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Kinetic Studies of the Metabolism of Foreign Organic Compounds

1. THE FORMATION OF BENZOIC ACID FROM BENZAMIDE, TOLUENE, BENZYL ALCOHOL AND BENZALDEHYDE AND ITS CONJUGATION WITH GLYCINE AND GLUCURONIC ACID IN THE RABBIT

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Investigations carried out in this laboratory in recent years (summarized by Thorpe, 1950) have dealt with the factors determining the metabolic pathways followed by foreign aromatic compounds when several such pathways are available. While it is to be expected that all possible reactions will occur simultaneously, the proportions of the dose which actually follow the various routes differ greatly. Many factors may theoretically contribute to these differences, e.g. the efficiency of the necessary enzyme systems, the availability of conjugating molecules (e.g. glycine) and the availability of free energy for synthetic reactions. All such factors, taken together, determine the velocity of each individual reaction in the intact animal, so that knowledge of the relevant velocity constants is likely to prove of value in considering the detailed metabolism of foreign organic compounds. Since such information does not appear to be available in the literature we have planned a series of investigations in which we hope to compare the velocities of typical metabolic reactions which foreign organic compounds may undergo in the rabbit. In this first investigation the formation of benzoic acid from various precursors and its conjugation with glycine and glucuronic acid have been studied. The method consisted essentially of analysis of the individual urine samples passed by rabbits which had received the compounds under investigation. The metabolites estimated were ether-soluble acid (in some experiments fractionated into benzoic and hippuric acids) and ester glucuronide.

When a compound is administered to a rabbit it may undergo several consecutive processes, e.g. absorption, conversion of potential centre (see Bray, Ryman & Thorpe, 1948), conjugation and excretion. Preliminary experiments showed that absorption appeared to be virtually complete within about 2·5 hr., so that if measurements were made only on those portions of the excretion curves corresponding to times after this, the effect of absorption could be neglected. For the rest it was assumed that the curves obtained would represent the slowest process of the series. Thus if the amount of the compound administered is the only limiting factor then it would be expected that the reaction being studied would be of the first order.

METHODS

Animals, diet and dosage. Rabbits of not more than 2 kg. weight were used, since larger animals were liable to pass urine samples greater in volume than the capacity of the receivers used (140 ml.). They were maintained on the constant diet of rabbit pellets and water customary in this laboratory (Bray, Ryman & Thorpe, 1947). The compounds were administered by stomach tube as solutions or suspensions in water.

Collection of urine samples. For this purpose a fraction collector was constructed. It consisted of a brass turntable which carried round its periphery forty-eight receiving tubes each of capacity 140 ml. Its movement was weight-activated and controlled by an escapement operated by an electromagnet activated by impulses from a timing clock. In this way the receiver could be changed automatically at predetermined time intervals ranging from 1 min. to 1 hr. The rabbit was housed in a metabolism cage fitted into a tinned-copper collecting funnel of the usual type, the spout of which was connected to a glass tube delivering into the receivers beneath.

Estimation of metabolites. Ether-soluble acid was determined as previously described (Bray, Neale & Thorpe, 1946), fractionation being achieved by extraction of ether-soluble material with light petroleum (b.p. 40–60°) using three successive volumes of 25 ml. The method was adapted for use on blood samples (2 ml.). These were collected from a marginal ear vein directly into H_2SO_4 (15 ml., 0·083 N) and subsequently made up to 25 ml. with acid of the same strength. A sample of this (24 ml.) was transferred to an extractor and the protein precipitated by means of sodium tungstate (3 ml., 10% w/v). The resulting mixture was allowed to stand for 5 min., further acidified by the addition of 2 N-H_2SO_4 (5 ml.) and continuously extracted with ether for 6 hr. The ether-soluble acid was titrated with NaOH (0·01 N). Fractionation was carried out as for urinary acid. Control experiments showed that benzoic and hippuric acids added to normal rabbit blood (100–500 mg./100 ml. blood) could be recovered by this method to extents of 101 and 105%, respectively (ranges 96–109% and 100–109%, respectively). Ester glucuronide in urine was estimated by the reducing method previously described (Bray *et al.* 1946).

