69. HISTIDINE IN URINE. 2

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IN a previous paper [1940] a method has been described for quantitative estimation of histidine in urine. Numerous estimations have been carried out and values corresponding to 2–80 mg. histidine monohydrochloride per 100 ml. have been found in normal urines. These figures were unexpectedly high, and so it was decided to investigate further the nature of the substance or substances responsible for the bromine colour test.

EXPERIMENTAL

1. *Purification of the substance giving the bromine colour test*

Urine, in portions of 200–300 ml., was shaken with 20 g. purified animal charcoal for 25 min. The urine was then filtered through a Büchner funnel and the filtrate poured into a separate flask. The charcoal, which remained in the Büchner funnel, was washed with $N/10$ H_2SO_4 until all the histidine adsorbed by the charcoal was eluted and the last washing became negative to the colour test. The first urine filtrate, which was kept separate, was again shaken with charcoal, filtered and the filtrate kept separate, the charcoal being washed with $N/10$ H_2SO_4 . This procedure was repeated until the original urine was free from histidine. The combined washings were then evaporated *in vacuo* to about 100 ml. and treated with hot saturated baryta until neutral to phenol red. The precipitated barium sulphate was centrifuged down, the supernatant fluid decanted, the precipitate washed with hot distilled water and again centrifuged. The combined supernatant fluids were evaporated *in vacuo* to about 50 ml. and 5 ml. NH_2SO_4 were added. To this an excess of $AgNO_3$, dissolved in distilled water, was added (6–8 g. were usually required). After precipitation was completed, the mixture was filtered, the precipitate of silver chloride being washed with distilled water. The clear filtrate was brought to pH about 8.0 with $2N$ $NaOH$ and left overnight in the refrigerator. The precipitate was filtered off and taken up in distilled water. It was then acidified with dilute H_2SO_4 and decomposed with H_2S for about 12 hr. The mixture was filtered, the precipitate washed and the combined filtrates evaporated *in vacuo* to a low bulk. This purified solution was analysed by the bromine reaction and diluted until it corresponded with a 1% solution of histidine monohydrochloride. The purified material was used for further investigation.

2. *The biological breakdown of the substance in urine*

It has been shown by Edlbacher [1926] that liver contains an enzyme, histidase, which breaks down histidine. The activities of this enzyme in breaking down histidine and the substance excreted in urine have been compared.

• The liver was passed through a Latapie mincer, then ground with sand and suspended in $M/15$ Na phosphate buffer of pH 8.0, so that 4 ml. of the suspension contained 500 mg. of liver brei. The suspension was centrifuged for 20 min.,

the supernatant fluid decanted and used as enzyme solution. 4 ml. were used for each experiment. The activity of the enzyme was noted, comparing the breakdown of 10 mg. histidine monohydrochloride with that of the corresponding amount of the purified urine solution over varying periods. After incubation in a water bath at 40° the mixture was precipitated with 5 ml. of

Fig. 1. Rat liver histidase activity. — Histidine HCl. --- Urine histidine.

20% trichloroacetic acid and centrifuged; the supernatant fluids were decanted and the precipitate twice washed with distilled water. The fluids were collected and made up to a known volume. The clear solutions were analysed by the bromine reaction and the results obtained are shown graphically in Fig. 1.

3. Pharmacological test of the substance in urine

Hanke & Koessler [1922; 1924] studied the formation of histamine from histidine by various types of *Bact. coli*. Recently Gale [1940] has followed this breakdown under more favourable conditions in manometric experiments. Using suspensions of 'resting bacteria' [Quastel & Whetham, 1924] he measured the activity of the decarboxylase for various amino-acids.

Fig. 2. Decarboxylation of histidine. — Histidine HCl. --- Urine histidine.

The decarboxylation of the substance in urine has been followed by this method, using a standard histidine solution as control. After the manometric experiments were concluded the solutions were tested pharmacologically.

Fig. 3. Fowl caecum. (1) 0.1 μ g. histamine HCl. (2) 0.1 μ g. histamine from histidine HCl. (3) 0.1 μ g. histamine from urine histidine. Lower figure: 0.2 μ g. histamine HCl.

Fig. 4. Fowl caecum. (4) 0.1 μ g. histamine from urine histidine. (5) 0.1 μ g. histamine from urine histidine + histaminase. (6) 0.1 μ g. histamine HCl. (7) 0.1 μ g. histamine HCl + histaminase. All incubated for 24 hr. at 37° and then tested.

