Kinetic Studies of the Metabolism of Foreign Organic Compounds

3. THE CONJUGATION OF PHENOLS WITH GLUCURONIC ACID

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It is well known that phenols, whether formed *in vivo* or administered as such, are excreted by many species largely conjugated with glucuronic and sulphuric acids. The extent of the conjugation with glucuronic acid has been examined for many phenols and by many workers, the result usually being recorded as a percentage of the dose excreted as glucuronide in 24 or 48 hr. Several of the glucuronides have been isolated from urine in pure form. Studies of the formation and hydrolysis of phenylglucuronides by tissue extracts or slices have also been made, but the mechanism of glucuronide formation *in vivo* is as yet obscure. The present paper records the results of an investigation of the kinetics of glucuronide formation. This aspect does not appear to have been investigated previously.

METHODS

Animals, diet and collection of urine specimens. The rabbits, does of average weight 2·8 kg., were maintained on the standard diet of rabbit pellets used previously (Bray, Ryman & Thorpe, 1947). The apparatus for the collection of urine specimens has been described (Bray, Thorpe & White, 1951).

Dosage. The compounds were administered by stomach tube as solutions or suspensions in water, at dose levels usually between 0·15 and 0·45 g./kg. Since many of the experiments described here also formed part of the study of the kinetics of ethereal sulphate formation described in the next paper, Na_2SO_3 or L-cystine was often administered with the phenol. This did not appear to affect the kinetics of glucuronide formation.

Materials. *p*-Cyanophenol was prepared by a Sandmeyer reaction from *p*-aminophenol; *p*-hydroxybenzamide as described by Bray *et al.* (1947), *p*-hydroxybenzenesulphonamide by decomposition of the diazonium salt of *p*-aminobenzenesulphonamide; *p*-hydroxyphenylurea as described by Bray, Lake & Thorpe (1949). Hydroxybenzoxazolone was isolated from urines of rabbits dosed with benzoxazolone (Bray, Clowes & Thorpe, 1952*a*). Other phenols were purchased.

Estimation of metabolites

Methods used for the estimation of glucuronic acid, inorganic and ethereal sulphates and free phenols have been described in the previous paper (Bray, Humphris, Thorpe, White & Wood, 1952*b*). Estimation of glucuronides in blood was carried out on a trichloroacetic acid filtrate of whole blood. Xylenylglucuronides were also estimated by determination of the ether-soluble acid on a sodium tungstate filtrate of whole blood.

p-*Hydroxybenzoic acid.* The above method for phenols is unsuitable for the estimation of *p*-hydroxybenzoic acid which gives only a feeble colour with the Folin and Ciocalteu reagent. The method to be described is based upon the colour formed when *p*-hydroxybenzoic acid is coupled with diazotized *p*-nitraniline.

The following reagents were required: *p*-nitraniline solution (3 mg./l. in 0·8 N-HCl); sodium nitrite solution (5% w/v in water); ethanol (96%); diazotized *p*-nitraniline solution (*p*-nitraniline, 25 ml., and sodium nitrite, 1·5 ml., mixed immediately before use); sodium carbonate solution (Na_2CO_3, 5% w/v in water). Urine (20 ml.) acidified with H_2SO_4 (2 ml. 2 N) was continuously extracted with ether for 6 hr. After removal of ether, the extract was titrated with NaOH (0·02 N) using phenol red as indicator. This gave the total ether-soluble acid. The solution was diluted so that the concentration of *p*-hydroxybenzoic acid was between 0·2 and 2 mg./100 ml. This solution (5 ml.) was mixed with ethanol (5 ml.) and diazotized *p*-nitraniline (1 ml.) and left for 2 min. Sodium carbonate solution (3 ml.) was added and the solution diluted to 25 ml. with water. The absorption value was read immediately on a Spekker photoelectric absorptiometer using a blue filter (Chance OB1).

