Kinetic Studies on the Metabolism of Foreign Organic Compounds

6. REACTIONS OF SOME NUCLEAR-SUBSTITUTED BENZOIC ACIDS, BENZAMIDES AND TOLUENES IN THE RABBIT

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The previous papers of this series (Bray, Thorpe & White, 1951, 1952*e*; Bray, Humphris, Thorpe, White & Wood, 1952*b, c, d*) reported a study of the kinetics *in vivo* of some reactions involving benzoic acid and some of its precursors, certain precursors of phenols and a series of substituted phenols. A mathematical model was worked out which could account for the effect upon metabolic fate of dose level, nuclear substituents, quantitative differences between the metabolism of precursors and their conversion products, and species differences. These investigations have now been extended by studying the metabolism in the rabbit of various nuclear substituted toluenes, benzamides and benzoic acids. It has been found that the metabolic processes involved follow kinetics similar to those already reported and values are given for the appropriate velocity constants. There appeared to be no correlation between these values and the size or electronic nature of the substituents.

MATERIALS AND METHODS

Materials. *p*-Acetamidobenzoic acid was prepared by the acetylation of *p*-aminobenzoic acid with acetic anhydride and *p*-methoxytoluene by methylation of *p*-cresol using methyl sulphate and NaOH. *p*-Cyanobenzamide was prepared by treatment of methyl *p*-cyanobenzoate with NH_3 in ethanol at 100° for 8 hr. in a sealed tube. *p*-Nitro-, *p*-chloro-, *p*-fluoro- and *p*-methoxy-benzamides were obtained by the action of aqueous NH_3 (sp.gr. 0.88) on the corresponding acid chlorides, prepared by the action of PCl_5 on the appropriate acids. *p*-Toluamide was prepared by the action of H_2O_2 on *p*-tolunitrile (Noller, 1933) and *p*-cyanobenzoic acid by a Sandmeyer reaction on *p*-aminobenzoic acid. The other compounds investigated were purchased. Melting points and properties of all compounds agreed with those described in the literature.

Methods. The plan of the experiments, the collection of urine samples and the analytical methods were as described in earlier papers (Bray *et al.* 1951, 1952*b, c, d*). Glycine conjugates were estimated by the method of Bray, Clowes, Thorpe, White & Wood (1952*a*). The velocity constants were calculated by graphical methods. Both 'log' and 'tangent' methods were used as convenient: when both were applied to the same results values in good agreement were obtained.

Symbols. First-order velocity constants for the formation of hippuric acids and of glucuronic acids are denoted by k_h

and k_g respectively and those for the conversion of benzamide and of toluene into benzoic acid by $k_{b, \text{am}}$ and $k_{b, \text{tol}}$; the zero-order velocity constant for hippuric acid formation is denoted by v_h . The symbol, k_c , used in the discussion, refers to the first-order velocity constant for conjugation of a phenol with sulphuric acid.

RESULTS

The analytical results and the values of the velocity constants obtained are summarized in Tables 1 and 2.

Glycine conjugation. It has already been shown (Bray *et al.* 1951) that, at dose levels of 0.3–1.3 g./kg., the rate of conjugation of benzoic acid with glycine was invariably independent of the body level. From the present results it appears that the conjugation of substituted benzoic acids having values for k_h greater than 0.2 hr.^{-1} follows the kinetics of a zero-order reaction unless glycine is administered simultaneously, when the conjugation follows the kinetics of a first-order reaction. This justifies the grouping together of glycine and sulphate conjugation as processes for which there is a critical body level at which the kinetics change from those of a zero to those of a first-order reaction (Bray *et al.* 1952*e*). An example of a typical first-order curve for the conjugation of *p*-toluic acid with glycine is given in Fig. 1; in this experiment glycine was administered simultaneously with the dose. When the same dose of *p*-toluic acid was administered without glycine, the excretion curve for conjugated glycine was linear, as was found (Bray *et al.* 1951) in all experiments with unsubstituted benzoic acid even when glycine was administered with it. The values of v_h are different for different compounds and all are smaller than the average found for benzoic acid itself.

