XLVI. A STUDY OF SOME BIOCHEMICAL TESTS.

No. 2. THE ADAMKIEWICZ PROTEIN REACTION. THE MECHANISM OF THE HOPKINS-COLE TEST FOR TRYPTOPHAN. A NEW COLOUR TEST FOR GLYOXYLIC ACID.

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In 1874 Adamkiewicz published [1874] a description of the colour phenomena due to the action of strong sulphuric acid on egg-albumin. In some experiments he used acetic acid as a solvent, and he observed in many such cases that a purple colour was produced on the addition of the sulphuric acid. He considered this colour to be due to "a special influence of the acetic acid upon the colour already produced in the sulphuric acid protein solution."

Udranszky [1888] described the test as a furfural reaction, which explanation was generally accepted till Hopkins and Cole [1901, 1, 2] published the first of their well-known papers, in which they showed that the colour was due to an impurity in the acetic acid. They isolated glyoxylic acid from several samples of acetic acid and showed that in the presence of sulphuric acid it gave a purple colour with certain proteins, the facts thus discovered leading later to the isolation of the amino-acid, tryptophan, which is responsible for the reaction.

Cole [1903] investigated the various protein colour tests with aldehydes, or bodies that gave rise to aldehydes, and proved that the majority of these reactions depended on the presence of tryptophan. He drew attention to the importance of the presence of an oxidising agent in the production of the colours.

Hopkins and Cole's explanation was criticised by Rosenheim [1906], who described a colour reaction between tryptophan and formaldehyde in sulphuric acid containing a trace of an oxidiser, which was similar to that given by glyoxylic acid. Rosenheim considered that the Hopkins-Cole test depended on the presence of formaldehyde in the acetic acid or arising from the reagents used. He remarked upon the ease with which glyoxylic acid is decomposed at high temperature into formaldehyde and carbon dioxide.

Dakin [1907] investigated the tests, and found that glyoxylic acid prepared by different methods gave the test. He thought it "hard to believe that these uniformly positive results with different specimens of glyoxylic acid are due to impurities." Glyoxylic acid preparations in many cases gave the test directly, formaldehyde always required an oxidising agent. Dakin discussed the possibilities of the production of glyoxylic acid from formaldehyde by an aldol type of condensation followed by oxidation. Kondo (1906) described a test for indole similar in many respects to the Rosenheim test. Formaldehyde with indole gives a crimson colour in presence of strong sulphuric acid.

Research was done in the following years on the general chemistry of bodies related to tryptophan, which will be referred to later, as it does not directly concern the Hopkins-Cole reaction [Blumenthal, 1909].

In 1913 Homer published the results of work on aldehyde derivatives of tryptophan [1913]. She believed that the colour bodies were due to an interaction between the aldehyde and the imino-group of the tryptophan. She concluded from the general behaviour of these bodies that the Hopkins-Cole test was due to formaldehyde.

In the same year Mottram [1913] described a failure of the glyoxylic test owing to an excess of oxidising agents in the sulphuric acid used. This was confirmed by Breidahl in 1915, who stated that such acids could be made suitable for the test by the addition of small quantities of reducing agents [1915]. These papers emphasised the fact that a trace of an oxidising agent is necessary in the majority of cases; if the oxidising agent be too strong or too weak the test will not succeed.

Voisenet in 1918, from similarities in appearance and behaviour towards reagents, concluded that the colour produced in the Hopkins-Cole and the Rosenheim tests was the same substance, and that in each test it was due to formaldehyde [1918].

The following research was undertaken originally with the object of finding a satisfactory method for estimating tryptophan colorimetrically. Although such a method has not yet been found, some information has been obtained about the mechanism of the tryptophan colour tests. The first question investigated was the nature of the aldehydes concerned in the Hopkins-Cole and Rosenheim tests with a view to deciding the long-standing glyoxylic acid and formaldehyde controversy.

The Hopkins-Cole Reaction in the absence of Formaldehyde. If formaldehyde were liberated from the glyoxylic acid during the process of the test it should be possible to detect its presence, since there are colour tests for formaldehyde in presence of sulphuric acid that are more delicate than the reaction between formaldehyde and tryptophan. Should formaldehyde be detected under the conditions of the Hopkins-Cole reaction, it probably enters into the test. On the other hand, should the test be obtained under conditions that are such that formaldehyde cannot be detected in the reacting solutions, it is strong evidence that the reaction is due to glyoxylic acid.

