CXLVII. STUDIES ON BILE PIGMENTS.

III. THE QUANTITATIVE DETERMINATION OF UROBILIN AND UROBILINOGEN IN URINE AND FAECES.

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In a previous paper [Naumann, 1936] the qualitative tests for urobilinogen and urobilin have been studied. In the following the quantitative aspect of the problem is treated.

A basis for the estimation of urobilin and related compounds was first introduced by Terwen [1924; 1925]. The main features of this method consist in determining the sum of urobilinogen and urobilin as urobilinogen after reduction by means of a ferrous salt in alkaline solution, the extraction of the total chromogen with ether or light petroleum, the timing of the aldehyde reaction, the replacement of the hydrochloric acid by acetic acid by adding sodium acetate, and the comparison with a phenolphthalein standard in an Autenrieth colorimeter. The method has been examined and partly modified by Greppi [1926], Fuerth and Singer [1929], Heilmeyer [1931], Watson [1931], Royer [1934] and Christophe [1934].

Terwen's method is subject to the disadvantage of the non-specificity of the aldehyde reaction as explained formerly. Many of the interfering substances, *e.g.* indole and phenol derivatives, phenazone, Extr. Rhei *etc.*, are extracted by ether and light petroleum; the replacement of the hydrochloric acid by acetic acid only partly eliminates this source of error. A further disturbance is caused by the presence of much bilirubin which is extracted partly by ether, whilst light petroleum does not extract bilirubin, but at the same time fails to extract urobilinogen completely. Also the final colour in the presence of interfering substances is more orange or brownish, which renders the colorimetric comparison rather difficult.

EXPERIMENTAL.

Influence of protein and mucus on the ether extraction.

Yet a further source of error caused by the presence of protein in urine is the incompleteness of the urobilinogen extraction (see Table I). If the albuminuria is not excessive the loss can be presumed to be less than 20 %. A much greater

Table I. *Influence of the presence of protein and mucus on the extractability of urobilinogen.*

Amount of urine tested	Approx. % of protein	Urobilinogen content in mg./100 ml.	Loss %
10 ml. urine without addition	—	0.083	—
10 ml. urine + 0.1 ml. serum	0.1	0.072	13
10 ml. urine + 0.5 ml. serum	0.5	0.068	18
10 ml. urine + 1.0 ml. serum	1	0.065	22
10 ml. urine + 2.0 ml. serum	2	0.062	25
10 ml. urine + 1.0 ml. sputum	—	0.054	35
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loss is caused by the presence of mucus which in a rather small quantity can cause a deficiency of 35%. Thus the presence of protein disturbs in two ways: (1) by interfering with the aldehyde reaction; (2) by preventing complete extraction of the urobilinogen.

The reduction of urobilin by ferrous ammonium sulphate (Mohr's salt).

Greppi [1926] states that the reduction is not always successful but will be effective after repetition, and Adler [1927] and Royer [1934] point out that urobilinogen is partly destroyed during reduction, whilst the latter found that if ferrous sulphate is used according to Watson [1931] there is no destruction. If the iron reduction is to be effective the following conditions must be ensured:

- (1) Bilirubin is not transformed into urobilinogen.
- (2) Urobilinogen is not destroyed.
- (3) Urobilin is reduced completely.

Watson [1931] states that bilirubin is partly reduced to urobilinogen when ferrous sulphate is used and removes the bilirubin with $\text{Ca}(\text{OH})_2$ before reducing. However, under the conditions of Terwen's method and the modification described below no positive aldehyde test was obtained either directly or after extraction with light petroleum, although the bilirubin solutions showed a lighter colour after reduction. It can thus be assumed that with Mohr's salt reduction of bilirubin to urobilinogen does not take place.

Fig. 1. Reduction of aqueous faecal extract with Mohr's salt under varying conditions of concentration, time and temperature.

In order to investigate whether urobilinogen is destroyed and whether urobilin is completely reduced it was necessary to determine the conditions of reduction. Fig. 1 shows the effect on reducing experiments with aqueous faecal extracts of variation of the concentration of Mohr's salt solution (Curve I), of the time of reduction (Curve II) and of the temperature (Curve III). In the curves I, II and III the estimated amount of urobilinogen is plotted against ml. of Mohr's salt solution added to 2 ml. of faecal extract, against time of reduction, and temperature in °C. of the reaction mixture respectively. From these curves the following optimum conditions are deduced:

- (1) The optimum concentration is 2 volumes of diluted faeces + 1 volume of Mohr's salt solution.

