XI. ON THE COLOUR REACTIONS OF CERTAIN INDOLE DERIVATIVES, AND THEIR SIGNIFICANCE WITH REGARD TO THE GLYOXYLIC REACTION.

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In the preceding paper [Homer, 1913] the preparation and properties of several new indole derivatives have been described in detail.

The condensation products formed between tryptophane and nascent formaldehyde, formaldehyde and glyoxylic acid respectively in the absence of a condensing agent, are colourless crystalline compounds. The reaction takes place between the $-CHO$ group of the aldehyde and the $-NH_2$ group of tryptophane.

In the presence of condensing agents, such as zinc chloride or an alcoholic solution of hydrochloric acid gas, indole derivatives and pyrrole, by virtue of the $-NH$ group in the nucleus, combine with trioxymethylene to form coloured substances; in the presence of concentrated sulphuric acid a solution of formaldehyde reacts with indole and pyrrole derivatives to give colour reactions.

In the case of the condensation of glyoxal with tryptophane, the presence of an oxidising agent is necessary, and the resulting product, which is coloured, no longer gives the glyoxylic reaction.

The results of an investigation of the colour reactions of the above-mentioned substances have been embodied in the following tables, and afford special interest with regard to the mechanism of (a) the Adamkiewicz reaction for the detection of proteins and (b) Hopkins and Cole's glyoxylic reaction.

SECTION I.

(In the following reactions, air-free water has been used in every case.)

TABLE I. *Colour reactions produced by the action of pure concentrated sulphuric acid on the formaldehyde, and glyoxylic acid condensation products of tryptophane, on the ether oxidation substance and on tryptophane.*

Action of concentrated sulphuric acid	Tryptophane	Formaldehyde product	Glyoxylic condensation product	Ether oxidation substance
A. On the solid :				
i. In the cold ...	None	Purple ¹	Yellow	None
ii. On warming ...	Slight darkening. Finally charring	Colour intensified. Finally charring	Colour intensified. Finally charring	Slight purple tint
B. On the solid suspended in water :				
i. Directly treated with the acid	None	Purple	Yellow	None
ii. Heated to 90°, cooled and then treated with the acid	None	Purple colour intensified	Yellow	None
iii. In the presence of one drop of a 1/200 solution of formalin in water	Brown	Purple colour intensified	Purple	Purple
iv. In the presence of a trace of glyoxylic acid	Purple	Purple	Purple	Purple
v. In the presence of a trace of ferric chloride	Orange red	Very intense purple	Brown	Green
vi. In the presence of a trace of hydrogen peroxide	Orange red	Brown	Claret	Green

¹ The term "purple" has been used for colours that are red with a tinge of blue in them, or vice versa.

TABLE II. *The colour reactions produced by the action of concentrated sulphuric acid on pyrrole and indole derivatives in presence of glyoxal.*

Action of concentrated sulphuric acid	Pyrrole	Indole	Skatole	Indole-propionic acid	Tryptophane
In the presence of :					
i. A trace of glyoxal ...	Reddish brown	Indian red	Burnt Sienna	Reddish brown	Claret
to which has been added a trace of :					
(a) Ferric chloride ...	Greenish tint	Greenish tint	Reddish brown	Deep orange which rapidly changes to purple madder tint	Claret colour intensified
(b) Hydrogen peroxide...					
ii. An infinitesimal trace of glyoxal	Yellow brown	Very delicate shade of Indian red	Reddish brown	Yellowish brown with a tinge of red	Russet
to which has been added a trace of :					
(a) Ferric chloride ...	Intense blue changing to bluish purple	Green	Reddish brown	Orange red which rapidly changes to a purple madder	Russet colour intensified
(b) Hydrogen peroxide...	Green	Green	Magenta		

TABLE III. *The colour reactions produced by the action of pure concentrated sulphuric acid on pyrrole and various indole derivatives in the presence of (A) formaldehyde, and (B) glyoxylic acid.*

