A RAPID METHOD FOR THE DETERMINATION OF HIPPURIC ACID IN URINE.*

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In making benzoate tests for renal efficiency we were confronted with the necessity for having a rapid and accurate method for the determination of hippuric acid in urine. The Folin-Flanders method which we have been using required more time than was thought necessary. By means of this method analyses could be made in 9 or 10 hours when necessary, but with the routine of teaching and other university work 24 hours were usually required. It was our object to devise a method which would conserve the accuracy of the Folin-Flanders (1) method, but one which could be completed within 2 or 3 hours and be as applicable for hospital routine work as are any of the other modern biochemical methods.

A careful review of the more recent methods for the determination of hippuric acid shows that at present there is only one method which fulfils the requirements of accuracy and simplicity. This is the method of Folin and Flanders. Two other methods which appeared at about the same time, Steenbock's (2) and Hryntschak's (3) methods meet the requirements of accuracy fairly well but are too tedious to compete with the Folin-Flanders method. Ito's (4) method appearing 4 years later is more complicated than those mentioned above and does not represent an advance in this field. Steenbock's and Hryntschak's procedures depend upon the isolation and weighing of benzoic acid, which are accompanied by slight losses, more in the latter method than in the former, and are necessary only in those cases in which benzoic acid cannot be directly titrated, as for instance, in the presence of other titratable

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acids. Since there are no other acids present in the final extraction and titration stages of the Folin-Flanders method, titration in this case is not only easier to accomplish but more accurate.

Folin and Flanders proved that their method gave quantitative results with pure hippuric acid solutions which we have confirmed many times in the last few years. They did not compare their method, as applied to urine with any other procedure of analysis, nor as far as we can find, has any other investigator. They have assumed, however, that their method gives the most accurate results of any method devised up to that time. We have proved in the experiments which are directly to follow that the Folin-Flanders method does correctly estimate the amount of hippuric acid that can be extracted directly from urine by means of ethyl acetate.

Experiment I.—0.561 gm. of pure sodium hippurate was dissolved in 100 cc. of water, 1 cc. of concentrated nitric acid added, and the mixture then extracted with ten 50 cc. portions of ethyl acetate, shaking exactly 2 minutes each time. The aqueous mixture left was then filtered, the filtrate evaporated to dryness over night on the steam bath with 10 cc. more of 5 per cent sodium hydroxide than that required for neutralization of the nitric acid present. The residue was then analyzed for any remaining hippuric acid by the Folin-Flanders method. The titration value was 0.07 cc., which is the ordinary blank of the method. The hippuric acid was completely extracted by this procedure.

100 cc. of urine, the hippuric acid titration value of which was 13.58 cc. of one-tenth normal sodium ethylate were acidified with 2 cc. of concentrated nitric acid and extracted with ten 50 cc. portions of ethyl acetate, shaking 2 minutes each time. The combined extracts were washed with two 200 cc. portions of the Folin-Flanders sodium chloride solution and then steam distilled until all of the ethyl acetate and approximately 300 cc. of water had passed over. The aqueous solution of hippuric acid remaining in the distilling flask was quantitatively transferred to a casserole and analyzed according to the Folin-Flanders method. The titration value was 13.43 cc. of one-tenth normal sodium ethylate, agreeing with the value obtained directly as well as duplicates can usually be obtained by this method.

Experimental Methods of Analysis.

Our problem resolved itself into increasing the speed of the hydrolysis of hippuric acid either by acids or alkalies and the effective oxidation of urinary pigments and other disturbing substances. Without going into the details of many experiments
carried out it may be stated that by using 15 gm. of solid sodium hydroxide in hydrolyzing the hippuric acid of 100 cc. of urine at the boiling point for 30 minutes and subsequently acidifying, extracting, and titrating, results were obtained that were, in one experiment, 22 per cent higher than the known titration value for this specimen of urine. It was also found that values from 10 to 33 per cent higher than those obtained by the Folin-Flanders method resulted when urine was boiled with an equal volume of a mixture of concentrated nitric and sulfuric acids for 30 minutes in a process that gave 100 per cent recovery when applied to solutions of pure hippuric acid. Oxidation of the urine with alkaline potassium permanganate after the plan of Hryntschak was tried and yielded such promising results that the details of one typical experiment are given below:

Experiment 2.—50 cc. of urine were boiled with 7.5 gm. of solid sodium hydroxide and 1.5 gm. of potassium permanganate for 30 minutes in a Kjeldahl flask with a rather closely fitting test-tube condenser in the neck. The flask was cooled and 50 cc. of concentrated nitric acid slowly poured down the side of the condenser. The brown mixture cleared up after boiling a few minutes, but this was continued for 30 minutes, then cooled and extracted as in the Folin-Flanders procedure using comparative amounts of the various materials; The titration value was 16.72 cc. of one-tenth normal sodium ethylate; by the regular Folin-Flanders method, 16.95 cc. In a series of 12 analyses made in this way it was found that values from 97 to 99 per cent of the Folin-Flanders figures could always be obtained when these were as large as 15 cc., but with lower values the error was sometimes as large as 25 per cent. This was believed to be due to the action of the potassium permanganate on the benzoic acid present as it was always most pronounced in the urines which were the most dilute and therefore containing less of the other substances to combine with the permanganate. It was difficult to estimate the correct amount of potassium permanganate to be added in each case and it frequently happened that 1.5 gm. were a greater amount than could be reduced beyond the manganate stage and 0.5 gm. portions of sodium bisulfite had to be added to complete the reduction. It was also found that if this method were applied to a pure solution of hippuric acid, allowing the potassium permanganate to act only 2 or 3 minutes before reducing it with sodium bisulfite that it was impossible to obtain more than 95 per cent of the theoretical amount. In Hryntschak's method the urine was boiled with 10 gm. of sodium hydroxide for 2.5 hours then 10 gm. of potassium permanganate were added and the boiling was continued for 6 or 7 minutes. The excess of permanganate was removed by adding about 15 gm. of sodium bisulfite prior to acidification and extraction. He subjected benzoic acid to the same conditions and was able to recover
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98.24 and 98.17 per cent in two experiments and concluded from this that potassium permanganate did not destroy any benzoic acid. This is contrary to our findings using the more sensitive titration method.

We were reluctant about giving up the use of potassium permanganate because the subsequent chloroform extracts were always practically colorless and remained so until the definite pink end-point of titration was reached. No decidedly yellow extracts such as are rather frequent in the Folin-Flanders method were ever encountered. It was found by one of us that if a small quantity of magnesium oxide were present the effect of the permanganate in decreasing the titration value was prevented. The details of the method as we have adopted it follow:

Description of the Method.

50 cc. of urine are treated with 7.5 gm. of sodium hydroxide and 0.5 gm. of magnesium oxide in a 500 or 800 cc. Kjeldahl flask. This mixture is boiled at such a rate as to bring its volume down to approximately 25 cc. in the course of half an hour. At the end of this time, while still at the boiling temperature, 10 cc. of a 7 per cent solution of potassium permanganate (a solution approximately saturated at room temperature) is added, care being taken to rinse down any that may remain on the neck of the flask with the smallest possible amount of water since no unaltered permanganate must be present when the acid is subsequently added. The flask with its brown contents is twirled gently for a minute or two, cooled under the tap, a fairly closely fitting test-tube condenser placed in the neck and 30 cc. of concentrated nitric acid slowly poured in down the side of the condenser. The mixture, which rapidly clears up on the addition of the acid, is now gently boiled for 45 minutes (30 minutes are sufficient for accurate results, but a less colored, more easily titratable extract is obtained by boiling it 45 minutes) with a good current of water flowing through the condenser, cooled under the tap, and the extraction with chloroform carried out approximately according to the Folin-Flanders method. The condenser is rinsed down with 25 cc. of water to remove any benzoic acid sublimed on the bottom of the condenser, the contents of the flask are transferred to a 500 cc. separatory funnel containing 25 gm. of solid ammonium sulfate. The flask is rinsed with 20 cc. of water which is poured into the separatory funnel. After dissolving the ammonium sulfate the benzoic acid is extracted successively with one 50 cc., one 35 cc.,
and two 25 cc. portions of neutral, well washed chloroform. The first 2 portions of chloroform are used to rinse the Kjeldahl flask. The combined extracts in a second separatory funnel are washed once with 100 cc. of the Folin-Flanders salt solution (containing 1.0 cc. of concentrated HCl in 2 liters of saturated NaCl solution) and drawn off through a dry filter paper into a dry Erlenmeyer flask. The separatory funnel from which the extract was drawn is rinsed with 20 cc. of chloroform. This is drawn off into a small beaker to which the wet filter paper had been transferred. The paper is rinsed with the chloroform and the latter is poured through a dry filter into the main bulk of extract in the Erlenmeyer flask. 4 drops of 1 per cent phenolphthalein in absolute alcohol are added and the benzoic acid solution titrated to a faint, but definite pink with tenth normal sodium ethylate. The preparation and standardization of this alkali solution are adequately described in the original paper of Folin and Flanders.

