4. Incubation of homogenates in acetate buffer leads to a progressive release of enzyme from the granules to the solution. This does not occur in citrate buffer.

5. In concentrated homogenates, the presence of the insoluble enzyme fraction can lead to false results for the total activity.

6. The pH-activity curves for the hydrolysis of phenolphthalein glucuronide in acetate buffer by the

soluble and insoluble fractions are identical in having peaks at pH 4.5 and 5.2.

7. Saccharate inhibits the enzyme in the granules without affecting its release to the solution on incubation in acetate buffer.

8. The significance of these findings in the assay of glucuronidase is discussed, with particular reference to tissues in varying states of proliferation.

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The Micro-estimation of Benzoic and Hippuric Acids in Biological Material

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In a study of the aromatization of cyclohexanecarboxylic acid and related compounds by animal tissues (Beer, Dickens & Pearson, 1950) it was necessary to be able to estimate small amounts of benzoic or hippuric acids in presence of other organic acids, and particularly of the unaromatized substrate employed. For this purpose existing methods were not applicable, and in this paper a method is described which has been found adequate for the conditions of the above experiments. The method is based on the nitration of benzoic acid at room temperature, reduction of the resulting mononitrobenzoic acid to aminobenzoic acid, diazotization of the latter and coupling with a colour-forming base. The estimation is completed by colorimetric estimation of the resulting dye and is here described for application to samples containing 0.05–10 mg. benzoic acid or equivalent amounts of hippuric acid.

Older methods of estimation of benzoic and hippuric acids

Urinary hippuric acid has been estimated by isolation and weighing (Friedmann, 1911); a method only suitable for large quantities. The titrimetric

procedures of Folin & Flanders (1912) and Kingsbury & Swanson (1921) required continuous extraction of the urine with ether and titration of the benzoic acid liberated on hydrolysis of the hippuric acid. Quick (1926) determined instead the amino nitrogen by formol titration. Griffith (1926; see also Hawk & Bergeim, 1938) destroyed traces of urea in the ether extract and then estimated hippuric acid nitrogen by Kjeldahl analysis. None of these methods is suitable for the present purpose, and the latter two methods cannot be used for estimation of benzoic acid in any case.

Colorimetric methods depending on aromatic structure would obviously be preferable. Nicholls (1928) oxidized benzoic acid by hydrogen peroxide to salicylic acid which can be colorimetrically estimated; this oxidation proceeds only to the extent of about 10%, and is therefore an unsatisfactory basis for a quantitative method.

The most promising published micromethod for benzoic acid estimation appeared to be that of Waelsch & Klepetar (1935) and Waelsch & Busztin (1937). This is based on the hot nitration of benzoic acid to give a dinitrobenzoic acid, thought (Mohler,

1890) to be 3:5-dinitrobenzoic acid. Grossfield (1915) reduced the nitration product in alkaline solution with hydroxylamine to give a red colour, and the reaction was developed by the Prague workers into a quantitative test for amounts of 0.1–50 mg. benzoic acid. This reaction was also found to be unsuitable for the present purpose since *cyclohexane* compounds (and many other substances also) char to such an extent in the hot nitrating mixture that no reading of the final colour is possible. Moreover, the method involves the selection of a time of maximum colour development (10–55 min.) at which readings must be taken, which is dependent on the actual amount of benzoic acid present. Further drawbacks were found to be the non-linearity of colour development with concentration of benzoic acid and the fact that we have found (Beer, Dickens & Salmony, 1950) that the true colour former in this reaction appears to be mainly traces of 2:5-dinitrobenzoic acid and not the 3:5-dinitrobenzoic acid formed as the main product in the hot nitration of benzoic acid.

EXPERIMENTAL

Method for micro-estimation of benzoic and hippuric acids

Reagents. Nitrating mixture: a solution of 10 g. A.R. KNO_3 in 100 ml. H_2SO_4 , 98% (w/w). TiCl_3 (13–15%, w/v) in protected burette. NaNO_2 A.R. 1 g./10 ml. water; freshly prepared solution.

Coupling reagent: *N*-(1-naphthyl)-ethylenediamine hydrochloride, 0.5 g./100 ml. *N*-HCl solution used within 2 days of dissolving. *iso*Amyl alcohol, 'purified' or 'milk-testing' grade. NaOH (40 g./100 ml. water; approx. 9.6*N*) and dilute (approx. 0.1*N*) solution. Sodium carbonate 20% (w/v) Na_2CO_3 ; solid urea and tartaric acid; HCl, 32% (w/w).

