16. HISTIDINE DETECTION AND ESTIMATION IN URINE

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DURING the last 10 years many attempts have been made to detect changes in the liver metabolism of patients suffering from mental disorders. For example, in this hospital investigations have been carried out which indicate changes in the sugar tolerance of manic depressive patients [McCowan & Quastel, 1931], while diminution of hippuric acid excretion after benzoate administration has been shown by Quastel & Wales [1938] to take place in catatonic patients.

Schimmelpfeng [1937] stated that there is an increased histidine excretion in melancholic patients and he correlated this with disturbances of the liver function.

The object of this work was to try to confirm and to follow up this statement of Schimmelpfeng.

Detection and estimation of histidine

Histidine was first isolated by Engeland [1908] in very small quantities from 40 l. of normal urine. Hunter [1922] isolated 0.1 g. histidine from 1 l. of the urine of a patient suffering from measles. Armstrong & Walker [1932] isolated from 10 l. of pregnancy urine 0.2 g. of histidine monohydrochloride and stated that there was much loss during the isolation.

For the detection of histidine in urine two colour tests have been evolved. Firstly there is the diazo reaction of Pauly [1904], a modification of which by Koessler & Hanke [1919; 1924] is generally used. However, this reaction is not considered to be specific because, as stated by Koessler & Hanke [1919], Hunter [1925] and others, iminazoles, certain purines, phenols, urine pigments and tyrosine give this reaction as well as histidine. Therefore they interfere with a quantitative determination of the latter. Again, this reaction is very easily interfered with by small amounts of metallic salts, e.g. copper, cobalt; also by some amino-acids, alcohols and uric acid. All these facts render the application of this very sensitive method unsuitable for a quantitative estimation of histidine in urine.

The second colour test used for histidine is the bromine reaction first described by Knoop [1908]. Hunter [1922] and Kapeller-Adler [1933] have modified this test and have increased its sensitivity. Since this test is considered by many authors [Knoop, 1908; Hoppe-Seyler & Tierfelder, 1924; Armstrong & Walker, 1932; Kapeller-Adler, 1936; Tschopp & Tschopp, 1938] as specific for histidine attempts have been made by many workers to apply it to human urine especially since Voge [1929] and Kapeller-Adler [1933] suggested it as a means for the early detection of pregnancy. Many contradictory results have been published since then about histidine excretion in urine. Tschopp & Tschopp [1938], considering these results as well as those of their own investigations, came to the conclusion that histidine is excreted sometimes in normal but more frequently in some pathological conditions. This was especially so in liver diseases and allergic conditions. They also used the bromine test as described by Kapeller-Adler [1936].

In principle the test, as applied qualitatively by Kapeller-Adler [1936], Schimmelpfeng [1937], Tschopp & Tschopp [1938] and many others, is as follows:

To 5 ml. of urine are added drop by drop the bromine reagent until a slight excess of bromine is indicated by starch-iodide paper. After 10 min. ammonia-ammonium carbonate solution is added, then the test tube is placed in boiling water for a short period. A red-violet colour indicates the presence of histidine.

The disadvantages of this method are as follows: In the first place the pigmentation of the urine renders the value of the colour test sometimes rather uncertain. In the second place the development of the colour is often inhibited by precipitation of phosphates after the alkali treatment. This was already observed by Kapeller-Adler [1933]. Addition of fairly large quantities of histidine to urines rich in phosphates quite often did not give a corresponding colour reaction. Thirdly there are substances in urine which sometimes greatly increase the colour produced by bromination.

This was proved by experiments on specially treated normal urines. The phosphates were first removed with baryta. The pH was adjusted to 7.4, then all pigments and finally all the histidine were removed with animal charcoal. After readjusting the pH to that of a standard histidine solution, an equal amount of histidine was added to each sample. Application of the bromine test resulted generally in an increased depth of colour and often in a different shade from that given by the standard. The increase of colour varied from sample to sample.

Effect of urates on the bromine colour test

On further investigation of the above phenomenon it was found that the addition of urates to a pure histidine solution greatly increased the sensitivity of the colour reaction and also affected the colour itself.