Glyoxylic acid is more stable in strong sulphuric acid than has been generally supposed. When calcium glyoxylate was added to concentrated sulphuric acid and the mixture warmed gently, decomposition did not begin until the temperature rose above 50°. Carbon dioxide was then evolved, and formaldehyde was recognised in the solution by means of the thiophen test [Fearon, 1918]. The Hopkins-Cole test may be carried out at temperatures below that of the decomposition of the glyoxylate and in the absence of water, by using solid tryptophan and calcium glyoxylate. The characteristic colour develops under conditions where formaldehyde can be shown to be absent. (In passing, it may be noted that if the sulphuric acid be contaminated with nitrous acid an ivy-green colour may be formed on warming the solution, this is due to an entirely different reaction which will be discussed in a later communication.)

Various samples of acetic acid which gave the reaction were tested for formaldehyde, alone and under the conditions of the test, with negative results in nearly every case.

To detect the presence of formaldehyde in excess of sulphuric acid the following tests were employed.

The Thiophen Test (1918). This test will show one part of formaldehyde in half a million parts of sulphuric acid. The colour with formaldehyde is distinctive and much more delicate than the colour with glyoxylic acid.

The delicacy of Rosenheim's test for tryptophan is about 1 in 200,000 at most.

The Pyrogallol-aldehyde Reaction, described in the present paper.

This is an extremely sensitive test for aldehydes in presence of sulphuric acid; it will show 1 part formaldehyde in over 1,000,000 parts water, and may be used to show the presence of formaldehyde in mixtures containing glyoxylic acid.

As the result of a series of experiments on the Hopkins-Cole test under various conditions and with different preparations of glyoxylic acid, it is concluded that the test can be obtained in the absence of formaldehyde.

The Rosenheim Test in the absence of Glyoxylic Acid. Although glyoxylic acid gives many of the general aldehyde reactions, there is no simple colour test for distinguishing it from other aldehydes in presence of sulphuric acid. The test current in the text-books is simply the reverse of the Hopkins-Cole reaction and open to the same criticism of uncertainty as regards the aldehyde. This test was introduced by Eppinger [1905] and modified by Schloss [1906].

A NEW COLOUR TEST FOR GLYOXYLIC ACID.

The reagent is 1 % pyrogallol in pure concentrated sulphuric acid, which must be free from any nitrous contamination, otherwise the solution turns brown and is useless. The reagent will not keep for long, after a few days a white condensation product is deposited.

When a trace of calcium glyoxylate, or a couple of drops of reduced oxalic acid solution are added to the reagent, a deep blue colour develops on gentle warming. Dilution with water changes the blue to a deep carmine which becomes blue again on addition of excess of sulphuric acid.

The blue colour is not given by oxalic acid or any of its simple derivatives which do not give rise to glyoxylic acid under the condition of the test.

With the aliphatic aldehydes the reagent gives various shades of red. The presence of glyoxylic acid along with formaldehyde can be shown by the production of a purple due to the formation of two colours. The test is useful for detecting aldehydes in ether, as mentioned later. Syrupy phosphoric acid may be used instead of sulphuric acid as a condensing agent, in which case a red colour is obtained with all the aldehydes on warming.

The delicacy of the test is greatest for formaldehyde; with glyoxylic acid it will show less than one part in a thousand, with weaker solutions the colour takes some time to develop.

The chemistry of the test has only been briefly investigated as yet. The coloured condensation compounds are probably derivatives of xanthene. When separated from the sulphuric acid they show many of the properties of xanthidrol, such as forming typical insoluble compounds with urea in acid solution. The colour is probably due to the formation of the pyrone ring [Palazzo and Onorato, 1905, and references].

On applying the pyrogallol-reagent to solutions of formaldehyde in sulphuric acid in the presence of oxidising agents, under the conditions of the Rosenheim test, no evidence whatever could be obtained of the formation of glyoxylic acid, though on addition to the mixture it could be detected. This supports Rosenheim's view that the test depends on formaldehyde alone.

From a purely chemical aspect, it is not improbable that both formaldehyde and glyoxylic acid should react with tryptophan to give distinct derivatives, though subsequent investigation showed that the glyoxylic derivative readily lost carbon dioxide and changed into the corresponding formaldehyde derivative as might be expected. The fact that the Hopkins-Cole test is considered by many investigators to be more reliable than the Rosenheim test would be explained by the greater tendency, as subsequently observed, of the formaldehyde to react still further with the condensation products. A disadvantage of both tests is the local heating at the junction of the liquids;

this can be greatly reduced by employing glacial acetic acid as a solvent for the glyoxylate or the formaldehyde as the case may be. As will be shown later, an oxidising agent is necessary in both tests, this is usually supplied by the sulphuric acid [Mottram, 1913].

