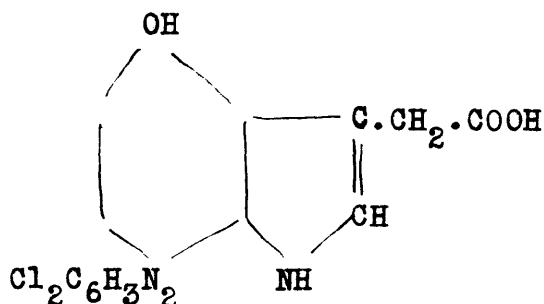
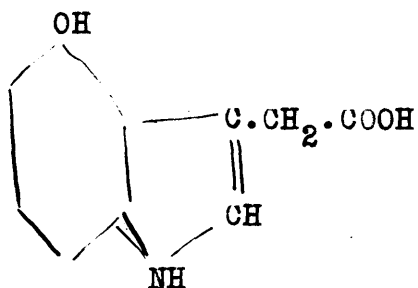
From ^{an} investigation of urine from tuberculous patients Hermanns and Sachs [1921, 1] concluded that the substance giving the diazo-reaction is a sulphuric acid ether of an oxidation product of tyrosine. Their evidence rests on the finding that the ether extract of the urine gives a greenish ferric chloride test, a positive Millon's test, besides decolorising bromine and permanganate solutions, and reducing ammoniacal silver. The presence of sulphur was indicated by finding it in an ether-pyridine extract of the urine after addition of diazotised dichloroaniline in alkaline solution. The dye, however, was not obtained in a pure state.

Hermanns [1922] prepared a dye as follows: 10 litres of urine from tuberculosis was made acid to Congo red and heated for 5 minutes on a gauze. (This procedure is to hydrolyse the sulphuric acid ether supposed to be present by Hermanns and Sachs.) The urine was then cooled and made alkaline with sodium hydroxide and sufficient dichlorodiazobenzene added. The dye, recovered from the ether-pyridine extract, was then extracted in a Soxhlet apparatus with ether. From the latter it was precipitated with petrol-ether. By repeated solution in ether and precipitation with petrol-ether, dark red irregular prisms were finally obtained, which on microanalysis led to the formula $C_{14}H_9N_2Cl_2O_4$; the original substance thus having the composition $C_8H_7O_4$. The substance they thus regard as a polyphenol with probably a cumarone nucleus:



Hermanns and Sachs [1921,2] applied a similar procedure to urine from a patient with carcinoma of the liver. The substance finally crystallised in short dark-red needles. An isomer, which crystallised ^{is} in wedges, but gave the same analysis, was also present. The formula of the coupled

product was found to be $C_{10}H_8NO_3 \cdot C_6H_3N_2Cl_2$, from which the original substance is represented by $C_{10}H_9NO_3$. Hermanns and Sachs thus regard the original substance and dye formed from it as represented:



Hydroxylindole acetic acid.

The substance gave a uroscosein reaction with nitrous acid.

Hermanns and Sachs further observe that this dye is not sensitive to acids; the scarlet-red colour obtained by coupling in an alkaline medium remains red, even on heating, when the solution has been made acid.

The writer has made various attempts to isolate the substance giving Ehrlich's diazo-reaction, but so far without success. In the course of this work, however, there have emerged certain indications as to the probable nature of the substance, and a new method has been suggested for performing the test, whereby this type of diazo-reaction is rendered quite distinct from all other types with which we are concerned. This

test has already been briefly described by the writer [Hunter, 1925] (see Paper No. 5).

It is of some interest to observe the behaviour of urochromogen towards different diazo-reagents, and using different alkalies. [Note. Urochromogen is here used merely as a convenient term for the substance, and without prejudice as to its relationship to other urinary pigments]. Thus, we may take Ehrlich's diazo-reagent which contains no free nitrous acid, (see pg. 55) and the Koessler and Hanke diazo-reagent which has an excess of nitrous acid, (see pg. 99) ; we may choose as alkalies ammonium hydroxide, sodium carbonate and sodium hydroxide.

If 1 cc. of Ehrlich's reagent is taken in 3 tubes and the first made alkaline with ammonium hydroxide, the second with sodium carbonate, and the third with excess of sodium hydroxide, and a drop of positive urine added to each tube, we find that in the first two tubes a bright red colour immediately flashes up and fades quickly, but no coupling takes place in the third tube. Of course, a slight yellow colour slowly develops in the first two tubes through the coupling of the diazo-compound with the excess of sulphanilic acid in solution.

If we repeat the procedure with the Koessler and Hanke reagent the same findings are obtained.

If we now take 1 cc. of Ehrlich's reagent in 3 tubes and add to each 2 drops of the urine no colour appears in the acid solution. If we then make alkaline as before with ammonia, sodium carbonate, and sodium hydroxide, we immediately obtain colours in all tubes; but in presence of either ammonia or sodium carbonate the colour is unstable, whereas in presence of sodium hydroxide the colour is relatively stable.

If to 1 cc. of the Koessler and Hanke reagent we add 2 drops of urine ~~then~~ a distinct yellow colour quickly appears. If alkali is then added no red colour is obtained. The same yellow colour is obtained on adding the urine to a dilute solution of nitrous acid.

We may conclude from these tests that the substance giving Ehrlich's reaction is destroyed by nitrous acid. As in Ehrlich's technique the substance is first in contact with the acid diazo-reagent it is clear why he emphasised that the reagent used for his test should not contain free nitrous acid.

In the second place the azo-colour produced is very unstable in an excess of weak alkali but relatively stable in presence of strong alkali. An excess of nitrous acid in the reagent has no effect on the test provided the reagent is made alkaline before the addition of the urine.

The substance does not couple in presence of excess of strong alkali.

Its speed of coupling is, however, so rapid that if the substance is already dissolved in the reagent, coupling takes place before an excess of alkali can be added.

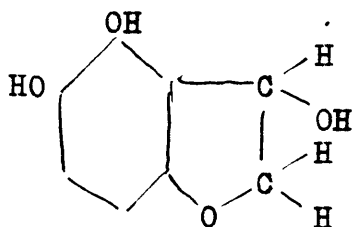
The yellow colour obtained with nitrous acid is probably related to the fact that the substance gives a marked xanthoproteic acid test.

The writer has previously suggested that for qualitative purposes the Ehrlich test is best performed by adding a single drop of urine to the Koessler and Hanke reagent made alkaline with sodium carbonate. The great instability of the azo-colour produced from urochromogen thus differentiates this type of reaction from the other types described. The diazo-reactions obtained in native urine in alkaline solution were divided by the writer into two types called for convenience Type A and Type B, but only with the latter are we here concerned. The urochromogen reaction and certain of its relationships are described under "Type B" in the following excerpt from my paper ^{No.5.} ~~Hunter 1925, I~~. (Note: "Type A" diazo-reaction is not a "type" in the sense under which I have in this thesis endeavoured to systematise diazo-reactions).

~~(Insert Excerpt)~~

There appears no reason to believe that there is more than one substance to which we may attribute the true Ehrlich diazo-reaction, or the modification of it described in Paper No. 5. The urochrome derivative obtained from the urine in carcinoma of the liver by Hermanns and Sachs does not give a typical Ehrlich reaction as its colour is not sensitive to acid; a finding in accordance with the behaviour of an indole derivative, (see pg. 91).

The suggestion of Hermanns that the substance is a coumarone derivative appears to the writer of much interest as coumarone contains an ethylene linkage and may form a compound of the type described by Baudisch. The composition of the substance as found by Hermanns could thus readily be formulated as follows:



the formula demanding a composition of $C_8H_8O_4$ instead of $C_8H_7O_4$ given by Hermanns. Such a substance would most probably give all of the reactions which have been described for urochromogen. The work of Hermanns, however, will require to be confirmed before the above formulation of urochromogen can be taken as more than a suggestion.

8. Miscellaneous Diazo-Reactions.

A. Peptones and Proteins.

Petri [1884] observed that when an acid solution of diazobenzene-*p*-sulphonic acid was allowed to act on an aqueous solution of peptone no colour appeared^s, but when made alkaline a yellow to dark brown-red colour developed^s. Ammonium hydroxide was found to give more colour than fixed alkali. The colour disappears on acidification. Colours were thus obtained [Petri, 1883-'84] from serum protein, commercial albumin, casein, and egg white. By the use of metallic reducers as sodium amalgam ~~and~~ or zinc dust along with fixed alkali, fuchsin-red colours were obtained as in the aldehyde test of Penzoldt and Fischer. Petri also took precautions to exclude carbohydrate from the materials tested.

As the writer has shown (pg. 141) that the amino acid tyrosine gives the Penzoldt aldehyde test in presence of metallic reducing agents, the findings of Petri with peptones and proteins may thus be accounted for.

Pauly [1904] placed considerable emphasis on the possibilities of the diazo-reaction for the detection of histidine and tyrosine in protein. He found a positive reaction in all proteins from which

histidine or tyrosine had been isolated and a negative reaction in others from which they had been proved to be absent. The following table, with slight modification, is given by Pauly:

Protein	Histidine %	Tyrosine %	Millon	Diazo-Reaction
Sturine	12.9	0	-	+
Histone (Thymus)	1.21	6.31	+	+
Edestin	2.19	2.13	+	+
Casein	2.6	4.5	+	+
Salmin	0	0	-	-
Scombrin	0	0	-	-
Clupein	0	0	-	-
Protamine	?	?	Hardly recognisable	Yellow with reddish tinge
Cyclopterin (Seehase)	0	8.3	Clear reaction	Red orange

Pauly also tested the majority of the amino acids and found that they generally give slight^{ly} yellow colours by his method.