Glucuronic acid conjugation. In every case the glucuronic acid conjugation followed first-order reaction kinetics. Most of the values for k_g are of the same order as that for benzoic acid (0.08 hr.^{-1}), except that for *p*-methoxybenzoic acid (0.22 hr.^{-1}) which is appreciably greater. It should, perhaps, be mentioned that in some of these experiments the percentage of the dose conjugated with glucuronic

Table 1. *Velocity constants for glycine and glucuronic acid conjugation of p-substituted benzoic acids by the rabbit*

Dose level 0.4 g./kg. Where values for both zero- and first-order velocity constants are given for the same experiment, the former is evaluated from the first part of the curve and the latter from the last part. Values in italics are for experiments in which 3-4 equivalents of glycine were given with the dose. The percentages recorded under 'excreted unconjugated' are the differences between those for the total ether-soluble acid and the ether-soluble conjugates, i.e. glycine conjugates + ether-soluble glucuronic acid conjugates. (The glucuronic acid conjugates of the halogenobenzoic acids and of *p*-toluic acid are ether-soluble.) The means are geometric. Values (mean) for benzoic acid are from Bray *et al.* (1951).

Substituent	Conjugation with glycine (% of dose)	v_h (mg. glycine/hr.)	k_h (hr. ⁻¹)	Conjugation with glucuronic acid (% of dose)	k_g (hr. ⁻¹)	Excreted unconjugated (% of dose)	Rate of linear excretion of unconjugated acid (mg./hr.)
None	83	163	—	15	0.08	1	—
<i>p</i> -CN	35	—	0.17	0	—	68	—
	26	—	0.23	2	0.013	78	—
	33	—	0.13	2	0.008	53	—
	46	—	0.23	3	0.014	65	—
Mean	—	—	0.19	—	0.011	—	—
<i>p</i> -NO ₂	6	—	0.04	6	0.04	70	—
	8	—	0.03	6	0.02	88	—
	20	—	0.09	6	0.02	78	—
	13	—	0.06	1	0.04	80	—
Mean	—	—	0.05	—	0.03	—	—
<i>p</i> -OCH ₃	39	30	—	59	0.17	0	—
	33	36	—	59	0.36	0	—
	41	31	—	52	0.29	3	—
	50	—	0.21	43	0.20	8	—
	52	—	0.16	40	0.13	0	—
Mean	—	—	0.18	—	0.22	—	—
<i>p</i> -I	35	17	—	53	0.14	12	—
	46	20	—	39	0.09	0	—
	77	—	1.2	9	0.03	3	—
	65	16	—	12	—	0	—
	62	—	1.1	18	0.18	0	—
Mean	—	—	1.1	—	0.09	—	—
<i>p</i> -Cl	83	91	0.38	9	0.04	8	—
	78	107	—	3	—	4	—
	88	—	0.45	3	0.02	4	—
	82	—	0.39	4	0.02	11	—
	91	—	0.36	5	0.02	8	—
Mean	—	—	0.39	—	0.02	—	—
<i>p</i> -F	83	108	1.2	7	0.10	5	—
	84	—	1.3	4	0.06	8	—
	88	—	0.88	2	0.02	6	—
	68	—	1.22	2	0.04	25	—
	80	—	1.0	4	0.04	9	—
	76	—	1.04	2	0.03	12	—
Mean	—	—	1.1	—	0.04	—	—
<i>p</i> -CH ₃	47	55	—	17	0.06	46	—
	57	55	—	23	—	30	—
	68	—	0.43	8	0.05	34	—
	76	—	0.37	12	0.07	13	—
Mean	—	—	0.4	—	0.06	—	—
<i>p</i> -NHCOCH ₃	2	—	0.01*	0	0.002*	77	125
	10	—		1		92	100
	7	—		1		82	90
	3	—		1		89	100

* Excretion of conjugates too small for accurate evaluation.

acid and, therefore, the actual amount of glucuronic acid determined was very small. These values may be subject to a greater experimental error than those for experiments in which a larger percentage of the dose was excreted as glucuronide.