THE MECHANISM OF THE HOPKINS-COLE REACTION.

Having concluded from the previous experiments that both glyoxylic acid and formaldehyde gave coloured derivatives with tryptophan, which is confirmed by subsequent research, the nature of the reaction was next investigated. Whilst it is well known that indole and its methyl derivatives yield with different aldehydes a large number of coloured derivatives some of which have been investigated, the aldehyde reactions of tryptophan are known chiefly as qualitative colour tests and the exact nature of the compounds formed never seems to have been fully investigated. This is due partly to the difficulty of obtaining tryptophan in quantities sufficient for extended chemical research and partly to the difficulties of isolating the coloured compounds or their precursors in a stable form.

A number of colour reactions between tryptophan and various aldehydes have been described [Rohde, 1905; Sasaki, 1910], but for the purposes of the present paper it will be sufficient to consider three only: formaldehyde, glyoxylic acid and benzaldehyde. It will also be necessary to consider the reactions of these aldehydes with some substances related to tryptophan, namely: indole, scatole, and carbazole.

PREPARATION AND ISOLATION OF THE ALDEHYDE CONDENSATION DERIVATIVES OF INDOLE, SCATOLE, TRYPTOPHAN AND CARBAZOLE.

Condensation. Strong sulphuric acid is quite unsuited for use as a condensing agent for these substances. It may contain a large number of active impurities; it acts as an oxidising agent in a variable manner, the heat it is apt to develop may destroy the condensation products. Fischer [1889] employed alcohol and hydrochloric acid gas for his work on the indoles. Homer and others used zinc chloride. Neither of these reagents was found to be quite satisfactory, and most of the present work was done by means of pure glacial acetic acid saturated with hydrochloric acid gas. This gave good results as a condensing agent, and is free from the objections to sulphuric acid. Phosphoric acid was used in a few particular cases. The glacial acetic acid was purified by fractionation until it no longer gave a colour with tryptophan or any of the aldehyde reagents. When pure it will keep for a long time if moisture and light are excluded, and the bottle be kept filled, as the changes leading to the formation of aldehydes appear to occur in the vapour over the liquid.

Acetic acid so prepared will be referred to in the present paper as "pure" acetic acid.

The hydrochloric acid gas for saturation was prepared by the action of pure sulphuric acid on pure dry sodium chloride. If the acid be badly contaminated with nitrous impurities some of them may pass over into the condensation mixture and spoil the preparation.

The aldehydes were used in the following forms:

Glyoxylic acid as the calcium salt. $(\text{CHO} \cdot \text{COO})_2 \text{Ca}$, $2\text{H}_2\text{O}$.

Formaldehyde as paraformaldehyde. The ordinary solutions of "formol" are unsuitable owing to the presence of methylal and other derivatives [Werner, 1917].

Benzaldehyde as a solution in "pure" acetic acid.

One of the first facts observed during the preparation of the colour condensation compounds by the above method was that an oxidising agent was necessary in every case. The condensation products as formed are colourless or pale yellow; on oxidation the characteristic colour is produced. This fact escapes notice when sulphuric acid is used as a condensing agent since it acts as an oxidiser as well.

For the oxidation a fresh 1 % solution of H_2O_2 in "pure" acetic acid was used. In some instances the oxidation was allowed to proceed spontaneously in the air overnight.

Notes on the Condensation. It is best to use an excess of the solvent and to pass in the HCl gas slowly, shaking at intervals. After the solution has been saturated, gas is passed in for an additional quarter of an hour. A few cc. of the solution are withdrawn in a test-tube and one drop of the 1 % H_2O_2 added, and the tube well shaken, when the colour should develop in a few minutes. The H_2O_2 is added carefully up to the point of maximum depth of colour, too much will discharge the colour altogether. The approximate quantity of H_2O_2 is noted. Passage of HCl into the mixture is continued until no increase in depth of colour is seen on removing and testing the samples. It is sometimes useful to pass a current of air into the mixture to remove excess of HCl before the final oxidation.

