XXVI. THE ESTIMATION OF HIPPURIC AND PHENACETURIC ACIDS IN URINE.

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HIPPURIC acid formation, as an example of a simple synthetic process occurring in the body, has long excited interest, and methods for the isolation and estimation of this acid are very numerous. In the course of an investigation on the metabolism of phenyl-fatty acids, existing methods were reviewed and a comparison of the reliability of some of them was attempted.

The methods of estimation fall into three main classes. In the earlier the hippuric acid was extracted from the urine by some solvent and subsequently purified with as little loss as possible. In a few cases it was decomposed, after extraction, by boiling with either acid or alkali and the benzoic acid so produced isolated and weighed. The best of these methods and probably the only accurate one, that of Dakin [1910], is much too long to be of use where a number of analyses have to be performed.

In the second type of method the glycine from the extracted hippuric acid, after hydrolysis, is estimated by a formaldehyde titration [Henriques and Sørensen, 1909; Quick, 1926] or by a Kjeldahl estimation [Snapper and Laqueur, 1923]. These methods differentiate hippuric acid from benzoic acid and benzoylglycuronic acid, both of which may occur in urine, and are only to be recommended where error would be introduced by mistaking benzoylglycuronic acid for hippuric acid.

In the third type of method, hippuric acid is decomposed before extraction by boiling the urine with alkali or acid, the benzoic acid so produced being extracted and either weighed or titrated. The most rapid method is Kingsbury and Swanson's modification [1921] of Folin and Flanders's original method [1912], in which a washed chloroform extract is titrated with standard sodium ethoxide. This method was found to give good results with urines containing a not too small amount of hippuric acid but in the case of normal dog's urine, which contains on an average only 0.08 g. of hippuric acid *per diem* (equivalent to 4.5 cc. 0.1 *N* sodium ethoxide), it was found that the chloroform extract was much too yellow to enable a titration to be performed with any degree of accuracy. The method of Hryntschak [1912] in which the urinary pigments are oxidised by means of potassium permanganate prior to ether extraction was found to yield pure samples of benzoic acid and to be moderately accurate. It cannot, however, be considered to give quantitative

results when the amount of benzoic acid present is only small, in view of the observations of Kingsbury and Swanson on the effect of potassium permanganate in reducing the titration value under such circumstances. In the method of Steenbock [1912] which was worked out on cow's urine, the urine, after boiling with caustic soda to hydrolyse the hippuric acid with the addition of hydrogen peroxide to oxidise pigments, is rendered acid and extracted with ether. The benzoic acid in the residue after evaporation of the ether is sublimed out in a suitable apparatus, collected and weighed. This method was found, at first, to give not very satisfactory results, the quantitative sublimation of benzoic acid in particular affording much difficulty. As, however, it was clear that this method offered certain advantages over all others, especially for the investigation mentioned above, various modifications were introduced until ultimately a satisfactory technique was evolved. The advantages are twofold.

In the first place, if hippuric acid and phenaceturic acid are present together in urine, an investigation of their properties as well as of the properties of benzoic and phenylacetic acids and their salts showed that there was too much similarity to hope for any quantitative separation based on relative solubilities. The suggestion of Steenbock that, in his method, a titration of the sublimate with standard alkali gives a clue to the relative amounts of benzoic and phenylacetic acids present seemed to indicate the most satisfactory lines on which to work. The second advantage of this method is its applicability to the estimation of the small amounts of hippuric acid normally found in dog's urine. As already mentioned it is just under these circumstances that other possible methods become unreliable.

The method as described by Steenbock was modified in only one respect in the part prior to sublimation. The benzoic or phenylacetic acid is extracted from a solution fairly strongly acidified with sulphuric acid and a certain amount of the aqueous layer is inevitably carried over with the ether. In this way traces of sulphuric acid may be introduced into the sublimation tube and attack the benzoic acid on heating. To avoid this the ethereal solution was washed with 10–15 cc. of Folin-Flanders salt solution (saturated sodium chloride with 0.5 cc. concentrated hydrochloric acid to a litre).

The efficient sublimation of benzoic and phenylacetic acids offered most difficulty. Steenbock recommends a condensing tube 25 cm. long and 9 mm. bore with bulbs 3 cm. in diameter blown at 9 mm. intervals and filled with glass wool. If the benzoic acid was merely to be weighed this method served admirably, but where it was desired to titrate the sublimed acid it was found very difficult to wash it out completely from the glass wool and if the latter was removed from the apparatus and suspended in the solution during the titration low results were obtained owing to its slight alkalinity. Glass wool after washing loses its power as a filter. Cotton wool was therefore adopted as a filter and was found to be more efficient than glass wool in every respect. A thickness of 5–7 cm. is quite sufficient to retain the acids, whereas, a

column of at least 20 cm. of glass wool is required. Cotton wool as bought was found to be slightly acid. It was, therefore, washed several times with distilled water followed by 90 % alcohol just neutral to phenolphthalein and dried, when 1.25 g. (the approximate amount used in an experiment) suspended in neutral alcohol did not require more than 0.20 cc. *N*/20 caustic soda to render the alcohol and wool distinctly alkaline to phenolphthalein. Specimens from each batch of prepared wool were tested and a constant of 0.10 to 0.20 cc. deducted from the burette reading according to the result.