Hydrolysis of amides. The results in Table 2 show that the hydrolysis of all the amides studied followed first-order kinetics with respect to the body level of amide. The values obtained for the velocity constant ranged from 0.7 hr.^{-1} for *p*-fluorobenzamide to 0.23 hr.^{-1} for *p*-nitrobenzamide, as compared with 0.32 hr.^{-1} for benzamide. *In vitro*, however, *p*-nitrobenzamide has always been more readily hydrolysed than other amides (e.g. Bray, James, Thorpe & Wasdell, 1950*a*).

Oxidation of toluenes. This process also followed first-order reaction kinetics for all the compounds examined (Table 2).

DISCUSSION

In studies of the metabolism of foreign organic compounds satisfactory interpretation of analyses of 24 hr. urines is difficult (see Bray *et al.* 1952*e*), and kinetic studies seem to offer a more promising method of investigation. A knowledge of the type of kinetics involved assists the interpretation of the variation of metabolic fate with varying dose level;

Fig. 1. Excretion curve for the conjugate of *p*-toluic acid with glycine in the rabbit. Glycine (4 g.) was administered with *p*-toluic acid (1 g.). The curve is the theoretical one for $k_a = 0.37$ and the points those obtained by experiment.

the variation in the metabolic fate of different nuclear-substituted derivatives depends on the numerical value of the velocity constants. Further, there is the possibility that in the numerical values of velocity constants there may be regularities which can be correlated with other known properties; such observations might permit the prediction of metabolic fates. For example, it has already been shown (Bray *et al.* 1952*c*), that there is a correlation between the rate of glucuronic acid conjugation of phenols and the size of the substituent in the *para* position.

The velocity constants for the formation of ester glucuronides from substituted benzoic acids show no correlation with size or electronic influence of the substituent group. Quick's (1932) generalization, relating to the dog, that 'there is a fairly constant, though not absolute, correlation between the pK of an acid and the rate with which it is excreted combined with glycuronic acid' is not supported by our measurements of k_e values in the rabbit. It is, however, likely that the k_e values for a given acid would be different for the dog and for the rabbit so that a comparison between different species is not valid (see Bray *et al.* 1952*e*).

Quick (1932) noted that in the dog, *para* substitution of benzoic acid had comparatively little effect on the percentage of a dose excreted as glycine conjugate. In the rabbit there is considerable variation in the percentage of the dose excreted in this form, e.g. from 6% for *p*-acetamidobenzoic acid to 84% for *p*-chlorobenzoic acid, which is comparable with 83% for unsubstituted benzoic acid. Without exception, the *para* substituents which we have studied diminish the velocity constant k_a . Although there is no reason to believe that the amount of glycine potentially available in the body is less than that when unsubstituted benzoic acid is conjugated, the values of v_h for *para* substituted benzoic acids are smaller. The reason for this is unknown but two possibilities may be mentioned. Either substituted benzoic acids may inhibit the mobilization of glycine for this purpose or it may be that the first- and zero-order kinetics measured in these experiments are in fact related to Michaelis-type kinetics of an enzyme or enzymes concerned in the metabolic process. Thus the zero-order velocity constant may correspond to the 'maximum velocity' and the first-order constant directly to that of Michaelis kinetics. Hearon (1952) has pointed out that, even in multienzyme systems in the steady state, variations in the overall rate, which occur when the concentration of only one substrate is varied, will follow Michaelis kinetics.

It should be noted that our interpretation of the results is based upon the assumption that the transition from zero- to first-order kinetics as body level falls is abrupt and that intermediate stages

which have fractional order kinetics are ignored (cf. Bray *et al.* 1951). In the earlier experiments the curves obtained were either almost entirely linear or entirely exponential. In the present work, however, some curves were obtained which had both linear and exponential portions of such length that both zero- and first-order velocity constants could be evaluated in a single experiment. An example of this transition from zero- to first-order kinetics is

given in Fig. 2, which also shows linear and exponential curves of the type more usually obtained. Curves *A* and *B* are entirely exponential from the time at which absorption can be assumed to be complete. Curve *D* is linear until excretion is practically complete, or, if a transition does occur, it occupies so short a time that the shape of the curve cannot be accurately determined. Curve *C* is linear up to 3 hr. but after that it is exponential.