Since urines contain different quantities of urates and other sensitizing agents, different samples of urines containing even the same quantity of histidine may show different intensities of colour.

Effect of substances other than urates on the bromine test

It was found in the case of patients who had had somnifaine¹ treatment, that the apparent histidine excretion was high. Investigation showed that this apparent increase was due to the excretion of somnifaine, which itself has an intensifying action on the bromine colour test. Thus the property of urates in intensifying the colour test is shared by other molecules, in particular by some unsaturated fatty acids and unsaturated alcohols. (The sensitizing agent in somnifaine is the unsaturated molecule allylisopropylbarbituric acid.)

It was then decided to modify the existing method of histidine detection by adding to all urines an excess of urates. This procedure resulted in the elimination of all differences in intensity and shade of the colour reaction due to the presence of varying quantities of sensitizing agents in the urines, and thus made quantitative estimation of histidine possible.

METHOD

Chemicals required

(1) Histidine standard solution. 100 mg. histidine monohydrochloride are dissolved in 1 l. of $N/10$ H_2SO_4 .

(2) 5% sodium urate suspension. 25 g. sodium urate are added to 500 ml. of distilled water. This is left to stand for 1 day. The suspension is shaken before use.

¹ Somnifaine is a narcotic containing diethylbarbituric acid and allylisopropylbarbituric acid.

(3) Bromine-acetic acid solution. 2 ml. of bromine are added to 100 ml. of glacial acetic acid, then made up to 300 ml. with distilled water.

(4) Arsenious oxide solution. This is a saturated solution of As_2O_3 in a 10% NH_3 .

(5) Na_2CO_3 (anhydrous).

(6) Saturated baryta.

(7) $N \text{ H}_2\text{SO}_4$.

(8) Animal charcoal purified (for decolorizing purposes).

Procedure

(1) 6 ml. of urine are pipetted into a test tube and 3 ml. of baryta added. The mixture is now filtered.

(2) To the filtrate from above 3 ml. of $N \text{ H}_2\text{SO}_4$ and about 100 mg. of charcoal are added. The mixture is again filtered.

(3) 6 ml. of the clear, colourless filtrate and also 6 ml. of the histidine standard are each treated with 2.5 ml. urate suspension. Bromine in small portions is added until a slight excess is indicated by a faint persistent yellow colour.

(4) The solutions are kept for 10 min. with occasional shaking. The excess of bromine is removed by 1-2 drops of arsenious oxide, when the solutions should again become colourless. If this is not so, the procedure up to this point should be repeated with a fresh sample of urine, using a little more charcoal.

(5) The test tubes are placed in boiling water and, in order to prevent excess of frothing and of dilution, small quantities of solid Na_2CO_3 are added until no more CO_2 bubbles are produced. The test tubes are shaken from time to time. 5-7 min. are required to complete the reaction.

(6) The test tubes are taken from the water bath and after standing 10 min. the orange-red colours are compared in the colorimeter. The values thus obtained for the amount of histidine in the urine must be multiplied by 2, because to the 6 ml. of urine 3 ml. of baryta and 3 ml. of H_2SO_4 were added.

Rationale of the method

For reasons already stated the phosphates are removed with baryta. The excess of baryta is removed and the solution brought to an acidity roughly corresponding to the pH of the standard. It has been found that charcoal, when added in small quantities to a solution of this acidity, absorbs histidine to a negligible extent. The actual amount of charcoal to be used depends on the activity of the charcoal sample. The smallest possible amount resulting in a colourless filtrate is taken.

The use of arsenious oxide is recommended by Conrad & Berg [1937]. It must be added since the presence of an excess of bromine considerably diminishes the colour. Kapeller-Adler suggested that this is due to a further attack of the histidine molecule by the excess of bromine. However, Conrad & Berg [1937] and Woolley & Peterson [1937] have shown that if the excess of bromine is removed before the alkali is added the colour development is not inhibited. Hence it seems improbable that much of the histidine is further attacked during the 10 min. contact with the bromine. Woolley & Peterson therefore supposed that the histidine is not attacked by bromine itself, but that conversion of histidine into the corresponding aldehyde takes place by the action of NH_4OBr , which is produced by the addition of ammonia. It is more probable, however, that this inhibition is due to the fact that bromine oxidizes the molecule responsible for

the colour. This was shown by addition of bromine after the colour had already completely developed. Similarly decoloration took place with oxidizing agents such as H_2O_2 .