Action of concentrated sulphuric acid	Pyrrole	Indole	Skatole	Indole-propionic acid	Tryptophane	Glyoxylic condensation product	Ether oxidation substance
A. i. In the presence of 1 drop of a solution of 1 c.c. of formalin in 10 c.c. of water	Red brown	Purple	Brown	Brown	Brown	Purple	Purple
ii. In the presence of 1 drop of a 1/200 solution of formalin in water	Red brown	Purple	Brown	Brown	Brown	Purple	Purple
to which has been added a trace of an oxidising agent	Green	Purple	Purple	Purple	Purple	Purple	Purple
iii. In the presence of 1 drop of a 1/2000 solution of formalin in water	Yellowish brown	Purple	Purple	Purple	Purple	Purple	Purple
iv. In the presence of 1 drop of a solution of formaldehyde made by dissolving 1 part of trioxymethylene in 1000 parts boiling water	Red brown	Purple	Brown	Brown	Brown	Purple	Purple
v. In presence of 1 drop of the solution used in iv, diluted ten times	Yellowish brown	Purple	Purple	Purple	Purple	Purple	Purple
B. In the presence of glyoxylic acid	Red brown	Purple	Purple	Purple	Purple	Purple	Purple

TABLE IV. *The colour reactions produced by the direct action of pure concentrated sulphuric acid on the trioxymethylene condensation derivatives of indole, skatole and tryptophane.*

(a) Formed in the presence of zinc chloride as condensing agent.

(b) Formed in the presence of an alcoholic solution of hydrochloric acid gas.

Action of concentrated sulphuric acid	Indole derivative	Skatole derivative	Tryptophane derivative
(a) On the solid	Purple	Brown	Brown
(b) On the solid	Purple	Brown	Brown

Tables Va and Vb illustrate the masking effect due to the presence of trioxymethylene.

TABLE V a. *Colour reactions produced by the action of pure concentrated sulphuric acid on aqueous solutions or suspensions of indole derivatives.*

Action of pure concentrated sulphuric acid	Indole	Skatole	Indole-propionic acid	Tryptophane	Glyoxylic condensation product and ether oxidation substance
i. In the presence of 1 drop of a 1/200 solution of formalin in water to which has been added an oxidising agent	Purple	Brown	Brown	Brown	Purple
	Purple	Purple	Purple	Purple	Purple
ii. In the presence of a trace of trioxymethylene	Purple	Brown	Brown	Brown	Purple
iii. In the presence of 1 drop of a 1/200 solution of formalin in water, an oxidising agent, and a trace of trioxymethylene, cf. i (above)	Purple	Brown	Momentary appearance of purple colour, immediately masked by brown colour	Brown	Purple

TABLE V b. *Colour reactions produced by the action of concentrated sulphuric acid, in which has been dissolved a trace of trioxymethylene, on aqueous solutions or suspensions of indole derivatives.*

Action of concentrated sulphuric acid, containing a trace of trioxymethylene	Indole	Skatole	Indole-propionic acid	Tryptophane	Glyoxylic condensation product and ether oxidation substance
i. Directly on the aqueous solution or suspension	Purple	Brown	Brown	Brown	Purple
ii. In the presence of an oxidising agent	Purple	Brown	Momentary appearance of purple, very soon masked by predominating brown	Brown	Purple
iii. In the presence of an oxidising agent and 1 drop of a 1/200 solution of formalin in water	Purple	Brown	Momentary appearance of purple, very soon masked by predominating brown	Brown	Purple

Adamkiewicz [1874] demonstrated that a violet colour is produced by the action of concentrated sulphuric acid on an acetic acid solution of protein. Hopkins and Cole [1900; 1902] showed that the protein reacts by virtue of its tryptophane groupings, and that *pure* acetic acid does not give the colour test. As a result of their investigations they were led to the conclusion that the reactive impurity in the acetic acid was glyoxylic acid.

Therefore, in the Adamkiewicz reaction, Hopkins and Cole regarded

glyoxylic acid as the substance essential to the formation of the characteristic violet colour. Rosenheim [1906] however, points out that the colour produced by the action of formaldehyde on proteins in presence of sulphuric acid and oxidising agents [Voisenet 1905] is identical with that produced in the Adamkiewicz reaction. He criticises Hopkins and Cole's view as to the importance of glyoxylic acid *per se*; he suggests that even with glyoxylic acid an oxidising agent is essential, and that although the latter may not have been added, yet the glyoxylic acid itself, however carefully prepared, is always contaminated with sufficient hydrogen peroxide for the reaction to take place. On the other hand Dakin [1909], noticing that the colours produced by the action of glyoxylic acid and formaldehyde are identical, suggests that in the case of formaldehyde, under the influence of acids, an aldol condensation and subsequent oxidation to glyoxylic acid take place.

