LV. STUDIES ON BILE PIGMENTS.

I. A STUDY OF EHRLICH'S TEST FOR UROBILINOGEN AND SCHLESINGER'S REACTION FOR UROBILIN.

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THE study of bile pigment derivatives is important because these substances represent the end-products of haemoglobin metabolism. The destruction of haemoglobin both normal and pathological can thus be measured by quantitative determination of the urobilin and urobilinogen in urine and faeces. On the other hand, when pathological destruction of haemoglobin can be excluded, urobilin- or urobilinogen-uria indicates failure of the liver to take up these substances from the portal circulation. Thus the test is valuable in the study of both blood and liver diseases. In the present work the Ehrlich-Neubauer colour reaction for urobilinogen with *p*-dimethylaminobenzaldehyde in HCl and the Jaffé-Schlesinger fluorescence reaction with zinc-acetate-alcohol for urobilin have been studied as qualitative urine tests.

Neubauer [1903] identified urobilinogen as the reacting substance in Ehrlich's aldehyde test but described also positive reactions with other pyrrole derivatives such as reduced haematin and haemopyrrole. Thomas [1907] summarised the chemical properties of urobilin and urobilinogen and mentions indole and tryptophan as interfering substances. Fischer and Meyer-Betz [1911; 1912] first isolated urobilinogen from a pathological urine and assumed this substance to be identical with mesobilirubinogen prepared from bilirubin. Watson [1932; 1933] and Lemberg [1934] however stated that urobilinogen is not identical with mesobilirubinogen which differs from the former in its characteristic violet iron reaction. The quantitative estimation has been greatly improved by Charnass [1909] who first applied Ehrlich's test quantitatively instead of using the less stable urobilin reactions. Terwen [1924] devised a quantitative method introducing ferrous salts for the reduction of urobilin.

EXPERIMENTAL.

Ehrlich's test with p-dimethylaminobenzaldehyde in HCl (aldehyde test).

For the application of the aldehyde test to urine it is essential to observe the following standardised conditions. Fasting urine should be used in order to avoid errors due to digestive urobilinogenuria and dilution of the urine. Even so a heavy meal as long as 2 days before can cause a marked increase of urobilinogen so that in doubtful cases the test should be repeated. The urine must be fresh or can be kept in the cold for a day in a dark bottle filled up to the cork. The reagent is prepared by dissolving 1 g. of pure *p*-dimethylaminobenzaldehyde in 100 ml. of conc. HCl and kept in a dropping bottle delivering 20 drops per ml. Neither dilution of the HCl nor the prescription of Niemann [1925] for avoiding the faint yellow tinge of the reagent is of advantage in testing the urine.

The final concentration of HCl in the urine is of the greatest importance for a correct test, since excess destroys the urobilinogen and the urobilinogen-aldehyde product and hydrolyses protein and indole derivatives transforming them into more reactive compounds. Since the reagent is made up in conc. HCl the final concentration of the latter will depend on the quantity of reagent added, since a single drop of the reagent provides a great excess of aldehyde for complete reaction; the effect of varying final concentrations of reagent may therefore be ascribed to variation in HCl concentration. In Table I is shown the effect of varying quantities of reagent on the degree of coloration as measured in an Autenrieth colorimeter with Terwen's standard solution. For the comparison of the urine test a compensatory trough filled with suitably diluted urine is used as a light filter. In column 1 the quantities of reagent added to 2 ml. of urine or faeces extract are shown, in column 2 the final concentrations of HCl in % and in columns 3-5 readings of the scale multiplied by the dilution rate.

The aqueous faecal extract and the urine with increased urobilinogen both show the highest figure on addition of 0.15 ml. of aldehyde reagent corresponding to a concentration of 2.5 % or 0.69 *N* HCl. Normal urine, however, shows no peak, but a steady increase up to a concentration of 7.2 % HCl which can be explained only by the hydrolysis of interfering substances. The latter play a minor rôle in both the other solutions with higher urobilinogen contents. The importance of interfering substances especially in normal urine may be demonstrated by carrying out the same procedure in ether extracts of the urines and faecal extracts; the readings obtained in this way were 42 for the faecal extract, 32 for the urine with increased urobilinogen and 17 for normal urine. Since urobilinogen is completely extracted by ether the difference in the results obtained can only be explained by the presence of interfering substances which are not extracted by ether.

Table I. *Influence of different concentrations of HCl on the aldehyde test.*

ml. of reagent	Final concentration of HCl %	Diluted aqueous extract of normal faeces	Diluted urine with increased urobilinogen	Normal urine
0.025	0.4	34	41	22
0.05	0.9	44	65	30
0.075	1.3	47	72	31
0.1	1.7	48	74	36
0.125	2.1	50	68	37
0.15	2.5	57	76	42
0.2	3.3	55	68	45
0.25	4.0	52	55	47
0.3	4.7	46	53	51
0.35	5.4	—	—	52
0.4	6.0	—	—	59
0.45	6.6	—	—	55
0.5	7.2	—	—	59

The effects of temperature and time factors on the aldehyde test are shown in Table II. The determination is the same as in Table I, 0.15 ml. of reagent being added to 2 ml. of aqueous faecal extract. The temperature was measured before and after each series of readings and the mean temperature given in the Table. The course of the aldehyde reaction is more rapid at higher temperatures but at the same time the destructive and hydrolytic effects of the acid increase at an even higher rate. It is therefore quite erroneous to boil the urine as is done sometimes, when a reaction fails to occur in the cold. Optimum conditions of

temperature and time appear to be 20° and 5 min. after mixing. Freshly passed urine is better cooled first to room temperature.