The writer has not had the opportunity of testing any proteins free from tyrosine and histidine but in his experience the colours obtained from such substances as casein and edestin can hardly be regarded as characteristic for histidine or tyrosine, although it remains probable that the colours, such as they are, arise from the presence of histidine

or tyrosine in the protein.

Dr. Blythe A. Eagles has recently called my attention to a peculiar diazo-reaction given by pure zein, which is apparently allied to the diazo-reaction described for thioliminazoles, and is given markedly by zein presumably through the presence in this protein of a thioliminazole (see pg. 123) . When a solution of zein in 50 per cent. alcohol is tested as for thioliminazoles (see ~~Paper No. 8~~ p. 5) a yellow-brown colour is first given, followed on the addition of sodium hydroxide by a clear though faint purplish-red colour.

When tested as for iminazoles (see ~~Paper No. 5~~ p. 27) a yellowish colour with a pinkish tinge rapidly develops, the pink colour fading in about 10 seconds.

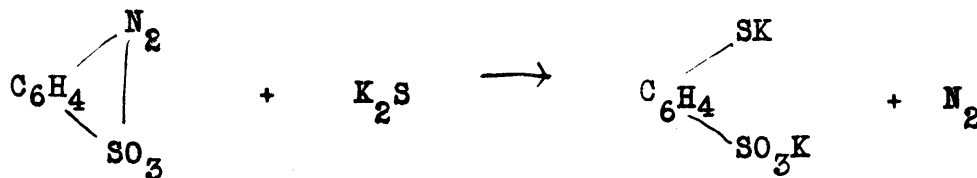
The writer has tested in the same manner casein, commercial egg albumen, pea globulin, edestin, and fibrin, but none of these behaves as zein. With the iminazole method all give gradually increasing colours until a yellowish-orange shade is reached, indicating that histidine or tyrosine dominates the reaction in these proteins. Commercial peptone and urease were found to behave similarly.

B. Interfering Substances.

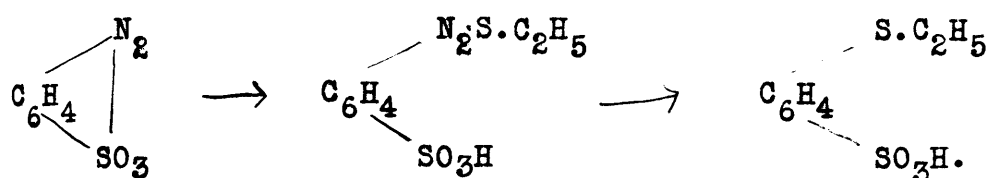
Substances may interfere with diazo-reactions in two main ways: (a)

they may give a colour in contact with the diazo-reagents, (b) they may or may not give a colour with the diazo-reagents but they inhibit or affect the colour produced by other substances. Koessler and Hanke [1919] have examined a number of common substances from this point of view, and found that urea, NaCl, KCl, Na_2SO_4 , KH_2PO_4 , sodium acetate, and sodium citrate, do not interfere up to a concentration of 5 per cent. with the estimation of histidine. Glycerol, glucose, ethyl alcohol, methyl alcohol, and amyl alcohol in high concentration in histidine solutions, lead to yellow colours and inhibit colour production to varying degrees. Ammonium salts and keto bodies were found to show the most marked interference. The writer, [Hunter 1922] (see Paper No. 2), in relation to the estimation of carnosine in muscle, found that such substances as metallic sulphides, hydrogen peroxide, and formaldehyde, are very active interfering substances.

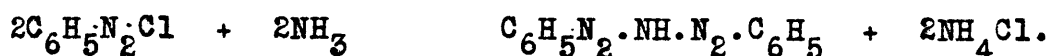
The fact that sulphides give a yellow colour with diazo-compounds may be accounted for by the reaction:



Mercaptans act similarly forming an unstable intermediate compound:



It may also be noted that concentrated ammonia acts according to the following equation:



which substance readily decomposed to a mixture of phenol, aniline, and nitrogen [see Cain 1920 p.66], where the action of other substances on diazo-compounds is considered.

More important perhaps than the reagents considered above, are the substances in biological material which interfere with the particular type of diazo-reaction in which we may be interested. The differentiation of the types of diazo-reaction here attempted by the writer may be of some help in the application of diazo-reactions to general biological material, though different material requires special study ^{with regard} ~~from the point~~ ^{to} ~~of view of~~ substances reacting with diazo-compounds and substances interfering with such reactions. Urine provides perhaps a good example and for this reason will have special consideration in Part III. Here,

however, we may ask why bilirubin even when present in relatively high concentration in urine, is not detectable by the diazo-reagents. We can only assume that the failure of the reaction is due to interfering substances, for as shown (pg. 75) bilirubin can be satisfactorily tested for in urine which has been suitably fractionated. Such an interference does not occur in blood serum. It appears to the writer, however, that the hypobilirubinaemia found in uraemia on the evidence of the diazo-reaction, might be profitably re-examined on the assumption that uraemic serum like urine contains substances inhibiting colour production.

In the same way a normal urine rarely shows a characteristic Pauly or Koessler and Hanke test for iminazoles, but after suitable fractionation a perfect iminazole type of diazo-reaction is obtained (See Paper ^{No. 5} ~~inserted~~ pg. . In this case protein-free blood extracts behave as urine, for after treatment with basic lead acetate, blood also shows a characteristic iminazole test. It appears to the writer that reduced glutathione and ergothioneine are active interfering substances in blood, and small amounts of disulphide compounds in urine probably play a great part in the interference with the tests for bilirubin and for iminazoles.

It would appear to the writer that the principle of Clemens [1899]

that the substance in urine or other medium having the greatest affinity for the diazo-compound couples preferentially, has very little application in practice; for, when an excess of diazo-compound is used, as is necessarily so in the diazo-reactions with which we are concerned, all substances react which are capable of reacting under the conditions, and mixed colours, which cannot be interpreted, are obtained. In the use of diazo-reactions the material must be fractionated in such a way that the reaction is obtained in a typical manner. Of course a typical Ehrlich reaction is obtainable in certain urines from typhoid and other fevers because the amount of urochromogen present is in relatively high concentration, but in normal urine, where the concentration of urochromogen is low the interfering substances exert a relatively great effect and no test is obtained.

C. The Diazo-Reaction as a Test for Nitrite.

Diazotisation of a suitable aromatic amine such as sulphanilic acid occurs at almost infinite dilution of nitrous acid, and coupling with another suitable aromatic amine or phenol at like dilution of diazo-compound; so that exceedingly sensitive tests for nitrite are rendered possible. The tests are also specific for nitrite.

There is of course, a very wide choice of diazotisable and coupling constituents for the test. One substance may be used for both purposes as ^{phenylene diamine} ~~m-diaminobenzene~~ [Griess, 1878]. Griess [1879] ~~however,~~ later proposed a combination of sulphanilic acid and α -naphthylamine. Ilosvay [1889] used as a reagent 0.5 gm. of α -naphthylamine in the same solvent. To remove a bluish fluorescence he boiled 0.1 gm. of the naphthylamine with 20 cc. of water, filtered, and made to 150 cc. with dil. acetic acid. To 20 cc. of the solution to be tested Ilosvay added 2-3 cc. of the sulphanilic acid solution, (in the presence of very small amounts of nitrite, heated to 70 - 80°) and added some of the naphthylamine solution. The nitrous acid of course, must not be present in such large amount as to diazotise all of both constituents added.

Lunge [1889] used the sulphanilic acid and naphthylamine in one solution kept free from air contamination. Sulphanilic acid is so much more readily diazotised than naphthylamine that the same dye is formed as in Ilosvay's procedure. Ilosvay and Lunge found the reaction to be sensitive to 1 in 1000 million parts of nitrous acid.

The test described is perhaps as satisfactory as any other but the methods of Meldola [1884] and of Riegler [1897] may also be referred to.

Part IIIDiazo-Reactions of Urine.

Ehrlich added to a test-tube third filled with urine an equal volume of diazo-reagent. Any colour developed in the acid medium he called a primary colour. He then made alkaline with excess of ammonium hydroxide, and any colour developed in the alkaline medium he called a secondary colour. In pathological urines he recognised two distinct kinds of diazo-reaction in acid solution and in alkaline solution. These may for convenience be tabulated as follows:

Primary Reactions, 1. Primary darkening → bilirubin

2. Primary yolk colour → urobilinogen

Secondary Reactions, 1. Secondary yolk colour → aromatic hydroxy acids.

2. Red reaction → obtained by a substance in the urine in typhoid fever, miliary tuberculosis, and other febrile conditions.

He further recognised, that by the use of potassium hydroxide as alkali, glucose gives a colour on long standing, and also that with a concentrated solution of diazo-compound, such as that employed by Penzoldt with ammonia as alkali, ethylacetoacetate gives a red colour

which is changed to a red-violet by the addition of a large excess of hydrochloric acid.

The primary diazo-reactions of urine with bilirubin and with urobilinogen have already been discussed on pp. 75-84, respectively.

The secondary eigeln or yolk colour Ehrlich found to be obtained after ingestion of large doses of tannin, gallic acid, and similar substances, and depends on the presence of aromatic hydroxy compounds.

(shortly discussed p. 106) .

The secondary red colour or true Ehrlich diazo reaction has been discussed (p. 144).