From Table I it is obvious that formaldehyde *per se* plays an important part in the colour reaction, since of the three condensation products, the only one which will give the colour without the addition of glyoxylic acid or formaldehyde, is the formaldehyde condensation product. Further it has been shown that this product is readily hydrolysed with liberation of formaldehyde; at the line of junction of concentrated acid and water such hydrolysis would take place. A proof of this assumption lies in the observation that after boiling this product with water and so ensuring hydrolysis, the colour reaction is still more marked (Table I, B ii).

Were the essential factor glyoxylic acid *per se*, the glyoxylic condensation product ought to give the colour reaction with sulphuric acid, but it will not do so unless formaldehyde or more glyoxylic acid be added. Now glyoxylic acid itself is decomposed by sulphuric acid with the formation of carbon dioxide and formaldehyde¹. It therefore seems reasonable to assume that glyoxylic acid is able to take part in the colour reaction by virtue of its decomposition into formaldehyde, and not, as Rosenheim suggests, because of contamination with hydrogen peroxide. The fact that the colour reaction is not given by glyoxal (Table II) and glycollic aldehyde, neither of which gives formaldehyde on treatment with acid, is in support of this view.

On the other hand, if formaldehyde be the essential factor, then some explanation must be offered to account for the generally acknowledged uncertainty of the colour reaction when that reagent is used, whereas with glyoxylic acid there is no difficulty experienced in carrying out the test for indole derivatives.

¹ A small quantity of glyoxylic acid was heated with 5 c.c. of water and 5 c.c. of concentrated sulphuric acid. The gases evolved were found to contain formaldehyde and carbon dioxide.

According to Hopkins and Cole, Dakin, and Rosenheim, the presence of an oxidising agent is necessary for the Adamkiewicz reaction to be carried out with formaldehyde.

In the present investigation samples of pure sulphuric acid (Kahlbaum and others) were specially procured and carefully selected so that they did not induce a purple colour with tryptophane and a 1/500 solution of formalin in air-free water. The addition of a trace of ferric chloride solution to the reacting liquids induced the purple colour to appear. Samples of sulphuric acid contaminated with a trace of an oxidising agent caused the appearance of the purple colour with the use of a 1/100, and in some cases a 1/10 solution of formalin in water.

A study of Table III shows that, using a 1/200 solution of formalin in water, without the addition of an oxidising agent, indole, indolealdehyde, indoleacetic acid, and the condensation products of tryptophane gave a purple colour, skatole, tryptophane and indolepropionic acid gave brown colourations. The addition of a trace of an oxidising agent to the latter was necessary for the production of the purple colour. In this connexion the following observations have been made:

1. In applying the formaldehyde test to tryptophane and skatole, if previous to the addition of the above-mentioned formaldehyde solution a trace of indolealdehyde be added, then the presence of the concentrated sulphuric acid will induce the formation of the characteristic purple colour, even though no oxidising agent has been added. This experiment shows that for the production of the purple colour the indole derivative and not the formaldehyde is attacked by the oxidising agent.

2. If to an aqueous solution of tryptophane one or two drops of formalin be added, the liquid boiled, cooled, diluted and treated with concentrated sulphuric acid, then the characteristic purple is produced, but is soon masked by the brown colouration usually noticed.

In this experiment the presence of one of the condensation products of tryptophane which gives a purple colour with formaldehyde has been assured.

In order to ascertain whether this purple colour reaction was due to an aldol condensation and oxidation of formaldehyde to glyoxylic acid [Dakin, 1909], the formalin itself was boiled, cooled, diluted to 1/200 and added to a cold aqueous solution of tryptophane. There was no production of a purple colour.

It will be seen from Table III, A iii and v that if the formaldehyde be diluted to the order of 1 part to 2000 or 20,000 parts of water, then the

purple colour is formed with skatole, tryptophane and indolepropionic acid without the addition of an oxidising agent. This result was obtained with Kahlbaum's and Merck's formalin, and also with a solution of formaldehyde made by dissolving trioxymethylene in boiling water which had been previously boiled for some time to expel air. The substances to be tested were dissolved in air-free water. Even with such large dilution, there is in the case of tryptophane a certain amount of brown colouration produced, but not sufficient to mask the purple colour. As the concentration of the formalin is increased, so the brown colour preponderates and masks the purple.

