IV. THE DIAZO REACTION IN URINE.

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THE extensive literature on the "diaz reaction" in urine leaves many problems still unsolved. [For literature *v.* Neubauer-Huppert, 1913.] Ever since it was found that some urines gave an orange or red colour in an alkaline solution of diazotised sulphanilic acid there have been periodic attempts to isolate the substance responsible for the reaction, but so far none of these has been quite successful. Only two colour-giving substances have, as far as the writer can find, been isolated from urine and identified. The first was urocanic acid isolated by Jaffé [1874] and later found by Siegfried [1898] in dogs' urine—the dogs probably presenting a rare anomaly of metabolism. The second substance was histidine, isolated in small amount by Engeland [1908] from 40 litres of human urine. Again, in 1922 the writer [Hunter, 1922, 2] isolated 0.1 g. of histidine as monohydrochloride from a litre of measles urine in which it was estimated that there was 0.31 g. present.

Many suggestions have been made as to the cause of the diazo reaction in urine. Clemens [quoted by von Noorden, 1907] claims to have isolated the sodium salt of the substance responsible for the reaction. Others assume it to be due to chromoxy-proteic acids or to ill-defined members of this group of substances [Neubauer-Huppert, 1913]. Weiss [1920] is emphatic that urochromogen is responsible for the diazo reaction, whilst Hermanns and Sachs [1921] consider that an oxidation product of tyrosine is responsible for the reaction in tuberculous patients and hydroxyindoleacetic acid was indicated in a urine from a patient suffering from cancer of the liver.

Ehrlich first applied the reaction to urine but his procedure has been many times modified and this adds to the difficulty of interpreting the findings by his method. Ehrlich's diazo reagent consisted of a mixture of two solutions [Ehrlich, 1883]:

(a) 5 g. of sulphanilic acid with 50 cc. conc. HCl made to 1 l. with water.

(b) 5 g. of sodium nitrite made to 1 l. with water.

The solution for carrying out the test was made by mixing 50 cc. of solution (a) with 1 cc. of solution (b).

In performing the test [Ehrlich, 1884] a quantity of urine was taken and an equal quantity of reagent added. If a colour was produced at this stage—what was called a "primary colour reaction"—it indicated the presence of

bilirubin. Strong ammonia was then added, and if the test was positive a "secondary colour reaction" was obtained and a red foam on shaking, regarded as of special significance in typhoid, measles, certain types of tuberculosis and other disorders.

There are thus two distinct types of diazo reaction according to Ehrlich—the primary colour reaction in acid solution and the secondary colour reaction in alkaline solution—parallel with the fact that aromatic amines couple with diazonium salts in acid solution and phenols in alkaline solution.

The primary colour reaction of Ehrlich appears to be very specific for bilirubin and this test has recently been used by van Bergh and others [McNee, 1923] in blood serum to distinguish certain types of jaundice. The diazo reaction in acid solution will not be further dealt with in this paper as there has never been any doubt about the substance responsible for it. It is the substances in urine which give a colour with diazonium salts in alkaline solution which have been wholly responsible for differences of opinion in the literature on the diazo reaction in urine; and it is with the diazo reaction in alkaline solution that the writer here purposes to deal. Hitherto only one type of diazo reaction in alkaline solution has been recognised, but, as the sequel will show, there are what may be regarded as two quite distinct types of diazo reaction in alkaline solution.

The writer has had the advantage of using a much more satisfactory diazo reagent than that used by the older investigators on this subject, namely that of Koessler and Hanke [1919], and has reviewed elsewhere the main substances which give a colour with the reagent [Hunter, 1922, 1]. These include phenols, iminazoles, certain purines, tyrosine, sulphides and ammonium salts. The reagent is thus very unspecific but no less so than the older reagents. With careful handling of the solution to be tested the non-specificity of the reagent is perhaps more of an advantage than a disadvantage as it permits the use of a very delicate reagent in the estimation of a wide variety of substances. Its reaction in urine will be considered here only from a qualitative aspect.

The diazo reagent, according to Koessler and Hanke [1919], consists of two stock solutions:

(a) 9.0 g. of sulphanilic acid with 90 cc. of 37 % HCl made to 1 l. with water.

(b) 50.0 g. of 90 % sodium nitrite made to 1 l. with water.