It is perhaps clear from what has gone before that the manner of performing the test and the reagent used is of much importance. Since Engeland [1908] isolated histidine from human urine it has been generally believed that iminazoles play a large part in the so-called diazo-reaction of urine. The subject of iminazoles has already been discussed but it may here be mentioned that histidine, for example, does not give a red colour in presence of sodium carbonate or ammonia with Ehrlich's diazo-reagent, but merely a yellow or yellow-orange. It appears that the colour production is interfered with by the excess of sulphanilic acid

in Ehrlich's reagent . Using the Pauly method or the Koessler and Hanke reagent characteristic iminazole tests are sometimes obtained in urine and may be confused with the positive Ehrlich reaction.

[Hunter 1922] . The following excerpt from this paper^{No 3,}_{3A} represents an early attempt to fractionate substances giving the diazo-reaction in a urine with a very marked iminazole test:

"Five hundred c.cm. of a mixed sample of measles urine were quantitatively fractionated by lead acetate, lead acetate and baryta, and by mercuric acetate. A portion of the last fraction was further precipitated with silver nitrate, followed by silver nitrate and baryta. The colour value developed by the Koessler and Hanke diazo-reagent was measured in the various fractions. The colour standard employed by these workers for histidine was here used as a standard. An orange light-filter placed over the eyepiece of the Duboscq colorimeter was consistently used throughout the measurements in order to obtain a match with the yellow colour developed in certain fractions. The total colour value of the original 500 c.cm. of urine was found to be 86.500 mm.

"The lead acetate fraction had a total colour value of 770 mm., the

lead acetate baryta fraction a total colour value of 4,400 mm., the mercuric acetate fraction a total colour value of 87,500 mm., and the final filtrate a total colour value of 10,000 mm. The sum-total colour values of the various fractions is greater than the original for the reason that there are certain substances in the original urine which inhibit colour production. The portion of the mercury fraction taken for the silver baryta separation had an original total colour value of 12,600 mm. In the silver baryta fraction there were recovered 12,500 mm., or almost 100 per cent. The silver fraction and the final filtrate gave no colour.

"From 1 litre of urine the silver baryta fraction was obtained as above. Creatinine was separated by precipitation with picric acid, the filtrate re-treated with silver baryta, and the latter fraction finally precipitated with mercuric chloride. From this well-washed precipitate there was obtained about 0.1 gram of pure histidine monohydrochloride. (The amount actually present per litre was estimated at 0.31 gram). It had the following properties: melting-point, 253 to 254; melting-point, with decomposition, of the picrolonate, 231 to 233. The microscopic appearance of both these *agreed with authentic descriptions and with the same salts of histidine* salts of γ -histidine obtained from a different origin. There was found 16.9 per cent (calculated 17 per cent.) Cl. The molecular colour value was 114.5

million mm. It was positive to Knoop's test.

"The substance mainly responsible for the colour not accounted for by histidine was not isolated, but partly purified and relatively concentrated solutions gave reactions indicating a phenolic constitution."

A further attempt to compare the amount of colour given by native urines as contrasted with the amount of colour obtainable from the iminazole fraction of the same urines is contained in the following excerpt

from Paper
Hunter,

No. 5

~~1925. "Type A" reaction is explained on p.~~

28

G. HUNTER

1. Alkaline Diazo Reaction, Type A.

Some kind of Type A reaction is obtained in all urines. As a rule the more red the colour obtained, the more iminazole is present. Yet it must be emphasised that this method of qualitative examination is a very poor index of the actual amount of iminazole in urine. This is so because of interfering substances in untreated urine. It has previously been shown [Hunter, 1922, 1] that certain purines interfere with colour production, but the presence of uric acid in urine is alone insufficient to account for the great inhibition of colour development which generally prevails. This is clearly seen by a consideration of the 5 urines in Table I. The amount of iminazole present in each was determined by the recent method of Koessler and Hanke [1924], the amounts taken to obtain the readings given below representing 0.05 cc. of original urine. Alongside these are given the approximate readings obtained from 0.05 cc. of untreated urine. The test cylinder in each case is set at 20 mm. and the cylinder containing the histidine colour standard [Koessler and Hanke, 1919] moved to match.

Table I.

Urine No.	Standard cylinder		Test cylinder
	Untreated urine mm.	Prepared urine mm.	
1	4.5	14.5	20
2	6.5	29.5	"
3	6.5	27.5	"
4	2.0	4.9	"
5	1.0	1.7	"

It will thus be seen that although urines Nos. 2 and 3 show higher readings than Nos. 1, 4 and 5 by both methods, the readings in Nos. 2 and 3 for the untreated urine represent only about $\frac{1}{5}$ of the colour obtainable from the iminazoles actually present in the urines.

The classification of Ehrlich of the different kinds of diazo-reaction obtainable in pathological urine by his method, useful as it is as a guide, provides little indication of the numerous substances present even in normal urine, capable of coupling with diazo-compounds. Thus in fresh urine we have at least urobilinogen and indoxyl which react in acid solution with diazo-compounds. There are probably other compounds related to indole. So that even in acid solution the diazo-reaction of normal urine is not a simple one. And in alkaline solution we have to consider phenols, iminazoles, purines, keto-enolic substances, and urochromogen, without the certainty that normal urine does not also contain small amounts of thioliminazoles and pyrimidines. In other words the subject amounts of diazo-reactions in urine covers practically the whole subject with which we have dealt, and the futility of drawing conclusions from the behaviour of untreated urine with diazo-compounds, except in rare pathological conditions where one constituent is in great excess, becomes apparent.

At present, however, diazo-reagents provide a ready means for the detection of indoxyl (p. 94), bilirubin, and urochromogen ~~in urine~~, and also for the estimation of iminazoles *in urine*.

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THE ESTIMATION OF CARNOSINE IN
MUSCLE EXTRACT
(PRELIMINARY NOTE)

BY

GEORGE HUNTER

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LXXXVI. THE ESTIMATION OF CARNOSINE IN MUSCLE EXTRACT. (PRELIMINARY NOTE)

By GEORGE HUNTER, *Strang-Steel Scholar,*
Institute of Physiology, University of Glasgow.

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WITH a view to the study of the physiological significance of carnosine, it was first necessary to devise a method of estimation applicable to small quantities of material, which, at the same time, would give more accurate results than have hitherto been obtained with this substance [*vide* von Fürth and Hryntschak, 1914]. A general method of estimation of iminazole derivatives by a modification of the diazo reaction described by Koessler and Hanke [1919]—but not applied by those workers to carnosine—has here been tried as a likely means of affording a reliable indication of the carnosine content of muscle¹. Before the method could be satisfactorily tested, it was first necessary to prepare pure carnosine and for comparative purposes also histidine.

Histidine monohydrochloride was prepared from ox-blood by the method of Fränkel [1907]. M.P.T. 253°, with softening and loss of water at 175–180°.

Kjeldahl. Nitrogen. Found 19.79 %. Calculated 20.05 %.

Preparation of Carnosine. This was obtained from Lemco. The most successful method found for the preparation was that adopted by Baumann and Ingwaldsen [1918] with the use of mercury acetate followed by the usual silver-baryta precipitation. A very readily crystallisable product is obtained if, after the addition of excess silver nitrate solution and before filtration of the purine fraction, barium hydroxide solution is added to more than neutralise the nitric acid set free by the use of the silver nitrate reagent. Otherwise, part of the purine group remains dissolved and escapes precipitation. This incomplete separation of the purine fraction from the carnosine fraction probably accounts for much of the difficulty workers have experienced in the isolation of pure carnosine [*vide* Gulewitsch, 1913; Smorodinez, 1913]. On first attempting the preparation this difficulty was met with. On precipitating the carnosine fraction, which refused to crystallise from alcohol, with the above precaution the product was readily purified. The purine part which

¹ Clifford [1921], since the commencement of this research, has applied a modified Koessler and Hanke procedure to the estimation of carnosine. As to both treatment of material and results found, the writer is so much at variance with the above worker that a publication at the present stage of this research has been deemed desirable.

separated here was treated with hydrogen sulphide, concentrated, and a syrup separated on treatment with alcohol. This appears at least presumptive evidence for the validity of the foregoing suggestion of the cause of the difficulty. The substance was first isolated as carnosine nitrate. This agreed in properties with those described by Gulewitsch and others [*vide* Gulewitsch and Amiradžibi, 1900; Gulewitsch, 1913]. M.P.T. 220°.

The free base was prepared from part of the above product by reprecipitation with silver nitrate and barium hydroxide, and concentrated without neutralisation. The carnosine thus obtained was similar to that of Gulewitsch; M.P.T. 260° with decomposition.

Part of the carnosine was used to prepare the copper-salt according to the directions of Mauthner [*vide* von Fürth and Hryntschak, 1914]. Deep-blue hexagonal crystals about 1 mm. in greatest length were obtained.

Solutions of 0.1 % carnosine were made from the free base and from the carnosine nitrate after these had been dried in desiccator to constant weight. Equal quantities of these tested colorimetrically agreed almost perfectly.

It was further intended to make a standard solution from the copper salt but the difficulty of getting this to constant weight, along with the rapidity with which it took up moisture when dry, led to the acceptance of the value as confirmed by the free base and its nitrate.

THE COLORIMETRIC METHOD.

Working mainly from certain theoretical considerations of the properties of diazonium compounds and their mode of coupling, Koessler and Hanke have, undoubtedly, greatly enhanced the value of the diazo reaction as a method of estimating iminazole derivatives. The use of a relatively stable colour-standard is only a small part of the improvement they have effected on the previously somewhat haphazard way of performing the diazo test.