The calculated amount of 1 % H_2O_2 is then added to the mixture, which is well shaken till oxidation is complete. It is then poured into a measuring cylinder, three times its volume of purified ether added, and the mixture shaken. The pigment will be precipitated in small flakes. If precipitation is not complete, another volume of ether may be added, but it is advisable not to exceed four volumes if a pure preparation is required, as otherwise the other substances may be precipitated.

Isolation of the Condensation Products. This proved to be one of the most difficult parts of the investigation. It was necessary to free the products from unchanged aldehyde, and indole derivatives, and from the condensing agent. Fractional precipitation with *pure* ether was finally adopted and gave satisfactory results. It was found that if three to four volumes of ether were added to one volume of the acetic acid solution of the pigment and the mixture well shaken, the latter came down as a flocculent precipitate and the aldehydes

and unchanged indole derivatives were left in the ethereal solution, as was also the calcium chloride which was formed when calcium glyoxylate was employed.

The purity of the ether is of great importance. It was found that merely washing and drying it was not sufficient. It should also be freed from aldehydes and related substances by distillation from sodium hydroxide, after having been left in contact with it for at least 24 hours. If the ether contains aldehydes it will react with the precipitated pigment on standing, converting it into a greenish paste, which may resinify on drying.

For this reason the coloured compounds should be filtered from the ether as soon as they are precipitated. The pyrogallol test is useful for detecting aldehydes in samples of ether.

List of Pigments isolated. The following are the colour derivatives obtained by the condensation of indole, scatole, tryptophan and carbazole with the three aldehydes, formaldehyde, glyoxylic acid and benzaldehyde respectively.

(The figures placed below the colour denote the number of molecules interacting.)

ALDEHYDE	CH ₂ O	CHO.COOH	C ₆ H ₅ .CHO
Indole	Red	Red	Red
	2I + F	2I + G	2I + B
Scatole	Red	Red	Purple
	2S + F	2S + G	2S + B
Tryptophan	Red	Red	Blue
	2T + F	2T + G	2T + B
Tryptophan	Blue	Blue	—
	2T + 3F	2T + 3G	
Carbazole	Blue	Blue	Blue
	2C + F	2C + G	2C + B

No attempt has been made to describe the shade in the above list, anything varying from rose to carmine has been grouped as "red" and similarly for the other colours. The qualitative differences are quite definite in many cases.

Many other aldehyde condensation derivatives were prepared, but the above are the most important in connection with the discussion which follows on the chemical constitution of these bodies.

General Properties of the Condensation Derivatives. Both the leuco-compounds and the pigments can be obtained by the ether precipitation method. The leuco-compounds are unstable and soon oxidise to form the pigments.

As precipitated the pigments are all hydrochlorides of nearly colourless bases. The pigments are soluble in strong mineral acids, in the lower aliphatic acids, in esters such as ethyl chloride, in phenol to a less extent. The solubility varies somewhat with the different pigments. The red and blue derivatives of tryptophan are hydrolysed at once when water is added to their solution in the HCl-acetic condensation mixture. The scatole and carbazole pigments are much more stable, the carbazole blues being precipitated on the addition of excess of water. Addition of alkali discharges the colour of the

pigments, as might be expected; in most cases the base is precipitated from the alkaline solution. The pigments were purified by repeated solution in "pure" acetic acid and precipitation with pure ether. They were then rapidly dried in a current of air. When pure they are amorphous deeply coloured powders.

Melting Points. These were all high, in the region of 300° , depending on the rate of heating. The tryptophan reds and blues tend to lose CO_2 on heating.

If the pigments are dissolved in absolute alcohol and treated with dry NH_3 , they are decolorised and gradually precipitated from solution as bases, which in some cases are microcrystalline. Addition of strong acid to the bases restores the original colour of the pigments. The bases were also obtained during an attempt to crystallise the pigments from alcohol. The alcohol apparently combined with the HCl of the pigment and removed it to form ethyl chloride.

Molecular weights. The molecular weights of the bases were determined by the freezing point method with comparatively concordant results, using acetic acid or phenol as solvents. It has been shown that compounds of related indigotin type do not tend to form associated molecules in these solvents [Beckmann and Sabel, 1906].

The pigments are the salts of these bases.

Analyses. For the present preliminary work, the only element estimated was nitrogen. This was done by a modified Kjeldahl method, using mercury as catalyst instead of copper, and drawing the fumes by means of a Folin absorber into a wash-bottle, so that a control could be kept on any nitrogen which might escape combustion. These bodies can be analysed satisfactorily by the Kjeldahl method provided that the heating is continued for a considerable time after the mixture has cleared [cf. Dakin and Dudley, 1914].

