REFERENCES

- Bray, H. G., Clowes, R. C. & Thorpe, W. V. (1952*a*). *Biochem. J.* **51**, 70.
 Bray, H. G., Humphris, B. G., Thorpe, W. V., White, K. & Wood, P. B. (1952*b*). *Biochem. J.* **52**, 412.
 Bray, H. G., Humphris, B. G., Thorpe, W. V., White, K. & Wood, P. B. (1952*c*). *Biochem. J.* **52**, 419.
 Bray, H. G., Lake, H. J. & Thorpe, W. V. (1949). *Biochem. J.* **44**, 136.
 Bray, H. G., Ryman, B. E. & Thorpe, W. V. (1947). *Biochem. J.* **41**, 212.
 Bray, H. G., Thorpe, W. V. & White, K. (1951). *Biochem. J.* **48**, 88.
 Bray, H. G., Thorpe, W. V. & White, K. (1952). *Biochem. J.* **52**, 423.
 Hammett, L. P. (1937). *J. Amer. chem. Soc.* **59**, 96.
 Williams, R. T. (1938). *Biochem. J.* **32**, 878.

Kinetic Studies of the Metabolism of Foreign Organic Compounds

4. THE CONJUGATION OF PHENOLS WITH SULPHURIC ACID

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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 ($\text{Na}_2\text{SO}_3 \cdot 7\text{H}_2\text{O}$) 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	3-9 hr. after dose
L-Cystine	1.5	50-150	50, 55
L-Cystine	1.0		32, 33
$\text{Na}_2\text{SO}_3 \cdot 7\text{H}_2\text{O}$	2 + 1		39, 27
$\text{Na}_2\text{SO}_3 \cdot 7\text{H}_2\text{O}$	2		37, 48
DL-Methionine	1	0-25	34, 38
$\text{Na}_2\text{S}_2\text{O}_8 \cdot 5\text{H}_2\text{O}$	1		30
Sulphur	1		15, 15
$\text{Na}_2\text{SO}_4 \cdot 10\text{H}_2\text{O}$	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

approximately 14 hr. after the rabbits had ingested the normal daily ration of pellets. By this time the excretion of inorganic and ethereal sulphate had fallen to a low and constant level (about 5 mg. inorganic and 1 mg. ethereal $\text{SO}_3/\text{hr.}$). Rabbits in this state will be referred to as 'fasting' animals. It can be seen that sulphate precursors vary considerably in their rates of conversion to inorganic sulphate. Sodium sulphite and L-cystine were most effective. With most phenols either could be used, but with *p*-hydroxybenzamide and *p*-chlorophenol only cystine could be given since the simultaneous administration of sodium sulphite caused death even with doses which caused no toxic effects when either was given separately.

Estimation of metabolites. The methods used for urine have been described previously (Bray, Humphris, Thorpe, White & Wood, 1952a). Inorganic and ethereal sulphates were determined in blood as follows: blood (about 1 ml.) from a puncture in an ear vein was mixed with potassium oxalate solution (5 ml. 0.5% w/v) in a tared beaker. After weighing, trichloroacetic acid (2.5 ml. 10%, w/v) was stirred in and the mixture filtered. Sulphates were estimated in the filtrate as for urine.

RESULTS

Throughout this investigation it has been assumed that the rate of formation of ethereal sulphate does not exceed the rate of its excretion, i.e. that there is

Fig. 1. Excretion of ethereal sulphate after administration of *p*-hydroxybenzamide (3.6 g.) to a fasting rabbit.

no appreciable accumulation of ethereal sulphate in the body. Determination of the blood level of ethereal sulphate after the administration of various phenols has shown that even when rapid excretion was occurring the average increase in the blood level was approximately 2 mg. $\text{SO}_3/100$ ml. This is so close to the experimental error of the method that it is of doubtful significance. After administration of a phenol to a fasting rabbit the rate of ethereal sulphate formation is approximately constant until almost the whole of the dose is eliminated. This is illustrated in Fig. 1 which shows the excretion curve of ethereal sulphate after

the administration of *p*-hydroxybenzamide. This constant rate, designated v_s , is of the same order for all phenols examined. The average value for 22 experiments was 12.5 (s.d. ± 5.1) mg. $\text{SO}_3/\text{hr.}$ (4.5 mg. $\text{SO}_3/\text{kg.}/\text{hr.}$). Since the glucuronide conjugation which is occurring simultaneously proceeds at a rate which is proportional to the body level of the phenol (Bray *et al.* 1952b) the ratio of the rate of formation of glucuronide to that of ethereal sulphate (g/s) will decrease throughout the excretion period. This is illustrated in Fig. 2 which was obtained in the same experiment as Fig. 1.

