The Measurement of Glucuronide Synthesis by Tissue Preparations

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To measure the ability of surviving tissue slices to form glucuronides of compounds such as menthol and phenol, Lipschitz & Bueding (1939) determined ether-soluble compounds giving the Tollens colour reaction for glucuronic acid. Whilst this procedure yielded valuable information in their hands, it is inconvenient for routine purposes, and the lack of specificity in the colour reaction can be a serious disadvantage. It must also be borne in mind that not all glucuronides are as ether-soluble as that derived from menthol. De Meio & Arnolt (1944), in their study of phenol conjugation by tissue slices, assumed the figure for combined phenol given by the method of Theis & Benedict (1924) to represent phenylsulphuric acid and phenylglucuronide. As shown below, it is doubtful whether the results of De Meio & Arnolt (1944) have any bearing on the problem of glucuronide synthesis.

It was considered that the study of the biological mechanism of glucuronide formation required a sensitive reaction, specific for a glucuronide in the presence of a large excess of the hydroxy compound. Following a suggestion made by Dr R. T. Williams (private communication), the possibility was investigated of determining *o*-aminophenyl-β-D-glucuronide (Williams, 1943) in the presence of free *o*-aminophenol by the reaction described by Bratton & Marshall (1939) for the estimation of sulphonamides. If the reaction was carried out according to their directions, in strongly acid solutions, the bluish pink given by the glucuronide was slow to develop, and when it had reached its full intensity an appreciable colour of similar shade was seen in parallel experiments with the free phenol. By careful control of pH it was found possible, not only to shorten the period for complete colour development with the glucuronide, but to eliminate interference by free *o*-aminophenol. To avoid having to adjust the pH of the solution after removal of proteins, methods of protein precipitation at the pH chosen for colour development were studied. Application of the complete procedure to the measurement of glucuronide synthesis by mouse-liver slices is described below.

EXPERIMENTAL

The colour reaction

Diazotization and coupling with naphthylethylenediamine were carried out as described by Bratton & Marshall (1939). To 2 ml. of the test solution were added 1 ml. acid or buffer, followed at suitable intervals by 1 ml. of each of the following: 0·1% $NaNO_2$, 0·5% ammonium sulphamate and 0·1% naphthylethylenediamine dihydrochloride. In later experiments, the concentration of $NaNO_2$ was reduced to half. When 1 ml. of N-HCl or N-trichloroacetic acid was added to the reaction mixture, quantities of the order of 100 μg. *o*-aminophenol present as the glucuronide could readily be distinguished from larger amounts of the free phenol by the pink colour seen immediately after adding the coupling reagent. It was possible to detect much smaller amounts of the glucuronide if colour development was allowed to proceed to completion, the process being accelerated by warming, but under such conditions 100 μg. free *o*-aminophenol gave a strong pink colour. Table 1 shows the

Table 1. *Extinction coefficients, using different filters, of the azo colours produced by* o-*aminophenylglucuronide and by* o-*aminophenol*

(Colour development for 18 hr. at 37° in presence of HCl. 12 μg. *o*-aminophenylglucuronide (calc. as free phenol) and 120 μg. *o*-aminophenol. Extinction coefficients measured with Hilger Spekker absorptiometer.)

Ilford filter no.	Extinction coefficients: *o*-Aminophenylglucuronide	Extinction coefficients: *o*-Aminophenol
601	0·024	0·053
602	0·075	0·077
603	0·168	0·120
604	0·312	0·202
605	0·432	0·312
606	0·337	0·322
607	0·160	0·232
608	0·012	0·048

extinction coefficients with different filters in the Hilger Spekker absorptiometer after 18 hr. colour development in HCl at 37°, and it can be seen that no filter permitted distinction between the two compounds. Ilford filter no. 605 (yellow-green) was selected for further work.

Freshly prepared aqueous solutions of pure *o*-aminophenol were colourless, but on standing there was rapid formation of an ether-soluble brown pigment giving appreciable readings in the Spekker absorptiometer. Shaking the solution in an atmosphere of O_2 hastened pigment formation. Interference from this source was greatly reduced or entirely prevented by adding ascorbic acid to the solution. Ascorbic acid itself had no effect on the colour reaction for the glucuronide. The initial reaction of *o*-aminophenol with HNO_2 gives a pale yellow solution. This colour does not register on the Spekker absorptiometer with Ilford filter no. 605.