The results of the nitrogen estimations were used to check the formulae of the condensation derivatives deduced from the molecular weights, sufficient tryptophan not being at my disposal for a complete analysis of the various pigments.

CHEMICAL CONSTITUTION OF THE CONDENSATION DERIVATIVES.

Up to the present time no structural formulae appear to have been suggested for the tryptophan pigments. From the preliminary examination of these bodies it is possible to assign formulae to them which will satisfy the requirements of the Hopkins-Cole and Rosenheim tests without attempting the more ambitious task of giving a complete account of the chemistry of these interesting condensations.

From determinations of the molecular weights of the colour bases, it appears that all the pigments prepared are the salts of compounds formed by the condensation of two molecules of the indole derivative with one of the aldehyde, except in the case of tryptophan blue, where condensation occurs with three molecules of the aldehyde.

In theory the aldehyde may unite with the indole derivative in three ways:

- (1) by replacing hydrogen in the benzene ring,
- (2) by replacing hydrogen in the pyrrole ring,
- (3) by replacing hydrogen in the imino-group.

The fact that all the pigments arise from oxidation of *leuco*-condensation bodies is, on the quinonoid theory of colour and constitution, strong evidence for union in either of the ways (1) or (2). The subsequent removal of hydrogen from the aldehyde residue and from one of the imino-groups occurs on oxidation and leads to the formation of the double bonds¹.

Fischer [1889] gives the following formula for the aldehyde derivatives of α -methylindole (methylketole), the union taking place by the aldehyde replacing the hydrogen atoms of the β - or 3-carbon atom of the pyrrole groups.

Freund and Lebach have prepared and investigated several aldehyde derivatives of α -methylindole (methylketole). They accept the Fischer formula, which, as they point out [1905], accounts for the production of a colour by oxidation.

Although Fischer's formula is satisfactory when applied to the aldehyde condensation derivatives of indole and methylindole, it is not so applicable to the 3-substituted indoles, such as scatole and tryptophan. Here the 3-carbon is already in union with another group and it is unlikely that under the conditions of the condensation the aldehyde displaces these groups, and even if it did, this would not explain the fact that the scatole-benzaldehyde compound is *deep purple* while the corresponding indole-benzaldehyde compound is *red*.

Again, if condensation did occur in the pyrrole ring in scatole-benzaldehyde purple, it is hard to account for the existence of the leuco-precursor. Freund and Lebach accept the formula for the aldehyde derivatives of scatole on the lines of Fischer, though they note its inability to explain the formation of the colour on oxidation.

If the aldehyde condenses with the carbons in the *benzene* ring, instead of the pyrrole ring, all these objections can be overcome. The existence of both leuco-compounds and pigments can be easily explained in the case of all the substances examined, and the general properties of the pigments can be accounted for.

The di- and tri-phenylmethane dyes in common use are quinonoid salts of bases of this type. One of their disadvantages is their sensitiveness to alkalis,

¹ The formulae are printed so as to show that the nitrogen of the imino-group is in union with two different carbon atoms.

which reconvert them into the colourless bases. It will be seen that the pigments described in the present paper have many properties in common with the di-phenylmethane dyes.

The carbazole pigments are similar in many respects to the indole derivatives. Here condensation with the aldehyde has almost certainly taken place in the benzene ring, as the pigments are produced by oxidation of leuco-compounds. The greater stability of the carbazole blues may be explained as being due to the absence of the mobile hydrogen atom of the 2-unsubstituted indoles, which is capable of changing its position from the nitrogen to the neighbouring carbon atom, and *vice versa*. The carbazole ring is stable and the imino-hydrogen is held by the presence of the benzene rings on either side. This tendency on the part of the indoles to undergo tautomeric change has been studied by various investigators [Weissgerber, 1913]. In assigning formulae to the compounds mentioned in the present paper, the hypothesis that aldehyde condensation takes place in the benzene ring has been applied. According to this, when the indole derivative reacts with the aldehyde in the presence of a condensing agent, water is eliminated and the aldehyde links up two molecules of the indole derivatives by uniting with the carbon atoms of their benzene rings to form the leuco-compound, this on oxidation loses one hydrogen atom from the aldehyde residue and one from an imino-group, with the production of the quinonoid configuration in the molecule and the formation of a colour.