A calibration curve was constructed using solutions containing 0·01–0·10 mg. *p*-hydroxybenzoic acid/5 ml. Within this range the curve was linear, and, when allowance was made for the reagent blank, passed through the origin. The slope was approximately 0·01 mg. acid ≡ 0·1 drum reading. The average recovery of *p*-hydroxybenzoic acid added to normal urine was 102% (range 96–110%). The difference between the total ether-soluble acid and the *p*-hydroxybenzoic acid values may be taken as representing *p*-hydroxyhippuric acid since this compound gives no significant colour under the above conditions.

p-*Hydroxybenzamide.* The titrated ether-soluble acid solution prepared as for the estimation of *p*-hydroxybenzoic acid (above) was diluted to 100 ml. with water. The diluted solution (5 ml.) was mixed with H_2SO_4 (5 ml. 10 N) in a test tube (16 × 150 mm.) and heated in boiling water for 2 hr. using a 'cold finger' as condenser. The mixture was cooled, treated with NaOH (10 ml. 5 N), again cooled and diluted to 25 ml. The *p*-hydroxybenzoic acid in this solution (further diluted if necessary in order to obtain a concentration of 0·01–0·1 mg. *p*-hydroxybenzoic acid/5 ml.) was estimated as described above.

Plan of experiments

This was in general as described previously (Bray *et al.* 1951), except that since the excretion of administered compounds was complete within 6–16 hr. the ordinary food

ration could be withheld until the end of the experiment. Phenols were administered about 14 hr. after ingestion of the previous day's food ration, when base-line excretion of the metabolites studied was constant. In the early stages of an experiment water was given at a rate of about 100 ml./hr. After 4 hr. the amount was reduced to that necessary to ensure the passing of a sufficient number of urine samples.

RESULTS

For most of the phenols used, studies of the detailed metabolic fate had already been made. Where this information was not available it was confirmed that virtually the whole dose could be accounted for as free phenol, glucuronide and ethereal sulphate. The treatment of analytical data and the calculation of velocity constants were as previously described (Bray *et al.* 1951).

It was found that the rate of excretion of glucuronide was proportional to the body level of the phenol. The velocity constants (k_g) were calculated chiefly by means of the 'tangent' method. The rate of excretion of glucuronide, obtained from the

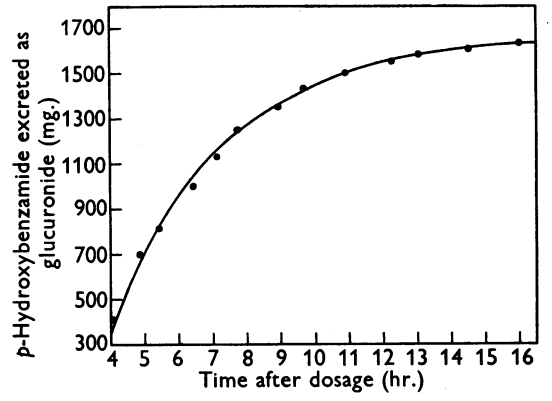

Fig. 1. Excretion of glucuronide by the rabbit after administration of *p*-hydroxybenzamide (1·3 g./kg.). The curve is the theoretical curve for $k_g = 0{\cdot}18$ hr.$^{-1}$ and the points those obtained by experiment.

glucuronide 'difference curve' (see Bray *et al.* 1951, p. 89) was plotted against the body level of the phenol, taken from the total difference curve for all estimated metabolites. The slope of the line gave an approximate value of k_g. A more accurate value was deduced by constructing theoretical glucuronide difference curves for values of k_g close to the approximate one and checking the fit of the experimentally determined points. An example, from an experiment with *p*-hydroxybenzamide, is shown in Fig. 1. In experiments where sulphite or cystine was also administered the sulphate conjugation also follows first-order kinetics (see Bray, Humphris, Thorpe, White & Wood, 1952*c*), so that both the 'log' and 'tangent' methods (see Bray *et al.* 1951) may be used independently for deriving k_g. This can be seen from Table 1 which shows some values for various phenols obtained by the two methods. The mean values of k_g for all the phenols studied are given in Table 2. The application of graphical methods depends upon certain conditions. The rate of excretion should not be taken as the rate of formation if there is appreciable accumulation in the body of the excretion products. It was found for various phenols that the blood level of the glucuronide, even when this metabolite was being excreted rapidly, showed only random deviation (less than ± 4 mg./100 ml.) about the base-line value (average 30 mg./100 ml.). As in the benzoic acid study (Bray *et al.* 1951), only experimental points obtained after absorption was complete, i.e. about 2 hr. after dosage, were used for calculation of velocity constants from the excretion curves.

Table 1. *Comparison of the velocity constants, k_g, for glucuronide conjugation of various phenols in the rabbit derived by 'log' and 'tangent' methods, expressed as hr.$^{-1}$*

(Dose level 0·25 g./kg.)

