LXXIX. A MODIFIED METHOD FOR THE DETERMINATION OF HIPPURIC ACID AND FREE BENZOIC ACID IN THE URINE OF CATTLE.

BY FREDERICK JOHN WARTH AND
NIRMAL CHANDRA DAS GUPTA.

*From the Animal Nutrition Section, Imperial Department
of Agriculture in India, Bangalore.*

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HIPPURIC acid is usually estimated by hydrolysing the substance and determining one or other of the constituents. If hydrolysis is effected with acid the resulting amino-acid is determined. If alkali is used for the hydrolysis the resulting benzoic acid is determined. For work on which we are engaged, the existing methods have not been found convenient. Our experience with procedures in which acid hydrolysis is employed has been unsatisfactory generally. In all cases the hydrolysed liquid requires lengthy manipulation to prepare it for the determination. We have also found the formaldehyde titration method unreliable with our urine samples, although we introduced a refinement by titrating with the aid of a comparator. The best known methods, in which alkaline hydrolysis is employed, are those of Folin and Flanders [1912] and of Kingsbury and Swanson [1921]. We have found the method of Folin and Flanders satisfactory for small amounts of hippuric acid but not so good when considerable quantities have to be determined. We have tested the method of alkaline hydrolysis recommended by Kingsbury and Swanson and find it entirely satisfactory. These authors boil 50 cc. of liquid with 7.5 g. NaOH in a Kjeldahl flask for half an hour, reducing the volume to 25 cc. during this time. We find that this amount of alkali is just sufficient to ensure hydrolysis. For safety we prefer to boil down twice.

Folin and Flanders, and Kingsbury and Swanson employ similar methods for determining the benzoic acid liberated by hydrolysis. The liquid is oxidised with nitric acid under a reflux condenser to destroy colouring matters and emulsifying substances. The benzoic acid is then completely extracted by shaking out repeatedly with chloroform and the chloroform solution is washed and titrated. We have observed the emulsifying action of the raw hydrolysate upon chloroform and agree with the above-mentioned authors that clean and complete extraction is impossible without preliminary oxidation. The time and labour involved in the oxidation are, however, a serious drawback to the

process. Therefore, as a possible means of avoiding this lengthy operation entirely, the senior author proposed the following new procedure.

After alkaline hydrolysis the liquid is acidified, made to a definite volume and shaken with a definite volume of immiscible non-volatile solvent. Kerosene was proposed for this purpose. The benzoic acid in the solvent is determined by titrating an aliquot part. The benzoic acid remaining in the aqueous layer is calculated from previously determined partition coefficients. By this means the necessity for complete extraction is avoided. We have tested this procedure and have obtained entirely satisfactory results with it. The experimental work falls under three heads.

1. A study of the partition coefficients of benzoic acid.
2. The determination of hippuric acid in urine.
3. The determination of free benzoic acid in urine.

PARTITION COEFFICIENTS OF BENZOIC ACID.

1. *Partition between water and different solvents.* Tests were made with kerosene, toluene and chloroform. The results are shown in Chart 1. The chart brings out two points.

(a) The partition of benzoic acid between water and any of these solvents is a function of the concentration. The proportion of benzoic acid passing into the organic solvent increases rapidly with the concentration.

(b) Chloroform and toluene are very efficient solvents for benzoic acid, chloroform being slightly superior to toluene. Kerosene has a low efficiency, especially when the concentration of benzoic acid is low.

2. *The effect of mineral acids and salts on the partition of benzoic acid.* As salts and mineral acids must always be present after hydrolysis, it was necessary to determine their effect on the partition. Typical experimental results are given in Chart 2. In these tests the organic solvent (kerosene) was the constant and the aqueous layer the variable. The sodium chloride solutions all contained 1 % HCl. The chart shows that the efficiency of extraction increases regularly with increasing concentration of sodium chloride. The increase is so great that kerosene with saturated salt solution becomes as effective as chloroform with water. This property of sodium chloride is of great practical value and has been made use of by us in our method for determining benzoic acid. Presumably other salts exert a similar influence. For example $(\text{NH}_4)_2\text{SO}_4$ has been used for the same purpose by Delprat and Whipple [1921]. We have confined our tests to sodium chloride because its solubility is almost unaffected by small changes in temperature, a property which makes it particularly suitable for our purpose.

3. *Effect of temperature on the partition of benzoic acid.* To observe the effect of temperature, the partitions at 20° and 30° were compared. The temperature factor was considerable in the case of water without mineral acid and salt. It became less marked as the salinity increased. In every case, the partition was less favourable at the higher temperature. The results were perfectly regular, but it is only necessary to quote the figures obtained with saturated sodium chloride, since, for practical purposes, only saturated solutions are used by us now. The figures are included in Table I.

