INTRODUCTION.

Since 1824 when Wöhler first called attention to the fact that the formation of hippuric acid represented a synthetic reaction by the animal body, this substance has occupied an interesting place in biological chemistry. In recent years it has received special attention from the fact that the study of its production may throw light upon certain phases of nitrogen metabolism. However, owing to the difficulty of its quantitative estimation, the progress of such work has been slow.

Bunge and Schmiedeberg first developed a method which with modifications has been in use in various laboratories in preference to all others. While the method, in that it aims to isolate the acid in pure form and to weigh it as such, is a desirable one, it still leaves much to be desired because of its difficulty of manipulation, its tediousness, and its lack of accuracy. This is very evident by the far from simple modifications that have been suggested from time to time.

F. Blumenthal sought to avoid the error, incident to the impossibility of crystallizing the hippuric acid quantitatively from urine, by determining the nitrogen in the residue. He did this on the

1 Published with the permission of the Director of the Agricultural Experiment Station.
3 Dakin: This Journal, vii, p. 106, 1910; Ringer: ibid., x, p. 328, 1911.
4 Maly's Jahresbericht, xxx, p. 303, 1910.
supposition that the impurities were not nitrogen containing substances. Henriques and Sörensen\textsuperscript{5} used the formol titration on the glycocoll liberated by boiling the hippuric acid from an ethyl acetate extract with concentrated hydrochloric acid.

R. Cohn\textsuperscript{6} decomposed the hippuric acid from an ethyl acetate extract of the evaporated urine by boiling under a reflux for five hours with concentrated HCl. The liberated benzoic acid was weighed as such upon volatilization of an ether extract in a tared beaker. Pfeiffer, Bloch and Reicke\textsuperscript{7} decomposed the hippuric acid by long continued distillation with H\textsubscript{2}SO\textsubscript{4}, and finally titrated the benzoic acid in the distillate. Jaarsveld and Stokvis\textsuperscript{8} decomposed the hippuric acid, extracted with ethyl acetate, by boiling with strong NaOH. The benzoic acid was shaken out with petroleum ether, and weighed after volatilization of the ether at room temperature.

W. Wiechowski\textsuperscript{9} sought to purify the benzoic acid, obtained by boiling hippuric acid with NaOH, by a steam distillation. The distillate was made alkaline, evaporated to a small volume, acidified, and then extracted with petroleum ether.

Without going into individual criticisms of these various methods, the use of Bunge and Schmiedeberg's method in preference to all others shows the unsatisfactory manner in which their details have been worked out. Jaarsveld and Stokvis admit that they were unable to obtain a pure benzoic acid and while Wiechowski secured a purer product by steam distillation, it is practically an impossibility to steam distill benzoic acid quantitatively. The determination of hippuric acid as benzoic acid by sublimation seemed to offer the most satisfactory plan for further experimentation. To make this applicable to urine it was necessary to take into consideration, (a) the occurrence in urine of non-conjugated benzoic acid (benzoates); (b) the occurrence in urine of conjugated benzoic acids, which, like hippuric, yield benzoic acid in the method of decomposition adopted; (c) the decomposition of hippuric acid and its quantitative recovery as benzoic acid.