Plan of experiments. The overall plan was similar to that used in most metabolic investigations on the intact animal. A 'baseline' was determined during the day before the main experiment. Since the ingestion of food was followed by a sudden increase in the baseline value the experiments were carried out on fasting animals, beginning at approx. 10.30 a.m. The food ration was given at approx. 7 p.m. when most of the administered compound had been excreted. The importance of a constant food ration, adequate yet small enough to be ingested almost immediately, is obvious. Rabbits normally urinate only once or twice during the day time, and in order to obtain a sufficient number of urine samples during the experiment water (100 ml.) was given three or four times in the course of the day. Occasionally a rabbit passes a small urine sample when the bladder is full, shortly before the main sample. This leads to an error in the corresponding point, but since the values plotted are cumulative this is corrected in the next point (e.g. see Figs. 1 and 3, points at 5 hr. 20 min.). Two or three blood samples were withdrawn during the experiment. It will be seen from Table 1 that in some experiments glycine was given with the compound under investigation. Griffith & Lewis (1923) found that the average rate of excretion of conjugated benzoic acid by the rabbit on a normal diet was 68·7 mg./hr./kg. and after administration of glycine (3 equiv.) 111·7 mg./hr./kg. Consequently, we administered glycine (usually 3–5 equiv.) with the precursor in experiments in which the dose was relatively large to obtain rapid formation and excretion of hippuric acid and to prevent the accumulation of benzoic acid in the blood.

RESULTS

The treatment of the experimental results is explained by means of the protocols and calculation of the results of two typical experiments. The experiments can be divided into two groups: (1) the formation and conjugation of benzoic acid formed from administered toluene, benzyl alcohol, benzaldehyde and benzamide, and (2) the conjugation of benzoic acid administered as such. It should be noted that results have been expressed as benzoic acid throughout.

(1) *Exp.* 23, *rabbit no.* 301

Day 1. Baseline determined. Total excretion of ether-soluble acid during 23 hr., 399 mg. benzoic acid.

Day 2. Dose toluene (1·3 g.) + glycine (3 g.), at 11.10 hr. Water (100 ml.) given at 11.10, 12.30, 15.30 and 16.50 hr. Urine samples passed at 11.10, 14.15, 16.30, 16.50, 17.50, 19.45, 03.10 and 09.00 hr. Blood samples taken at 12.30 and 15.30 hr.

No increase was observed in the ether-soluble acid content of either sample of blood, and there was no increase in the ester glucuronide content of the urine throughout the experiment. The curves in Fig. 1

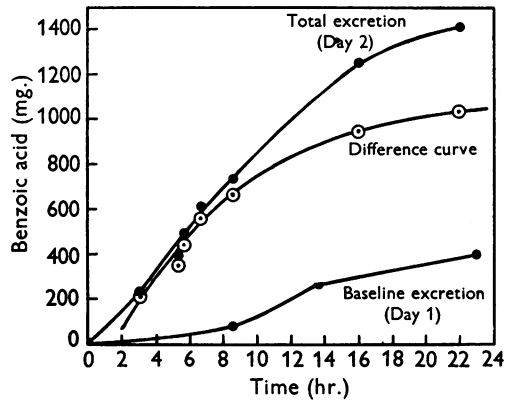

Fig. 1. Excretion of ether-soluble acid (expressed as benzoic acid) by rabbit no. 301 after administration of toluene (1·3 g.).

show the excretion of ether-soluble acid (expressed as benzoic acid) on days 1 and 2 and the 'difference curve', i.e. the baseline values obtained on day 1 have been subtracted from the results of day 2 and so represent the excretion of ether-soluble acid (expressed as benzoic acid), derived from the toluene administered.

Calculation of results

If the conversion of the precursor to benzoic acid is a first order reaction, then

$$db/dt = k_b a, \quad (1)$$

where a and b are the amounts of precursor and benzoic acid in the body, and k_b the velocity constant for formation of benzoic acid. If the precursor undergoes other processes in addition to the formation of benzoic acid, and assuming a first-order mechanism for these, then

$$-da/dt = Ka, \quad (2)$$

where K is the velocity constant for total excretion by all routes. Thus

$$k_b/K = E_b/E = E_{max.}/\text{dose}, \quad (3)$$

where E_b is the amount of benzoic acid (and its conjugates) excreted in a given time, E the total amount

of precursor in all its forms excreted in the same time, and $E_{max.}$ the final amount of benzoic acid (and its conjugates) excreted. Therefore