4. *Standard partition coefficients with saturated sodium chloride in 1 % HCl.* The preliminary experiments having shown that saturated sodium chloride was the best working medium, more exact determinations, covering a wider range, have been carried out with saturated solutions. The results are shown in Table I, which, in graphic form, is our working basis for the determination of benzoic acid.

Table I. *Standard partition determinations.*

Kerosene and saturated NaCl in 1 % HCl 20°		Kerosene and saturated NaCl in 1 % HCl 30°		Kerosene and saturated NaCl in 2 % HCl 30°		Toluene and saturated NaCl in 1 % HCl 30°	
K	W	K	W	K	W	T	W
3.40	0.43	3.20	0.47	3.30	0.49	7.8	0.24
7.80	0.68	7.50	0.73	7.45	0.81	16.2	0.35
17.50	1.01	16.75	1.14	16.70	1.27	33.2	0.54
27.40	1.30	26.40	1.48	26.35	1.64	51.0	0.56
42.70	1.66	41.50	1.88	41.30	2.11	68.4	0.67
58.30	1.96	57.00	2.20	56.80	2.46	—	—

The figures in this table represent corresponding equilibrium concentrations in the two phases. The concentration is expressed as cc. $N/7$ benzoic acid per 100 cc. liquid.

The points of practical importance in these figures are as follows.

(a) The temperature factor is small. It is unnecessary to use a thermostat as long as the laboratory temperature lies between 20° and 30°. The actual temperature is noted and the partition for that temperature is read off from a large scale graph.

(b) The concentration of mineral acid has a perceptible but not an appreciable effect.

(c) The figures for kerosene are so favourable that there is generally no advantage in using toluene.

THE DETERMINATION OF HIPPURIC ACID.

The steps in the new procedure are the following.

1. *Hydrolysis with NaOH.* This has been described above.

2. *Preparation of the solution.* The liquid in the Kjeldahl flask is acidified and then made just alkaline. Solid sodium chloride is added in excess and shaken. The liquid is transferred to a 110 cc. flask, saturated sodium chloride being used for rinsing. The liquid is made up to 110 cc. with saturated sodium chloride. 100 cc. are pipetted out into a shaking bottle and 10 cc. of 10 % HCl saturated with sodium chloride are added. Benzoic acid is thus liberated in a known volume of saturated sodium chloride solution. Some solid sodium chloride and a definite volume of kerosene (or toluene) are added and the bottle is shaken at room temperature. The entire contents of the bottle are poured into a separating funnel and allowed to stand until the two liquids have separated. Kerosene and toluene are found to separate better than chloroform. The aqueous layer is run out and the kerosene is transferred to a clean, dry beaker and allowed to stand for some time. All the remaining water separates out on the sides of the vessel and a perfectly clean kerosene solution of benzoic acid is obtained. We have titrated many such solutions and find that there is no contamination with mineral acid. Washing is unnecessary, but it is advisable to filter the kerosene through a dry filter even if it appears to be quite clear. An aliquot part of the filtrate is titrated.

3. *Titration of kerosene and toluene solutions of benzoic acid.* An equal volume of alcohol is added to the liquid in an Erlenmeyer flask. The contents are mixed with a rotary motion and titrated with aqueous standard alkali. The toluene and alcohol form a clear homogeneous mixture which is very easily titrated. The kerosene and alcohol do not mix completely, a milky emulsion being obtained. Theoretically such a two-phase liquid might not be expected to titrate either rapidly or completely, but practical experience with many standard solutions of benzoic acid shows that the method always yields quantitative results. It may be mentioned here that we have abandoned the use of alcoholic potash for chloroform titrations also, and have adopted the above procedure instead. With chloroform, however, more alcohol is required and the titration is slower.

EXAMPLES.

1. 0.801 g. pure hippuric acid, treated as described, required 26.90 cc. *N*/7 alkali.

The graph shows that the aqueous layer must contain 1.40 cc. *N*/7 benzoic acid per 100 cc. or 1.54 cc. in 110 cc. Hence 0.7999 g. hippuric acid is found, which agrees very well with the quantity taken.

2. *Tests with urine.* The following figures from four separate tests on the same sample of urine show that duplicates give concordant results. 50 cc. of the kerosene extract gave titrations of 7.25, 7.25, 7.30 and 7.25 cc., respectively.