Fig. 2. The relation between the rates of excretion of glucuronide and ethereal sulphate, expressed as g/s , after administration of *p*-hydroxybenzamide to the rabbit (3.6 g.). Inset: the same ratio obtained in an experiment in which phenol (0.56 g.) was given together with $\text{Na}_2\text{SO}_3 \cdot 7\text{H}_2\text{O}$ (1 g.) 2 hr. after a dose (2 g.) of the latter.

Table 2. Excretion of ethereal sulphate in 24 hr. after administration of various phenols with and without sulphate precursor, expressed as percentage of the dose

Compound	(Dose level 0.25 g./kg.)	
	Without sulphate precursor	With sulphate precursor
Phenol	15, 16	29, 23, 28, 32
2:6-Xylen-1-ol	12, 9, 16	33, 31
3:4-Xylen-1-ol	13, 11, 12	29, 33
Quinol	18	45
<i>m</i> -Methoxyphenol	12	22
<i>p</i> -Methoxyphenol	10, 15	32, 31, 43, 30
<i>p</i> -Chlorophenol	10, 12	33, 33, 27
<i>p</i> -Hydroxybenzamide	17, 7	40, 36
Hydroxybenzoxazolone	28, 28	40, 52
<i>p</i> -Hydroxybenzenesulphonamide	8, 19	28, 75

When the phenol is administered together with sufficient sulphate precursor different results are obtained; the percentage of the dose excreted as ethereal sulphate is increased (see Table 2) and that as glucuronide is decreased. Ethereal sulphate is formed at a rate proportional to the body level of

the phenol and the ratio g/s^* does not steadily decrease throughout the period of elimination. This is illustrated in the inset of Fig. 2 which shows the values for the ratio g/s for phenol administered with sulphite. Fig. 3 shows the excretion curves for ethereal sulphate and glucuronide when *p*-chlorophenol is administered alone and with L-cystine. Fig. 4 shows experimental points obtained with *p*-methoxyphenol plotted together with a theoretical curve calculated for the given value for the first-order velocity constant for ethereal sulphate formation (k_s).*

Fig. 3. The effect upon the conjugation of *p*-chlorophenol with glucuronic and sulphuric acids of administration of L-cystine to the rabbit. ●—●, excretion curves after administration of *p*-chlorophenol (0.56 g.) alone; ●---●, excretion curves after administration of *p*-chlorophenol (0.56 g.) 2 hr. after L-cystine (1.5 g.).

An accurate value for k_s could not be derived by means of the graphical methods (Bray, Thorpe & White, 1951) since the maximum amount of sulphate precursor which could be safely administered did not supply sufficient sulphate ion for the conjugation of more than small doses of phenols. This difficulty is accentuated by the more rapid excretion of phenols under conditions where the kinetics of sulphate conjugation follow those of the first-order reactions, so that only a small number of

experimental points can be obtained. However, no such limitation exists for the determination of k_g and the constant value of the ratio g/s (designated R), which is characteristic of the first-order kinetics. Both these values can be determined by experiments of types 1, 2 and 3. Clearly R will be numerically equal to the ratio k_g/k_s . It was found that the ratio g/s was steady throughout experiments of type 1 and approximately equal to those obtained in experiments of types 2 and 3 when the ratio of the rate of excretion of inorganic sulphate to that of ethereal sulphate was greater than 2. Consequently, this was adopted as a criterion of adequacy of sulphate ion for conjugation, and values of g/s determined under these conditions were taken as R .

Values of R were obtained from type 1 experiments only when a small dose of a phenol was given with a sulphate precursor and from all experiments

Fig. 4. Excretion of ethereal sulphate by the rabbit after administration of *p*-methoxyphenol and sulphite. The curve is the theoretical one for $k_s=0.60 \text{ hr.}^{-1}$ and the points are those obtained by experiment. Dosage: *p*-methoxyphenol (0.56 g.) with $\text{Na}_2\text{SO}_3 \cdot 7\text{H}_2\text{O}$ (1 g.) 2 hr. after giving $\text{Na}_2\text{SO}_3 \cdot 7\text{H}_2\text{O}$ (2 g.).

of types 2 and 3. These were calculated by taking the mean of the values for the ratio for each urine specimen. In type 1 experiments most of the experimental points relate to high body levels of the phenol; in those of type 2 a low body level was maintained continuously, while in experiments of type 3 the administration of a large dose of a phenol precursor gives a low body level of the phenol which changes only slowly (Bray *et al.* 1952*a, c*). The mean value of R for each phenol was calculated by taking the mean of the values for the ratio for each experiment (of all types). In all these calculations the geometric mean was used, i.e. the arithmetic mean of the logarithms of the ratio. Since the value of k_g for each phenol has been given as the geometric mean (Bray *et al.* 1952*b*) the derived values for k_s will also be geometric mean values. Since the variance of both k_g and R for any one phenol may be

* In a preliminary communication (Bray & White, 1951) the first-order velocity constant for ethereal sulphate formation was designated k_g and this ratio G/E . In view of the extension of these studies to other conjugation processes it is preferable to use the general symbol k_s for the first-order velocity constant of the 'second' conjugation reaction (in the present paper this applies to sulphate conjugation) and g/s for the ratio (see Bray, Thorpe & White, 1952*c*).