$$k_b/K = (E_b - E_{max.})/(E - \text{dose}). \quad (4)$$

Let $$E_{max.} - E_b = a_b$$

(i.e. the amount of toluene in the body which is destined for oxidation to benzoic acid). Then from (4), $k_b = Ka_b/a$, since dose $- E = a$. Hence

$$db/dt = Ka_b. \quad (5)$$

Integration of equation (5) gives

$$K = (\ln a_{b_1} - \ln a_{b_2})/(t_2 - t_1).$$

The total excretion E_b (free and conjugated benzoic acid, expressed as benzoic acid) is plotted against time (t) to give the 'difference' curve as shown in Fig. 2. An approximate asymptote ($E_{max.}$), representing the total excretion of toluene as benzoic acid

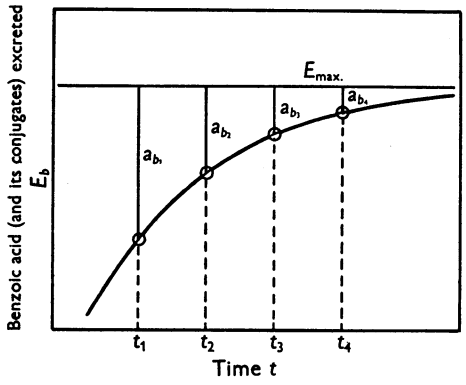

Fig. 2. Determination of a_b values from 'difference' curve (diagrammatic). ⊙ = Point determined by experiment.

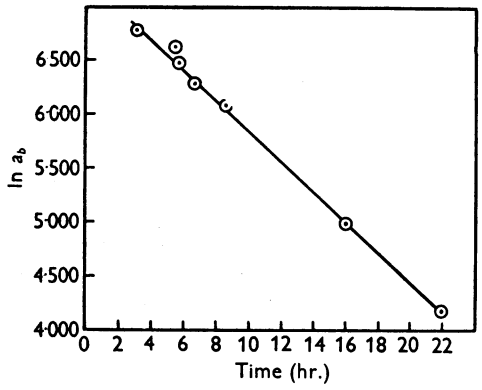

Fig. 3. Values of ln a_b for rabbit no. 301 plotted against t.

and its conjugates, is derived using the property of exponential curves whereby equal percentages of the amount of precursor remaining in the body are converted in equal periods of time. Values of a_b, i.e. ($E_{max.} - E_b$), corresponding to various times, t_1, t_2, t_3, etc., i.e. a_{b_1}, a_{b_2}, a_{b_3}, etc., are determined and ln a_b plotted against t as in Fig. 3. For a first-order reaction this graph should be linear, and by a process of successive approximations the value for $E_{max.}$ is determined which will in fact fulfil this condition. The slope of this line is $-K$. By then drawing the graph of E_b and t derived from K the fit of the experimentally determined points can be ascertained. In most experiments these conditions were fulfilled within reasonable experimental error. The following values were obtained by this treatment of the results of Exp. 23: $K = 0{\cdot}14$ hr.$^{-1}$; $E_{max.} = 1090$ mg. benzoic acid (= 63·5 % of dose) and

$$k_b = 0{\cdot}14 \times 63{\cdot}5/100 = 0{\cdot}09 \text{ hr.}^{-1}.$$

In the case of toluene a part of the dose is known to be eliminated via the lungs and approximately 60 % is converted to benzoic acid. Since the experimental results fit an exponential curve it follows that elimination of the other 40 % is according to a first-order reaction.

Application to other precursors of benzoic acid. If excretion unchanged takes place, as in the expiration of toluene, k_b may be calculated from K as in the above example, e.g. if 63·5 % of the dose is excreted as benzoic acid and its conjugates, then $k_b = 0{\cdot}635K$.

If modification of the potential centre for conjugation takes place before excretion in some form not estimated, then K may be taken as the velocity constant for the conversion of the potential centre (k_b). If the unknown processes are not of the first order, the error involved in the above assumption will be reflected in the excretion curves and the 'fit' with the theoretical curve will be correspondingly less close. It will be seen from Table 1 that approximately 70–90 % of doses of the compounds fed (with the exception of toluene) were in fact excreted as benzoic acid and its conjugates. Since the fate of the remainder is uncertain K is taken as k_b and any error incurred will be relatively small.