These experiments, taken in conjunction with the fact that the formaldehyde condensation product is the only one which gives the Adamkiewicz reaction without the addition of glyoxylic acid or formaldehyde, seem to be conclusive as to the importance of formaldehyde *per se* as a factor in this reaction. But at the same time, in performing the Adamkiewicz colour reaction the fact that concentrated sulphuric acid causes the condensation of formaldehyde to trioxymethylene must be taken into consideration. In the previous paper it has been shown that trioxymethylene, in the presence of a condensing agent, reacts with indole and indoleacetic acid to give purple derivatives; with skatole, indolepropionic acid and tryptophane to give brown coloured substances. In the former cases, therefore, the secondary action with trioxymethylene will not interfere with the purple colour formation, but in the latter cases the brown colour due to the formation of trioxymethylene derivatives will preponderate. The correctness of this assumption is demonstrated by the results described in Tables V *a* and V *b*. It will be noticed that a trace of trioxymethylene interferes with the appearance of the purple colour which would normally be produced under the conditions of the experiment (V *a* iii and V *b* iii). The masking effect due to the formation of trioxymethylene by the action of concentrated sulphuric acid on the formaldehyde may be obviated:

1. By using the formaldehyde solution excessively dilute, whereby the formation of trioxymethylene is delayed, and the brown colouration due to the condensation of certain indole derivatives with trioxymethylene does not interfere with the Adamkiewicz test.

2. By the addition of a trace of an oxidising agent, whereby the formation of an indole derivative, which will give the colour test, is induced.

3. In the case of tryptophane, by ensuring the previous formation of one of the three condensation products described above (p. 121).

4. By the use of glyoxylic acid as the source of formaldehyde instead

of formaldehyde itself (Hopkins and Cole's modification of the Adamkiewicz reaction).

The author suggests that at the line of junction of the concentrated sulphuric acid and the aqueous layer containing a trace of glyoxylic acid and the indole derivative, decomposition of the glyoxylic acid takes place with the liberation of formaldehyde. The vivid colour reaction may be assigned to one of the following causes:

1. The amount of formaldehyde produced under the conditions of the experiment is never more than a trace, and the corresponding trioxymethylene formation does not interfere with the colour reaction (Table III).

2. The formaldehyde is liberated in the nascent state, and is therefore intensely reactive.

Either of these explanations can be offered to account for the vivid colour reaction when the formaldehyde condensation product of tryptophane is acted upon by concentrated sulphuric acid without the previous addition of formaldehyde, glyoxylic acid or an oxidising agent. But that the nascent condition of the formaldehyde is not essential to the colour reaction is deduced from the fact that the addition of a 1/10 solution of formalin in water to the ether oxidation substance or the glyoxylic condensation product gives rise in the presence of concentrated sulphuric acid, to the formation of a purple colour.

3. In the glyoxylic reaction with tryptophane, the colour reaction is probably influenced by the action of the formaldehyde, liberated from the glyoxylic acid, on the glyoxylic condensation product formed with tryptophane.

SECTION II.

The following experiments show that the colour reaction induced by the action of concentrated sulphuric acid on solutions of indole derivatives to which formaldehyde and an oxidising agent have been added, is of a much more complex nature than when formaldehyde alone is used.

In Section I (p. 121) it was demonstrated that the so-called "glyoxylic" colour reactions could be obtained by the action of concentrated sulphuric acid on solutions of tryptophane and skatole to which had been added traces of indolealdehyde and formaldehyde. Therefore in the Adamkiewicz test the part played by the oxidising agent is to convert the tryptophane and skatole into some indole derivative which will react with formaldehyde to form compounds capable of giving purple colour reactions with sulphuric acid and not to oxidise the formaldehyde as Dakin suggests.