As a quantitative method for the estimation of histidine the diazo reaction had been used by Weisz and Ssobolew [1914] and their method was adopted for the estimation of carnosine in mammalian muscle by von Fürth and Hryntschak. The much more consistent results obtainable by the Koessler and Hanke procedure testify to its superiority over any previous method.

The method has been adopted here almost *in toto* for the estimation of carnosine and found to give very satisfactory results in pure solutions of that substance. Different proportions of methyl-orange and Congo red had of course to be employed to get a match for the carnosine solutions. A very suitable match for pure carnosine solutions was obtained with 0.10 cc. methyl-orange and 0.25 cc. Congo red per 100 cc. water. These amounts were taken from 0.1 % stock methyl-orange and from 0.5 % stock Congo red.

Molecular colour values. To test the purity of the above-mentioned histidine compound and at the same time to verify the accuracy of the method, the molecular colour value of histidine was determined according to the (CR-MO)

colour-standard employed by Koessler and Hanke. This was found to be approximately 117,000,000 mm. as compared with 114,000,000 mm. found by those workers. The slight discrepancy between these results can be accounted for by experimental error, along with the possibility that there might be slight differences in the quality of the methyl-orange and Congo red employed.

The molecular colour value of carnosine was found to be 152,000,000 mm. against the same indicator.

Question as to the linearity of colour production. Clifford [1921] questions the direct proportionality between colour-production and the amount of iminazole in solution. The linear relationship, as set forth in the ideal tables of Koessler and Hanke, was tested for both histidine and carnosine by the writer and accepted within the limits of experimental error before the publication of the paper above referred to. It was very carefully retested on Clifford's figures coming to his notice, but the conclusion reached was merely a re-confirmation of Koessler and Hanke's results. Histidine was tested only within the limits of 0.01 mg. and 0.05 mg. histidine monohydrochloride in the test cylinder, whilst carnosine was tested between 0.005 mg. and 0.2 mg. The following table shows the results for carnosine, using a difference of 0.005 mg. carnosine between each reading up to 0.05 mg.

Carnosine in test cylinder mg.											
0.005	0.010	0.015	0.020	0.025	0.030	0.035	0.040	0.045	0.050	0.1	0.2
Reading of standard cylinder mm.											
3.2	5.7	8.9	11.8	14.8	17.5	20.8	23.8	26.9	30.0	59.7	120
Calculated reading of standard cylinder mm.											
3	6	9	12	15	18	21	24	27	30	60	120

When 0.1 mg. and 0.2 mg. carnosine were present in the test cylinder, the plunger was set at 10 mm. and 5 mm. respectively, and the reading of the standard cylinder multiplied by 2 or 4 gave the value equivalent to the test cylinder set at 20 mm.

The results for histidine within the limits tested were similar in their linearity.

Clifford finds colour values greater than those given by calculation on the linearity basis, in the more concentrated iminazole solutions. Exactly the opposite results were found by the writer, when the usual concentration of diazonium sulphonate reagent was used for widely differing concentrations of carnosine. Experiments here showed that the time required for maximum colour development increased with the amount of carnosine present so long as that maximum was the theoretical value. This was especially marked when more than 0.045 mg. carnosine was used. To give a few practical examples, it may be noted that with 0.045 mg. the theoretical value was reached only after standing for 12 to 14 minutes; with 0.05 mg. it was almost reached after 20 minutes; with 0.1 mg. and 0.2 mg. the highest readings were obtained only after 15 and 10 minutes respectively, but both of these were short of the

theoretical. With 0.1 mg. this shortage was 7 mm., *i.e.* approximately 12 %. With 0.2 mg. the reading was 24 mm. low, *i.e.* 20 %. If, however, a more concentrated diazonium reagent is used, the time necessary for maximum colour development is decreased, and the actual maximum is then approximately theoretical as shown in the foregoing table.

These results are quite in accordance with the mass action law. In presence of a relatively large amount of carnosine the diazonium sulphonate is in great measure used up, with the result that the speed of coupling under these conditions becomes low, and at a certain point practically nil. All the diazonium compound is not, however, used up with 0.1 mg. carnosine as the behaviour with 0.2 mg. shows. Here, the presence of a higher concentration of carnosine accounts for a greater amount of coupling, yet a similar equilibrium becomes established before complete coupling of the carnosine has taken place.

These facts merely indicate that a definite excess of diazonium sulphonate is necessary to complete the chemical reaction in one direction. They do not at all affect the validity of the linear relationship.

It is difficult to account for the deviation found by Clifford.

The best results were obtained when the test cylinder contained 0.02 mg.-0.04 mg. carnosine. The maximum is reached in 8-12 minutes after mixing, and is stable at those concentrations for approximately another 8 minutes.

No appreciable colour was developed by the interaction of the sodium carbonate used (Merck's) with the diazo reagent alone, even after 20 minutes.

CERTAIN CONSIDERATIONS PRELIMINARY TO THE ESTIMATION OF CARNOSINE IN MUSCLE EXTRACT.

Filter paper adsorbs carnosine. Koessler and Hanke [1919] show that iminazoles are adsorbed by animal charcoal. This fact suggested that the use of filter paper might vitiate the results in such a microchemical method as that here employed. The adsorptive effect of filter paper was shown in the following way.

20 cc. of a cold solution of carnosine with a colour value of 13.9 mm. per cc. were run from a pipette through a 15 cm. diam. dry filter paper into a dry measuring cylinder. 1 cc. of this filtrate was then tested and found to have a colour value of only 11.5 mm. This process was thrice repeated, through three papers, and 1 cc. of the filtrate gave successively colour values of 10.0 mm., 8.5 mm., and 7.7 mm. That is, the total loss in colour value was 6.2 mm. per cc., or approximately 44 %.

In order to determine to what extent this loss was recoverable by washing, 10 cc. solution of carnosine with colour value of 18.0 mm. per cc. were added to four dry filter papers as above but folded into one funnel. The results are expressed in the following table:

Added to filter	Volume of filtrate cc.	Colour value of filtrate per cc. mm.	Total colour value of filtrate mm.
10 cc. carnosine solution	3.2	8.5	27.2
10 cc. water	9.2	5.5	50.6
20 cc. „	19.8	2.5	49.5
40 cc. „	evaporated to 27.0	1.0	27.0

The loss as shown in the first filtrate is 9.5 mm. per cc., *i.e.* approximately 53 %. The sum total of colour values of filtrates is 154.3 mm. The total colour value of the original solution is 180 mm. so that, after washing with water to the extent of seven times the volume of the original carnosine solution, there yet remains 14 % of the original carnosine held by the filter paper.

For these reasons, glass wool, which shows no adsorptive properties, has been adopted for filtration purposes.

The effect of heat on the colour value of pure carnosine solutions. Several clean, dry duroglass test-tubes were constricted to a neck of about 3 mm. diameter about 3 cm. from the bottom. A horizontal mark was made with a file at the constriction. (The object of constricting the tubes was to obtain more accurate measurement.) The tubes were filled to the mark with solutions of carnosine of known colour value and set on a boiling water-bath for 1 hour. They were then removed, cooled, and made up to the mark with water. A number of solutions of carnosine with an original colour value of 19.5 mm. per cc. showed after this treatment an average loss in colour value of 1 mm. per cc. Similarly a number of solutions of carnosine nitrate with an original colour value of 18.5 mm. per cc. showed after the same treatment an average loss of 0.5 mm. No change of tint in the colour developed was observable in these tests.

It is probable from these results that a process of extraction which involves prolonged boiling may be harmful to the carnosine.

METHOD OF EXTRACTION EMPLOYED.

The most consistent results have been obtained by the following procedure. A small quantity of muscle, say 1.5 g., is extracted with about 20 cc. water on a bath at 70°. After 30 minutes the solution is filtered through glass wool into a small Buchner flask. The meat residue is transferred to a mortar, and ground with glass to a fine pulp. This is again extracted with 20 cc. water for 15 minutes, poured through the same filter and sucked as dry as possible. The top layer of wool and meat residue is re-transferred to beaker, stirred up with a further 20 cc. water and heated another 15 minutes. It is then re-filtered and washed twice with 10 cc. warm water, sucking as dry as possible between washings. After this treatment the final filtrate should give no diazo reaction.

One extraction, found by Clifford [1921] to be sufficient, has never given satisfactory results in the writer's hands. After extracting the finely ground tissue once with a large volume of water, followed by copious washing, the

colour value has shown a loss of 6 to 10 % reckoned as carnosine, as compared with that from the same meat extracted as directed above.

The total milky filtrate—representing about 50 cc. per g. of meat used—is poured into a suitable beaker. The reaction is made slightly acid with dilute acetic acid and the beaker set on the water-bath at 70° for a few minutes. Under these conditions the proteins are very effectively precipitated. The coagulum settles quickly and is readily caught in the glass wool filter. The contents of the filter are washed thrice with a little warm water. The final filtrate should be quite clear and entirely negative to all protein tests.

The results are practically unaltered if instead of the above method excess of lead acetate followed by excess of disodium phosphate is used to precipitate the protein. Thus three samples from a piece of lean muscle, extracted as above, showed on reckoning the total colour value as carnosine 0.355 %, 0.369 % and 0.380 %. In the first two samples the proteins were precipitated with lead acetate and sodium phosphate; in the last by the acetic acid method. The acetic acid method was finally adopted because it is more simple and gives a smaller precipitate by which less carnosine is likely to be adsorbed.

Further it would appear that there are no substances in muscle extract as prepared above which inhibit the diazo colour production; thus 1 cc. meat extract with colour value 22.0 mm. per cc. was mixed with 1 cc. carnosine solution 18 mm. per cc., and 1 cc. of the mixture tested. This was found to have a colour value of 20 mm. which is the theoretical number.