Sensitivity of the method and experimental error

The sensitivity of the bromine reaction, which is described by Hunter [1922] as 1 : 10,000 and by Tschopp & Tschopp [1938] as 1 : 6250, is increased by this modification to 1 : 100,000.

The experimental error is about 10 % according to controls in which known quantities of histidine have been added to the urine (see Table 1).

Table 1

Case	Histidine originally in urine mg./100 ml.	Histidine added to 6 ml. urine mg.	Histidine calculated mg./100 ml.	Histidine estimated mg./100 ml.	Experimental error %
M. N.	9.1	0.8	22.4	21.6	-3.5
F. T.	17.3	0.8	30.6	27.9	-8.8
R. E.	75.5	1.0	92.1	90.3	-1.9
D. J.	20.3	1.3	42.0	38.8	-7.6

Results

More than 400 samples of urine of some 180 persons have been examined. Morning urine or 24 hr. specimens have been used. It has been found that in the urines of normal persons as well as in those of all kinds of mental disorders so far investigated the bromine test proved to be positive. The colours obtained by this test were on an average equivalent to that of a 20 mg./100 ml. histidine monohydrochloride solution. However, the figures varied between 2 and 80 mg./100 ml. (see Table 2 for typical results).

Table 2. *Results of normal cases and patients with mental disorder*

Case	Sex	Diagnosis	Histidine mg./100 ml.	Urea g./100 ml.
D. W.	F.	Normal	19	1.6
D. J.	M.	Normal	21	2.1
G. J. J.	M.	Normal	64	—
A. S.	F.	Normal	6	0.9
R. E.	M.	Normal	75	2.3
W. H.	M.	Melancholia and arteriosclerosis	36	1.9
C. D.	F.	Melancholia	18	1.3
L. A. D.	M.	Melancholia	10	1.1
H. A.	F.	Agitated melancholia	23	—
W. D.	F.	Melancholia and diabetes	12	—
Li.	M.	Melancholia	50	—
A. M.	F.	Dementia paranoides	17	1.6
S. R. (negro)	M.	Schizophrenia	51	—
O. R.	M.	Schizophrenia	33	1.7
S. M.	F.	Schizophrenia	11	—
R. J.	F.	Senile dementia	7	0.9
S. M.	F.	Mania	23	2.0
M. S.	F.	Primary dementia (jaundice)	48	—

A proportionality between histidine and urea excretions has been observed as well as a certain proportionality to the specific gravity. But this does not account for the large individual differences. In three cases the histidine excretion was followed up for 14 days and compared with the urea excretion. The examinations were carried out twice daily in the first week, the first time on the morning urine, the second time 2 hr. after lunch.

Fig. 1. Normal case. Histidine excretion —; urea excretion — — —

Fig. 2. Normal case. Histidine excretion —; urea excretion — — —

Fig. 3. Melancholic patient. Histidine excretion —; urea excretion — — —

The first two of these persons were normal and the third was a melancholic patient. The results are shown in Figs. 1-3.

These results do not support the conclusion of Schimmelpfeng [1937] that melancholic patients excrete abnormally large quantities of histidine.

Results of pregnant cases

The urines of 27 pregnant women have been investigated. Six of these urines showed definitely high values (Table 3).

Table 3

Case	Age years	Duration of pregnancy months	Histidine mg./100 ml.	Urea gm./100 ml.	Specific gravity
A. S.	23	6	162	1.4	1018
T. M.	20	6	109	1.3	1022
K.	24	4	97	1.4	1024
M. C.	28	3	97	1.4	1023
G. L.	30	3	96	2.0	1023
J. D.	?	3	107	1.3	1018

Although the other urines showed figures within the limit of normal values, it must be noted that the urea content and the specific gravity of these urines were extremely low (see Table 4).