Procedure. (1) The sample, which should not exceed 10 ml. in volume or contain more than 10 mg. benzoic (or hippuric) acid, is measured into a 20 ml. glass or porcelain crucible, made alkaline if necessary with a few drops excess of 20% sodium carbonate and evaporated to dryness on a steam bath. The drying is completed in the oven for 30 min. at a temperature not exceeding 100°.

(2) The cooled, perfectly dry residue is nitrated at room temperature for 30 min. by addition of 1.0 ml. nitrating mixture, with stirring to ensure solution. After this time the contents of the crucible are cautiously diluted with 3.5 ml. water and cooled. Partial neutralization is then effected by the addition of 2.5 ml. of the concentrated sodium hydroxide solution (see *Reagents*).

(3) The contents of the crucible are transferred with 3 ml. water to a 50 ml. separating funnel, or to a test tube, 20 × 130 mm., provided with a glass or rubber stopper and in addition with a suitable double-bored rubber stopper carrying a pair of fine glass tubes for blowing out the top layer (as in a wash-

bottle): the depth of immersion of the longer glass tube is adjustable and its lower end is turned upwards. The use of these tubes is more convenient if series of analyses are done simultaneously, as they are readily rotated for mixing the contents by attaching them to a rotating disk provided with a double row of spring clips. Each tube has its own blow-out head as described above.

*iso*Amyl alcohol (10 ml.) is added to each tube or separating funnel, and, after thorough mixing, the upper layer of amyl alcohol is transferred to a 50 ml. separating funnel. A second extraction with 10 ml. amyl alcohol is added to the first. By using tubes of appropriate size emulsions which may form with some biological extracts are quickly separated by centrifugation: the above size is convenient for the small bench-type angle centrifuge used by us.

(4) The combined amyl alcohol extracts are washed by thorough shaking in the separating funnel with three successive portions each of 5 ml. water, the aqueous washings being rejected. The nitration product is then extracted from the amyl alcohol by thoroughly shaking with two portions, each of 5 ml. approx. 0.1*N*-sodium hydroxide. These extracts, which must be alkaline in reaction, are transferred to a 50 ml. pyrex boiling tube.

(5) To the contents of the tube are added 15 ml. concentrated hydrochloric acid, 12 g. tartaric acid crystals and, after solution, 1 ml. titanous chloride solution. The whole is placed in a boiling-water bath for exactly 15 min., when the tube and contents are cooled to below 5° in ice. Diazotization follows, by gradual addition of 1 ml. 10% sodium nitrate and letting stand 30 min. in the ice bath.

(6) While the tube is still cooled in ice, urea (7 g.) is cautiously added to remove excess nitrous acid. It is best to pour the total quantity of solid urea into the tube without stirring, allow it to settle, and then stir to solution. This will avoid loss by frothing. After standing 10 min. the solution should be free of nitrous acid.

(7) The coupling reagent (5 ml.) is then added, the mixture removed from the ice bath and let stand 30 min. before being made up to exactly 100 ml. with water and 10 ml. concentrated hydrochloric acid. After a further 60 min. for colour stabilization, the density of the red colour is estimated in an absorptiometer provided with a green light source (Ilford spectrum green light filter no. 604, transmission 5000–5400 Å. used with 1 cm. depth cell in the Hilger absorptiometer). If the amount in the original sample does not exceed 10 mg., solutions of too intense colour for direct measurement may, if necessary, be quantitatively diluted with water before reading, the readings obtained being multiplied by the appropriate dilution factor.

(8) Reagent blank. This is determined for each batch of reagents by blank estimations put through

the full procedure. The blank density reading with various batches (1 cm. cell, green filter) was 0.07–0.10 corresponding to a total amount of 'benzoic acid' of 0.035–0.050 mg. in our analyses. The use of pure ethanol (see below) for deproteinization did not affect this blank.

(9) Calculation. A calibration curve is prepared by estimations on known amounts of benzoic and hippuric acid over the range 0.05–10 mg. (see Fig. 1).

Fig. 1. The estimation of aqueous solutions of pure benzoic acid (○) and hippuric acid (●). Abscissae: amount in mg. of benzoic acid or of benzoic acid equivalent of the hippuric acid taken. Ordinates: absorptiometer density readings multiplied by dilution factor, corrected for reagent blank (see text).

This is sufficiently close to a straight line for a constant factor to be employed for calculation, the mean value of our calibration factor being 0.50. If the observed optical density (1 cm. cell, green filter) is d , the dilution factor (if any) is F and the density in the reagent blank is b , then:

$$\text{mg. benzoic acid in sample} = 0.50 (dF - b),$$

where 0.50 is the empirical value of the calibration factor determined as described above. Fig. 1 shows that hippuric acid is estimated by this method as the equivalent amount of benzoic acid; a point of practical convenience in the present method.