These pigments, especially the benzaldehyde derivatives, and their leuco-precursors are comparable on this hypothesis with malachite green.

EXPERIMENTAL.

The Scatole Derivatives¹.

(1) <i>Scatole formaldehyde red.</i>	$\text{NH} \cdot \text{C}_6\text{H}_4(\text{CH}_3) \cdot \text{CH} : \overline{\text{C}_6\text{H}_4(\text{CH}_3)} : \text{NX}$
M.W. of base, 272.	
(2) <i>Scatole glyoxylic red.</i>	$\text{NH} \cdot \text{C}_6\text{H}_4(\text{CH}_3) \cdot \text{C}(\text{COOH}) : \overline{\text{C}_6\text{H}_4(\text{CH}_3)} : \text{NX}$
M.W. of base, 316.	
(3) <i>Scatole benzaldehyde purple.</i>	$\text{NH} \cdot \text{C}_6\text{H}_4(\text{CH}_3) \cdot \text{C}(\text{C}_6\text{H}_5) : \overline{\text{C}_6\text{H}_4(\text{CH}_3)} : \text{NX}$
M.W. of base, 348.	

Two molecular parts of scatole and one of the aldehyde are dissolved in excess of "pure" acetic acid and condensed by means of dry hydrochloric acid gas as described. The condensation and subsequent oxidation should be done at a temperature not above 20°, otherwise the scatole itself may be oxidised with the formation of a red-brown pigment. The condensation compounds are precipitated by ether and purified as already described. They are amorphous intensely-coloured powders, fairly soluble in dilute HCl and H₂SO₄.

¹ The formulae are written so as to show the union of the nitrogen atom with two different carbon atoms. X represents the acid which combines with the pigment base to form the coloured salt. In the present paper X represents HCl.

The benzaldehyde derivative is the one least hydrolysed by dilution with water. They are all decolorised and precipitated by alkalis, boiling with which reconverts them in part to the original scatole. As prepared by the above method the yield is nearly quantitative.

General formula suggested for these compounds:

The Tryptophan Derivatives

- (4) *Tryptophan formaldehyde red.*
M.W. of base (average), 418.

- (5) *Tryptophan glyoxylic red.*
M.W. of base, above 420.

- (6) *Tryptophan benzaldehyde blue.*
M.W. of base, 494.

Two molecular parts of tryptophan are condensed with one part of the aldehyde as previously described. The preparation must be carried out in one stage, as the compounds may undergo decomposition if left for long in the condensing reagent. The temperature should not rise above 15°. If the aldehyde be added rapidly at the start in preparing tryptophan formaldehyde red, it may interact with the NH group to form a methylene derivative $\text{—N}=\text{CH}_2$, but this does not affect the general formula type given above. This methylenation of the amino-group is an intermediate stage in the formation of the blue compounds to be described later.

The tryptophan reds are the most unstable of the pigments prepared. They readily lose CO_2 on heating, and for this reason it is difficult to isolate tryptophan glyoxylic red, although there is evidence for its existence in solution. It is very difficult to obtain these derivatives in a pure state, apart from tryptophan benzaldehyde blue. The product from the ether precipitation appears to be a varying mixture of the true tryptophan condensation product with condensation products from decarboxylated tryptophan and tryptophan, in which the amino-group has been methylenated. However, there seems to be little doubt that all these reactions are modifications of the condensation along the lines of the formulae given.

Tryptophan benzaldehyde blue is one of the more stable pigments, and seems to be well suited for colorimetric work as well as being a good reagent

for qualitative tests. Cole in 1903 drew attention to the clear colour produced. As obtained by the above method, the pigment is a deep blue powder with a faint lustre. It melts just above 310° , and rapidly decomposes according to the rate of heating. It is soluble in the solvents mentioned, but has not been crystallised from any. Like the previous tryptophan derivatives it is largely hydrolysed on diluting the acetic acid solution with water. Readings of the molecular weight of the pure base in acetic acid were on the average below 500 and just above 490, which corresponds to the theoretical value, 494, required by the formula.

(7) *Tryptophan formaldehyde blue.*

M.W., base 1, 354 (average).

M.W., base 2, 444 (average).

(8) *Tryptophan glyoxylic blue.*

M.W., variable. Above 400.

Base 1 is derived from the decarboxylated tryptophan, Base 2 from ordinary tryptophan.