The effects of pH *and temperature on the colour development*

Fig. 1 shows the effect of varying the pH on the comparative intensities of the colours given by *o*-aminophenol

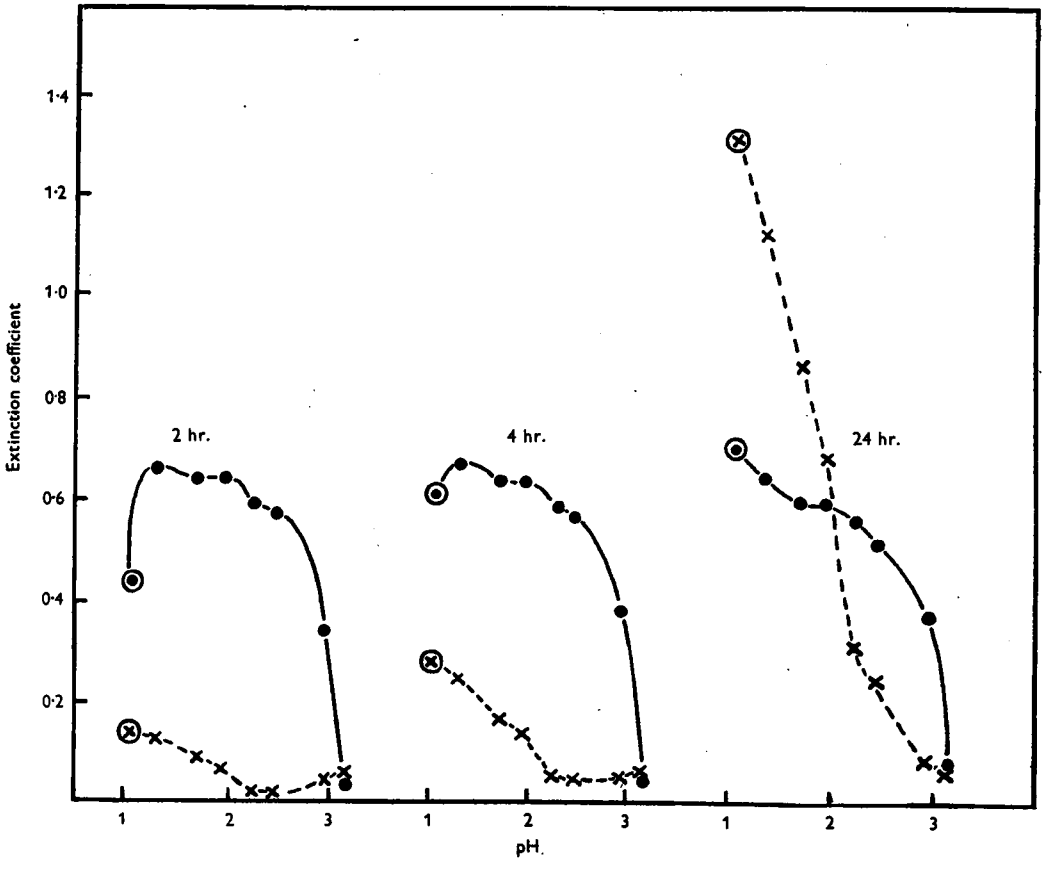

Fig. 1. The effect of pH on colour development after different periods at 25°. ●—●, 12 μg. *o*-aminophenol as glucuronide; ×—×, 120 μg. free *o*-aminophenol (no ascorbic acid present). Encircled points, pH adjusted with HCl; all others, phosphate buffer.