(2) *Exp.* 1, *rabbit no.* 309

Day 1. Baseline determined. Total excretion of ether-soluble acid in 5·7 hr. = 75 mg. (as benzoic acid). Total excretion of reducing material in 5·7 hr. = 17 mg. (as benzoic acid).

Day 2. Dose sodium benzoate (1·25 g.) at 09.00 hr. Water (100 ml.) at 09.00, 10.25, 12.20, 14.30 hr. Urine samples passed at 09.45, 10.25, 11.35, 11.55, 12.25, 12.50, 13.25, 14.05, 15.10, 16.10 hr. Blood samples taken at 11.35, 12.50, 15.10 hr.

The 'difference' excretion curves for ether-soluble acid and for glucuronide are shown in Figs. 4 and 5.

Blood analyses showed that during the experiment approximately 14 % of the free benzoic acid in the body was present in the blood, but no rise in the blood hippuric acid level was observed. (The blood

volume was taken as 6·7 % of the body weight, on the basis of four determinations on rabbits made for us by Dr M. E. Nutt and Miss D. M. Jackson by the dye (T-1824) method of Courtice & Gunton, 1949.)

The formation of hippuric acid took place at a constant rate (k_h), so that further mathematical treatment of these results is unnecessary. The formation of glucuronide can now be considered in detail.

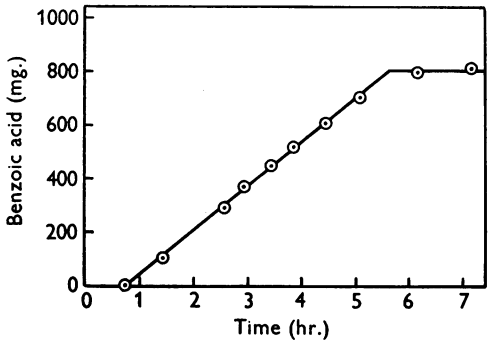

Fig. 4. 'Difference' excretion curve for ether-soluble acid (expressed as benzoic acid) for rabbit no. 309 after administration of sodium benzoate (1·25 g.).

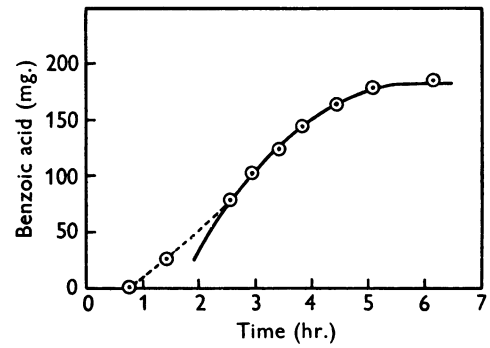

Fig. 5. 'Difference' excretion curve for glucuronide (expressed as benzoic acid) for rabbit no. 309 after administration of sodium benzoate (1·25 g.).

For a first-order reaction $dg/dt = k_g b$, where b is the total amount of benzoic acid present in the body, g the amount of glucuronide excreted and k_g the velocity constant for formation of benzoylglucuronide. The total excretion curve of E_b and t (E_b = ether-soluble acid and glucuronide) was plotted and also the glucuronide excretion curve of g and t.

Tangents to the second curve (dg/dt) were determined and plotted against b, which was determined from the first graph as the difference between the total excretion of benzoic acid ($E_{max.}$) and E_b. The slope of this line is k_g. The 'fit' of the curve (g, t) derived from k_g was checked with the experimental results. In Exp. 1 this mathematical treatment gave $k_g = 0{\cdot}11$ hr.$^{-1}$; $k_h = 168$ mg. hr.$^{-1}$.

Summary of results

Relevant results from all the experiments carried out are summarized in Table 1. It may be seen from the final column of Table 1 that straight-line excretion curves were sometimes obtained for hippuric acid after the administration of precursors as well as for benzoic acid itself. This happens when the initial rate of conversion of the precursor is greater than the maximum rate of glycine conjugation (see also Discussion, p. 94). Where these rates are approximately equal, small variations in k_h may be critical and either exponential curves or initially straight lines may be obtained. When the initial rate of conversion of the precursor exceeds the maximum rate of glycine conjugation benzoic acid would be expected to accumulate in the blood. This occurred in Exps. 17 and 18 with benzyl alcohol. In six experiments where benzoic acid was administered as sodium benzoate it was found that in fourteen determinations an average of 12 % (range 6–24 %) of the benzoic acid in the body was present in the blood. We made no attempt to determine the amount of glucuronide in the blood, owing to the practical difficulty of estimating small amounts in the presence of relatively large amounts of other reducing substances. Csonka (1924), however, found no increase in reducing material in the blood of the pig in 5 hr. following the administration of benzoic acid, although benzoylglucuronide was excreted during the first 6 hr. It may, therefore, be suggested that rapid excretion of benzoylglucuronide might also occur in the rabbit.