Table 3. Mean velocity constants, k_s , for sulphate conjugation of various phenols in the rabbit calculated from R and the k_g values

Compound	Weight and influence of substituent radical*	No. of exp.	log 100R		log 100 k_g †	log 100 k_s		P ‡	k_s § (hr. ⁻¹)
			Mean	S.D.		Mean	S.D.		
Phenol	1	7	2.205	0.1918	1.748	1.543	0.1594		0.35
Group 1									
2:6-Xylen-1-ol	2 × 15a	2	2.231	0.1082	1.543	1.312	0.1589	> 0.1	0.21
3:4-Xylen-1-ol	2 × 15a	2	2.233	0.2485	1.697	1.464	0.1816	> 0.1	0.29
Resorcinol	17a	3	2.214	0.1657	1.653	1.439	0.0134	> 0.1	0.27
Quinol	17a	4	2.023	0.1664	1.706	1.683	0.1593	> 0.1	0.48
<i>p</i> -Cyanophenol	26d	3	2.202	0.0687	1.721	1.519	0.1937	> 0.1	0.33
<i>m</i> -Methoxyphenol	31d	2	2.284	0.3264	1.902	1.718	0.3239	> 0.1	0.52
<i>p</i> -Methoxyphenol	31a	9	1.998	0.2714	1.733	1.735	0.2314	> 0.1	0.54
<i>p</i> -Chlorophenol	35.5d	4	2.206	0.2228	1.615	1.409	0.0831	> 0.1	0.26
Group 2									
<i>p</i> -Hydroxybenzamide	44d	3	1.017	0.2271	1.359	2.342	0.1459	0.01-0.1	2.2
<i>N</i> -(<i>p</i> -Hydroxyphenyl)-urea	59a	9	1.985	0.1182	1.023	1.038	0.0775	0.01-0.1	0.11
Hydroxybenzoxazolone	59	7	1.978	0.0699	1.244	1.266	0.2030	> 0.1	0.18
<i>p</i> -Hydroxybenzenesulphonamide	80d	3	0.859	0.5172	1.191	2.332	0.3877	0.01-0.1	2.1

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

† From previous paper (Bray *et al.* 1952b).

‡ The mean value of log 100 k_s was derived from the mean values of log 100 k_g and log 100R. The variance of log 100 k_s was taken as the variance of log 100R less the variance of log 100 k_g . Assuming a normal distribution, the value of P was calculated by comparing the difference in the mean values of log 100 k_s for phenol and the substituted phenol with the sum of the variances.

§ Derived from the arithmetic mean of log 100 k_s .

derived, the statistical significance of different values of k_s for different phenols may be assessed. The mean values of k_s found for several phenols are given in Table 3.

DISCUSSION

The administration, to a fasting animal, of a sulphate precursor increases the percentage sulphate conjugation of a phenol and transforms the conjugation process from one following zero-order kinetics to one following first-order kinetics. This indicates that the rate of sulphate conjugation is limited by the availability of sulphate and supports the view of the earlier workers already referred to that the sulphate ion is concerned in the conjugation process. Although it has been shown that the ethereal sulphate conjugation follows first-order kinetics with respect to the phenol when a certain amount of inorganic sulphate is available, the effect of a greater excess of sulphate ion could not be determined owing to the limitations of the analytical method.

It can be seen from Table 3 that the values of k_s for substituted phenols of group 1, like those for k_g (Bray *et al.* 1952b) do not differ significantly from that of phenol itself. Considering the phenols with larger substituent radicals (group 2), the value of k_s for *N*-(*p*-hydroxyphenyl)-urea is significantly lower than that of phenol, that for hydroxybenzoxazolone

is essentially the same, whilst those for *p*-hydroxybenzamide and *p*-hydroxybenzenesulphonamide are very much greater. The values of k_s for these four compounds are all significantly lower than that for phenol. It seems, therefore, that the value of k_s , unlike that of k_g , is not related to the size of the substituent. It can also be seen from Table 3 that the electronic nature of the substituent radicals does not appear to influence the value of k_s .