From the above results the following procedure is recommended for the aldehyde test:

0.15 ml. (3 drops) of 1% *p*-dimethylaminobenzaldehyde in conc. HCl is added to 2 ml. of fresh or suitably preserved urine at about 20° in a test-tube of 10 mm. diameter. The reaction is normal if after 5 min. an orange tinge is observed, increased if the colour is red, and negative if the colour is yellow.

Table II. *The aldehyde test at different times and temperatures.*

Times in minutes after addition of the reagent	11°	18°	29°	54°
0.5	13	22	24	34
1	17	31	34	49
1.5	21	34	37	40
2	27	38	40	36
2.5	30	38	40	34
3	31	39	42	30
3.5	34	39	41	28
4	36	40	—	30
4.5	37	41	—	27
5	36	41	—	27

The value of the aldehyde test in the urine is greatly restricted by its non-specificity in the presence of interfering substances (see Table III). The orange-coloured reaction of normal urine is caused by mixed red and yellow reactions. The two different phases of this reaction can be observed more distinctly by employing a ring test and adding the aldehyde reagent underneath the urine by means of a capillary tube. A yellow ring appears which after a time becomes

Table III. *The most important substances interfering with the aldehyde test in the urine.*

Substances	Colour of reaction
Indole	Red
Indican	Yellow
Skatole	Blue
Protein and protein derivatives	Red-orange to violet
Tryptophan	Yellowish to reddish
Pyrrole and pyrrole derivatives	Red
Urea	Yellow
Salvarsan	Yellow
Extr. Rhei	Brown
Phenazone	Salmon-coloured
Aldehydes and hexamethylentetramine	Inhibiting the urobilinogen aldehyde reaction

reddish at the lower surface. The yellow reaction is mainly due to urea and indican, the red to urobilinogen and indole. In cases of complete obstruction of the bile duct only a yellow reaction appears, but by using the ring test a faint reddish tint can be found which can be attributed to indole alone. A positive aldehyde test can be masked by pigments which do not themselves react such as bilirubin or drugs such as Extr. Sennae. On the other hand a reaction can be inhibited by the presence of aldehyde compounds such as formaldehyde, acetaldehyde and hexamethylentetramine which splits off formaldehyde in the presence of acids. Such inhibiting substances are present also to a small extent in normal urine, as it was found that the reaction of aqueous faecal extract is decreased by addition of normal urine and very markedly by that of some

pathological urines. In the presence of albumin the reaction is delayed for hours and gives finally a strong brownish or violet colour. These facts demonstrate that the aldehyde test is not sufficient for the identification of urobilinogen in the urine.

The Jaffé-Schlesinger test for urobilin.

This test is based on the green fluorescence produced by zinc acetate in alcohol with urobilin. The same precautions regarding the general conditions of the urine as were given previously for the aldehyde reaction should be observed. The original direction by Schlesinger [1903] to use as reagent a suspension of 10 % zinc acetate in absolute alcohol appears most suitable. Neither the modification given by Adler [1922] nor that given by Elzso [1934] gives better results. In order to perceive smaller degrees of fluorescence the urine-alcohol filtrate must be viewed in a sufficiently deep fluid column against black velvet with a strong side light and compared with a control tube of approximately the same shade, wherein the fluorescence is eliminated by acidification. The test is carried out in the following way:

10 ml. of fresh or suitably preserved urine are mixed with the same vol. of a well-shaken suspension of 10 % zinc acetate in absolute alcohol and filtered. Two equal portions of the filtrate are poured into test tubes of 10 mm. diameter to a height of 10 cm. and to one of them are added a few drops of HCl. Both tubes are observed on black velvet with a strong side light the upper limit of the luminous cone being about 1 cm. below the surface. A slight bluish Tyndall phenomenon is sometimes observed, but only a greenish tinge indicates a positive reaction. Then a drop of *N*/10 iodine is added to each tube, transforming the urobilinogen that may be present into urobilin.

By means of this technique a faint fluorescence can be detected always in normal fasting urine even without addition of iodine. Probably this small amount of urobilin is produced from urobilinogen during the test. As to specificity the only similar reaction known is a red fluorescence of pure bilirubin with zinc acetate-alcohol and iodine as described by Auché [1908]. In applying the Jaffé-Schlesinger test to urine however interference by bilirubin does not occur, nor does a higher content of other pigments prevent the perception of even a faint fluorescence. The only significant interfering substance is albumin which adsorbs small amounts of pigments. Thus it is evident that for detection of both urobilin and urobilinogen the fluorescence test is more reliable than the aldehyde test. If both tests are used in conjunction a positive fluorescence test will prove that a positive aldehyde reaction is due actually to urobilinogen.

SUMMARY.

1. The conditions of Ehrlich's aldehyde test in fasting urine regarding concentration of the reagent, temperature and time have been investigated and a suitable technique has been devised.
2. The non-specificity of the aldehyde reaction has been emphasised and the most important interfering substances have been mentioned.
3. The orange-coloured reaction of normal urine has been explained as combined yellow and red reactions, the former being due mainly to urea and indican, the latter to urobilinogen and indole.
4. A suitable technique for the Jaffé-Schlesinger test has been devised so that traces of urobilin and urobilinogen can be detected in normal urine.
5. The fluorescence test has been found to be specific and therefore more reliable than the aldehyde reaction.

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