If it should be necessary to distinguish between benzoic and hippuric acids (it was not necessary in the work for which this method was devised), this could be done by separate estimations on the extracted acids of total benzoic acid (free and conjugated) by the above method, and also of glycine nitrogen as for example in the method of Griffith (1926).

Accuracy. The blanks on reagents were well duplicated, twenty-eight estimations on one set of reagents giving a mean value of $d = 0.104 \pm \text{s.e. } 0.013$.

This would correspond to 0.052 ± 0.007 mg. benzoic acid, and is always subtracted from the readings as explained above.

There was more variation in the estimation of pure solutions of benzoic and hippuric acids, as would be expected in view of the fairly large number of operations concerned. Nevertheless, the method has proved more reliable than previous methods in our experience. The mean percentage errors were: over the range 0.1–1.0 mg. benzoic acid, -6% (19 observations); hippuric acid, $+3\%$ (10 observations); over the range 1–10 mg., benzoic acid, $+4\%$ (23 observations); hippuric acid, -0.1% (12 observations). Individual single estimations are scattered as shown in Fig. 1, but the above calculations show that there would be no practical advantage in using different factors for the calculation in different regions of the 0.1–10 mg. range or for the two different acids (all results are expressed as the equivalent amount of benzoic acid whichever acid is actually being estimated).

Application to tissue slices and homogenates

For the estimation of benzoic acid present in tissue preparations after incubation in manometric vessels, the proteins are first removed by precipitation with 60% ethanol. The whole contents of the vessel (after removal of any carbon dioxide absorbent used) are rinsed out or diluted in the vessel with a measured volume of water to make 6.0 ml. total volume, which is made to 15 ml. with a sufficient quantity of pure absolute ethanol to give a final concentration of 60% (v/v). After standing for at least 2 hr., and filtering through paper, a measured portion (usually 10 ml.) of the filtrate is transferred to a 20 ml. crucible. If interfering substances such as partially hydrogenated derivatives of benzoic acid are present they must be eliminated by bromination, otherwise the 10 ml. ethanolic filtrate is made just alkaline by addition of a few drops of 20% sodium carbonate and evaporated to dryness. From this stage the analysis is as described above.

For removal of interfering substances, the 10 ml. ethanolic filtrate is adjusted, if necessary, to pH 6.8, using bromothymol blue as external indicator. Saturated bromine water (2 ml.) is then added, and after 30 min. at room temperature the excess bromine is blown off by a fine but rapid stream of nitrogen: the time for this should not exceed 15 min. The solution is then made just alkaline and evaporated as described in the preceding paragraph.

The recovery by this method of added benzoic and hippuric acids from samples of tissue slices is shown in Table 1.

Application to blood. To the samples of blood (5 ml.) 2 ml. water and sufficient absolute ethanol (10.5 ml.) are added to make the final volume after mixing 17 ml. After standing for 2 hr., measured

Table 1. *Recovery of added benzoic and hippuric acids from rabbit-tissue slices*

(Pooled tissue slices of moist wt. 300 mg. suspended in phosphate-Ringer solution. Benzoic (0.61 mg.) or hippuric acid (equiv. to 0.61 mg. benzoic acid) added as shown.)

Acid added	Liver		Kidney	
	Benzoic acid found, total (mg.)	Recovery (%)	Benzoic acid found, total (mg.)	Recovery (%)
None	0.028	—	0.027	—
None	0.033	—	0.040	—
None	0.040	—	0.032	—
Benzoic	0.630	97.7	0.725	113.4
Benzoic	0.557	85.7	0.635	98.7
Benzoic	0.645	100.0	—	—
Benzoic	0.601	93.0	—	—
Hippuric	0.609	94.3	0.615	95.5
Hippuric	0.616	95.4	0.675	105.3
Hippuric	0.616	95.4	0.660	102.8

portions (5–10 ml.) of the filtrate are taken for analysis, for which purpose they are made slightly alkaline and evaporated as already described.

Table 2. *Recovery of added benzoic and hippuric acids from rabbit blood*

(To 5 ml. samples of blood the stated amounts of sodium salts of benzoic or hippuric acid (expressed as the equiv. amount of benzoic acid) were added, and the analysis completed as in the text.)