SUMMARY

1. The kinetics of the conjugation of phenols with sulphuric acid have been studied in the rabbit.

2. In the fasting rabbit the rate of ethereal sulphate formation is independent of the amount of phenol present above a certain body level, but the simultaneous administration of a compound, e.g. sodium sulphite or L-cystine, which is converted to sulphate *in vivo*, changes the kinetics of the process to those of a first-order reaction.

3. The effect of various substituents on the value of the velocity constant for sulphate conjugation was determined and does not appear to be related to the size or electronic influence of the substituent group.

We are indebted to Dr Colin White for advice upon the statistical treatment of our results.

REFERENCES

- Bray, H. G., Humphris, B. G., Thorpe, W. V., White, K. & Wood, P. B. (1952*a*). *Biochem. J.* **52**, 412.
 Bray, H. G., Humphris, B. G., Thorpe, W. V., White, K. & Wood, P. B. (1952*b*). *Biochem. J.* **52**, 416.
 Bray, H. G., Thorpe, W. V. & White, K. (1951). *Biochem. J.* **48**, 88.
 Bray, H. G., Thorpe, W. V. & White, K. (1952*c*). *Biochem. J.* **52**, 423.
 Bray, H. G. & White, K. (1951). *Biochem. J.* **48**, xliii.
 Hammett, L. P. (1937). *J. Amer. chem. Soc.* **59**, 96.
 Hele, T. S. (1924). *Biochem. J.* **18**, 110.
 Hele, T. S. (1931). *Biochem. J.* **25**, 1736.
 Laidlaw, J. C. & Young, L. (1948). *Biochem. J.* **42**, 1.
 Tauber, S. (1895). *Arch. exp. Path. Pharmac.* **38**, 197.

Kinetic Studies of the Metabolism of Foreign Organic Compounds

5. A MATHEMATICAL MODEL EXPRESSING THE METABOLIC FATE OF PHENOLS, BENZOIC ACIDS AND THEIR PRECURSORS

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Previous papers from this laboratory have recorded the results of studies of the kinetics of the reactions which benzoic acid, phenols and compounds which give rise to them, undergo in the rabbit (Bray, Thorpe & White, 1951; Bray, Humphris, Thorpe, White & Wood, 1952*a, b, c*). These results have provided a basis for the construction of a mathematical model expressing the quantitative fate of such compounds. This model is in accord with experimental results obtained previously both in this and other laboratories. Experiments with aniline, acetanilide, *o*- and *p*-aminophenols, *o*- and *p*-hydroxyacetanilides and *o*-, *m*- and *p*-hydroxybenzaldehydes have been carried out as further tests of the validity of the model.

THEORETICAL

In this section mathematical expressions are derived representing the fate of compounds such as phenols, benzoic acids and their precursors undergoing metabolic reactions according to a defined pattern of kinetics.

In the previous paper (Bray *et al.* 1952*c*) it was shown that for small doses of a phenol the rate of conjugation with sulphate is proportional to the body level of the phenol, while for large doses the rate is constant and equal to the maximum rate of supply of sulphate ion. In the present paper it will be assumed that the transition from first-order to zero-order kinetics is abrupt. Thus, with increasing body level of the phenol, the rate of conjugation with sulphate increases in proportion until the maximum rate at which sulphate can be supplied is reached.

The magnitude of this critical body level will be fixed by the values of the first- and zero-order velocity constants for conjugation. Although such transitions are not encountered in purely chemical systems, our experimental results suggest that they may occur in the rabbit. The mechanism seems to be related to the tendency to maintain a certain blood level of sulphate ion, essentially by means of the kidney. Similar kinetic considerations appear to apply also to glycine conjugation.

The basic mathematical treatment of such a system has already been presented for benzoic acid (Bray *et al.* 1951). In this the critical body level of benzoic acid was equated to zero since the very high value of the first-order velocity constant for glycine conjugation rendered the critical value negligibly small. This treatment is now extended to represent the metabolism of compounds for which this approximation cannot be made. The substance formed, actually or theoretically from the precursor, whether it be a phenol, a benzoic acid or some other compound, is described as the intermediate.

Symbols

A = dose level of precursor; $A_{crit.}$ = critical dose level of precursor; B = dose level of intermediate; $B_{crit.}$ = critical dose level of intermediate; b = amount of intermediate, free and conjugated, at time t ; g = amount of glucuronide at time t ; s = amount of second conjugate at time t (e.g. an ethereal sulphate for phenols, a hippuric acid for benzoic acids); $m = g + s$; T = time at which critical body level of free intermediate is reached; k_1 = first-order velocity constant for conversion of precursor