XVII. THE BROMINE REACTION OF PREGNANCY URINE.

BY ARTHUR RILEY ARMSTRONG AND ERNEST WALKER.

From the Department of Biochemistry, Oxford.

(Received January 1st, 1932.)

SOME two years ago Voge [1929] suggested that Knoop's test for histidine, when applied to urine, offered a simple means of testing for pregnancy. With the efficacy of this test we are not concerned in this communication. Reports upon it have been supplied by Burt-White [1930] and Voge [1930]. We became interested in this reaction at the instigation of Dr R. Greene, who has been applying it clinically and will be publishing his results shortly. In an attempt to render the reaction more decisive we were led to inquire whether the substance in urine giving a positive Knoop test was, as is always assumed, histidine. As a result, we have found that the substance concerned is histidine and we have no evidence that any other substance is involved in the reaction.

Knoop's test consists in adding bromine water to the solution under test to the point of maximum absorption, avoiding excess. On heating, a port wine-red colour develops which fades slowly on standing, a black particulate precipitate being deposited. The reaction works best in faintly acid solution; the colour is modified but not inhibited by the presence of strong acids. Hunter [1922, 1] has rendered the reaction more sensitive.

Isolation of histidine.

After some preliminary experiments a Kossel silver fractionation was adopted, with slight modifications, the bulk of the substance being found in the histidine fraction and a little in the arginine fraction. The process is very similar to that described by Hunter [1922, 2].

The urine used was always a mixed sample and was frequently contaminated with debris. It was early found desirable to give it a preliminary clearing with baryta, which (a) rendered filtration more rapid, (b) removed phosphates which would otherwise use up silver and (c) removed a certain amount of pigment. Hot saturated baryta was added to the urine to the point of maximum precipitation. The precipitate was allowed to settle and the mixture filtered on a Büchner funnel, the precipitate being added last. Strong nitric acid was then added to the filtrate until the reaction was just acid to Congo red. Silver nitrate was added in the form of a 60 % solution until no further precipitation took place. The precipitate (mainly silver chloride) was allowed to settle and

was then filtered off through a fluted filter-paper, filtering the supernatant fluid first. More silver nitrate solution was added to the filtrate until it gave the brown spot test with baryta. Strong NaOH solution was then added to bring the solution to p_H 7.4. The precipitate was allowed to settle; the supernatant liquid was decanted and the silver precipitate washed twice with distilled water, by decantation. It was then suspended in water, made acid, and the silver fractionation repeated. The silver precipitate so obtained was again suspended in water, made acid to Congo red with sulphuric acid, and treated with hydrogen sulphide. The silver sulphide was filtered off and the filtrate aerated and finally just boiled to expel the hydrogen sulphide. Whilst still hot an appropriate amount (about 1 g. per 100 cc. of liquid) of charcoal was added, with stirring. The mixture was filtered, and the sulphuric acid was removed from the filtrate quantitatively with just sufficient baryta. The clear colourless filtrate was then concentrated by distillation *in vacuo* to about 1/30 of its volume. At this stage it was found desirable to treat the concentrate with picric acid in order to remove creatinine, which otherwise followed the fractionation through to the end. Difficulties were encountered in trying to remove the excess of picric acid by ether extraction, and in the end it was necessary to resort to a further silver precipitation, the silver salt being subjected to a very thorough washing by decantation in order to remove picric acid. The silver salt was decomposed in the usual way, the filtrate freed from sulphuric acid and baryta and concentrated almost to dryness by distillation *in vacuo*. The syrupy residue was transferred to a beaker, the distilling flask being rinsed with a small amount of 50 % HCl and the rinsings were added to the main fraction. Boiling 97 % alcohol was then added to the syrup and the mixture kept boiling for ten minutes. If at all coloured, a small amount of charcoal was added at this stage. The solution was filtered hot. When cool, ether was added to the filtrate until permanent cloudiness was obtained. If now left at 0° the product usually separated as a syrup. Such a syrup was dried and redissolved in hot 90 % alcohol containing 5 % HCl, from which the crystalline product separated on cooling. This was the general scheme for the separation of the substance giving the Knoop reaction. It was frequently necessary at the last stage to dissolve in alcohol and to precipitate several times with ether, to vary the strength of the alcohol, or to extract the syrup with absolute alcohol, in order to obtain a crystalline product. An attempt to obtain a dihydrochloride by the method used by Vickery and Leavenworth [1928] for histidine was only partially successful, a mixture of mono- and di-hydrochlorides being apparently obtained.