Addition		Benzoic acid found, total (mg.)	Recovery (%)
Acid	Amount of benzoic acid equiv. (mg.)		
None	—	0.125	—
None	—	0.119	—
Benzoic	1	0.993	87
Benzoic	2	2.084	98
Hippuric	1	0.976	86
Hippuric	2	1.880	88

Table 2 illustrates the recovery of added benzoic and hippuric acids from whole blood: there is a slight loss, possibly due to adsorption on the voluminous precipitate. It is possible that part of the values ob-

tained on the original blood may be due to substances other than benzoic or hippuric acid, e.g. *p*-aminobenzoic acid, the amount (about 2 mg./100 ml. blood) being too small for identification by isolation.

Application to urine. Owing to the presence of phenols and other interfering substances in urine, preliminary treatment with bromine is necessary. The urine (5 ml.) is adjusted to pH 6.8 with bromothymol blue as external indicator. Saturated bromine water (5 ml.) is added and after standing for 30 min. the mixture is filtered. The filtrate, which must smell strongly of bromine, is freed from excess of the latter by passing a brisk stream of nitrogen bubbles through it for 15 min. The whole, or a measured portion if abnormally high amounts of benzoic or hippuric acids are known to be present, is transferred to a separating funnel (or, better, the stoppered extraction tubes described above) with 1 ml. *N*-hydrochloric acid and water to make a total volume of 10 ml. It is then extracted by shaking with three successive portions each of 10 ml. *iso*amyl alcohol. The combined extracts are washed once in a separating funnel with 5 ml. water, and then the benzoic and hippuric acids are shaken out with two portions each 5 ml. approx. 0.1 *N*-sodium hydroxide. The combined alkaline extracts are evaporated to

Table 3. *Recovery of added benzoic and hippuric acids from rabbit urine*

(With bromination (see text). 5 ml. portions of urine were taken with the addition as shown below. Suitable measured portions were taken for analysis, the results being expressed as total benzoic acid in 5 ml. urine.)

Benzoic acid			Hippuric acid		
Added (mg.)	Benzoic acid found, total (mg.)	Recovery (%)	Equiv. benzoic acid added (mg.)	Benzoic acid found, total (mg.)	Recovery (%)
0	1.57	—	0	1.6	—
1.22	2.72	94	17.04	18.5	99
2.44	4.01	100	27.2	28.9	100
3.66	4.76	87	34.1	32.1	90
			34.1	36.0	101
			34.1	37.0	104

dryness in a 20 ml. crucible and the analysis proceeds from this stage as described above.

The recovery of added benzoic and hippuric acids from urine is illustrated by Table 3. It should be noted that in some species part of the benzoic acid excreted in the urine may exist as a benzoyl glucuronide (see Williams, 1947). In such cases it will be necessary to hydrolyse the benzoyl linkage by alkaline hydrolysis before proceeding with the above estimation.

Interfering substances

The presence of any aromatic substance capable of undergoing nitration in the cold to give an acidic nitro compound will interfere in the application of this method. Phenols are also sufficiently acidic after nitration to be extracted by the 0.1N-sodium hydroxide employed, and the bromination method described above must precede the analysis if phenols are present. With some phenols this may not be adequate, especially if abnormal amounts are present, and such specimens would not be suitable for analysis by the present method without special modification.

Among unsaturated derivatives of cyclohexanecarboxylic acid, which may yield traces of aromatic acid during the reaction with the nitrating mixture, Δ^1 -, Δ^2 - and Δ^3 -cyclohexenecarboxylic acids are largely eliminated by the usual preliminary bromina-

tion treatment (Table 4). The monohydroxycyclohexanecarboxylic acids also interfere, presumably because they are first dehydrated by the sulphuric acid in the nitrating mixture and then partially aromatized and nitrated. cycloHexanecarboxylic acid itself gives no measurable amount of aromatic compound (Table 4).

Notes on the method

Nitration of benzoic acid by potassium nitrate and sulphuric acid without heat yields a mixture of mononitro derivatives, in the approximate proportion: *ortho*, 18%; *meta*, 80%; *para*, 2% (cf. Houben, 1924). If *m*-nitrobenzoic acid is dissolved in the cold nitrating mixture used in stage 2 of the present method (see above) and put through the entire process it is recovered to the average extent of 89.4%, as compared with the direct reduction of an equimolar amount of *m*-nitrobenzoic acid in stage 5 followed by colour development (stages 6 and 7). *m*-Aminobenzoic acid, after diazotization and dye-coupling, yields within 1% the same colour as the direct reduction of *m*-nitrobenzoic acid followed by diazotization and dye-coupling. *m*-Nitrohippuric acid yields, molecule for molecule, the same colour density as *m*-nitrobenzoic acid. Benzoic or hippuric acids put through the entire process yield an average of 86% of the colour given by direct colour develop-

Table 4. Colorimetric equivalents (expressed as the amount of benzoic acid (mg.) required to give the same density readings) of 1 mg. quantity of various substances

(Ilford spectrum green light filter: 1 cm. cell depth.)