\textsuperscript{5} Zeitschr. f. physiol. Chem., lxii, p. 327.
\textsuperscript{6} Festschrift für Jaffé, Braunschweig, iii, p. 327, 1901.
\textsuperscript{7} Maly's Jahresbericht, xxxii, p. 364, 1903.
\textsuperscript{8} Archiv f. exp. Path. u. Pharm., x, p. 71, 1879.
\textsuperscript{9} Hofmeister's Beträge, vii, p. 263, 1896.
Non-conjugated benzoic acid in fresh and apparently normal urines has been reported from time to time, but apparently this varies with different animals.\textsuperscript{10} Jaarsveld and Stokvis\textsuperscript{11} were unable to find any in normal human urine, which observation has been verified by Dakin,\textsuperscript{12} Lewinski,\textsuperscript{13} and Seo.\textsuperscript{14} Lewinski, however, noted exceptions upon the administration of large amounts of sodium benzoate. Brugsch and co-workers,\textsuperscript{15} on the other hand, report finding free benzoic acid in dog urines on feeding sodium benzoate. Apparently then, until the occurrence of non-conjugated benzoic acid has been more thoroughly investigated, in a determination of total benzoic acid in urine as hippuric acid a preliminary test for free benzoic acid should first be made. Of special significance in this connection is the observation of Seo,\textsuperscript{16} that, in urine, on standing, decomposition of hippuric acid rapidly sets in, due to bacterial action. Schmiedeberg\textsuperscript{17} and Minkowski\textsuperscript{18} showed that the same decomposition could be caused by an enzyme, histozyme, occurring in the kidneys of dogs and pigs. If it is true that the free benzoic acid in urines originates from a decomposition of the hippuric acid, subsequent to its excretion by the kidneys, it would be permissible to calculate it as hippuric acid.

Very few benzoic acid complexes besides hippuric acid are known to occur in urine. On feeding very large amounts of benzoic acid to animals, apparently exceeding their synthetic capacity to form hippuric acid, urines have been observed to rotate polarized light to the right instead of to the left, and to possess strong reducing power. This has been shown by Magnus-Levy\textsuperscript{19} to be due to the presence of benzoyl glycuronic acid which he was able to detect in sheep urines only when very large amounts of benzoic acid were fed. With rabbits and dogs, a still smaller

\textsuperscript{10} Hammarsten's \textit{Physiological Chemistry}, p. 657, 1911.
\textsuperscript{11} Archiv f. exp. Path. u. Pharm., v, p. 278, 1879.
\textsuperscript{12} This Journal, vii, p. 103, 1909.
\textsuperscript{13} Archiv f. exp. Path. u. Pharm., lxi, p. 88, 1909.
\textsuperscript{14} Ibid., lviii, p. 440, 1908.
\textsuperscript{15} Zeitschr. f. exp. Path. u. Therapie, iii, p. 663, 1906; v, p. 731, 1909.
\textsuperscript{16} Loc. cit.
\textsuperscript{17} Archiv f. exp. Path u. Pharm., xiv, p. 379, 1881.
\textsuperscript{18} Ibid., xvii, p. 453, 1883.
\textsuperscript{19} Biochem. Zeitschr., vi, p. 502, 1907.
tendency to form this acid was observed. Dakin\textsuperscript{20} was able to find traces of it in human urine when administering 10 grams of sodium benzoate. When present, it is readily detected in urines by its reducing power, its non-fermentability, and its dextro-rotation. In such abnormal urines a method based on the determination of conjugated benzoic acid as hippuric is not applicable. Ornithuric acid discovered by Jaffé\textsuperscript{21} on feeding benzoic acid to birds need not be considered here as it has not yet been isolated, so far as the writer is aware, from the urines of mammals.

**EXPERIMENTAL.**

Benzoic acid was determined essentially according to the method proposed by Dakin\textsuperscript{22} for qualitative work. Steam distillation was attempted but inasmuch as the solubility, as well as the boiling point of a substance, is a factor of its volatility, it was practically an impossibility to quantitatively distill the benzoic acid; even when as much as 4 to 5 liters of distillate were collected, only 90 per cent of the total benzoic acid was accounted for. On decreasing the solubility of the acid by means of phosphoric acid, a very weak acid as judged by its ability to saponify esters, slight decomposition of the hippuric acid resulted. Direct extraction of the benzoic acid by means of petroleum ether was also attempted, but finally abandoned in favor of benzol. Two hundred cubic centimeters of urine (cow urines only were used in all experimental work) acidified with phosphoric acid were extracted for twelve hours with 90 per cent benzol (purified by washing with dilute alkali and then with water and redistilling). By shaking the benzol solution with dilute alkali, all benzoic and hippuric acids in solution were taken up. After neutralizing, the water extract was evaporated to dryness, taken up with 25 cc. of water, transferred to a separatory funnel, a few grams of sodium chloride added, and shaken out with freshly distilled petroleum ether (B. P., 40°). Though benzoic acid is much more soluble in ethylether, petroleum ether was used since this does not dissolve the traces of hippuric acid (about 0.013 gram) extracted by the benzol. One hundred