The yield of crystalline material was very small, 0.2 g. being obtained from some ten litres of urine. There was undoubtedly much wastage, the syrupy non-crystallisable residue rejected in the end stages being very rich in material giving a positive Knoop reaction. It is probably safe to assume that the initial concentration was of the order 0.7 to 1.0 g. in the ten litres.

Identification.

There could be little doubt from its general properties and its occurrence in the histidine fraction of the silver precipitation that the substance isolated was histidine. The crystalline appearance of the hydrochloride (thick rhombic prisms) afforded confirmation of this. The melting point was 251° (the melting point of histidine monohydrochloride is usually given as $251\text{--}252^{\circ}$). In addition it showed that curious effervescence at $165\text{--}170^{\circ}$ without melting, which is characteristic of histidine hydrochloride. A mixed melting-point with a purchased specimen of histidine hydrochloride showed no depression. There was, however, always the possibility of the substance being methylhistidine; the melting-point of the hydrochloride of this compound is not known to us and the melting-point of the base itself is given [Linneweh, Keil and Hoppe-Seyler, 1929] as $248\text{--}252^{\circ}$; the melting-point of histidine itself is 253° .

Analyses were therefore carried out. The results were:

N: 20.6, 19.55 (selves), 18.4 (Schoeller) %.

N: calculated, 20.05 %.

Cl: found, 17.36 (selves), 16.67 (Schoeller) %.

Cl: calculated, 16.94 %.

The slightly low result for nitrogen by Schoeller is discrepant. Our nitrogen analyses were done by the micro-Kjeldahl method and with a purchased specimen of histidine we obtained 19.17 % N. We conclude therefore that the substance isolated is histidine.

Specificity of Knoop's reaction.

In the course of this work we have carried out a large number of Knoop tests and we have applied this reaction to many substances other than histidine. In particular we have tested many compounds closely related structurally to histidine (*i.e.* glyoxaline derivatives) and we have in other cases culled from the literature references to the application of this test. No other substance in our experience gives a Knoop reaction similar to that of histidine and the only instance quoted in the literature is that of methylhistidine [Linneweh *et al.* 1929]. Thus α -hydroxy- β -iminazolypropionic acid in our hands gives no Knoop test; nor, on the other hand, does iminazolyacetic acid [Stewart, 1923]. Fargher and Pyman's [1921] methylhistidine however does not give a positive Knoop test; but in this case the methyl group is attached to the amino-group, whereas in Linneweh, Keil and Hoppe-Seyler's methylhistidine the methyl group is attached to the ring. A few substances have been met which give with bromine water a reaction of a different type; *e.g.* adrenaline gives a rose-red colour, but quickly and without the application of heat. Tyramine likewise gives a somewhat similar reaction, but this may be differentiated by the initial formation of a cloudy solution. Knoop [1908] himself tested several glyoxaline derivatives and concluded that the only substance

other than histidine giving a positive test was histamine; but he adds "... doch ist das Verhalten hier etwas abweichend." That is precisely our experience, the reaction with histamine being quite different, because (a) the colour obtained is violet and (b) it can be readily extracted with amyl alcohol (with histidine, this is not the case). Apart, therefore, from methylhistidine, we are convinced that with due care and experience Knoop's test is specific for histidine.

SUMMARY.

1. The substance in pregnancy urine giving a positive reaction with bromine water (Knoop's test) has been isolated and identified as histidine.
2. Knoop's test is considered to be specific for histidine and *N*-methylhistidine.

We are indebted to Prof. Peters for his advice and encouragement.

REFERENCES.

- Burt-White (1930). *Proc. Roy. Soc. Med.* **23**, 639.
Fargher and Pyman (1921). *J. Chem. Soc.* **119**, 734.
Hunter (1922, 1). *Biochem. J.* **16**, 637.
—— (1922, 2). *Brit. Med. J.* ii, 751.
Knoop (1908). *Hofmeisters Beiträge*, **11**, 396.
Linneweh, Keil and Hoppe-Seyler (1929). *Z. physiol. Chem.* **183**, 11.
Robertson (1927). *J. Amer. Chem. Soc.* **49**, 2893.
Stewart (1923). *Biochem. J.* **17**, 130.
Vickery and Leavenworth (1928). *J. Biol. Chem.* **78**, 627.
Voge (1929). *Brit. Med. J.* ii, 829.
—— (1930). *Proc. Roy. Soc. Med.* **23**, 638.