The Tryptophan Blues. It was confirmed at an early stage in the present research that the Hopkins-Cole test and the Rosenheim test each gave two distinct colours with tryptophan according to the amount of aldehyde used, a fact previously noticed by Mottram [1913] in the former test. With the minimum amount of aldehyde and the test-tube kept cool, the colour in each test is *carmine*. Using more aldehyde and allowing the temperature of the mixture to rise, the colour is *violet* to *blue*. On studying the reaction using "pure" acetic acid and HCl gas, it was found that tryptophan is able to give two distinct coloured derivatives when condensed with formaldehyde or with glyoxylic acid. One class has been briefly referred to above as the tryptophan reds.

When two parts of tryptophan are condensed with three parts of formaldehyde or of glyoxylic acid at a temperature between 50° and 70° , the resulting compound on oxidation is deep blue. Also, when tryptophan red was dissolved in "pure" acetic acid saturated with HCl and left for a couple of days, it gradually changed into the blue compound. This change did not occur in acetic acid alone. The change is accompanied by the liberation of some free tryptophan, as was shown by extracting the mixture with ether containing HCl.

Investigation of the molecular weight of the HCl-free base indicates that the pigment is a di-molecular derivative of tryptophan like the other substances investigated. It was thought probable that the additional molecules of the aldehyde reacted with the *amino*-group. This was shown to be so by the preparation of some indole-hydroxypropionic acid by the action of the calculated amount of NaNO_2 on tryptophan in HCl solution and removal of any excess of HNO_2 by means of urea. This substance only gave a red aldehyde derivative under the above conditions. From this it was concluded that for the production of the *blue* pigment interaction was necessary between the aldehyde and the amino-group of the tryptophan. The first effect of such reaction would be to produce a methylene derivative of the amino-group,

—N = CH₂ which would not pass into a methyl group unless the aldehyde were in considerable excess or a reducing agent were present [Werner, 1917]. Neither of these conditions obtains in the preparation as described above. It is, however, hard to understand why the methylenation of the amino-groups should change the red pigment into a blue one, and there is evidence that tryptophan red may contain such substances without alteration of colour. It is probable that there is an additional condensation. Bearing in mind the fact that the corresponding aldehyde derivatives of carbazole are all deep *blue* in colour, and that the H atom attached to the α - or 2-carbon of indole is labile, it does not seem unreasonable to suppose that a condensation might occur between the carbon of the methylene substituting group in the tryptophan and the carbon atom of the indole group to form a three-ring molecule of the carbazole type, having a pyridine nucleus instead of one of the benzene nuclei. Such a body might be formed as follows:

This ring should resemble carbazole in many of its properties. Perkin and Robinson [1919] have found a similar ring in the harmine series. They have shown that the base C₁₂H₁₀N₂ prepared by Hopkins and Cole [1903] by the oxidation of tryptophan with ferric chloride is harman [Fischer, 1901]. Consequently there is convincing evidence that closure of the tryptophan side-chain can occur. Perkin and Robinson advance the theory that harmine originates in nature by an acetaldehyde condensation with hydroxytryptophan, a substance already identified by Abderhalden and Baumann [1908]. Additional evidence for the closure of the tryptophan side-chain has been obtained in connection with other reactions which I hope to describe in a future communication.

On this hypothesis, the formula for tryptophan blue is:

The quinonoid salt of the colourless base

* Or H, if decarboxylated.

There appears to be very little difference between tryptophan blue prepared from formaldehyde or from glyoxylic acid. Loss of CO₂ occurs during the preparation and the final pigment obtained is probably the same in each case. Unless the condensation be carried out thoroughly, the pigment pre-

cipitated by the ether is very impure, containing, as well as the blue derivative, a varying amount of purple material which probably represents an earlier stage in the condensation when the side chain of only one of the tryptophan molecules has closed up. The pigment should not be precipitated until it has stood for some days, by which time most of the substances will have condensed and oxidised spontaneously to the blue.

The blue is precipitated more readily by ether than the red or the purple colouring matters, but, even so, it is almost impossible to separate them completely.

Tryptophan formaldehyde blue resembles the benzaldehyde blue in some respects and the carbazole blues in others.

When purified it is moderately stable and has been obtained in solution in dilute HCl. Like the other tryptophan derivatives, it melts in the region of 300°, the exact temperature depending somewhat on the rate of heating.

As usually carried out, the Hopkins-Cole test results in a mixture of tryptophan *red* and *blue*, the proportions depending on the conditions. For this reason it is unsatisfactory as the basis of colorimetric work, as two solutions of the same strength may give different shades if the temperature be at all different. Fasal [1912] noticed this difference in tint during his work on the estimation of tryptophan, but considered it entirely due to the amount of colouring matter formed.