It is more difficult to prove that the method does not introduce systematic errors when applied to urine analysis. As pure hippuric acid yields theoretical results, a systematic error in urine analysis can only be due to urinary constituents. These constituents may, conceivably, cause errors in two ways. Firstly, the partition of benzoic acid may be altered. Secondly, titratable impurities may be extracted by the kerosene. The first error can be checked with certainty by carrying out duplicate analyses with different quantities of urine. The second error will be revealed in the same way unless the impurity happens to have exactly the same partition coefficients as benzoic acid, which is a very unlikely circumstance.

Accordingly four lots of 50 cc. and four lots of 25 cc. urine were submitted to the process. The same amount of alkali was used in all cases and the liquids were all made up to the same volume. The following results were obtained.

Table II. *Hippuric acid found (g. per 100 cc. urine).*

	50 cc. tests	25 cc. tests
1.	1.285	1.287
2.	1.285	1.287
3.	1.304	1.298
4.	1.285	1.298

The figures are a fair representation of the accuracy and reliability of the method and indicate that there is no appreciable systematic error in the determinations.

THE DETERMINATION OF FREE BENZOIC ACID IN URINE.

In determining small quantities of free benzoic acid by any of the existing methods, the question arises whether the titration obtained is due to benzoic acid or not. By our partition method some indirect information can be gained on this point. It should be noted here that whenever kerosene (or toluene) extraction is spoken of in the sequel it refers to the extraction of a liquid saturated with sodium chloride and containing 1 % HCl. We have frequently carried out kerosene extractions of unhydrolysed urine and have obtained perceptible amounts of acidity from perfectly fresh urine. The quantities are very small, ranging generally between 3 and 5 drops of *N*/7 acid in 50 cc. urine. Is this benzoic acid? Duplicate extractions with kerosene and toluene were

carried out. Toluene was found to indicate a higher percentage acidity. Therefore either our partition coefficients have broken down at this extreme range, or the substance extracted is not benzoic acid. To investigate this discrepancy, minute quantities of benzoic acid were added to urine, which was then extracted as usual. Blank determinations on the urine alone were made simultaneously. The same sample of urine was tested with both solvents. The results obtained are given in Table III.

Table III.

	cc. <i>N</i> /7 benzoic acid added to urine	cc. <i>N</i> /7 acidity found in urine	Excess over blank
Kerosene extraction	0	0.58	0
	1.35	1.83	1.25
	2.70	3.19	2.61
Toluene extraction	0	0.89	0
	1.00	2.04	1.15
	2.00	3.01	2.12

The figures show that very small amounts of added benzoic acid can be accounted for satisfactorily. This proves that the standard partition coefficients must apply with a fair degree of accuracy to urine. We are inclined to conclude from this experiment that the blank titration with toluene does not represent an equivalent amount of free benzoic acid, but is, at least partly, due to some other substance which is more soluble in toluene than in kerosene. Chloroform is liable to take up considerably more titratable impurities than toluene. Extractions were carried out with the three solvents on a sample of fresh urine. In each case 80 cc. of urine and 60 cc. of solvent were used. After separation, 50 cc. of the solvent were titrated (Table IV).

Table IV.

	Acidity in 50 cc. solvent	Acidity in 60 cc. solvent	Computed free benzoic acid in urine
Chloroform test	1.80	2.16	>2.16
Toluene test	0.40	0.48	0.50
Kerosene test	0.15	0.18	0.20

Judging by the results of Table IV it seems certain that the titration obtained with chloroform in this test cannot be due to benzoic acid. Our conclusion is that kerosene is probably the safest solvent to use when free benzoic acid has to be determined.

Concerning the determination of free benzoic acid by means of kerosene, the figures of Table III show that 1.3 cc. *N*/7 free benzoic acid in 100 cc. of urine can be easily determined by this method.

Finally a word must be said regarding the relative merits of the three solvents. Kerosene has many points in its favour. It is, for our purpose, non-volatile; it separates most easily and most completely from the aqueous layer; it takes up a minimum amount of titratable impurity; its low cost is a further point in its favour. The disadvantages of kerosene are that it is indefinite in

composition and that its solvent power for benzoic acid is low. Toluene has a greater solvent power but, to set off this advantage, it may take up more titratable impurity; it is also somewhat volatile. Chloroform is too volatile to be of use for our method.

SUMMARY.

1. A modified method for the determination of hippuric acid is described. The feature of the method consists in the estimation of the liberated benzoic acid with the aid of partition coefficients. A tedious operation of oxidation is eliminated by this means.

2. The method, when applied to unhydrolysed urine, gives some indication that titratable impurities are likely to be determined with the free benzoic acid.

3. Kerosene possesses certain advantages as a solvent in the determination of benzoic acid.

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