It has been shown that velocity constants of enzymic reactions increase with increasing temperature (see Wilson, 1949). We did not determine the body temperature of our experimental animals and it may, in fact, have varied, since Griffith (1938) observed that the administration of benzoic and hippuric acids to rats caused a fall in body temperature. It may be significant that vasoconstriction was observed in the ears of some animals after giving benzoic acid. While we found some variation in the individual values for k for each reaction, most variation occurred in the values found for benzoylglucuronide formation, which were much greater than could be accounted for by experimental error.

DISCUSSION

Conjugation and excretion of benzoic acid

Hippuric acid formation takes place at a constant rate and the acid is excreted immediately up to a limiting rate which appears to vary considerably between different rabbits. In two experiments (Exps. 4 and 5, Table 1), in which hippuric acid was found in the blood after feeding benzoic acid and glycine, values of k_h of 270 and 480 mg. hr.$^{-1}$ (as

Table 1. *Kinetics of formation and conjugation of benzoic acid in the rabbit*

Exp. no.	Rabbit no.	Dose (g.)*	Percentage of dose accounted for (*A*)	Percentage of *A* excreted as: Glucuronide	Ether-soluble acid	Hippuric acid	Benzoic acid	k_g (hr.$^{-1}$)	k_h (mg. hr.$^{-1}$)	k_b (hr.$^{-1}$)	Remarks (see footnote)
						Sodium benzoate					
1	309	1·25	95	19	81	78	3	0·11	168	—	*b, d*
2	309	1·25	92	16	84	83	1	0·09	163	—	*b, d*
3	263	0·84	81	14	86	86	0	0·05	115	—	*b, d*
4	301	3·0 (2)	108	12	88	87	1	0·08	480	—	*a, b, d*
5	302	2·5 (4)	72	—	100	—	—	—	270	—	*a, b, d, e, h*
6	299	1·25	76	—	100	—	—	—	142	—	*b, e, d, h*
							Average	**0·08**			
						Benzamide					
7	288	0·8	88	5	95	—	—	—	—	0·30	*b, f*^{5}*, g*
8	263	0·8	67	6	94	—	—	—	—	—	*b, e, f*^{7}
9	301	0·8 (5)	69	0	100	—	—	—	—	0·35	—
10	304	0·8 (5)	86	2	98	—	—	—	—	0·32	*f*^{2}
11	308	0·7 (6)	84	0	100	—	—	—	—	0·29	—
									Average	**0·32**	
						Benzaldehyde					
12	301	1·0	90	5	95	—	—	—	—	0·30	*b, f*^{6}
13	262	1·5 (3)	98	0	100	—	—	—	—	0·20	—
14	299	1·0 (4)	80	0	100	—	—	—	—	0·50	—
15	309	1·0 (4)	86	1	99	—	—	—	—	—	*e*
16	299	0·5 (8)	87	0	100	—	—	—	—	—	*e*
									Average	**0·33**	
						Benzyl alcohol					
17	306	1·0	82	24	76	—	—	—	—	0·80	*b, c, d*
18	306	1·6 (3)	79	9	91	—	—	—	—	0·90	*b, c, d*
19	301	0·5 (8)	52	3	97	—	—	—	—	1·00	*f*^{2}
20	302	0·5 (4)	84	2	98	—	1	—	—	1·00	*f*$^{2\cdot5}$
							Average of Exps. 19 and 20			**1·00**	
						Toluene					
21	279	1·3	45	0	100	—	—	—	—	0·16	—
22	299	1·0	63	0	100	—	—	—	—	0·11	—
23	301	1·3 (3)	64	0	100	—	—	—	—	0·09	—
24	301	1·3 (3)	58	0	100	—	1	—	—	0·09	—
25	303	2·6	40	4	96	—	—	—	—	—	*e, h*
									Average	**0·11**	