The method as ultimately adopted is as follows. 50 to 100 cc. of urine, concentrated to the latter volume if necessary, are taken and sufficient sodium hydroxide is added to make a 20 % solution of this reagent. The mixture is boiled for from 3 to 4 hours in a 500 cc. Kjeldahl flask with a condenser over a low flame. During this period up to 30 cc. of 20 vols. hydrogen peroxide are added in portions if the urine is concentrated or darkly coloured. This reagent must be avoided if it is desired to search for higher homologues of phenylacetic acid in the sublimate. The contents of the flask are then cooled and 50 % sulphuric acid added down the condenser till the mixture is faintly acid to litmus. Bromine water may now be added to precipitate phenolic bodies if these are present. The solution is filtered through

Fig. 1.

a Büchner funnel, the flask and funnel being carefully washed with three or four separate quantities of water. Sulphuric acid (50 %) is now added to render the solution strongly acid and the solution shaken out five times with ether using 150 cc. in all. The ethereal solution is transferred to a separating funnel, washed with 10 to 15 cc. of the saturated salt solution already described and then run in portions into a U-tube immersed in water at 50° through which a current of air is drawn. When all the ether is evaporated the U-tube is evacuated whilst still in the water-bath, when any water present passes off. It is now attached to a sublimation tube and the acids present sublimed.

The apparatus is represented in Fig. 1. The U-tube of diameter 2.5 cm. is immersed in a bath of molten paraffin wax and the temperature maintained at 130°. The sublimation tube for the reception of the sublimed acids is 23 cm. long and 3 cm. in diameter, and is drawn out at one end to

a diameter of 0.5 cm. so as to pass through a rubber bung into a guard tube filled with glass wool. This is weighed before and after the experiment and serves to make sure that the whole of the acids have been stopped by the cotton wool. A layer of cotton wool about 5 cm. in length is placed at the end of the sublimation tube so that half the length is in the wider and half in the narrower part. The U-tube passes into the sublimation tube through a narrow band of rubber, cut out from a rubber bung. Air, dried by passage through a sulphuric acid wash-bottle, is drawn through the apparatus by a filter pump at not too rapid a rate. Any acid condensing on the horizontal part of the U-tube is driven over from time to time by a small flame. The sublimation tube (with cotton wool) is allowed to stand overnight in a desiccator containing calcium chloride prior to the experiment and during the experiment is weighed at intervals till constancy is attained. It is again allowed to stand overnight in the desiccator before the final weighing. The length of time required for efficient sublimation is from 4 to 6 hours according to the amount of foreign material mixed with the crude acids. After the final weighing, the benzoic or phenylacetic acid, or mixture of the two, is transferred to a wide-mouthed flask along with the cotton wool and neutral 90 % alcohol added. The sublimation tube is thoroughly washed with alcohol, the solution added to that in the flask and the whole titrated with *N*/20 caustic soda free from carbon dioxide with efficient stirring. This serves as a check on the weight if the pure acids are present alone and, with mixtures of benzoic and phenylacetic acid, it serves to determine the relative amount of each. To calculate this the weight of sublimate corresponding to the titration if all were benzoic acid and if all were phenylacetic acid is found. The actual weight of sublimate lies between these figures and the proportion of either acid can be found by the simple mixture rule. In a similar way higher sublimable homologues of phenylacetic acid (β -phenylpropionic acid sublimes readily under the above conditions) or sublimable hydroxy- and keto-acids when present mixed with phenylacetic acid can be detected.

If it is desired to estimate free benzoic or phenylacetic acid as distinct from their conjugated forms the method described in Steenbock's original paper may be employed.

Duplicate experiments on different samples of urine by this method giving 0.2 to 0.3 g. of sublimate were found to agree to within 0.007 g. Using a dog's urine of known hippuric acid content an average of 97 % of added hippuric acid was recoverable as benzoic acid. The accuracy is, therefore, as great as is claimed for other methods.

With a weight of sublimate of 0.2 g., an error of 0.1 cc. *N*/20 caustic soda represents, when calculating the relative amounts of benzoic and phenylacetic acids present, a 3 % error. As an error of 0.25 cc. was found to be possible in the titration an accuracy of more than 7.5 % in this figure cannot be claimed.

SUMMARY.

(1) A method for the accurate estimation of the total benzoic acid normally present in urine is described.

(2) By means of this method the relative amounts of phenylacetic and benzoic acids, when occurring together in urine, can be estimated.

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