We have found that the following treatment of the chloroform used in this method insures a product that is reliable as far as its neutrality is concerned:

New chloroform, which is of the U. S. P. grade and contains about 0.75 per cent of ethyl alcohol, should be washed with an equal volume of distilled water twice before being used for the extraction of benzoic acid. Chloroform which has been used in analysis and therefore contains sodium benzoate and alcohol is first filtered through a dry filter paper which removes a considerable part of the sodium benzoate in those determinations in which the titration figure was fairly large. It is now washed successively with equal volumes of tap water, once; tap water containing 5 to 10 cc. of a saturated solution of NaOH, twice; tap water, twice; and distilled water, once; six washings in all. Since the accuracy of this method depends primarily upon the use of a sample of chloroform which not only reacts neutral when tested, but which must remain neutral after being shaken with nitric acid, we have used the test which follows to determine this point:

155 cc. of chloroform, the amount used in an analysis, washed as described above, are shaken with dilute nitric acid, washed with 100 cc. of the Folin-Flanders salt solution, filtered through a dry paper, and titrated. The titration of this amount of chloroform suitable for use should not exceed 0.10 cc. of tenth normal sodium ethylate.
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The application of this method or that of Folin and Flanders requires the removal of protein from the urine when this is present, as in nephritic urines. Directions for doing this have already been published, but perhaps may be repeated here.

The albuminous urine is collected in 2 per cent nitric acid which was found by Raiziss and Dubin (5) to be effective in preventing the hydrolysis of hippuric acid. 15 cc. of this dilute nitric acid are sufficient for a 3 hour nephritic urine. 50 cc. of this urine, treated with 3 or 4 drops of 0.1 per cent methyl red solution in alcohol, are brought to the first definite yellow by the addition of approximately normal NaOH. The solution is then boiled, and during the boiling sufficient one-tenth normal HCl is added to produce the first definite red color. This procedure removes the albumin nearly quantitatively so that there is no increase in the resulting titration, as has already been shown (6). The coagulum of albumin on the filter paper is washed twice with 50 cc. of boiling water. The combined washings and main bulk of filtrate are evaporated rapidly over a free flame in an 800 cc. Kjeldahl flask after being made slightly alkaline to methyl red by the addition of a small amount of dilute alkali. Bumping and frothing, should they occur, are checked by adding a glass pearl and a drop of caprylic alcohol. By supporting the funnel in the neck of the flask by means of a slice of a large cork stopper the filtration and evaporation are continued simultaneously. When the contents of the flask have been evaporated to approximately 50 cc., 7.5 gm. of NaOH and 0.5 gm. of MgO are added and the analysis made according to the directions already given.

In Table I are given the comparative results with various specimens of urine, normal and pathological, obtained by the new method and by that of Folin and Flanders. In a series of approximately half of the determinations one of us used one method and the other, the other method. The results of neither of us were known to the other until all the determinations of this series had been made, when they were compared. No. 19 in Table I is a comparison of the two methods with 50 cc. aliquots of a pure sodium hippurate solution. The only modification in this case was the reduction of the permanganate with 0.5 gm. of sodium bisulfite as a substitute for the urinary constituents which ordinarily function in this manner, prior to the acid treatment. It is noted that the agreement
between the two methods is good, as close in general as duplicates can be made by the older method, and that duplicates by the new method, where they have been made show a very close agreement.

### TABLE I.

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*Urines designated by "P." are pathological specimens. All others are normal.

†50 cc. of a sodium hippurate solution were used.

A few duplicate determinations have been made several days apart with no evidence of loss of hippuric acid in acid urines at room temperature preserved with a small amount of a 10 per cent solution of thymol in chloroform.
CONCLUSION.

An accurate, rapid method for the determination of hippuric acid in urine is described which requires about 2 hours for completion with normal urine and about 3 hours with urine containing albumin.

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F. B. Kingsbury and W. W. Swanson


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