The total colour value of muscle extract as obtained by the methods outlined above, is consistent for the same material to within 3–4 % of the colour value reckoned as carnosine in meat. Thus three pieces of ox muscle yielded 0.388 %, 0.378 % and 0.379 %. Another sample of ox muscle gave 0.614 %, 0.606 % and 0.620 %. These materials were purchased from a butcher. A cat muscle in three portions showed 0.280 %, 0.287 % and 0.293 %.

As yet, however, the method does not appear to be entirely satisfactory for the estimation of carnosine in muscle extract. Carnosine is, undoubtedly, responsible for the production of the great bulk of the colour in these muscle extracts, but the writer has good reason to believe that certain other substances contribute to a considerable degree to the yielding of the total diazo value. Further investigation is in progress on this point.

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Paper No 2

THE ESTIMATION OF CARNOSINE IN MUSCLE
EXTRACT—A CRITICAL STUDY

BY

GEORGE HUNTER

[FROM THE BIOCHEMICAL JOURNAL, Vol. XVI, No. 5, 1922]



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LXVI. THE ESTIMATION OF CARNOSINE IN MUSCLE EXTRACT—A CRITICAL STUDY.

By GEORGE HUNTER, *Strang-Steel Scholar.*

Institute of Physiology, University of Glasgow.

(Received July 17th, 1922.)

IN a recent communication [Hunter, 1921] a colorimetric method for the estimation of carnosine in muscle extract was generally outlined. This was based on a method of estimation of iminazoles devised by Koessler and Hanke [1919, 1]. This method depended on the coupling of carnosine with diazotised sulphanilic acid in alkaline solution to form an azo dye. The colour produced was measured against a standard solution of a mixture of Congo red and methyl-orange. Pure solutions of carnosine were found to react very satisfactorily, and in different extracts of the same muscles the colour values were consistent. The accuracy of the method could not be doubted, but the certainty that carnosine was responsible for the total colour production was called in question.

The problem—of the extent to which carnosine is responsible for the production of the azo colour in muscle extracts—may be attacked in two ways: 1. By the elimination of all interfering substances. 2. By a direct method of confirming the carnosine content.

I. THE ELIMINATION OF INTERFERING SUBSTANCES.

Potentially interfering substances. It is well known that aromatic amines and phenols readily couple with a diazotised aromatic amine and are thus potentially interfering substances with the reagent here employed for the estimation of carnosine.

The iminazoles form another well defined group—of which carnosine is only one member—giving the diazo reaction.

The purines which contain the iminazole ring are generally described as substances giving a positive diazo test—this applies at least to the members adenine, hypoxanthine, and xanthine [*v.* Plimmer, 1918].

There are various other substances diversely referred to throughout biochemical literature as giving the diazo test. Of these may be mentioned thymine [Thierfelder, 1908], bilirubin [Neubauer-Huppert, 1913], urochro-

mogen and urobilinogen (Neubauer-Huppert), besides the "neutral-sulphur" compounds of urine (Neubauer-Huppert).

An exhaustive investigation into the behaviour of all substances—confined even to biochemical literature—towards the diazo reagent would probably be endless as well as fruitless. Attention will thus be confined only to those reagents ordinarily employed in the preparation of muscle aqueous extracts and to substances that may be present with a fair degree of probability in the extracts.

A. Reagents. Soluble chlorides, sulphates, nitrates, phosphates and sodium acetate, have been tested in relatively high concentrations and found to have no effect on the diazo reagent.

Ammonia and its salts not only interfere with the production of colour from other substances such as histidine [Koessler and Hanke, 1919, 2] but when added alone to the diazo reagent give a yellow colour.

Soluble sulphides give a colour in low concentration. The presence of sulphur in cystine probably accounts for the similar behaviour of that substance towards the diazo reagent.

Ethyl alcohol was found to give no colour with the reagent though Koessler and Hanke find that it inhibits colour production.

Hydrogen peroxide, formaldehyde and acetone all give yellow colours.

Tannic acid in very low concentration gives a marked colour. This is accounted for by its phenolic constituents.

All solutions in which carnosine is to be estimated by means of the diazo reagent should thus be free from ammonium salts, sulphides, and tannic acid. Formaldehyde or phenols, such as thymol, should not be used as preservatives in this connection. Muscle tissue may be preserved in alcohol and extracts by a layer of toluene.

B. Muscle constituents. Certain normal muscle constituents which may possibly act as interfering substances in the extracts require a more detailed examination, as these are much less within the control of the worker than substances which may be used in the preparation of the extracts.

The presence of bilirubin, urochromogen and urobilinogen in muscle extracts is too unlikely to claim further consideration for those substances. Thymine may also be dismissed on account of the difficulty with which it is liberated by hydrolysis from nucleic acid [v. Jones, 1920].

(1) *Phenols and aromatic amines.* The presence of these substances in protein-free extracts has not been shown by any of the tests used to detect them. Extracts from several types of muscle were shaken in acid solution with ether. The ether extracts were evaporated to dryness and the slight residues taken up in small quantities of water. These gave no diazo reaction nor has Millon's test been found positive either in the extracts themselves or in the ether fractions.

It is known that aromatic amines couple with the diazo reagent in acid solutions, whereas iminazoles and phenols require a weak alkaline medium before

a colour is produced. Thus if a little 1. 2. 4-diaminotoluidine is added to the reagent without the addition of sodium carbonate, a strong orange colour is developed. If an iminazole or phenol is added under the same conditions no colour is produced¹.

Various extracts have been tested with the acid reagent but all with negative results. It may thus be concluded that there are no aromatic amines contributing to the azo colour developed in meat extracts².

(2) *Tyrosine*. This substance, which is not extractable by ether, has received considerable attention from the point of view of the diazo reaction. Totani [1915] devised a method to distinguish the azo colour developed by histidine from that developed by tyrosine. The colours were first reduced with zinc and hydrochloric acid, twice the volume of 25 % ammonia added and in both cases a golden yellow colour was obtained. In the case of histidine the addition of hydrogen peroxide changed the colour to lemon yellow whereas the colour was destroyed in the case of tyrosine. A dilution greater than 1 : 20,000 histidine was necessary to get the characteristic final colour.

Among the various substances considered by Totani no mention is made of iminazoles other than histidine, nor of purines, nor of aromatic amines nor of phenols other than tyrosine.

On repetition of Totani's procedure with histidine alongside carnosine, the two substances went through approximately the same colour changes. The final colour was not destroyed and so the presence of tyrosine could not be detected by this method.

Tyrosine gives a positive Millon's test; histidine and carnosine are negative to Millon's. Tyrosine gives a faint Millon's test at a dilution of 1 : 25,000.

A still more delicate test for tyrosine—on which is also founded a method of estimation—has recently been devised by Hanke and Koessler [1922]. This depends on a further modification of the diazo reaction and is approximately as delicate as that reaction is for carnosine. For quantitative purposes the procedure is the same as that adopted for iminazoles and phenols. The test cylinder is allowed to stand for exactly 5½ minutes after the tyrosine solution has been added to the alkaline reagent. This gives rise to a primary yellow colour the intensity of which is not directly proportional to the amount of tyrosine used.

2 cc. of 3*N* sodium hydroxide solution are now added and the contents of the cylinder mixed. This gives rise to a colour intensification with a change of tint towards the red.

One minute after the addition of the sodium hydroxide 0.10 cc. of a 20 % solution of hydroxylamine hydrochloride is added and rapidly mixed. After

¹ The aromatic amines also give a colour in alkaline solutions.

² This test would also eliminate bilirubin which gives a blue colour in acid solution [Ehrlich, quoted by Neubauer-Huppert].

a latent period of 5 to 10 seconds an intense bluish red colour rapidly develops. This colour is stable and is directly proportional to the amount of tyrosine present.

Hanke and Koessler note that this intensification is also given by substances capable of a keto-enol tautomerism such as acetaldehyde, acetone and aceto-acetic acid.

It was decided to test this new modification on muscle extracts. It was first observed that the addition of sodium hydroxide and hydroxylamine hydrochloride in the manner above described to the azo colour developed by histidine or carnosine had the effect merely of a proportional dilution. Thus a solution of carnosine gave by the ordinary method a reading of 22.5 mm.; by the new method a reading of 18.3 mm., *i.e.* approximately the same as would be obtained by adding 2.1 cc. water to the cylinder. The following results were obtained on the extracts.

	Reading with diazo reagent mm.	Reading with diazo reagent, NaOH and NH ₂ OH . HCl mm.	Calculated reading— assuming proportional dilution mm.	Intensification %
Ox muscle	27.2	21.4	21.6	None
Cat „	18.0	16.5	14.3	15.4
Rabbit muscle	10.8	11.3	8.5	33.0
Otter „	15.5	18.5	12.3	50.4
Salmon „	13.7	21.5	10.8	100.0

The percentage of intensification of these extracts is in the order of the yellowness of their azo colours. Thus ox muscle extracts give a colour which matches the carnosine colour standard. The colour developed from cat muscle extract is generally slightly more yellow; that from rabbit almost matches the histidine colour standard; that from otter is still more yellow and that from salmon is so yellow as to be almost unmatchable.

The intensification colours were pink. They did not show the purplish tinge given by tyrosine. No Millon's reaction was obtained in concentrated extracts of salmon muscle and it is concluded that tyrosine is not responsible for the intensification obtained in that tissue.

The specific cause of this intensification has not been determined.