\textsuperscript{20} Loc. cit.

\textsuperscript{21} Ber. d. deutsch. chem. Gesell., x and xi.

\textsuperscript{22} Dakin: This Journal, vii, p. 107, 1909.
and fifty cubic centimeters used in five portions were found sufficient for 0.2 gram of benzoic acid. The extracted water solution, which contains hippuric acid, was added to the benzol extracted urine on which a conjugated benzoic acid determination could then be made. The petroleum ether extracts were united in a separatory funnel and allowed to stand till all traces of water in emulsified form could be separated. By running the ether, drop by drop, into a U-tube, through which a current of dry air was drawn with a suction pump, the ether was rapidly volatilized without any condensation of water. To facilitate the volatilization, which otherwise would be retarded by the cooling of the tube, it was immersed in a water bath at approximately 40°. At this temperature no appreciable loss of benzoic acid results in the time necessary for the complete volatilization of the ether and the drying of the residual benzoic acid. When thoroughly dry, the benzoic acid was purified by sublimation. The U-tube was suspended in an air oven, one arm connected with a drying bottle containing H2SO4, and the other with a tared condensing tube. The condensing tube consisted of a light glass tube (20 grams) 25 cm. long and 9 mm. bore with bulbs 3 cm. in diameter blown at 9 mm. intervals. These bulbs were filled with glass wool, which proved to be very efficient in condensing the benzoic acid from the current of air drawn through the apparatus by means of a suction pump. The large bore of the condenser made it possible to make connections with the arm of the U-tube inside the air bath by means of a small cork, thus preventing condensation and a clogging of the apparatus at the point of connection. By gradually bringing the air bath up to 130° the benzoic acid was quantitatively sublimed into the condenser, which at the end of the operation, lasting usually about one hour, was cooled in a desiccator and weighed.

By the above operation no benzoic acid was recovered in any of the cow urines examined, thus confirming the findings of others23 for human urines. That this was not due to faulty operations was shown by recovering 0.195 gram of 0.200 gram of benzoic acid which had been added to 200 cc. of urine.

With the absence of non-conjugated benzoic acid in cow urines which furthermore do not reduce Fehling's solution, it seemed en-
Determination of Benzoic Acid

tirely permissible to determine hippuric acid as benzoic if any practical methods of decomposition were found to be quantitative. Decomposition by boiling with HCl or H₂SO₄ were not only found difficult but liable to losses of benzoic acid by volatilization. Boiling with strong alkali was found much more efficacious.

One gram of hippuric acid was boiled for two hours with 50 cc. of 10 per cent NaOH under a reflux in a 500 cc. Jena Florence flask. After cooling, the solution was transferred to a separatory funnel, acidified with 50 per cent H₂SO₄ and shaken out consecutively with 50, 40, 20, 20, 20 cc. portions of ethyl ether. The ether was volatilized and the residual benzoic acid sublimed as previously outlined. Benzoic acid × 1.467 = hippuric acid.