* Glycine administered (equivalents) shown in brackets.

a Hippuric acid was shown to be present in the blood during the experiment.
b Hippuric acid excretion curve was initially linear.
c The fact that the excretion curve was initially linear made it impossible to calculate k_b from the experimental results by the general method described in the text. An approximate value for k_b was calculated from the observed glucuronide values using equation (9) of the appendix.
d Benzoic acid was shown to be present in the blood during the experiment.
e Velocity constants were not calculated owing to insufficient data.
f^n Glucuronide was excreted during first n hr.
g Value for k_b calculated in usual way but only approximate since hippuric acid excretion curve initially linear.
h Experiment concluded before completion of excretion.

benzoic acid) were found, assuming that hippuric acid was present only in blood and urine. For the three rabbits used in Exps. 1–3 and 6, in which benzoic acid alone was given, the average rate of formation and excretion of hippuric acid was 147 mg./hr. This agrees with the work of Griffith & Lewis (1923) and Csonka (1924), which suggests that the availability of glycine controls the rate of hippuric acid formation. Griffith & Lewis found that the dose of glycine giving a maximum rate of excretion of hippuric acid was approximately 3 equiv., but it should be noted that no determinations have been made of the maximum rate of hippuric acid formation.

The results given in this paper show that the conjugation of benzoic acid with glucuronic acid follows the kinetics of a first-order reaction with a velocity constant of 0·08 hr.$^{-1}$. It will be seen from Table 1 that the percentage of a dose of benzoic acid excreted conjugated with glucuronic acid depends on the dose level and whether or not glycine is administered with the dose. In a previous paper (Bray, Humphris & Thorpe, 1949), it was shown by a comparison of results obtained with benzoic acid and benzamide, and with the toluic acids, toluamides and xylenes, that the highest percentages of glucuronide conjugation were observed when the parent acids were fed, presumably because they gave rise to higher blood levels than did the administration of precursors. Glycine conjugation appeared to be affected to a much smaller extent. It was suggested that at higher blood levels the glycine conjugation mechanism was overwhelmed and that glucuronide conjugation was in the nature of a 'shock mechanism'. It is clear, however, from the results of the present investigation, that this is only partially true, at least for benzoic acid and its precursors. The rate of hippuric acid formation is independent of the amount of benzoic acid in the body probably for amounts down to 25 mg./kg. (by analogy with man, from data of Quick, 1931). If the amount of benzoic acid present in the body is sufficiently small, the rate of glycine conjugation will be proportional to it and the kinetics of the conjugation would show a change to those of a first-order process. These conditions would be expected to prevail when the rate of conversion of a benzoic acid precursor is less than the maximum rate of glycine conjugation, i.e. when the excretion of hippuric acid follows an exponential curve (see later section on conversion of potential centres). Under these conditions no significant glucuronide excretion was observed, but, making allowance for analytical error, as much as 3 % of the amount of the dose excreted may have been present as glucuronide. Thus a minimum value for the velocity constant for hippuric acid may be deduced, as follows: The ratio of glycine conjugation to glucuronide conjugation is 97 : 3; therefore, since $k_g = 0{\cdot}08$, the velocity constant for hippuric acid formation must be greater than $0{\cdot}08 \times 97/3 = 2{\cdot}6$. Glucuronic acid conjugation is proportional to the amount of benzoic acid in the body. Thus an increase in the percentage of the acid excreted conjugated with glucuronic acid is a necessary consequence of an increased blood level. It should be noted that the term 'blood level' is used in its widest sense, since we are not in a position to decide whether or not the relation between the true blood level and the total amount present in the body is linear. The blood level will depend on the magnitude of the dose of benzoic acid itself or on the dose level and rate of conversion of benzoic acid precursors. It is hoped to investigate the application of these considerations to toluic acid and its precursors.