Table 2. *Velocity constants for hydrolysis of p-substituted benzamides and oxidation of p-substituted toluenes in the rabbit*

Dose level 0.3 g./kg. Values in *italics* are for experiments in which 3-4 equivalents of glycine were given with the dose. The percentages recorded under 'total excreted as acid' are the sum of those for the total ether-soluble acid + acid conjugated with glucuronic acid unless glucuronic acid conjugates are ether-soluble (see Table 1). Means are geometric. Values (means) for benzamide and toluene from Bray *et al.* (1951).

Substituent	Benzamides			Toluenes		
	Total excreted as acid (% of dose)	Conjugation as ester glucuronide (% of dose)	$k_{b, am.}$ (hr. ⁻¹)	Total excreted as acid (% of dose)	Conjugation as ester glucuronide (% of dose)	$k_{b, tol.}$ (hr. ⁻¹)
None	—	—	0.32	—	—	0.11
<i>p</i> -CN	86	1	0.35	91	1	0.34
	82	1	0.33	92	1	0.28
	85	1	0.28	105	1	0.32
Mean	—	—	0.32	—	—	0.31
<i>p</i> -NO ₂	90	1	0.27	32*	1	0.04
	79	1	0.19	27*	1	0.08
Mean	—	—	0.23	—	—	0.06
<i>p</i> -OCH ₃	62	18	0.27	45†	2	0.09
	64	13	0.23	48†	2	0.12
	65	10	0.28	58†	5	0.14
Mean	—	—	0.28	—	—	0.12
<i>p</i> -Cl	94	1	0.68	83	1	0.26
	92	1	0.50	72	1	0.14
	104	1	0.53	64	1	0.11
	97	1	0.42	65	1	0.10
Mean	—	—	0.53	—	—	0.14
<i>p</i> -F	96	1	0.61	69	1	0.21
	95	1	0.70	55	1	0.19
	96	1	0.81	64	1	0.14
Mean	—	—	0.70	—	—	0.18
<i>p</i> -CH ₃	95	1	0.53	62	1	0.11
	92	1	0.37	83	1	0.15
	88	1	0.41	80	1	0.17
	81	1	0.41	—	—	—
	92	1	0.32	—	—	—
Mean	—	—	0.40	—	—	0.14
<i>p</i> -NHCOCH ₃	—	—	—	92	8	0.63
	—	—	—	91	8	0.56
Mean	—	—	—	—	—	0.59
<i>p</i> -SO ₂ NH ₂	—	—	—	97	1	0.25
	—	—	—	93	1	0.25
Mean	—	—	—	—	—	0.25

* Remainder of dose not accounted for.

† Remainder of dose mainly excreted as conjugates of *p*-cresol (Bray, Craddock & Thorpe, unpublished).

Where glycine availability is a limiting factor the actual shape of the excretion curve will depend on the relative body levels of glycine and conjugating acid at any given moment. In the experiment which provided curve *C* presumably the availability of glycine was the limiting factor during the first 3 hr. but after this time the body level of *p*-fluorobenzoic acid had diminished (60 % of the dose had already been excreted) to such an extent that the availability of glycine was then no longer a limiting factor. In all the experiments in which glycine was administered simultaneously with the dose this limiting factor was eliminated with the exception of one experiment with *p*-iodobenzoic acid (see Table 1).

The results for the hydrolysis of *para* substituted benzamides are, in general, such as might have been expected from results previously obtained *in vivo* and *in vitro* with liver extracts or slices (e.g. Bray *et al.* 1950*a*). There appears to be no correlation between the extent of amide hydrolysis and the electronic nature or size of the substituent group. The $k_{b, am}$ values for amides substituted with nitro, cyano and chloro groups, which are strongly deactivating in the *para* position, are of the same order as those for amides substituted with methoxy or methyl groups, which are activating in the *para* position. It can be calculated from the k_e and k_c values (Bray *et al.* 1952*c, d*) and the 24 hr. excretion

(Bray, Ryman & Thorpe, 1947) that *p*-hydroxybenzamide, in which the hydroxyl group is strongly activating, has the very low $k_{b, am}$ value of 0.05 hr.⁻¹. The very small extent of hydrolysis of this amide made a direct determination of the velocity constant impracticable. Another amide with a strongly activating substituent, *p*-aminobenzamide, could not be examined experimentally for the same reason; this amide is extensively metabolized by conjugation with acetic acid through the amino group (Bray, Lake, Neale, Thorpe & Wood, 1948).