(2) The optimum time of reduction is 3 hours at 20°.

(3) The optimum temperature is 35° for 5 min. of reduction.

However, as will be seen below these results are not always uniform.

Table II. *Comparison of different reducing methods.*

	Without reduction	Terwen's method	Watson's modification	Author's modification	
				3 hours	5 min. 35°
Urine No. 1	0.12	0.10	0.07	0.14	0.11
2	0.33	0.26	0.21	0.33	0.32
3	0.37	0.35	0.22	0.45	0.17
4	0.75	0.81	0.84	0.92	0.73
5	2.17	1.90	2.22	2.86	2.01
Faeces No. 1	11.65	11.88	13.38	15.41	18.00
2	28.60	33.80	36.40	34.40	35.30
3	33.10	40.80	35.40	38.30	34.40
4	106.50	83.40	96.00	96.30	108.20
5	333.50	247.30	396.20	284.50	388.30

Technique of tests.

From these results the following technique has been derived.

Test in faeces.

1 g. of the well mixed faeces is weighed on a microscope slide coverslip and ground together with the glass in a mortar with gradual addition of 9 ml. of water. 3 tests are then performed.

(a) *Test without reduction.* 0.2 ml. of the faecal mixture is washed quantitatively into 5 ml. of water placed in a separating funnel; 20 ml. of ether and 0.5 ml. of 20% tartaric acid are added. After shaking vigorously the aqueous phase is drawn off and the ether washed twice with small amounts of water. Then 5 drops of 1% *p*-dimethylaminobenzaldehyde in conc. HCl are added and shaken for 1½ min. 1 ml. of conc. sodium acetate is added and the coloured solution drawn off into a 10 ml. measuring cylinder, the ether washed with 20% acetic acid and the solution made up to 5 or 10 ml. or a greater volume according to the colour depth and compared with Terwen's phenolphthalein standard in an Autenrieth colorimeter.

To the remaining faecal mixture, 2.5 ml. of 16% ferrous ammonium sulphate and 2.5 ml. of 12% NaOH are added, well mixed and poured into two small test-tubes.

(b) *Test after reducing for 3 hours.* One of the two portions covered with a layer of paraffin oil is set aside in the dark for 3 hours and filtered. 0.2 ml. of the filtrate is worked up as described above.

(c) *Test after reducing for 5 min.* One of the two portions is put for 5 min. in a water-bath at 35° and then filtered immediately. 0.2 ml. of the filtrate is treated as described.

Calculation.

(a) $100 - x \times \frac{\text{final volume}}{0.2} \times 10 \times 0.4$ is the concentration of urobilinogen in mg./100 g. in the faeces without reduction, *i.e.* the actual urobilinogen content without urobilin. x is the reading of the scale of the Autenrieth colorimeter.

(b) and (c). $100 - x \times \frac{\text{final volume}}{0.2} \times 1.5 \times 10 \times 0.4 + 2\%$ (to allow for the 0.2 ml. taken away for (a) from the original faecal mixture) is the concentration of urobilinogen in mg./100 g. in the faeces after reduction, *i.e.* the concentration of urobilinogen + urobilin.

Test in urine.

(a) *Test without reduction.* 2 ml. of urine are placed in a separating funnel and treated correspondingly as described above.

(b) *Test after reduction.* To 9 ml. of urine are added 0.5 ml. of 16% ferrous ammonium sulphate and 0.5 ml. of 12% NaOH with good mixing. The mixture, placed in a small test-tube and covered with a layer of paraffin oil, is set aside for 3 hours in the dark and filtered. 2 ml. of the filtrate are treated as described.

Calculation.

(a) $100 - x \times \frac{\text{final volume}}{2} \times 0.4$ is the concentration of the actual urobilinogen in mg./100 ml. in the urine, if x is the reading on the Autenrieth colorimeter.

(b) $100 - x \times \frac{\text{final volume}}{2} \times 1.11 \times 0.4$ is the concentration of urobilinogen + urobilin in mg./100 ml. in the urine.

A comparison of the different reduction methods in Table II shows a 10–50 % better yield by the author's modification with the exception of faeces samples Nos. 2, 3 and 5. It must be considered, however, that differences up to 5 % are within the limit of error. The advantage of the described technique is more pronounced in the urine tests where only 3 hours' reduction gives good results.