The reagent is prepared by placing in a 50 cc. volumetric flask 1.5 cc. of solution (a) along with 1.5 cc. of solution (b). After 5 minutes 6.0 cc. of solution (b) are added and after another 5 minutes the flask is filled to the 50 cc. mark with cold water. It is recommended by the authors to keep the flask immersed in a freezing mixture, and if kept cold this reagent can be used for at least 24 hours.

A 1.1 % solution of pure anhydrous sodium carbonate is used as alkali.

When the reagent is used for quantitative measurements the sodium

carbonate solution and the diazo reagent are first mixed and then the solution to be tested is added.

This preliminary mixing of sodium carbonate and reagent has several advantages, and the mixture remains perfectly clear and colourless for about 30 minutes, so that all the colour developed is due to the test solution added.

The use of sodium carbonate as alkali is also important. Ehrlich used ammonia as alkali although it gives an orange yellow colour with the reagent in a few seconds. On the other hand, Pauly [1904] used solid diazobenzene sulphonate and sodium carbonate, but this mixture quickly turns orange.

Another important point emphasised by Koessler and Hanke is the order in which the solutions are used. The acid reagent has an excess of nitrous acid and may partially deaminate the substance to be tested before it has time to couple in the solution subsequently made alkaline. To prevent such a deamination it is necessary that the reagent be made alkaline before the addition of the test solution.

The reagent of Koessler and Hanke gives very little trouble if certain precautions are taken and the writer follows in general their procedure. The continuous use of ice is, however, not always convenient and can be dispensed with if the temperature of the tap water of the laboratory is less than 10°. The sulphanilic acid solution first put into the reagent flask is allowed to come to the temperature of the running water, then the sodium nitrite solution, preferably kept in the ice chest, is added. The mixing of the sulphanilic acid and sodium nitrite is the critical step in the making up of the reagent as diazotisation is a strongly exothermic reaction, so that the solutions should be as cold as possible before mixing. If attention is not paid to this point unsuitable reagents will often result.

When a sodium carbonate solution which has been made up for some time is used as alkali there is an abnormally rapid colour development, a maximum colour of too yellow a tint and of too low an intensity.

The reagent has been used for qualitative purposes in the following manner by the writer: 5 cc. of 1.1 % sodium carbonate are put in a test-tube and 2 cc. of the sulphanilic acid reagent added. The contents are mixed by shaking the tube but without inverting it. If any colour is developed at this stage it is probably due to a dirty test-tube. Within the space of about 1 minute *one drop of urine* is added. One of two distinct phenomena may occur at this stage: (1) *Alkaline diazo reaction, Type A*. In all urines a faint yellow colour is produced which goes on intensifying for the next 3 to 4 minutes. This colour does not generally develop further than a pale orange but may often show predominately a pink tinge and occasionally quite a marked red. (2) *Alkaline diazo reaction, Type B*. In urines from typhoid and measles a bright orange red colour appears in the solution immediately the drop of urine is added, but the colour disappears in a few seconds. (Thereafter there is an intensification of colour due to the substances responsible for Type A reaction.)

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 mm.
	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 $\frac{1}{3}$ of the colour obtainable from the iminazoles actually present in the urines.

On the other hand some urines give a redder colour by this direct method than one would be led to expect from the actual amount of iminazole present. This appears to arise from a decrease in the concentration of the substances inhibiting colour production. Thus the writer [Hunter, 1922, 2] was led to the conclusion that there was an increased excretion of histidine in urine from measles patients through finding a marked diazo reaction of this type, although as shown there were present 310 mg. of histidine monohydrochloride per litre, a not abnormal amount of iminazole as subsequent work has shown. This reaction, it should be noted, was also mistaken for the characteristic reaction given by measles urines before Type B reaction came to the writer's notice. Indeed, there is ample evidence in the literature that this confusion is common. Thus Neubauer and Huppert [1913] quote at least three workers who found a positive Ehrlich's diazo reaction in a fairly large proportion of normal urines. These positive reactions were probably due to the presence of iminazoles and almost certainly not due to the substance which gives the character-

istic test in typhoid and measles. For among several hundreds of normal urines the writer has failed to find one which gives a test of the type given in typhoid or measles.

Of course phenols in urine also contribute to the colour given in Type A reaction. Like the iminazoles the colours for phenols take definite if generally shorter times to develop to a maximum. Likewise purines and tyrosine intensify in colour, though even in pure solution the final colour intensity is not proportional to the amount of the purine or tyrosine present. Ammonium salts and sulphur compounds also give yellow colours, and there are probably several chromogens which either yield a colour in themselves or in some way prevent the diazo reagent from coupling with the iminazoles or phenols.