The values for the velocity constants for the oxidation of substituted toluenes to the corresponding benzoic acids show that there is no correlation between the extent of the oxidation and the nature of the substituent group. The velocity constant for *p*-acetotoluidide is conspicuously high. All the values of $k_{b, tol}$ are higher than that for toluene itself with the exception of that for *p*-nitrotoluene. With this compound, however, only about 30 % of the dose was accounted for. With *p*-methoxytoluene, in experiments without added glycine, about 25 % of the dose was excreted as conjugates of phenols, mainly *p*-cresol (Bray, Craddock & Thorpe, unpublished). The value of $k_{b, tol}$ for *p*-cresol, calculated from 24 hr. values (Bray, Thorpe & White, 1950*b*) and assuming k_e and k_c values are the same as those of phenol, is 0.08 hr.⁻¹.

From the compounds which have been examined in this investigation it would appear that the effect of *para* substituents upon the velocity constants for reactions involving the formation or conjugation of benzoic acids is not related to the size or electronic influence of the substituent group. This is in contrast to the effect of *para* substituents upon the velocity constants for the conjugation of phenols with glucuronic acid where it was found that small substituents had no significant effect on the value of k_e but large groups diminished this value.

SUMMARY

1. The kinetics of the conjugation of various *para* substituted benzoic acids with glycine and glucuronic acid, the oxidation of *para* substituted toluenes and the hydrolysis of *para* substituted benzamides have been investigated in the rabbit.

2. All the processes studied followed first-order reaction kinetics with respect to the administered compound except the glycine conjugation of *p*-methoxy-, *p*-iodo-, *p*-chloro-, *p*-fluoro- and *p*-methylbenzoic acids, administered as such under conditions of glycine inadequacy, when zero-order kinetics were followed.

3. Values for the velocity constants are given.

4. No correlation could be found between the magnitude of the velocity constants and the electronic nature or the size of the substituent groups.

Fig. 2. Typical curves for excretion of glycine conjugates. The compound and the dose administered to the rabbit are given in parentheses: A, exponential curve $k_h = 0.13$ (*p*-cyanobenzoic acid, 1 g. + glycine, 4 g.); B, exponential curve $k_h = 0.21$ (anisic acid, 1 g. + glycine, 4 g.); C, a curve which is initially linear but after 3 hr. is exponential, $k_h = 1.2$ (*p*-fluorobenzoic acid, 1 g.); D, a curve which is linear until excretion is almost complete (*p*-fluorobenzoic acid, 1 g.).

REFERENCES

- Bray, H. G., Clowes, R. C., Thorpe, W. V., White, K. & Wood, P. B. (1952a). *Biochem. J.* **50**, 583.
Bray, H. G., Humphris, B. G., Thorpe, W. V., White, K. & Wood, P. B. (1952b). *Biochem. J.* **52**, 412.
Bray, H. G., Humphris, B. G., Thorpe, W. V., White, K. & Wood, P. B. (1952c). *Biochem. J.* **52**, 416.
Bray, H. G., Humphris, B. G., Thorpe, W. V., White, K. & Wood, P. B. (1952d). *Biochem. J.* **52**, 419.
Bray, H. G., James, S. P., Thorpe, W. V. & Wasdell, M. R. (1950a). *Biochem. J.* **47**, 294.
Bray, H. G., Lake, H. J., Neale, F. C., Thorpe, W. V. & Wood, P. B. (1948). *Biochem. J.* **42**, 434.
Bray, H. G., Ryman, B. E. & Thorpe, W. V. (1947). *Biochem. J.* **41**, 212.
Bray, H. G., Thorpe, W. V. & White, K. (1950b). *Biochem. J.* **46**, 275.
Bray, H. G., Thorpe, W. V. & White, K. (1951). *Biochem. J.* **48**, 88.
Bray, H. G., Thorpe, W. V. & White, K. (1952e). *Biochem. J.* **52**, 423.
Hearon, J. Z. (1952). *Physiol. Rev.* **32**, 499.
Noller, C. R. (1933). *Org. Synth.* **13**, 94.
Quick, A. J. (1932). *J. biol. Chem.* **96**, 83.