<table>
<thead>
<tr>
<th>HIPPURIC ACID</th>
<th>BENZOIC ACID</th>
<th>HIPPURIC ACID</th>
<th>PER CENT HIPPURIC ACID</th>
</tr>
</thead>
<tbody>
<tr>
<td>WEIGHED OUT</td>
<td>RECOVERED</td>
<td>ACCOUNTED FOR</td>
<td>ACCOUNTED FOR</td>
</tr>
<tr>
<td>gram</td>
<td>gram</td>
<td>gram</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>1</td>
<td>0.677</td>
<td>0.9931</td>
</tr>
<tr>
<td>B</td>
<td>1</td>
<td>0.676</td>
<td>0.9916</td>
</tr>
</tbody>
</table>

The results prove beyond a doubt that the decomposition and recovery are quantitative. In urines, however, there are interfering substances which necessitated a procedure as subsequently outlined.

In a 500 cc. flask 100 cc. of urine were boiled for two hours over a low flame with 10 grams of NaOH, adding 25 cc. H₂O₂, a few cubic centimeters at a time, to oxidize coloring matters. After cooling, the solution was transferred to a 200 cc. volumetric flask, and slightly acidified to litmus with 50 per cent H₂SO₄. Bromine water was then added to a slight bromine odor, the solution made up to volume and filtered through a dry filter. Fifty cubic centimeters of the clear filtrate after acidification were shaken out with ether and sublimed as previously outlined, taking special precautions not to raise the temperature above 130°. High temperature may cause destructive distillation of some of the impurities extracted with the benzoic acid. In many cases sublimation at a low temperature may be facilitated by tilting the U-tube and thereby distributing the benzoic acid over a larger area. By the hydrogen peroxide treatment the coloring matters of all urines examined were readily oxidized to a pale straw color without loss or oxida-
tion of benzoic acid. Dakin in a practically neutral solution was able to oxidize benzoic acid to oxybenzoic acids by means of hydrogen peroxide. That these reactions did not proceed with the solution strongly alkaline was shown by negative tests for oxybenzoic acids with ferric chloride and with Millon's reagent. The bromine water was very efficient in precipitating phenols which otherwise would sublime with the benzoic acid. Strong acidity at this point should be avoided as it will cause the precipitation of benzoic acid which would be lost on filtering. Examples of typical results obtained are given in the following table which is supplemented with titration values for benzoic acid obtained by titrating the sublimate with $\frac{\text{NaOH}}{\text{NaOH}}$ using phenolphthalein as indicator.

<table>
<thead>
<tr>
<th>NUMBER OF URINE</th>
<th>BENZOIC IN FORM OF HIPPURIC ADDED</th>
<th>SUBLIMATE OBTAINED</th>
<th>CALCULATED AS HIPPURIC ACID</th>
<th>TITRATION VALUES FOR BENZOIC ACID</th>
<th>PER CENT OF ADDED HIPPURIC RECOVERED</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>gram</td>
<td>gram</td>
<td>gram</td>
<td>gram</td>
<td>100.6</td>
</tr>
<tr>
<td></td>
<td>0.1922</td>
<td>0.2321</td>
<td>0.1913</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.1938</td>
<td>0.2844</td>
<td>0.1931</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.2016</td>
<td>0.2959</td>
<td>0.2009</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.2036</td>
<td>0.2959</td>
<td>0.2015</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.3739</td>
<td>0.5488</td>
<td>0.3712</td>
<td></td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>0.170</td>
<td>0.3739</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

With the above examples as a type there can be no doubt as to the applicability of the method to cow urines. Under pathological conditions and with urines from other sources no statements can as yet be made though no serious difficulty is expected.

From the table it is seen that the titration values agree remarkably well with those corresponding for pure benzoic acid. This practically excludes the possibility of the presence of any sublimable homologues of benzoic acid, furthermore, the melting points of sublimates were found to correspond exactly to that of benzoic acid (121.5').

That homologues of benzoic acid do occur in urine was shown by Salkowski, who isolated 0.8 gram of phenaceturic acid from a liter of horse urine. Phenaceturic acid would, in the method of decomposition adopted for hippuric acid, yield phenylacetic acid which like benzoic acid is readily sublimable. The observa-

24 This Journal, iii, p. 419, 1907.
Determination of Benzoic Acid

Determinations on cow urines are entirely out of harmony with those of Vasiliu who, from the examination of the urines of sheep, comes to the conclusion that phenaceturic acid is almost as important a constituent of the urine of herbivora as hippuric acid.