and its glucuronide after different periods at 25°. Except for experiments at the lowest pH, in which N-HCl was used, the pH was adjusted by addition of approximately M-phosphate buffer. As elsewhere in this paper, the amount of glucuronide is expressed in terms of the *o*-aminophenol content. Above pH 2·25 no azo-dye formation was seen with the free phenol after 4 hr. incubation, the Spekker absorptiometer readings being accounted for entirely by the pigmented oxidation product. Above pH 2·50 the intensity of the colour given by the glucuronide fell sharply. On the basis of these results it seemed that pH 2·25–2·50 was the most favourable region for determining *o*-aminophenyl-glucuronide in the presence of excess of the free phenol. The use of agents other than phosphate for pH adjustment led to a similar conclusion. From Fig. 2 it can be seen that within the desired pH region colour development was complete with the glucuronide in 2 hr. at 25°. In the presence of HCl, on the other hand, the colour had not reached a maximum after 6 hr. incubation. Azo-dye formation by the free phenol below pH 2·25 can be clearly distinguished from oxidation alone at higher pH values in Fig. 2.

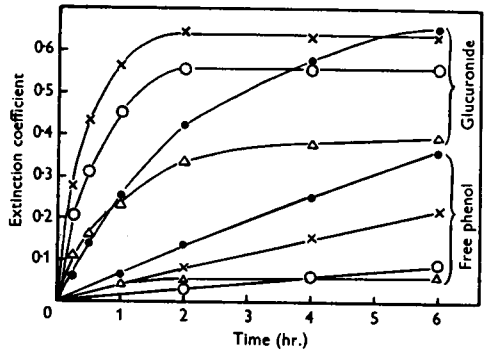

Fig. 2. The effect of pH on the rate of colour development at 25°. ●—●, pH 1·05 (HCl); ×—×, pH 1·68 (phosphate buffer); ○—○, pH 2·25 (phosphate buffer); △—△, pH 2·94 (phosphate buffer). 12 μg. *o*-aminophenol as glucuronide or 120 μg. free *o*-aminophenol (no ascorbic acid present).

Fig. 3 illustrates the effect of temperature on the colour development at pH 2·25. In general, increasing the temperature caused the maximum to be reached more quickly, but decreased the ultimate value, and fading occurred on prolonged incubation at high temperatures. Raising the temperature also increased the rate of oxidation of the free phenol in absence of ascorbic acid. At 25°, coupling of the diazotized glucuronide at pH 2·25 was as rapid as at 37°, and the former temperature was adopted for further work.

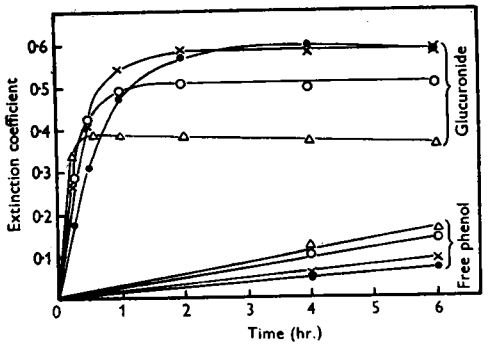

Fig. 3. The effect of temperature on colour development at pH 2·25. ●—●, 15°; ×—×, 25°; ○—○, 37°; △—△, 49°. 12 μg. *o*-aminophenol as glucuronide or 120 μg. free *o*-aminophenol (no ascorbic acid present).

The absorption curve for the glucuronide under the selected conditions of colour development (2 hr. at 25° and pH 2·25–2·50) was very similar to that shown in Table 1, and a straight-line relationship was observed between the absorptiometer readings with Ilford filter no. 605 and amounts of the glucuronide equivalent to 2–24 μg. *o*-aminophenol.

Protein precipitation

Solutions of sodium tungstate or trichloroacetic acid brought to pH 2·25 did not precipitate proteins until further additions of acid were made. The presence of phosphate buffer at the same pH did away with the necessity of making the precipitant more acid. Tungstic acid precipitated naphthylethylenediamine, but mixtures of trichloroacetic acid and phosphate buffer proved satisfactory in giving protein-free solutions in which the colour reaction could be carried out directly. Trichloroacetic acid was without effect on the colour reaction under these conditions, in spite of the fact that used alone for colour development at pH 1 it gave variable results for a known amount of glucuronide.