It has been found that there are considerable differences in the proportions of benzoic acid conjugated with glucuronic acid and glycine by different species. There is also a species difference in the rate of hippuric acid formation (for summary see Williams, 1947). Several suggestions have been put forward to account for the former. Thus Quick (1932) proposed immediate and complete conjugation of glucuronic acid in the dog, followed by partial hydrolysis and glycine conjugation. It can now be seen, however, that the species difference in the glucuronide : hippuric acid ratio can be accounted for solely in terms of the differences in rate of hippuric acid formation. Quick (1931) calculated that the approximate rates of glycine mobilization for man, dog, pig and rabbit were 9·0, 3·5, 15 and 24 mg./kg./hr., respectively. From these values the theoretical percentages of benzoic acid conjugated with glucuronic acid can be calculated, using the expressions (3), (7) and (9) derived in the Appendix, p. 95, taking

Table 2. *Species differences in the degree of conjugation of benzoic acid with glucuronic acid*

Species	Dose level (g./kg.)	Percentage of dose excreted as glucuronide		Rate of glycine mobilization (mg./kg./hr.)
		Calc.	Found	
Dog	0·45	68	66*	3·5*
Man	0·043	9	5†	9·0*
Pig	0·50	41	31‡	15·0*
Rabbit	0·50	24	19§	50·0§

* Quick (1931) † Unpublished results. ‡ Csonka (1924). § Present paper.

the velocity constant for glucuronide formation to be 0·08 as determined in this investigation for the rabbit. The results are given in Table 2. The value for k_h for the rabbit would appear to vary considerably in different individuals. For our present purpose we have used a value of 50 mg. glycine/kg./hr. which we observed for the rabbit (no. 309) used in the actual experiment. It can be seen that there is good agreement between the theoretical and experimentally determined values. It may be noted that some confusion has previously been caused (e.g. Quick, 1932) by the fact that man excretes benzoic acid almost entirely as hippuric acid and the dog mainly as glucuronide, although the rate of mobilization of glycine in both species is low compared with the pig and the rabbit. The difference in dose level accounts for this, since benzoic acid is normally administered to man at a very much lower dose level than to other animals.

Conversion of potential centres

It has been shown that the processes of conversion of benzamide, toluene and benzyl alcohol to benzoic acid follow the kinetics of first-order reactions with velocity constants 0·32, 0·11 and 1·0 respectively. An approximate value for benzaldehyde is 0·33. When the formation of benzoic acid takes place at a rate slower than that at which hippuric acid formation can occur (e.g. Fig. 1 in which the curve showing excretion of hippuric acid (expressed as benzoic acid) with time is exponential) the amount of glucuronide formed is insignificant. Where the rate of benzoic acid production exceeds the maximum rate of its conjugation with glycine (k_h) (i.e. when graph of hippuric acid excretion and time is initially linear) glucuronide formation occurs until the level of benzoic acid falls below that at which maximum glycine conjugation takes place (e.g. Exps. 7, 8, 12, 17 and 18 in Table 1).

The rate of benzoic acid formation is determined by the dose level and the velocity constant of the conversion process. The percentage of the dose excreted as glucuronide depends on the blood level of benzoic acid. Thus in summary it may be stated that the relative amounts of the metabolites of the benzoic acid precursors may be expressed as a function of velocity constants and the dose level at which the precursors are administered (see Appendix and Fig. 6).

Oxidation of benzaldehyde

The velocity constants for this process given in Table 1 are maximum values and approximate. Although the analytical results fitted an exponential curve when the graphical treatment previously described was applied, the deviation was greater than for the other reactions studied. A possible reason for this is the exceptional susceptibility of benzaldehyde to atmospheric oxidation. The substance was distilled *in vacuo* immediately before administration, but an appreciable amount may have been oxidized in the gut before absorption. Since the amount of glucuronide formed was negligible in Exps. 13, 14, 15 and 16, and was taken into account in calculating k_b for Exp. 12 (see Appendix, Fig. 6) it seems likely that the values for k_b are not greatly in error.

Mechanism of toluene oxidation

It is generally assumed that oxidation of the methyl group of toluene takes place as follows:

$$—CH_3 \rightarrow —CH_2OH \rightarrow —CHO \rightarrow —COOH.$$

The results presented above show that the velocity constant for the oxidation of benzyl alcohol is 1·0, while that for benzaldehyde is only 0·33. Thus it is unlikely that both compounds can be intermediate stages in the oxidation of toluene to benzoic acid. Direct evidence has been obtained by Jaffé (1878–9) and Bray, Thorpe & Wood (1949) that benzyl alcohol derivatives are intermediate stages in the oxidation of substituted toluenes, so that it seems reasonable to suggest that benzaldehyde as such is not an intermediate. It has been suggested that hydrate formation, followed by glucuronide conjugation, may precede oxidation or reduction of aldehydes (e.g. Lehmann & Knoefel, 1938; Sammons & Williams, 1946). If hydrate formation occurs with benzaldehyde it is unlikely that conjugation of the hydrate with glucuronic acid takes place, since it is then difficult to explain the observed metabolic results without suggesting a system of reactions for conversion of the conjugated hydrate into hippuric acid, for which there is no experimental evidence.