(3) *Purines*. These are normally present in muscle extracts so that a consideration of the individual members is rendered necessary. Adenine and guanine were prepared from commercial plant nucleic acid. This was hydrolysed by suspending in methyl alcohol and passing dry hydrochloric acid gas according to the method employed by Levene [1921] for animal nucleic acid. This process was found to work very satisfactorily. The precipitated chlorides of adenine and guanine were filtered off and separated according to methods described by Jones [1920].

Hypoxanthine nitrate and xanthine were prepared from parts of the adenine and guanine respectively by deamination and subsequent purification.

Cytosine and uracil were also prepared from the residues freed from methyl alcohol by further hydrolysis in the autoclave at 160° with 25 % sulphuric acid for five hours according to Jones [1920].

Neither of these pyrimidines gave a colour with the diazo reagent.

0.05 % solutions were made of adenine sulphate, of guanine chloride, of hypoxanthine nitrate and of xanthine—all in 1.1 % sodium carbonate solutions.

1 cc. of each of these was tested in the ordinary way. Guanine and xanthine gave marked colours. The adenine solution showed only a slight reaction and there was no colour in the case of hypoxanthine. With a tenth of the above amounts guanine and xanthine were still strongly positive whilst adenine and hypoxanthine were entirely negative. The intensities of the colour were approximately the same in like concentrations of guanine and xanthine. With 0.025 mg. of guanine hydrochloride in the cylinder a reading of 8.5 mm. was obtained with the histidine colour standard. With 0.05 mg. the reading was 13 mm. Xanthine in the same amounts gave the respective readings 8 mm. and 12.5 mm.¹ The colour production is not directly proportional to the amounts of guanine and xanthine in the cylinder; nor do guanine and xanthine, mixed with a known amount of carnosine, give proportional colours. Thus 1 cc. of a mixture of 1 cc. 18.6 mm. per cc. carnosine solution with 1 cc. 13.5 mm. per cc. guanine solution gave a reading of 13.2 mm. with the test cylinder set at 20 mm. This is 2.8 mm. short of the calculated reading of 16 mm. A similar result was found with xanthine. Adenine and hypoxanthine do not give a colour or inhibit colour production at this concentration, but they tend to make the colour due to carnosine too yellow. Thus with 1 cc. of a mixture of carnosine solution of the above concentration with 1 cc. 0.05 mg. per cc. of adenine sulphate solution, the calculated reading of 9.3 mm. was obtained. Hypoxanthine behaves similarly.

Guanine and xanthine are about half as sensitive towards the diazo reagent as carnosine. If present in appreciable amounts in muscle extract they must seriously affect the estimation of carnosine.

Uric acid in excess gives a slight yellowness to the diazo reagent.

To what extent are purines likely to interfere with the estimation of carnosine in muscle extracts? On the assumption that there is present in extract of meat free purine nitrogen to the extent of 0.045 % as quoted by Lusk [1921] it is unlikely that more than 0.020 % nitrogen represents purines that give a colour with the diazo reagent. If the nitrogen be taken as representing 42 % of the purine molecule—an average figure from adenine, guanine, hypoxanthine, xanthine and uric acid—the purines affecting the diazo reagent amount to about 0.05 %. In muscles with a carnosine content of less than, say 0.1 % the presence of such a proportion of purines would make the results worthless.

¹ The readings are only approximate as the colours are much more yellow than the standard.

To determine the actual purine interference under the conditions of extraction previously recommended, it was then considered necessary to carry out some quantitative fractionations¹.

A quantity of ox muscle was extracted at 70° with small amounts of water until the filtrate no longer gave a positive diazo test. The proteins were precipitated with excess of lead acetate and the excess of lead was removed with disodium phosphate. The filtrate was neutralised with sodium hydroxide and its colour value measured with the carnosine colour standard. This was found to be 1050 mm. per cc.

50 cc. of the extract were taken in a small beaker, made just acid with nitric acid, and silver nitrate was added till a drop of the solution on a tile gave a brown colour with baryta. The test drops were washed back into the beaker.

After settling, the contents of the beaker were filtered by suction through washed asbestos in a small Hirsch funnel. The precipitate was carefully washed with water and after sucking dry was transferred with the asbestos to a small beaker, stirred up with a little water and hydrogen sulphide passed to precipitate the silver. The contents were again filtered through asbestos in the same way and the hydrogen sulphide removed by prolonged aeration with the help of a pump. The filtrate was neutralised. This is the purine fraction.

To the filtrate from the purine fraction excess of solid finely ground baryta was added. The precipitate was allowed to settle, filtered as above, and washed with saturated baryta water. The silver was removed with hydrogen sulphide and the barium with slight excess of sulphuric acid. The slightly acid solution was aerated to remove the hydrogen sulphide and the solution was then neutralised with sodium hydroxide. This is the carnosine fraction.

The filtrate from the carnosine fraction was freed from barium and hydrogen sulphide and neutralised as above. This is the final filtrate.

The exact volume of each of the three fractions was noted. The colour value per cc. of each of the fractions was then measured and their total colour values calculated. The sum total colour value was then obtained and compared with the original, *i.e.* 50×1050 or 52,500 mm.

¹ All the carnosine can be extracted from muscle with much smaller proportions of water than recommended by the writer in his previous paper on this subject. A filtrate of about 50 cc. per g. of muscle used was there recommended mainly because the proteins are easily precipitated by slight acidification with acetic acid and heat under those conditions. In concentrated extracts this simple process is not effective and the use of lead acetate or other precipitant is necessitated. The number of washings, however, should not be reduced. There is no need to reduce the proportion of water to muscle when the extract is to be tested only for colour value unless the carnosine content is less than 0.1 %. With that amount in muscle a reading of 12 mm. will still be obtained with the 50 cc. per 1 g. proportion. But for precipitation purposes and other tests it is desirable to keep the filtrate small rather than have to evaporate it to a low volume.

The distribution of colour value is shown by the total colour values in mm. of the three fractions:

Purine fraction	...	1,560
Carnosine fraction	...	47,587
Final filtrate	2,200
Sum total	<u>51,347</u>

The unrecovered colour value is thus 1153 mm. or approximately 2 %. The purine fraction accounts for about 3 % and the final filtrate for about 4.5 %. The carnosine fraction represents almost 91 % of the original colour value.

The relatively small error due to purines by the gentle method of extraction recommended is further seen in the table to follow.

Three portions were taken from the same muscle and two extracted as above. The third was extracted at a temperature just under boiling point.

Aliquot portions of the filtrates from the lead precipitates were taken for total nitrogen estimation and for further fractionation into purine and carnosine fractions. The final filtrates were rejected without evaluation. The total nitrogen was also estimated in the carnosine fractions. In the table carnosine, nitrogen and purine (calculated as carnosine) are shown as percentages of the original muscle. The colour values of the purine fraction are at best only a rough estimate on account of the difficulty in comparing with a much redder standard. The error due to the purines in ox muscle is however small.

Meat No.	Carnosine from lead acetate filtrate	Total N from lead acetate fraction	Carnosine from carnosine fraction	Total N from carnosine fraction	Colour value in purine fraction	Difference in % carnosine in lead acetate filtrate and in carnosine fraction
1	0.517	0.530	0.498	0.246	0.004	0.019
2	0.512	0.546	0.495	0.230	0.012	0.017
3	0.533	0.653	0.487	0.258	0.014	0.046

Before considering other substances likely to interfere with the estimation it may be noted that the more drastic extraction process of muscle No. 3 results in a higher percentage colour value. The total nitrogen in this extract is also raised. In the carnosine fraction the percentage falls again into line with the other two samples. The loss is not accounted for by a higher purine value. Though the colour value of the lead acetate filtrate is raised by the more rigorous extraction, the actual percentage of carnosine is lowered. The lowering of colour value becomes obvious even in the lead acetate filtrate if boiling is prolonged. Thus two samples of muscle treated in the usual way showed an average of 0.639 % carnosine in the lead acetate filtrate, whilst three samples of the same muscle after boiling for about 30 minutes showed an average value of only 0.614 % or a fall of 0.025 % reckoned as carnosine.

In muscles 1 and 2 approximately 96 % of the colour is recovered in the carnosine fraction. Calculated as carnosine this accounts for about 50 % of

the nitrogen in the same fraction. There is no reason⁷ to doubt that carnosine is responsible for all the colour production in the carnosine fraction from ox muscle. In the case of rabbit muscle and notably salmon muscle, fractionation does not appreciably improve the match. Thus two portions of a rabbit muscle showed from the lead acetate filtrate colour values of 0.092 % and 0.099 % reckoned as carnosine. The carnosine fraction from the same muscles showed each a value of 0.072 % as carnosine. In the latter case the azo colour developed was still very yellow. The colour from salmon muscle extract is slightly more red in the carnosine fraction than in the lead acetate filtrate, yet it is far from satisfactory.

The precipitation process occupies much time and to obtain reliable quantitative results from it, great care must be exercised. With the additional possibility that the carnosine fraction may still contain other colour-giving substances, it would appear of little use to attempt the elimination of interfering substances by the silver-baryta precipitation except in very special cases.

(4) *Iminazoles*. Of this group only histidine and carnosine are known with certainty to occur in animal tissue. Histamine has been found in the intestinal mucosa by Barger and Dale where its presence is attributed to bacterial action [Barger, 1914]. Its isolation from the pituitary gland by Abel is accounted for by Hanke and Koessler through Abel's use of a commercial product. Hanke and Koessler [1920, 2] show that it is absent from fresh hypophysis cerebri.

In protein-free extract from fresh muscle there is no evidence that histidine is present provided the extraction process has been gentle. The ease with which carnosine may be hydrolysed (*v. later*), along with other factors to be considered, renders it important that there should be some ready means of detecting histidine in the presence of carnosine.