From Table II it is seen that the figures obtained by Terwen's method in all the urine samples except No. 4, and in two of the faeces samples are lower than those obtained without reduction, whilst the figures obtained with Watson's modification are lower only in three urines and one faeces sample. This can be explained only by a destructive action of the reducing process on the urobilinogen. On the other hand the 3 hours' reduction described gives a higher figure than is obtained without reduction in all urine samples except No. 2, where the figures are the same, and in the faeces samples figures obtained with either the 3 hours or the 5 min. technique are higher than without reduction. In the latter case the better yield must be considered to be the correct result. It must therefore be concluded that Terwen's method and to a smaller extent also Watson's modification effect a partial destruction of urobilinogen, which is prevented or at least greatly restricted by the technique described here.

The completeness of reduction of the urobilin is usually estimated by the spectroscopic test which depends greatly on the sensitivity of the instrument. A distinct urobilin band in Hartridge's spectroscope after reduction by either of the described methods has not been detected and has only been observed in a malaria case with an excessive excretion of urobilin. The problem has been investigated further by the following experiment.

A purified aqueous urobilinogen solution was prepared according to Terwen by treating a urobilinogen light petroleum extract with slightly alkaline water. The urobilinogen content of the almost colourless solution was 3.83 mg./100 ml. After standing for 24 hours in the dark the solution became slightly brown with a distinct urobilin spectrum and a urobilinogen content of 2.61 mg./100 ml. After reduction according to the author's modification the filtrate was only faintly yellow without a urobilin spectrum. The content was 3.84 mg./100 ml.

The latter experiment which is in accordance with similar experiments of Terwen supports the assumption that in general the reduction of urobilin will be complete unless a great excess of the pigment is present. In such cases faeces or urine must be diluted or a greater amount of iron solution used.

Stability of the phenolphthalein standard solution.

A disadvantage of Terwen's original method is the constant necessity of fresh preparation of the standard phenolphthalein solution which is supposed to keep stable not longer than a few hours. Observation of the phenolphthalein standard, prepared by dilution of 1 ml. of 0.05 % alcoholic phenolphthalein and 5 ml. conc. Na_2CO_3 plus distilled water up to 100 ml., showed however that the decrease of colour of such a solution takes place in a certain regular way. After a period of stability of about 5 hours the colour strength decreases slowly until a new stability is reached after about 18 hours. This definite value is maintained for about a month and decreases only very little during the course of a year.

This change is due to the influence of OH ions on the different compounds of the phenolphthalein group and is a general property of all phthaleins as pointed out by Kolthoff and Fischgold [1932]. The degree of decrease is about 15–30 % of the colour of the freshly prepared solution and depends on the purity of the phenolphthalein preparation. In view of the regular nature of this change it is possible for practical colorimetric purposes to use the phenolphthalein standard as a relatively stable one after an interval of a day provided that it is controlled at intervals of about a month. Such a phenolphthalein solution has been kept by the author in the wedge of the Autenrieth colorimeter for 17 months and no change has been noticed for about 8 months.

SUMMARY.

1. The quantitative estimation of urobilinogen and urobilin by the method of Terwen has been examined. It has been found that errors are caused by the unspecificity of the aldehyde reaction owing to interfering substances, particularly at a low urobilinogen content, and by the fact that the extraction is not complete in the presence of protein and mucus.

2. Investigation of Terwen's method of reducing urobilin by means of Mohr's salt has given the following results:

(a) The reduction is dependent on the time factor, the reducing action following a course with a peak after 3 hours at 20° or 5 min. at 35°.

(b) The reduction is dependent on a suitable relation between iron salt and urobilin concentration.

(c) In the course of the reduction urobilinogen is partly destroyed.

(d) The reduction is complete except in the presence of a great excess of urobilin.

(e) Bilirubin is not reduced to urobilinogen by Mohr's salt.

3. A modification of the reduction by using a suitable concentration of Mohr's salt and limiting the time of the reducing process gave 10–40 % better yields than the method of Terwen and Watson.

4. The phenolphthalein standard solution fades to the extent of 15–30 % during 18 hours after preparation and can be regarded afterwards as relatively stable for the period of a year provided that it is controlled at monthly intervals.

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