Compound	k_g found by 'log' method	k_g found by 'tangent' method
Phenol	0·59	0·56
Resorcinol	0·49	0·43
Quinol	0·49	0·47
p-Methoxyphenol	0·52	0·47
N-(*p*-Hydroxyphenyl)-urea	0·20	0·14

The percentage of the dose accounted for as glucuronide and sulphate was usually greater than 80. An exception was *p*-hydroxybenzamide, approximately 25 and 10 % of the dose of which was excreted as the free amide and acid respectively. On the assumption that failure to account for 100 % of the dose was due to the phenol being excreted as undetermined metabolites, the values of k_g derived graphically were corrected to give the value appropriate to 100 % recovery. Thus if the recovery were 85 % and the k_g found were 0·5 hr.$^{-1}$, then the true k_g was taken as 0·42 hr.$^{-1}$. Since the experiments were not continued until all traces of the phenol had been excreted, the 'percentage recovery' referred to is the value of the calculated asymptote, i.e. $E_{max.}$ (Bray *et al.* 1951, p. 90).

DISCUSSION

From the results recorded above it can be seen that conjugation of phenols with glucuronic acid follows first-order kinetics. Table 2 shows that the values of k_g for substituted phenols differ with the substituent, and it includes also P, the probabilities that the

Table 2. *Mean velocity constants, k_g, for glucuronide conjugation of various phenols in the rabbit*

Compound	Weight and influence of substituent radical*	No. of experiments	log $100k_g$ Mean	log $100k_g$ S.D.	P†	k_g‡ (hr.$^{-1}$)
Phenol	1	6	1·748	0·1067		0·56
Group 1						
2:6-Xylen-1-ol	$2 \times 15a$	4	1·543	0·1626	0·01–0·1	0·35
3:4-Xylen-1-ol	$2 \times 15a$	4	1·697	0·1696	>0·1	0·50
Catechol	$17a$	1	1·740	—	>0·1	0·55
Resorcinol	$17a$	2	1·653	0·0273	>0·1	0·45
Quinol	$17a$	2	1·706	0·0482	>0·1	0·51
p-Cyanophenol	$26d$	4	1·721	0·2056	>0·1	0·53
m-Methoxyphenol	$31d$	2	1·902	0·0383	>0·1	0·80
p-Methoxyphenol	$31a$	5	1·733	0·1414	>0·1	0·54
p-Chlorophenol	$35{\cdot}5d$	4	1·615	0·2067	>0·1	0·41
Group 2						
p-Hydroxybenzamide	$44d$	4	1·359	0·1741	<0·1	0·23
N-(*p*-Hydroxyphenyl)-urea	$59a$	4	1·023	0·1414	<0·1	0·11
Hydroxybenzoxazolone	59	4	1·244	0·2122	<0·1	0·18
p-Hydroxybenzenesulphonamide	$80d$	4	1·191	0·3118	<0·1	0·16
p-Benzylphenol	$91a$	4	1·179	0·2037	<0·1	0·15

* Activating radicals indicated by '*a*' and deactivating ones by '*d*'. (See Hammett, 1937.)

† The probability that the value of log $100k_g$ for the substituted phenol is the same as that for phenol, calculated by Student's '*t*' test.

‡ Derived from arithmetic mean of log 100 k_g values, i.e. geometric mean of k_g values.

differences from the value for phenol are significant. With one exception all the substituted phenols in group 1 have values of k_g essentially the same as that of phenol itself. 2:6-Xylen-1-ol is the only phenol of this group to have a significantly lower value of k_g. It may be relevant that this phenol has two *ortho* substituents. The phenols of group 2 all give values of k_g significantly smaller than that of phenol. The radical weights of the substituents in group 1 are smaller than those in group 2. Whether the difference in k_g values in groups 1 and 2 is due primarily to the size of the substituent group cannot be determined easily. Considering the *para*-substituted phenols in particular, the inclusion in each group of both strongly activating radicals (e.g. —OCH_3 and —$NHCONH_2$) and strongly de-activating radicals (e.g. —CN and —SO_2NH_2) suggests that the electronic influence is of little importance.

For simplicity, the phenols used for this study were selected because the substituents are metabolically inert, that is, they are modified slightly or not at all *in vivo*. As will be shown in the next paper (Bray *et al.* 1952*c*) sulphate conjugation in the fasting animal takes place at a uniform rate. For phenols containing metabolically inert substituents, therefore, the proportions of the dose excreted as ethereal sulphate and as glucuronide will be determined solely by the values of k_g. The percentages of the dose of a phenol excreted as the two conjugates are complementary, so that if the effect of a metabolically inert substituent is to decrease the percentage glucuronide conjugation, this will be accompanied by a corresponding increase in the sulphate conjugation. Since the percentage of the dose excreted as sulphate is usually less than that excreted as glucuronide, the variation in sulphate excretion will be relatively greater and more noticeable. The effect of substituents upon the percentage conjugation of phenols with sulphate was studied by Williams (1938), but from the foregoing it is clear that for metabolically inert substituents this was indirectly a study of the effect upon glucuronide conjugation. This is discussed further in a later paper (Bray, Thorpe & White, 1952).