It may be concluded that the development of a colour predominantly red, of the alkaline reaction A type, is due to the presence of iminazole. The red colour may indicate that the iminazole is present in abnormally large amounts or merely that it is present in normal amounts when the conditions in such a urine are favourable for the coupling of the diazonium salt with the iminazole present. Urocanic acid and histidine are the only two iminazoles so far isolated from urine, though there are probably other iminazoles responsible for Type A reactions.

Type A alkaline diazo reaction is likely to remain of little value as a biochemical test. The interpretation is still too uncertain, as the reagent is too unspecific and too easily interfered with to give reliable information even regarding iminazoles. The normal output of iminazoles in the urine is in the writer's opinion not yet definitely settled although the value of Koessler and Hanke's [1924] contribution in this direction is recognised. But apart from this, practically nothing is yet known of the physiological significance of either a high or a low iminazole excretion. Yet it does appear of importance to distinguish a reaction of Type A from a reaction of Type B such as is obtained in typhoid and measles urines, since Type B reaction holds promise of being a valuable test for certain pathological urines.

2. *Alkaline Diazo Reaction, Type B.*

As far as the writer is aware the alkaline diazo reaction Type B has not hitherto been observed. Its characteristic property is the almost instantaneous appearance of a bright orange red colour of only momentary duration. There is never any doubt as to whether the test is positive or not, as would appear to be in the case of a red foam according to Ehrlich's procedure. Within the past two years the writer has found this type of reaction in the urine of upwards of 30 different measles patients, and in about the same number of typhoid patients. It has been found to persist in some typhoid urines for nearly two weeks, but its onset and disappearance have not been sufficiently studied from a clinical standpoint to permit of discussion here.

A differentiation of two distinct types of diazo reaction in alkaline solution has hitherto been overlooked because of two main factors working together:

(a) with the more concentrated reagents of Ehrlich or Pauly the time of colour development is greatly reduced; (b) with the relatively large amounts of urine previously used such an excess of the substance responsible for Type B reaction is in the solution that a fading in colour is not readily observable. Further, the colours produced by iminazoles with the old reagents are less stable than those with the Koessler and Hanke reagents. It also appears that the Ehrlich reagent is less sensitive to the substance responsible for Type B reaction than is the new reagent. Thus, a sample of typhoid urine which showed a beautiful, though momentary, orange red colour when dropped into the new qualitative reagent as described above, when tested by the Ehrlich method showed a bright orange colour quickly, but on shaking, the foam was no more red than that obtainable from a urine containing about 0.04 % iminazole.

There is little doubt that the substance (or substances) responsible for Type B alkaline diazo reaction is new. From the fact that the diazo reaction has always been recognised as occurring in urines, especially from typhoid and measles, it is also most probably true that this substance was responsible for many of the so-called positive diazo reactions recorded in the older literature. Yet the possibility that iminazoles were responsible for many positive reactions can never be quite eliminated. The method suggested for carrying out the test cannot lead to this confusion.

Within the past two years the writer has made various unsuccessful attempts to isolate the substance from both typhoid and measles urines. The substance disappears in a few days from urine which is allowed to become alkaline, but may be kept for a longer period in acid urines. It is not precipitated by neutral lead acetate but is by mercuric acetate. It disappears on treatment with silver nitrate. It is readily soluble in ethyl alcohol and slowly extracted by butyl alcohol from acidified urine. It reduces alkaline potassium permanganate.

No suggestion of the nature of the substance was obtained until the appearance of a recent paper by Baudisch [1924] on the oxidation of pyrimidines by ferrous bicarbonate. (The only substance previously found by the writer to give a Type B reaction was adrenaline.) Baudisch's work was repeated on uracil. One litre of distilled water, 0.01 g. uracil and 5 g. sodium bicarbonate were boiled for 30 minutes in a 2 l. flask. A small test-tube containing 5 g. ferrous sulphate was then suspended in the neck of the flask. A rubber stopper, through which was a glass tube with a cock on the outside, was then tightly inserted into the neck of the flask with the cock open, and the boiling continued for 10 minutes. The flask was then removed from the flame, the cock closed, and the flask cooled under the tap. The flask was then inverted to mix the ferrous sulphate with the sodium bicarbonate solution. White ferrous bicarbonate was thus precipitated. The cock was then opened to admit air, the stopper removed and the contents of the flask shaken in contact with air until all the iron was changed to the red ferric hydroxide. The fluid was then

filtered and distilled *in vacuo* at 40° to a volume of about 10 cc. One drop of this solution gave with the diazo reagent a bright purplish colour of only momentary duration. The substance responsible for this test according to Baudisch is an unstable oxidation product of uracil, dihydroisobarbituric acid or a condensation product.