Table 4

Case	Age years	Duration of pregnancy month	Histidine mg./100 ml.	Urea g./100 ml.	Specific gravity
L. P.	22	6	39	0.9	1012
T. B.	?	3	44	0.7	1013
M. M.	37	4	63	1.3	1015
S. M.	28	3	12	0.4	1006
W. McD.	—	2	27	0.5	1007
Non-pregnant women:					
A. H.	56	—	28	2.2	1021
K. F.	37	—	56	2.6	1027
M. S.	27	—	10	1.5	1015

If the proportion of histidine to urea content and to specific gravity is taken into account the histidine excretion must be regarded as high. For the possible explanation of this phenomenon attention may be drawn to the investigations of Kapeller-Adler & Herrmann [1936] and Kapeller-Adler & Boxer [1937] in which they claim to have shown that gonadotropic hormones inhibit the action of histidase in liver brei. Also the urines of pregnant women treated by the alcohol precipitation method (Zondek) are stated to have a similar inhibitory action presumably due to the gonadotropic hormones present.

Discussion of the specificity of the bromine test

Knoop [1908], after examining a large number of substances with his test, concluded that only histamine besides histidine gave a positive reaction. Linneweh *et al.* [1929] stated that *N*-methylhistidine gave this reaction as well, and Kapeller-Adler confirmed this, but observed that only a fifth of the colour was obtained. Armstrong & Walker [1932] considered Knoop's test as specific for histidine and *N*-methylhistidine, although they mentioned that "a few substances have been met, which gave with bromine water a reaction of a

different type". Thus they described a reaction of bromine water with adrenaline and tyramine. With regard to histamine, they stated that the colour obtained can be extracted with amyl alcohol. Woolley & Peterson [1937] described a few substances which interfere with the histidine determination by the bromine reaction. They observed a brown shade if substances such as tyrosine and tryptophan were present.

These results can be confirmed in general. A number of other substances were found to react with bromine; some phenols (e.g. aminophenol), some alkaloids (e.g. morphine), tyrosine and tryptophan. The last two were mentioned by Woolley & Peterson as interfering substances. It was, however, found that they gave a reaction by themselves.

All these substances showed more or less marked differences from histidine in reacting with bromine. Some of them, such as morphine, were not decolorized by the addition of arsenious oxide after bromination. A yellow colour remained which was proportional to the amount of morphine present. The addition of sodium carbonate in the cold gave a reddish colour, some of which was readily extractable by amyl alcohol from the alkaline solution. This was not the case with histidine. The colour test for all substances except histidine mentioned above is characterized by this important difference. With regard to histamine it must be noted that a much less intense colour and a different shade are obtained by the modified bromine method.

Hence all substances investigated so far could be distinguished from histidine. The substance in urine giving the bromine reaction showed in every respect the characteristics of histidine. But considering the various kind of substances which have been found to give colour reactions with bromine, it is felt that this reaction may not be as completely specific for histidine as would appear from the literature. It is therefore possible that the unexpected high figures of histidine excretion in normal urine may perhaps be partly due to another substance the reaction of which with bromine has not yet been investigated.

Experiments are therefore in progress to isolate the histidine from urine, using a new method by which it is hoped large losses will be avoided.

SUMMARY

1. The application of present colorimetric methods for histidine detection and their difficulties are described.
2. A simple and rapid quantitative method for histidine estimation in urine by a modification of Knoop's bromine reaction is given.
3. The specificity of the bromine test is discussed.
4. When this reaction is applied to normal urine a colour is produced which corresponds to an average of 20 mg./100 ml. histidine monohydrochloride. The range of normal values lies between 2 mg./100 ml. and 80 mg./100 ml.
5. A higher excretion value in the urines of melancholic patients, as claimed by Schimmelpfeng, could not be confirmed.
6. Pregnancy urines usually show high histidine values when considered in relation to the urea excretion and to the specific gravity of the urine.

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