SUMMARY

1. The kinetics of the conjugation of phenol, catechol, resorcinol, quinol, *m*- and *p*-methoxy-, *p*-chloro- and *p*-cyano-phenols, and 2:6- and 3:4-xylen-1-ols with glucuronic acid in the rabbit have been studied.

2. The rate of glucuronide formation is proportional to the body level of the phenol.

3. Of the phenols examined, those with small substituent radicals have, with the exception of 2:6-xylen-1-ol, velocity constants, k_g, for glucuronide formation which do not differ significantly from that of phenol itself. Those with substituent radical weight greater than that of *p*-chlorophenol have significantly lower values for k_g.

4. For the *para*-substituted phenols examined the value of k_g does not appear to be related to the electronic influence of the substituent group.

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Previous investigations of the conjugation of phenols with sulphuric acid have been chiefly concerned with the percentage of a dose excreted as ethereal sulphate. The observation by various workers (e.g. Tauber, 1895; Hele, 1924, 1931) that simultaneous administration of compounds giving rise to inorganic sulphate *in vivo* increases the percentage sulphate conjugation, together with more direct evidence obtained by the use of radioactive sulphate (Laidlaw & Young, 1948), has shown that the sulphate ion participates in the synthesis and that the amount of sulphate available for conjugation in the normal animal limits the extent of this process. In the investigation now reported the kinetics of the ethereal sulphate conjugation have been studied. In the fasting rabbit the process takes place at a uniform rate, while in the presence of sufficient sulphate the rate is proportional to the body level of the phenol.

METHODS

The animals, their diet, the phenols used and the general plan of experiments and collection of urine specimens were as described in the previous paper (Bray, Humphris, Thorpe, White & Wood, 1952*b*).

Dosage. The experiments performed were of three types: (1) administration of a single dose of a phenol, alone or with a sulphate precursor; (2) administration of repeated small doses of a phenol, alone or with a sulphate precursor; (3) administration of a single dose of a phenol precursor, alone or with a sulphate precursor.

The doses of phenols ranged from 0·4 to 4·0 g. depending on the toxicity of the compound. In experiments of type 2 the phenol (50 mg.) was given each hour for 6 hr. The dose of sulphate precursor varied with the type of experiment. When L-cystine was used 1·5 g. was administered 2 hr. before the dose of the phenol. Larger doses could not be used owing to toxicity (3 g. may cause death within 48 hr.). In experiments of type 1, sodium sulphite ($Na_2SO_3.7H_2O$) was administered in doses of 2 and 1 g. an hour apart, the second dose simultaneously with the phenol. In type 2 experiments, a preliminary dose of sulphite (1 g.) was administered 1 hr. before the first dose of the phenol, and subsequently a small dose (100 mg.) was given each hour at the same time as the phenol throughout the experiment. Sodium sulphite (1 g.) was usually given with the phenol precursor in experiments of type 3.

Table 1. *Rates of excretion of inorganic sulphate after administration of sulphate precursors to rabbits 14 hr. after normal food ration*

Precursor	Dose (g.)	Mean rate (mg. SO_3/hr.) 0–3 hr. after dose	Mean rate (mg. SO_3/hr.) 3–9 hr. after dose
L-Cystine	1·5	50–150	50, 55
L-Cystine	1·0		32, 33
$Na_2SO_3.7H_2O$	2+1		39, 27
$Na_2SO_3.7H_2O$	2		37, 48
DL-Methionine	1	0–25	34, 38
$Na_2S_2O_3.5H_2O$	1		30
Sulphur	1		15, 15
$Na_2SO_4.10H_2O$	0·5		15
Taurine	1		7
Cabbage	100		24, 10
Cabbage	20		17
Pellets	25		19, 7
Bran, oats and cabbage (1:1:5)	70		5, 13

Choice of sulphate precursors. The rate of excretion of inorganic sulphate was determined after the administration of a number of sulphur-containing compounds and diets. The results are given in Table 1. The substances were given