Substance	Equivalent benzoic acid (mg.)		Colour obtained
	Without bromination	With bromination	
Benzoic acid	1.0	—	Magenta
Hippuric acid	1.0	—	Magenta
Phenol	Variable	—	Deep blue
Salicylic acid	0.08	—	Purple
<i>m</i> -Hydroxybenzoic acid	0.20	—	Magenta
<i>p</i> -Hydroxybenzoic acid	0.19	—	Green changing to blue
Tyrosine	0.01	—	(Very pale)
Phenylalanine	0.01	—	(Very pale)
Benzamide	0.01	—	Faint pink
<i>m</i> -Nitrobenzoic acid	0.83	—	—
<i>m</i> -Nitrohippuric acid	0.58	—	—
cycloHexanecarboxylic acid	0.00	—	—
Hexahydrohippuric acid	0.00	—	—
Hexahydrobenzamide	0.00	—	—
Δ^1 -cycloHexenecarboxylic acid	0.108	0.004	Magenta
Δ^2 -cycloHexenecarboxylic acid	0.108	0.014	Magenta
Δ^3 -cycloHexenecarboxylic acid	0.062	0.002	Magenta
1-Hydroxycyclohexanecarboxylic acid	0.02	—	Magenta
<i>cis</i> -2-Hydroxycyclohexanecarboxylic acid	0.03	—	Magenta
<i>trans</i> -2-Hydroxycyclohexanecarboxylic acid	0.00	—	Magenta
<i>cis</i> -3-Hydroxycyclohexanecarboxylic acid	0.02	—	Magenta
<i>trans</i> -3-Hydroxycyclohexanecarboxylic acid	0.03	—	Magenta
<i>cis</i> -4-Hydroxycyclohexanecarboxylic acid	0.04	0.027	Magenta
<i>trans</i> -4-Hydroxycyclohexanecarboxylic acid	0.03	0.023	Magenta
laevo-Quinic acid	0.00	—	—

ment from an equimolar amount of *m*-aminobenzoic acid. From these data it appears probable that there is a loss amounting only to a few per cent on nitration, and an overall loss which is nearly constant at about 14%, and occurs almost entirely in the nitration and extraction stages. The extraction loss might be reduced by the use of continuous extractors, but for serial analyses the inconvenience did not appear to be justified by the possible slight gain in accuracy. *iso*Amyl alcohol was adopted for this extraction because it is a satisfactory solvent for hippuric and benzoic acids, or their mononitro compounds. If only benzoic and not hippuric acid is present, ether may be used for extraction.

A further variation which has given satisfactory recoveries in trial experiments is to hydrolyse hippuric acid present in the original sample by boiling for 2 hr. after making alkaline with excess sodium hydroxide (about 2N), extracting the benzoic acid from the acidified solution by ether or light petroleum, and evaporating the extract to dryness in presence of a slight excess of 0.1N-alkali. The dry residue is then used for the analysis as described above. In particular cases this method may be advantageous in that the alkaline boiling may remove interfering substances or hydrolyse a glycuronide, but it is generally less convenient than the normal procedure.

Reduction in acid solution of the nitro acids follows the procedure applied by Eckert (1943) to the estimation of *p*-nitrobenzoic acid; the urea used

instead of ammonium sulphamate for removing excess nitrous acid appeared to be equally satisfactory. The colour-developing base chosen is that used by Bratton & Marshall (1939) for estimation of sulphonamide. The final colour obtained must be free from bluish tint and should closely match that given by alkaline dilute solutions of carmine. A bluish tint indicates either inadequate removal of nitrous acid or the presence of interfering substances, particularly phenolic compounds, in the original sample.

SUMMARY

1. A method has been developed for the micro-estimation of benzoic and hippuric acids in biological material. It is described for application to amounts of these acids within the range 0.05–10 mg.

2. The basis of the method is the nitration of these acids at room temperature, extraction of the resulting mononitro compounds, reduction to the corresponding amino compounds, followed by diazotization and finally coupling of the diazo compounds with a dye-forming base in acid solution. The amount of dye is closely proportional to the amount of benzoic or hippuric acid in the sample.

3. The application of the method to tissues, blood and urine is described.

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