The Baudisch oxidation by ferrous bicarbonate is apparently applicable to cyclic compounds containing an ethylene group. This group is common to the pyrimidines and is oxidised by ferrous bicarbonate by the addition of one molecule of water as follows:

Dihydroisobarbituric acid is unstable and condenses to a yellow-coloured pigment, a bimolecular pinacone-like compound, of an auto-oxidisable nature. The evaporated fluid (dihydroisobarbituric acid or the yellow pigment) reduces ammoniacal silver nitrate solution, gives a blue colour with phosphomolybdic acid and reduces methylene blue.

The writer obtained a reduction with ammoniacal silver nitrate, a blue colour with phosphomolybdic acid and a marked diazo reaction of Type B. The colour obtained with this oxidation product of uracil was however much more purple than that obtained from typhoid urines.

Indole contains an ethylene group like uracil and this was tested as described above for uracil. The final solution gave a reaction of the same type—a momentary orange colour which quickly faded to yellow. This colour was much more like that obtained from the typhoid urines but less bright in colour.

The iminazoles contain an ethylene group; histidine, therefore, was treated in the same manner. The final solution from histidine gave a reaction still of the same type, but the colour was much more yellow than that from typhoid urine. It may be noted here that after treatment with ferrous bicarbonate the solution showed no signs of a diazo reaction of Type A, a finding which shows that the iminazole ring suffers some change.

It cannot be concluded from these findings that the substance in typhoid urine is an oxidation product of indole, merely on the ground that the colour given by oxidised indole is nearer that from the typhoid urine than the other substances tested; for the substance in typhoid urine has not yet been obtained in sufficiently pure solutions to warrant the conclusion that an orange red is its characteristic colour, and, further, there is a wide variety of substances in the body presumably capable of the ferrous bicarbonate oxidation.

It is remarkable that the oxidation products of cytosine, uracil, indole and histidine all give a diazo reaction of Type B. This constancy of finding would suggest that Type B diazo reaction indicates some common atomic con-

figuration such as has been suggested by Baudisch for dihydroisobarbituric acid or the yellow pigment. (Whether it is dihydroisobarbituric acid or its condensation product which is responsible for the reaction does not appear quite clear from Baudisch's paper.) The substance responsible for Type B reaction in typhoid urines has thus probably a part of its molecule of the same configuration as the oxidation products of uracil or of other cyclic compounds with an ethylene linkage. The substance in typhoid urine reduces ammoniacal silver nitrate solution as does the oxidation product of uracil. Both substances are unstable in alkaline solution and reduce alkaline potassium permanganate.

M. Weiss's [1920] conclusion that the substance responsible for the diazo reaction in urine is urochromogen is supported by Baudisch's finding that the oxidation product of uracil condenses to a yellow pigment. It would however seem to be necessary to have a reliable analysis of pure urochrome and also an analysis of the substance in question before the hypothesis of Weiss can be regarded as an established fact.

Further details of the properties of the oxidation products of uracil, or of substances capable of this ferrous bicarbonate oxidation, may suggest new methods for the isolation of this substance from urine. If the substance does not actually prove of importance from a clinical point of view it is at least interesting as a pathological finding and may in time prove to be an exaggeration of some normal physiological process.

CONCLUSION.

An attempt has been made to systematise the different types of diazo reaction in normal and pathological urines. The Ehrlich diazo reaction in acid solution now used extensively to distinguish different types of jaundice, is only referred to as there has never been any question of its specificity in biological fluids for bilirubin. The diazo reaction in alkaline solution, or more specifically in sodium carbonate solution, has been divided into two types called for convenience Type A and Type B. Type A reaction is characterised by a gradual development of colour until a relatively stable maximum is reached. Type B reaction is characterised by an immediate development and a very rapid disappearance of the colour.

All urines give some form of Type A reaction. The substances in urine mainly responsible for it are iminazoles, phenols, purines and probably unknown chromogens. Type B reaction is given only by pathological urine, especially that from typhoid and measles. The substance responsible for Type B reaction is most probably a hitherto unisolated substance. Some indications of its probable nature have been suggested.

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