The lowering of titration values of the sublimate from that calculated for benzoic acid suggests the possibility of determining phenaceturic acid as well as hippuric acid. Other homologues of benzoic acid due to a longer side chain are not liable to occur in urine as it has been shown that phenyl propionic acid is oxidized in the body to benzoic acid. Phenylacetic acid differs in molecular weight from benzoic acid by one CH₂ group, therefore, any lowering of titration values can be calculated directly back to percentages of phenylacetic acid as their combined weights are known.

\[
\text{Wt. of sublimate} - (\text{cc. } \frac{M}{\text{NaOH}} \times \text{gms. benzoic acid in 1 cc. } \frac{\text{NaOH}}{M} \text{ solution}) = x \times \text{CH}_2: \text{C}_6\text{H}_5\text{CH}_2\text{COOH}
\]
\[x = \text{weight of phenyl acetic acid.}
\]
\[\text{Weight of sublimate} - x = \text{weight of benzoic acid.}
\]

To test the applicability of the method 1 gram of phenaceturic acid was added to 100 cc. of urine, of which the hippuric acid content was known, and the general method outlined for hippuric acid followed. The table shows the results obtained on an aliquot corresponding to 25 cc. of urine and 0.25 gram added phenaceturic acid.

<table>
<thead>
<tr>
<th>WEIGHT OF SUBLIMATE</th>
<th>TITRATION OF SUBLIMATE IN CARBONIC METER N (\frac{M}{\text{NaOH}})</th>
<th>WEIGHT BENZOIC ACID FROM HIPPURIC ACID</th>
<th>WEIGHT OF PHENYLACETIC ACID FROM PHENACETURIC ACID</th>
<th>WEIGHT OF BENZOIC ACID BY TITRATION</th>
<th>WEIGHT OF PHENYLACETIC ACID BY TITRATION</th>
</tr>
</thead>
<tbody>
<tr>
<td>A...............</td>
<td>0.3726</td>
<td>58.15</td>
<td>0.2030</td>
<td>0.1761</td>
<td>0.1988</td>
</tr>
<tr>
<td>B...............</td>
<td>0.3700</td>
<td>57.75</td>
<td>0.2030</td>
<td>0.1761</td>
<td>0.1981</td>
</tr>
</tbody>
</table>

While the above values agree remarkably well it must be remembered that the method is not without its limitations. The difference in molecular weight between benzoic and phenyl acetic acids is small and any impurities in the sublimate will materially affect the final values; a variation of 0.1 cc. of \(\frac{M}{\text{NaOH}}\) will mean a difference of 0.0068 gram of phenylacetic acid or, when multiplied

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26 Mitteilungen d. land. Instit. Breslau, iv, 1009.
by the conversion factor 1.4191, 0.0096 gram of phenaceturic acid. However, it is believed that this method for phenaceturic acid is far superior to fractional crystallization which up to the present time has been the only one in use. Where examination for unconjugated benzoic acid in the urine is to be made the method outlined was found entirely applicable, since phenaceturic like hippuric acid is practically insoluble in benzol.

SUMMARY.

Dakin's method for isolating benzoic acid was found to yield quantitative results when followed by sublimation.

Hippuric acid and phenaceturic acid occurring together can be determined respectively as benzoic and phenylacetic acid by sublimation followed by titration.

No salts of non-conjugated benzoic acid or of phenaceturic acid were found in cow urines.
QUANTITATIVE DETERMINATION OF BENZOIC, HIPPURIC, AND PHENACETURIC ACIDS IN URINE
H. Steenbock

J. Biol. Chem. 1912, 11:201-209.

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