The respiratory experiments have been carried out under the conditions laid down by Gale. The bacteria (*Bact. coli Esch.* Nat. Inst. of Type Cultures, no. 86) were grown in 2% glucose broth at 27° for 18 hr., centrifuged, well washed and suspended in distilled water. The dry weight was determined in each case by a photoelectric turbidimeter. 1 ml. of the bacteria suspension was pipetted into each Warburg vessel with 1 ml. of phthalate buffer (pH 4.0) and 0.7 ml. distilled water. 0.3 ml. of the histidine or urine solution was placed in the side cup. After equilibration in the thermostat at 30° the histidine was tipped in. The results were recorded graphically (Fig. 2).

After incubation the bacteria were centrifuged off, the clear solutions neutralized and diluted with Tyrode solution to a concentration of histamine 1 µg./ml. according to the theoretical amounts of histamine formation to be expected from the CO₂ output.

The solutions were then tested on the isolated caecum of the fowl [Barsoum & Gaddum, 1935]. The gut was suspended in a small water bath containing 8 ml. of a Tyrode glucose solution, kept at 37° and continuously aerated with oxygen. The amount of fluid tested did not exceed 0.2 ml. The contractions of the gut were recorded on a slowly rotating smoked drum and typical results are given in Fig. 3.

Histaminase (Torantil Bayer), which breaks down histamine, acts in the same way on the histamine produced from normal urine (Fig. 4).

4. Estimation of histidine by nitranilic acid

Block [1940] has shown that histidine is quantitatively recovered from solutions as nitranilate and he has used this for estimation of the amino-acid. This method was applied to the purified urine solution and quantitative recoveries, corresponding to the bromine colour test, have been obtained. The experimental procedure was as follows.

The purified urine solution was first treated with an excess of 95% alcohol and centrifuged. The precipitate was dissolved in distilled water, reprecipitated with alcohol and centrifuged. This process was repeated until the precipitate was free of histidine. The collected supernatant fluids were evaporated *in vacuo* to a few ml. and an equal amount of absolute methyl alcohol was added. To this the nitranilic acid, dissolved in distilled water, was added in excess. The mixture was left in the refrigerator overnight, and the centrifuged precipitate was then dried to constant weight. The result obtained is given in Table 1.

Table 1

	Histidine present mg.	Dry weight of precipitate mg.	Recovery %
Histidine HCl	40	71.8	97.5
Urine histidine	40	76.8	104.2

DISCUSSION

The experimental evidence presented above indicates that the greater part of the substance in urine reacting with bromine is histidine. Recent work on the subject [Sakaguchi, 1940; Langley, 1941] is in agreement with this conclusion.

Tschopp & Tschopp [1938] have investigated the excretion of histidine in various diseases, but as they were working with a qualitative method the results appear not to be conclusive.

A small number of quantitative histidine estimations have been made on patients with malignant tumours, relating the histidine excretion to the specific gravity and urea excretion; so far normal figures have always been obtained.

Two pathological conditions are of special interest with regard to histidine metabolism.

1. Experimental shock, produced by a procedure described elsewhere [Quastel & Racker, 1941]. Measurements of histamine formation *in vivo*, and by tissues anaerobically *in vitro*, have not, however, been convincing enough to justify any conclusion.

2. *Bact. coli* infection. Considering the large amounts of histidine excreted normally and the relatively small amount of histamine necessary to exert a pharmacological effect, investigation of the fate of urinary histidine seems to be indicated. The acidity of the urine prevailing in *Bact. coli* infection of the urinary tract should be a favourable factor for histamine formation. Because of lack of suitable clinical material this investigation has not yet been carried out.

It may be noted that in severe cases of toxæmia of pregnancy histamine has been found and isolated from urine by Kapeller-Adler [1941], thus indicating an altered histidine metabolism in this condition.

SUMMARY

1. The procedure of purification of the substance in urine, giving rise to the bromine colour test, is described.

2. The substance is broken down by liver histidase.

3. The decarboxylation of the substance has been followed in manometric experiments and the fluids have then been tested for their action on the isolated fowl's gut. If incubated with histaminase the effect on the gut is nullified.

4. The nitranilic acid precipitation method has been applied to the urine substance.

5. It is concluded that the colour produced by the bromine reaction is due to histidine. The clinical significance of this is discussed.

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