The effect of varying the o-aminophenol concentration on synthesis of the glucuronide by mouse-liver slices

The slices (about 10 mg. dry wt.), suspended in bicarbonate Ringer solution (Krebs & Henseleit, 1932), were shaken in an atmosphere of 5% CO_2 in O_2 at 37°. The solution contained 0·02 M-lactate (Lipschitz & Bueding, 1939), 0·001 M-ascorbic acid and varying concentrations of *o*-aminophenol, but no sulphate, $MgSO_4$ being replaced by an equivalent concentration of $MgCl_2$. Glucuronide synthesis was determined at varying periods for each concentration of *o*-aminophenol, slices from a single animal being used for each complete experiment. The course of the synthesis in representative experiments is shown in Fig. 4, results being expressed as mg. *o*-aminophenol conjugated by 1 g. dry weight of slices. Each point is the mean of three or four individual results.

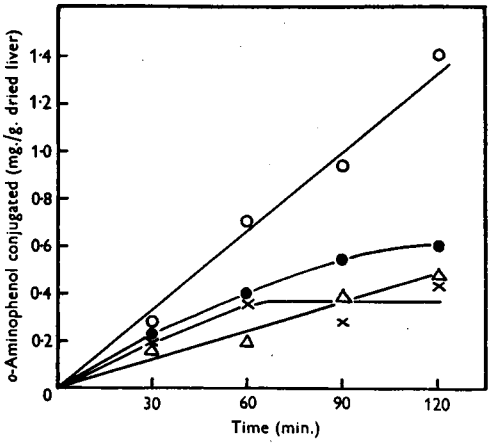

Fig. 4. The course of glucuronide synthesis by mouse-liver slices in various concentrations of *o*-aminophenol. △—△, 0·005%; ○—○, 0·0025%; ●—●, 0·00125%; ×—×, 0·0005%.

Variations in the initial slope may in part reflect individual variation in the ability of the liver to synthesize the glucuronide, but this factor can hardly explain all the differences in the shapes of the curves shown in Fig. 4. It appears that the rate of synthesis remained constant for 2 hr. in 0·0025% *o*-aminophenol. Reducing the concentration caused the rate of reaction to fall off after incubation for 1 hr. Whilst in 0·0035% *o*-aminophenol (results not shown) the reaction followed the same course as in 0·0025%, increasing the concentration to 0·005% caused an apparent decrease in the reaction rate. This is in agreement with the results of Lipschitz & Bueding (1939), who observed inhibition of glucuronide formation by excess alcohol or phenol.

Assay procedure for mouse-liver slices

Into 25 ml. conical flasks fitted with rubber stoppers were measured 2 ml. bicarbonate Ringer, containing 0·02 M-lactate, 0·001 M-ascorbic acid and 0·0025 % *o*-aminophenol, but no sulphate. Ascorbic acid and the phenol were added as the solids, in that order. The latter was purified by sublimation before use. After passing a brisk stream of 5% CO_2 in O_2 into the flasks for 1 min. they were stoppered and placed in a thermostatically controlled water bath at 37°. The contents were brought to atmospheric pressure by momentary release of the stoppers. Slices corresponding to approximately 10 mg. dry weight were dropped into each flask which was again gassed for 2 or 3 sec. before replacing the stopper. Shaking was commenced and continued for 1 hr. The slices were then removed for determination of the weight after drying at 110°. To the flask, 2 ml. of a solution containing sodium trichloroacetate and phosphate were immediately added. This solution was prepared daily by mixing equal volumes of stock M-trichloroacetate and M-phosphate, both adjusted to pH 2·25. The contents of the

flask were thoroughly mixed and poured into a 10 ml. centrifuge tube. After centrifuging, 3 ml. of the supernatant fluid were transferred to another tube, and to it was added 1 ml. 0·05% $NaNO_2$, followed after 3 min. by 1 ml. 0·5% ammonium sulphamate and, after another 2 min., by 1 ml. 0·1% naphthylethylenediamine dihydrochloride. The tubes were placed in a bath at 25° for 2 hr. and the intensities of the colours read with a Spekker absorptiometer against the reagents, using Ilford filter no. 605. Liver slices from normal mice gave zero readings after incubation in absence of *o*-aminophenol, as did the phenol itself in the concentration used above when ascorbic acid was present. Variation in a single liver in synthetic activity from slice to slice, however, made it advisable to do determinations in quadruplicate (see below).