As far as the writer is aware there is no known method of separating these substances. They both precipitate in the carnosine fraction in the "arginine separation." Though histidine is more readily precipitated with ammonium-silver than carnosine, yet the latter also precipitates in such relatively dilute solutions as 0.5 % carnosine. Nor could any test be found in the literature to distinguish the two substances.

Various colour tests for histidine were performed on solutions of carnosine. The main differences were found in their behaviour towards the biuret and Knoop tests.

The biuret reaction was found to be entirely negative even in about 30 % solution of carnosine and after standing at least one hour. This difference in behaviour is however of little value for discrimination purposes as relatively concentrated solutions of histidine must be used to get a positive biuret. Thus 1 % solution of histidine monohydrochloride gives the reaction in the cold only after standing for about $1\frac{1}{2}$ hours.

The writer has found Knoop's test to be the most useful way of detecting histidine in the presence of carnosine. Carnosine is quite negative to Knoop's

test. By a modification of the test as explained in the preceding paper, the writer has been able to increase both its delicacy and certainty as a test for histidine. The presence of histidine may be detected with certainty at a dilution of 1 : 10,000.

Though this reaction has not yet the delicacy that might be desired, its very specific nature makes it valuable for work of this kind. Knoop [1908] states that the reaction is also positive for histamine. No other substance, as far as the writer is aware, gives the reaction.

Certain muscle extracts suspected of histidine, were then tested by the modified Knoop's test. Extracts of rabbit and salmon which give notably yellow colours were tested in sufficient concentration but without giving an indubitable Knoop's test. It is thus concluded that histidine is not a factor in causing the yellow colour of those extracts.

In various cases of purchased butcher's meat a positive Knoop's test has been obtained. If the meat is allowed to become just noticeably putrid a very marked reaction is given. Histamine may in part be responsible for the positive test in such cases.

The test may perhaps be used to most advantage as an indirect test for carnosine where the presence of that substance is doubtful in any tissue or fluid. If a protein-free extract is at first negative to Knoop's test, and after hydrolysis (*v. later*) is positive to the test, it appears necessarily to follow that the unhydrolysed liquid contains carnosine.

The test can also be applied to elucidate some other problems that had arisen in the course of this work. The effect of heat on carnosine solutions will first be considered.

The effect of heat on solutions of carnosine.

In the writer's preliminary communication on the estimation of carnosine, a slight fall was noted in the colour values of solutions of carnosine heated on the water-bath for one hour. Similar experiments have since been repeated but the period of heating has been extended to four hours. Thus a number of solutions of carnosine nitrate with an original colour value of 22 mm. per cc. showed after four hours on a boiling water-bath an average value of 19.4 mm. per cc. or a fall of 2.6 mm. per cc.

10 cc. of a 0.5 % solution of carnosine as nitrate was brought to dryness three times in an open basin on a steam-bath. The total period of heating was about four hours. It was finally taken up in about 2 cc. of water and Knoop's test applied. A decided brown colour was developed indicating that in the process the carnosine had been partly hydrolysed, giving rise to histidine. This observation confirms the fall in colour value.

Though four hours is a relatively long period of heating, the fact that carnosine is thus destroyed in pure neutral solution indicates that care should be exercised in the process of extraction from muscle.

The fall in colour value as estimated in carnosine from 0.639 % to 0.614 %

on prolonged extraction of the muscle, previously noted (p. 646), may be accounted for by the destruction of carnosine. The occurrence of histidine in muscle extracts will thus arise more likely from the hydrolysis of carnosine than from the hydrolysis of histidine-containing proteins. For if the latter process occurred to a marked degree the colour values would tend to rise rather than to fall.

It will thus be observed that any process of evaporation at atmospheric pressure must result in at least a partial destruction of the substance.

Solutions of carnosine whether pure or in muscle extracts may be evaporated *in vacuo* to dryness with very little destruction. Thus 10 cc. of a neutral dilute meat extract were evaporated to dryness at 20 mm. pressure of mercury from a 200 cc. distilling flask on a water-bath about 60°. Exactly 10 cc. of water were then pipetted into the flask and rinsed round to dissolve the dried residue. The colour value of the original solution was 25.6 mm. per cc. whilst that of the dried substance taken up in 10 cc. of water was 25 mm. per cc. (The volume of solid in the original 10 cc. was regarded as negligible.) Repetition of this experiment still showed a loss in colour value of about 2 %.

This experiment at the same time goes to show that a very small proportion, if any, of the colour-producing substances in muscle extract is volatile. It serves to confirm the results obtained by ether extraction and Millon's test.

(5) *Other substances.* The effects of cystine, leucine and arginine on the diazo reagent have been measured by Hanke and Koessler [1920, 1]. These workers find that there is no interference so long as the ratio of cystine to histidine does not exceed 6 : 1, which is higher, they note, than has yet been found in any protein. Cystine may thus be dismissed as a probable interfering factor in muscle extracts.

The effects of arginine and leucine are less marked than those of cystine.

Creatine in large amounts gives a slight yellowness to the reagent.

Creatinine, urea and lactic acid have been found to have no effect on the reagent.

Such a process of elimination as has here been attempted must yield mainly negative conclusions. None of the substances most likely to interfere with the colour values has any very marked effect. In the case of ox muscle it has been shown that most probably less than 5 % of the colour value is not due to carnosine. In cat and frog extracts, judging merely from the colour development, the non-carnosine colour value is likely to lie within the same limit, but of salmon little can be said except that it contains carnosine. Any elimination method must necessarily be unsatisfactory unless the substance can be obtained pure; and many factors militate against that possibility in the case of carnosine. Among those may be noted the high solubility of the substance in water, its facility for adsorption, the inadequacy of the methods for its fractional precipitation and the ease with which it is hydrolysed. It is upon this last property that the writer has sought a more direct method for confirming the values.

II. HYDROLYSIS EXPERIMENTS.

(1) *The stability of histidine.* Histidine has been observed by Koessler and Hanke [1919, 2] to be remarkably stable towards hot concentrated hydrochloric acid. They observed that when 2 cc. of a 1 % solution of histidine dihydrochloride were heated on a boiling water-bath for 10 hours with 25 cc. concentrated hydrochloric acid the histidine was quite unchanged, as shown by the colour values recovered. Over a shorter interval the writer has confirmed those findings as shown by the following experiment.

Into each of five numbered test tubes there was introduced 1 cc. of a solution of histidine along with 3 cc. concentrated hydrochloric acid. These were then set on a boiling water-bath and one removed every 15 minutes. As each test tube was removed from the water-bath it was cooled, about 1 sq. mm. of litmus paper introduced, and the contents neutralised with sodium hydroxide. The contents were again cooled and poured into a 25 cc. flask. The test tube was repeatedly washed into the measuring flask, then water was added up to the mark. 1 cc. of this was taken for measuring the colour value against the histidine colour standard. The original value of 23 mm. was obtained by directly diluting 1 cc. of the original to 25 cc. and taking 1 cc. for the test. The colour values after periods of boiling lasting 15, 30, 45, 60 and 75 minutes were found respectively to be 23.2, 23.1, 23, 22.9 and 23 mm. High concentrations of sodium chloride affect neither the tint nor the intensity of the colour developed.

(2) *Hydrolysis of carnosine—theoretical considerations.* It thus seemed very probable that if carnosine were hydrolysed with hydrochloric acid, the colour value would continue to fall until the reaction was complete. If the diazo value of the solution were measured before hydrolysis and again after hydrolysis, the ratio of the colour values thus obtained should be that of their molecular colour values. The molecular colour value of histidine was previously found to be 117 million mm. as compared with 114 million mm. determined by Koessler and Hanke. A revised determination of the molecular colour value of carnosine has led the writer—mainly from increased skill in matching the histidine and carnosine colour standards—to the conclusion that the molecular colour value of carnosine is somewhat greater than the 152 million mm. previously published, viz. 161 million mm. This value is checked by the following considerations.

The histidine colour standard is a mixture of 0.22 cc. methyl-orange with 0.20 cc. Congo red in 100 cc. water. For convenience call this S_H .

The carnosine colour standard is a mixture of 0.10 cc. methyl-orange with 0.25 cc. Congo red in 100 cc. water. Call this S_C .

These amounts were taken from 0.1 % stock methyl-orange and from 0.5 % stock Congo red [v. Hunter, 1921].

On comparing in the colorimeter it is found that 24 mm. $S_C = 28.5$ mm. S_H .

From the table previously published [Hunter, 1921] 0.040 mg. of carnosine in the test cylinder set at 20 mm. showed a reading of 24 mm. S_C .

0.04 mg. of carnosine should thus give a reading of 28.5 mm. S_H .

The molecular colour value of carnosine is thus

$$\frac{28.5}{4} \times 226 \times 10^5,$$

or approximately 161 million mm. S_H .

Now, carnosine yields by theory on hydrolysis 68.5 % of histidine, *i.e.* 0.04 mg. of carnosine yields on hydrolysis 0.0274 mg. of histidine.

0.04 mg. of histidine monohydrochloride gives a reading of 22.4 mm. S_H .

[N.B. The molecular colour value of histidine is thus

$$\frac{22.4}{4} \times 209 \times 10^5,$$

i.e. approximately 117 million mm. S_H .]

Histidine monohydrochloride contains 74 % histidine, *i.e.* 0.04 mg. of histidine monohydrochloride contains 0.0296 mg. of histidine.