A study of the colour reactions of indolealdehyde was made:

*Colour reactions induced by the action of concentrated sulphuric acid
on solutions containing:*

	Colour produced.
(1) Indolealdehyde.	Pink. Colour becomes more intense on warming [Ellinger and Flamand, 1909].
(2) Indolealdehyde + one drop of a 1/100 solution of formalin.	Reddish purple.
(3) Indolealdehyde + trace of trioxymethylene.	,, ,,
(4) Indolealdehyde + a trace of an oxidising agent.	Bluish magenta.
(5) Indolealdehyde + tryptophane.	Orange red colour.
(6) Indolealdehyde + tryptophane + a trace of formaldehyde (1/100).	"Glyoxylic" colour at the line of junction of the acid and aqueous solution. Above this is seen an orange red colour.
(7) Indolealdehyde + tryptophane + an oxidising agent.	Magenta.
(8) Indolealdehyde + tryptophane + a trace of trioxymethylene.	"Glyoxylic" colour as in (6) but it is rapidly masked by the brown colour characteristic of the action of trioxymethylene on tryptophane.
(9) Indolealdehyde + skatole.	Reddish pink colour.
(10) Indolealdehyde + skatole + a trace of formaldehyde (1/100).	At the junction of the acid and solution a reddish purple colour is obtained (as in (11)). Above this layer the colour is reddish pink.
(11) Skatole + a trace of an oxidising agent + a trace of formaldehyde (1/100).	Reddish purple.

If the indolealdehyde in (10) be present in excess then the colour produced is practically that produced in (9), the purple being masked.

It is obvious from these experiments that where colour reactions are produced from tryptophane in the presence of an oxidising agent the secondary reactions with indolealdehyde have to be considered, and in these cases the actual shade of colour will depend upon the conditions of the experiment.

From (6) it will be seen that the "glyoxylic" colour is produced by the action of formaldehyde (*without the addition of an oxidising agent*) on the mixture of tryptophane and indolealdehyde. It may be that the indolealdehyde reacts with the amino-group of tryptophane to form a substance which then combines with formaldehyde as did the three condensation products (Section I, Table I).

An inspection of reactions (4) and (7) shows that in the presence of an oxidising agent concentrated sulphuric acid causes indolealdehyde to condense with more indolealdehyde (4) or with tryptophane (7) to form substances giving characteristic colour reactions which are not those of the "glyoxylic" reaction.

Although the chemical reactions involved in the actual process of the purple colour formation are at present unsolved yet it is clear that the condensation of indole nuclei is an essential feature of the reaction. In the case of those compounds which give the purple colour directly with formaldehyde the condensation takes place through the agency of the formaldehyde alone. But for those substances for which, on account of interference with the colour reaction due to the formation of trioxymethylene, an oxidising agent is used, the reaction also involves the condensation of indolealdehyde with the original indole derivative taken.

SUMMARY.

The formation of coloured condensation products from indole derivatives and certain aldehydes necessitates the use of condensing agents. The reaction takes place between the $-NH$ group of the indole nucleus and the $-CHO$ group of the aldehyde.

The evidence adduced in this paper is favourable to the Adamkiewicz reaction being primarily a formaldehyde reaction.

1. The formaldehyde condensation product of tryptophane gives the colour test directly with concentrated sulphuric acid, without the addition of an oxidising agent or of glyoxylic acid or of formaldehyde.

2. A trace of formaldehyde, without the addition of an oxidising agent, gives the colour reaction with indole derivatives.

3. When formaldehyde is used in testing for indole derivatives, the purple colour is often masked on account of further reactions taking place between the indole substance and the trioxymethylene formed from formaldehyde by the condensing power of the concentrated acid.

4. In Hopkins and Cole's modification of the test, glyoxylic acid reacts by virtue of its decomposition into formaldehyde, and for one of the two following reasons is the best reagent to use for the reaction:

(a) the formaldehyde is liberated in such small quantity that the formation of trioxymethylene does not take place to any appreciable extent,

(b) the formaldehyde so liberated is in the nascent state, and therefore more reactive.

5. In the case of tryptophane the colour test is often performed with formaldehyde in the presence of an oxidising agent. The part played by the latter is primarily to produce some oxidation product of tryptophane, e.g. indolealdehyde.

The reaction under these conditions is of a complex nature as, besides the

effects described above, (2) and (3), it involves the formation of coloured substances by the action of the concentrated acid on:

- (a) indolealdehyde and tryptophane.
- (b) indolealdehyde in the presence of an oxidising agent.
- (c) indolealdehyde and tryptophane in the presence of an oxidising agent.
- (d) indolealdehyde and tryptophane in the presence of formaldehyde.

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