Errors in the assay

To test the precipitation procedure, a large number of mouse-liver slices were shaken in bicarbonate Ringer solution of the composition described above except in that *o*-aminophenol was omitted. After 1 hr. the slices were removed and to 1 ml. samples of the residual liquid was added 1 ml. bicarbonate Ringer solution containing *o*-aminophenylglucuronide equivalent to 9·2 μg. of the free phenol. The removal of proteins and the colour reaction were carried out as described. The mean recovery in twenty two observations was 100%, and the standard deviation of a single observation from the mean 7%. This figure takes no account of the variable error arising from removal of slices in an actual assay. An overall measure of the variable error in the assay procedure, including that arising from variation from slice to slice in the synthetic activity of liver, was obtained from the individual results in a series of eight determinations, each done in quadruplicate, of the normal activity of adult mouse liver. The mean activity was 0·37 mg. *o*-aminophenol conjugated/g. dry wt./hr., and the standard deviation of a single observation from the mean 17%.

The identity of the compound giving the colour reaction

It was borne in mind that part of the *o*-aminophenol might be conjugated with sulphuric acid during the incubation with liver slices, and that the conjugate thus formed might give the colour reaction for *o*-aminophenylglucuronide. *o*-Aminophenylsulphuric acid was not available for study, but indirect evidence suggested that, if formed during the incubation, it did not interfere in the final determination.

In experiments in which mouse-liver slices were incubated with *o*-aminophenol in Ringer solution containing $MgSO_4$, the final readings were, if anything, slightly lower than those obtained with slices from the same animal in sulphate-free Ringer. This suggests that sulphate ion is not involved in the formation of the compound giving the colour reaction.

A comparative study of the stabilities of phenylglucuronide and phenylsulphuric acid to acid, using the colour reaction of Folin & Ciocalteu (see Kerr, Graham & Levvy, 1948), showed the former to be unchanged after 10 min. at 100° in N-HCl, while the latter was completely hydrolyzed under these conditions. In phosphate buffer, pH 2·25, the degree of hydrolysis of phenylsulphuric acid was 50% after 10 min. at 100°, and 25% after 2 hr. at 25°. *o*-Aminophenylglucuronide, studied by the colour reaction described above, resembled phenylglucuronide in its stability to acid. The molar concentrations in these experiments were of the same order as those dealt with elsewhere in this paper, and excess acid was neutralized before proceeding with the appropriate colour reaction. The compound giving the colour reaction after incubation of *o*-aminophenol with mouse-liver slices was just as stable to acid as the authentic glucuronide. In these experiments, the incubation was done on a larger scale than usual and protein was removed with trichloroacetic acid. Samples were heated with HCl for varying periods, and excess acid neutralized before adding the buffer for colour development. It is considered unlikely that *o*-aminophenylsulphuric acid would escape hydrolysis in such experiments. This is confirmed by the work of Burkhardt & Wood (1929), who found that although *o*-aminophenylsulphuric acid was 'rather more difficult' to hydrolyze than phenylsulphuric acid, hydrolysis was detectable after boiling for a few seconds in dilute mineral acid. They also showed that diazotization in N-HCl led to loss of the sulphate group.

DISCUSSION

De Meio & Arnolt (1944) observed tissue slices from one of their strains of rats to conjugate phenol in sulphate-free Ringer solution. Addition of sulphate, however, increased conjugation by liver, and the other strain of rats resembled guinea pigs (Bernheim & Bernheim, 1943) in that this ion was obligatory for phenol conjugation as determined by their method of assay. In the method of Theis & Benedict (1924) combined phenol is taken to be the difference between readings obtained before and after hydrolysis in 0·25 N-HCl for 10 min. at 100°. If, as appears to be the case, De Meio & Arnolt (1944) carried out their hydrolysis in this way, their results did not include phenol conjugated with glucuronic acid, since pure phenylglucuronide was unchanged after 10 min. at 100° in N-HCl. Unless some other acid-labile phenol derivative was present, the results of De Meio & Arnolt must be considered to apply to formation of the sulphate only. In this case it would appear that in one of their strains of rats the tissue slices either contained or produced sufficient sulphate ion for a limited degree of conjugation. In our own procedure, synthesis of the compound giving the colour reaction for *o*-aminophenylglucuronide was not stimulated by addition of sulphate, and this ion was omitted as a matter of routine.