0.01 mg. of histidine will thus give by calculation a reading of $\frac{22.4}{2.96}$ or 7.57 mm. S_H , and

0.0274 mg. of histidine will give 20.7 mm. S_H .

That is, a solution of carnosine giving an original reading of 28.5 mm. S_H will give after hydrolysis a reading of 20.7 mm. S_H .

The ratio of the final reading to the original reading should be the same as the ratio of the molecular colour value of histidine to the molecular colour value of carnosine.

Thus
$$\frac{20.7}{28.5} = \frac{117}{161} = 0.73.$$

For convenience this figure will be termed the *hydrolysis quotient*.

(3) *Experimental*. The results obtained from the actual hydrolysis of carnosine solutions are less satisfactory than one might expect from the above considerations. Thus a series of carnosine solutions were treated in exactly the same way as that described for histidine. 0.1 % solution of carnosine was used. The original value of 1 cc. from 25 cc. dilution was 28.5 mm. S_H . After hydrolysis for periods of 15, 30, 45, 60 and 75 minutes 1 cc. showed the respective colour values 20.4, 19, 18.2, 18.1 and 18 mm. S_H . After 15 minutes' hydrolysis the colour was more yellow than S_H .

Carnosine is thus very readily hydrolysed and under the above conditions part of the histidine is destroyed. After the initial sudden fall from 28.5 mm. to 20.4 mm. continued boiling lowers the value at a much diminished rate. After 30 minutes' boiling the values remain almost constant but the colours are too yellow—indicating some destruction. It would appear that in the course of the hydrolysis the histidine passes through an unstable phase in which the iminazole ring is readily disrupted. The histidine that emerges from that hypothetical intermediate condition resists further boiling.

Other hydrolytic agents, such as sodium hydroxide and acetic acid, besides

different conditions of temperature and concentration were tested, in the attempt to overcome the difficulty. Finally an approximately constant-boiling mixture of hydrochloric acid was used and the hydrolysis conducted at 90°. Under these conditions a strictly quantitative conversion is not yet attained, but a comparison of the results from various extracts under standard conditions would appear to be of some weight. The extracts hydrolysed were of such a concentration that 1 cc. of the final dilution gave approximately the same values. To the amount of extract to be hydrolysed exactly half its volume of concentrated hydrochloric acid was added. The following are the results from a solution of pure carnosine and from muscle extracts of cat, of ox, and of salmon. This ox extract was slightly positive to Knoop's test. All the readings were taken with the histidine standard.

Time mins.	Carnosine mm.	Cat muscle extract mm.	Ox muscle extract mm.	Salmon muscle extract mm.
Original	28.5	29.0	28.5	27.0
15	25.4	24.3	26.0	26.5
30	23.3	23.0	23.5	24.0
45	21.5	21.8	22.7	22.5
60	21.0	21.2	22.0	22.1
75	20.9	20.7	21.7	21.4
Hydrolysis quotients after 75 mins.	0.73	0.71	0.76	0.79

The carnosine solution and the cat muscle extract agree very well in both their rate of fall and in their hydrolysis quotients. The ox muscle quotient is slightly high, probably owing to the presence of either histidine or histamine in the original. The salmon extract quotient is still higher, but it is remarkable in face of the very yellow azo colour given by the original that the rate of fall accords so well with the others.

Although this method of hydrolysis is not sufficiently sensitive to show that a definite percentage of the colour is due to carnosine, it at least gives one a sense of assurance in the use of a very unspecific reagent for the estimation of carnosine in such a complex solution as muscle extract. It would further seem to indicate that carnosine is responsible for a very high percentage of the colour as measured from fresh muscle extracts treated only with lead acetate.

The specific cause of the yellow colour produced in such cases as salmon has not been found. In the case of rabbit muscle, it has been observed that the colour is more red when the carnosine content of the muscle is high and yellow when low. Some cat and frog muscles with a low carnosine content also showed a yellow colour. This is not surprising as the chances of interference are greatly increased when the test portions are less dilute.

Though purines certainly give rise to a small error and exact quantitative results cannot be got when the colour is not of the right tint, yet the total error in the lead acetate filtrate is too small to eliminate. With a better hydrolytic agent it might yet be possible to evaluate the error.

CARNOSINE CONTENT OF SOME MUSCLES.

With the method of extraction and treatment of the extract as previously described, the following results have been obtained. Each result represents the percentage colour value reckoned as carnosine in the fresh skeletal muscle. Further, each result represents the average of at least two results obtained from different pieces of the same muscle.

For convenience the amounts found are given from lowest to highest carnosine contents. The contents for four members of each species of animal are given.

Rabbit muscles	0.026	0.064	0.090	0.101	% carnosine
Frog "	0.107	0.128	0.142	0.280	" "
Cat "	0.123	0.203	0.336	0.380	" "
Ox "	0.340	0.400	0.515	0.640	" "

The results show that the carnosine content of muscle varies not only with the species of animal but varies greatly in different animals of the same species. The highest values obtained in the one species are two to four times greater than the lowest values in the same species. This finding is at variance with that of Clifford [1921, 2]. This worker finds, for example, ox muscle to have an almost constant carnosine content of 1.1 %. But apart from the constancy of the results found, the writer is compelled to question their accuracy. In a previous paper [1921, 1] Clifford finds that 0.02 mg. of histidine gives a value of 10.75 mm. measured with the Koessler and Hanke histidine standard. With these data—and assuming the author means 0.02 mg. of histidine *dihydrochloride*—the molecular colour value of the histidine is

$$\frac{10.75}{2} \times 228 \times 10^5,$$

which is approximately 122 million mm. S_H .

With the same colour standard Clifford records that 0.1 mg. of carnosine gives a reading of 30 mm. The molecular colour value of Clifford's carnosine is thus $30 \times 226 \times 10^4$ or approximately 68 million mm. As previously stated, the writer finds the molecular colour value of carnosine against this same colour standard to be 161 million mm. Assuming this figure represents 100 % pure carnosine, the carnosine employed by Clifford is thus only about 43 % pure. The carnosine content of the various muscles tested by Clifford should from this point of view be about 43 % of the values actually given.

SUMMARY.

1. Extracts to be tested for carnosine should be free from ammonium salts, sulphides, phenols and aldehydes.
2. The degree of yellowness of the azo colours developed from muscle extracts is in the order of the percentages of the intensification of the azo colours as given by sodium hydroxide and hydroxylamine.
3. In ox muscle purines are responsible for about 3 % of the colour value reckoned as carnosine. In the same tissue there is probably about other

2 % of the colour not due to carnosine. The error in the method due to colour-producing substances other than carnosine is probably about 5 % in ordinary muscles.

4. Histidine under certain circumstances may be present in muscle extracts.

5. A test has been found to distinguish histidine from carnosine.

6. A more certain means than the diazo reagent is given for the identification of carnosine in any tissue.

7. The fall in colour value of hydrolysed meat extracts agrees with that of pure solutions of carnosine.

8. The carnosine content of muscle varies with the species of animal and with different members of the same species.

The writer is much indebted to Prof. Cathcart for his inspiring guidance throughout this work.

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THE DIAZO-REACTION, WITH SPECIAL REFERENCE TO URINE
IN MEASLES.

.....
(Preliminary Note) *From B.M. J. / 7 751 (1922)*

by
GEORGE HUNTER, M.A., B.Sc.,
Institute of Physiology, University of Glasgow.

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The cause of the diazo-reaction in urine has been attributed to a variety of substances. Clemens (quoted by von Noorden (1) claims to have isolated the sodium salt of the substance responsible for the reaction. This substance contained 4.5 to 5.4 per cent. nitrogen. Others assume it to be due to chromoxy-proteic acids or to ill-defined members of this group of substances (Neubauer-Huppert (2). Weiss (3) is emphatic that urochromogen is the substance responsible for a positive diazo-test. Engeland (4) isolated from 40 litres of normal human urine a small amount of histidine. He thus suggests that iminazole derivatives primarily cause the diazo-reaction in urines. Hermanns and Sachs (5) consider that in urines from tuberculous patients an oxidation product of tyrosine is responsible for the diazo-reaction. In the urine of a patient with cancer of the liver these observers found oxyindolacetic acid.

The methods of carrying out the diazo-reaction in urine have been very varied. Suffice it to note here that, to avoid deaminization of colour giving amino-acids by the free nitrous acid of the diazo-reagent, the latter should be made alkaline before the addition of the urine to be tested. For reasons stated by Koessler and Hanke (6) sodium carbonate is for this purpose preferable to sodium hydroxide or to ammonium hydroxide.

-3-

retreated with silver baryta, and the latter fraction finally precipitated with mercuric chloride. From this well-washed precipitate there was obtained about 0.1 gram of pure histidine monohydrochloride. (The amount actually present per litre was estimated at 0.31 gram). It had the following properties: melting-point, 253 to 254; melting-point, with decomposition, of the picrolonate, 231 to 233. The microscopic appearance of both these salts agreed with authentic descriptions and with the same salts of histidine obtained from a different origin. There was found 36.9 per cent (calculated 17 per cent.) Cl. The molecular colour value was 114.5 million mm. (Hunter (7)). It was positive to Knoop's test.

The substance mainly responsible for the colour not accounted for by histidine was not isolated, but partly purified and relatively concentrated solutions gave reactions indicating a phenolic constitution.

CONCLUSIONS.

1. In the urine from patients suffering from measles, histidine is responsible for about 85 per cent. of the colour developed by the diazo-reagent.
2. In measles the output of histidine in the urine is increased.

The writer is indebted to Dr. Elliot, of Ruchill Hospital, Glasgow, for the material, and to Professor E.P. Cathcart for much help throughout the work.