For colorimetric work an aldehyde which only reacts in one way with the amino-acid is advisable, if not essential.

The above formula is in agreement with the experimental facts. It accounts for the existence of the leuco-form; for the three molecules of formaldehyde required; and for the colour and general properties of the pigment.

Only aldehydes that react readily with the amino-group, and are also capable of condensing with the indole nucleus, can give the carbolinine blue compound. Tryptophan benzaldehyde and salicyl aldehyde blues are simple condensation products and do not show any evidence of a closure of the tryptophan side-chain.

A study of the freezing-points of the bases obtained by removal of the HCl from the pigments indicated that the molecular weights of the formaldehyde and glyoxylic blues were probably the same, 352. Some preparations had higher molecular weights of the order of 400, which would be explained by the preparations not being fully decarboxylated. The pigment ultimately obtained in each case is one in which CO₂ has been lost by all the COOH groups. It will be seen that the condensation product formed by the closure of the tryptophan side-chain is derived from the ring nucleus:

Perkin and Robinson, who have described a similar ring, suggest the name "carboline" for this structure, indicating an analogy both to carbazole and quinoline. According to this nomenclature, tryptophan blue would be a carboline blue in which the C atoms of the pyridine nucleus are fully saturated, such a ring might be termed "carbolinine" by analogy to the relation between pyrrole and pyrroline. Tryptophan blue then would be "di-4-isocarbolininemethane," and the leuco-precursor would be, "di-4-carbolininemethane."

THE CARBAZOLE CONDENSATION DERIVATIVES.

Several of these bodies were prepared: the following are of most interest in connection with the present paper.

- (9) *Carbazole formaldehyde blue.*
- (10) *Carbazole glyoxylic blue.*
- (11) *Carbazole benzaldehyde blue.*

Only a brief reference will be made to these bodies.

Their preparation and principal properties resemble those of the pigments previously described. They are of different shades depending on the aldehyde and the solvent. They are more stable than the other pigments and will keep for a long time in aqueous solution. They are precipitated by alkalis. The melting points are high, the benzaldehyde blue beginning to decompose at 280°.

The general formula is probably:

This reaction is a good general test for the presence of carbazole; benzaldehyde is perhaps the most convenient aldehyde to work with, but the colours are good with all three.

A SIMPLE QUALITATIVE REAGENT FOR TRYPTOPHAN AND THE INDOLES.

The reagent is 10 % salicyl aldehyde in alcohol, which must be free from acetone. A small quantity of the liquid under examination is mixed with excess of strong hydrochloric acid. Four or five drops of the reagent are added and the mixture gently warmed for a few minutes, a drop of 10 % hydrogen peroxide is then added and the warming continued. An intense blue denotes tryptophan, a deep purple denotes scatole, while indole gives a bright carmine. If too much peroxide be added the colours are destroyed, otherwise, they are very stable and are not discharged on dilution with water. The reagent will

give a deep blue with less than 0.1 % tryptophan; with weak solutions care must be taken in the oxidation. By modifying the strength of the reagents the test can be made very delicate. In properties the pigments resemble very closely the benzaldehyde derivatives, their greater stability towards water is comparable with that of the malachite greens derived from substituted benzaldehyde. The fastness of these latter dyestuffs depends on the presence of a sulpho-group in the ortho-position to the methane carbon, and one is justified in assuming the hydroxyl of the salicyl aldehyde to operate in a similar manner.

Compare the behaviour of salicyl aldehyde and benzaldehyde when condensed with acetone in presence of hydrochloric acid.

SUMMARY.

(1) A new colour test for glyoxylic acid is described, depending on its interaction with pyrogallol in presence of sulphuric acid.

(2) The Hopkins-Cole test is due to glyoxylic acid, the Rosenheim test is due to formaldehyde. In each test at least two distinct colour derivatives of tryptophan are formed, a red and a blue.

(3) A method is described for the preparation of the aldehyde derivatives of indole, scatole, tryptophan and carbazole, and a number of these have been isolated.

(4) The possible formulae of these substances is discussed, and a general type of structure is suggested.

(5) The closure of the tryptophan side-chain is put forward as an explanation of the formation of tryptophan formaldehyde blue.

(6) Salicyl aldehyde is recommended as a useful qualitative reagent for the indole derivatives.

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