SUMMARY

1. Small amounts of *o*-aminophenylglucuronide can be determined colorimetrically after diazotizing and coupling with naphthylethylenediamine. Interference with the colour reaction by comparatively large amounts of *o*-aminophenol can be excluded if the colour is developed at pH 2·25, and

oxidation of *o*-aminophenol is avoided when ascorbic acid is present. Trichloroacetic acid solutions brought to pH 2·25 precipitate proteins if phosphate buffer at the same pH is also present, permitting colour development without further pH adjustment.

2. The colour reaction has been applied to the measurement of glucuronide synthesis by mouse-liver slices. Rates of synthesis of the order of 0·7 mg. *o*-aminophenol conjugated/g. dry weight of liver/hr. were obtained at the optimum concentration of 0·0025%.

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The Frequency Distribution of the Zinc Concentrations in the Dental Tissues of the Normal Population

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In earlier work the author demonstrated the presence of zinc in the dental tissues (Cruickshank, 1936) and studied the increases occurring in persons suffering from active tuberculosis (Cruickshank, 1940). The present paper deals with the way in which zinc concentrations of dental tissues vary throughout the 'normal' population. The experimental results indicate that the population sampled was heterogeneous and divisible into two (or three) groups, each with its characteristic zinc concentrations. The significance of these findings is briefly discussed.

EXPERIMENTAL

Population and material sampled. Over a period of years, the author collected 'sound' teeth extracted, for orthodontic reasons, from secondary school children attending the Public Dental Clinic at Cambridge. All specimens were carefully examined and only fully calcified pre-molars free from visible caries or defects were selected for analysis.

The children, boys and girls, ranged in age from 10 to 17 years, 80% falling between 12 and 15 years. They can be regarded as a random sample of the pre-adolescent secondary school population biased only in the sense that all had a degree of 'overcrowded' dentition calling for orthodontic extraction.

Zinc determinations. These were made by a modification of the dithizone extraction, ferricyanide-iodine titration technique of Sylvester & Hughes (1936) reduced to a micro scale in order to permit quadruplicate or quintuplicate analyses on the dentine and on the enamel of each single tooth specimen (Cruickshank, 1948). The standard deviation of the 250 standards (20 μg.) run during the tests was $\pm 3{\cdot}8\%$. Interbatch variations were eliminated by 'batch scattering' the multiple analysis for each specimen. The total number of zinc estimations was about 1250.

Preparation of specimens. Dentine: The 'root' of each pre-molar was cut off just below the crown with a fissure bur, dried to constant weight (105°), ashed in an electric furnace overnight (500°), cooled and pulverized. The four to five analyses were run on samples of this pulverized ash.

Enamel: The 'crown' was first heated in the electric furnace for 0·5 hr. at 270° (pyrometer controlled) to char the dentine. When cool the 'neck' was pressed lightly into a surface of plasticine, surrounded with a 1 in. length of 1 in. diameter rubber tubing, and the hole filled with plaster of Paris. When set, the plaster containing and supporting the 'crown' was removed and trimmed to expose the 'neck'. The charred dentine was then cut out with a rose-head bur. The remaining enamel 'cap' was freed by making a cruciform saw cut into the block and splitting apart; it was washed and ashed overnight (500°), and four to five samples of the pulverized ash were analyzed.

Examination of variables. It was intended to obtain experimental data for 100 persons but exigencies of the war reduced this to 70. The available data were first studied in relation to the known variables of (i) sex, (ii) age of the subject, (iii) position, and (iv) condition of the specimen. As already mentioned all specimens were 'pre-molars' (the zinc contents of incisors, canines, pre-molars and molars differ to some extent). The 'defectives' were sound teeth with no caries but with enamel of slightly less perfect