The Hydrolysis of *n*-Hexanamide by Rabbit-liver Extracts

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Previous papers from this laboratory (Bray, James, Ryman & Thorpe, 1948; Bray, James, Raffan, Ryman & Thorpe, 1949a; Bray, James, Thorpe, Wasdell & Wood, 1949b; Bray, James, Thorpe & Wasdell, 1950) have given a general account of the properties of an enzyme of rabbit liver which can hydrolyse the carbamoyl groups of a number of aliphatic and aromatic amides. This amidase appeared to be different from the more specific amidases (e.g. glutaminase), and results obtained *in vitro* were in many cases comparable with those found when the corresponding amides were administered to rabbits. Since the enzyme appeared to catalyse a simple reaction and it was active towards many readily accessible substrates, the kinetics of its action seemed worthy of study. In this paper an account is given of the hydrolysis of *n*-hexanamide, which can be regarded as a typical aliphatic amide.

EXPERIMENTAL

Materials. *n*-Hexanamide was purchased (British Drug Houses Ltd.) and was recrystallized once from water before use, m.p. 101°. The ω -phenyl derivatives of propionic, *n*-butyric, *n*-valeric and isohexanoic acids were prepared as previously described (Bray *et al.* 1950). Other carboxylic acids were purchased. Melting points of all the acids agreed with those stated in the literature.

Estimation of ammonia. The ammonia liberated by the action of the enzyme was estimated by microdiffusion (cf. Conway, 1939). The sample for analysis (1 ml.) was placed in the outer chamber of a Conway unit which contained saturated aqueous KBO₂ (1 ml.). The inner chamber contained 1 ml. of a solution of boric acid (0.5% in 20% (v/v) ethanol) which also contained bromocresol green (0.0033%)

and methyl red (0.0066%). The unit was sealed with tragacanth gum adhesive (Conway & O'Malley, 1942) and left at ordinary temperature for 2 hr. The contents of the centre chamber were then titrated to a pink colour with 0.01N-H₂SO₄. In control experiments recoveries of known amounts of ammonia ranged from 91.4 to 101.0%, the average recovery in seventeen experiments being 95.5%. *n*-Hexanamide was not significantly hydrolysed by KBO₂ under these conditions and the presence of the compounds used as inhibitors was without effect.

Preparation of digests. Rabbit-liver extract (1 ml.), prepared as described previously (Bray *et al.* 1948), was added to substrate contained in 5 ml. of buffer of appropriate pH. The buffer was a solution 0.06M with respect to KH₂PO₄, H₃BO₃, HCl and acetic acid, and was adjusted to the required pH by addition of 0.3N-NaOH (cf. Britton, 1942). Carboxylic acids used as inhibitors were adjusted to the appropriate pH by addition of NaOH.

The conditions were essentially as used previously, but two points require further comment. Bray *et al.* (1948) obtained preliminary evidence that dialysis had no effect upon the activity of the enzyme in liver extracts. These experiments have now been confirmed and extended (unpublished results). This suggests that no diffusible cofactor is involved in the action of the enzyme and, from a practical standpoint, implies that the effect of dilution is straightforward. It was also observed that the enzyme had little activity in salt-free solution. This has not been investigated further, but is of considerable theoretical interest. In this connexion it may be recalled that Bray *et al.* (1949a) showed that the substitution of a veronal for a phosphate buffer considerably reduced the activity of the enzyme towards *p*-nitrobenzamide.

Plan of experiments. Before mixing, the components of the digests were warmed to the temperature of the thermostat bath (37.2 ± 0.2° unless stated otherwise) and the time of mixing was taken as zero time for the experiment. Samples (1 ml.) were withdrawn at intervals and transferred to Conway units for estimation of ammonia. The