APPENDIX

When benzoic acid or a precursor is fed, a variable proportion of the dose is excreted as benzoylglucuronide. The amount formed may be expressed as a function of velocity constants and the dose level. In this appendix a general equation is derived. It is assumed that free benzoic acid and a precursor are introduced instantaneously into the body and that the precursor is transformed by a first-order reaction to benzoic acid; benzoic acid is conjugated with glucuronic acid according to a first-order reaction and with glycine at a constant maximum rate k_h. When no free benzoic acid is present and the rate of conversion of the precursor is less than k_h, it is assumed that no glucuronide is formed (see p. 93). A correction for the time during which absorption occurs in actual practice is derived and shown to be relatively small except for very small dose levels.

Symbols. a = amount of precursor in the body at time t; A = amount of precursor in the body at time $t=0$; b = amount of benzoic acid (free and conjugated) in the body at time t; B = amount of benzoic acid (free and conjugated) in the body at time $t=0$; m = amount of conjugated benzoic acid in the body at time t; g = amount of benzoylglucuronide in the body at time t; k_b = first-order reaction velocity constant for

precursor→benzoic acid; k_g=first order reaction velocity constant for benzoic acid→glucuronide; k_h=maximum rate of formation of hippuric acid. Let A precursor and B benzoic acid be introduced into the body instantaneously. Then

$$-da/dt = k_b a \quad \text{and} \quad a = Ae^{-k_b t}, \tag{1}$$

$$b = B + (A - a), \tag{2}$$

and by substitution from equation (1)

$$b = B + A(1 - e^{-k_b t}). \tag{3}$$

When $B>0$ and/or $-da/dt > k_h$

$$dm/dt = dg/dt + k_h, \tag{4}$$

and

$$dg/dt = k_g(b - m). \tag{5}$$

Substituting from equations (3) and (5) into (4)

$$dm/dt + k_g m = k_g(A + B) - k_g A e^{-k_b t} + k_h, \tag{6}$$

and on integration

$$m = \left(A + B + \frac{k_h}{k_g}\right)(1 - e^{-k_g t}) + \frac{k_g A}{k_g - k_b}(e^{-k_g t} - e^{-k_b t}). \tag{7}$$

Let the free benzoic acid in the body $(b - m)$ fall to zero at time T, i.e. $(b_T - m_T) = 0$.

Now equations (3) and (7) may be solved for T (by graph). The total amount of glucuronide formed, g_T, may be calculated:

$$g_T = m_T - k_h T = b_T - k_h T. \tag{8}$$

Substituting from equation (3)

$$g_T = A(1 - e^{-k_b T}) + B - k_h T. \tag{9}$$

Correction for time taken for absorption

Let k_{a_1}=rate of absorption of precursor $= A_0/t_a$; k_{a_2}=rate of absorption of benzoic acid $= B_0/t_a$; A_0=amount of precursor administered; B_0=amount of benzoic acid administered; t_a=duration of absorption (assumed to be same for A_0 and B_0). During absorption period

$$da/dt = k_{a_1} - k_b a \quad \text{or} \quad a = k_{a_1}/k_b\,(1 - e^{-k_b t}). \tag{10}$$

Let

$$k_{a_1} + k_{a_2} = k_a,$$

then

$$b = k_a t - a. \tag{11}$$

Substituting from (10)

$$b = k_a t - \frac{k_{a_1}}{k_b}(1 - e^{-k_b t}). \tag{12}$$

When

$$db/dt > k_h,$$

then

$$dm/dt = k_g(b - m) + k_h. \tag{13}$$

Assuming $db/dt > k_h$ initially, equation (13), substituting for b from (12), becomes

$$dm/dt + k_g m = k_g\left[k_a t - \frac{k_{a_1}}{k_b}(1 - e^{-k_b t})\right] + k_h. \tag{14}$$

On integration

$$m = \left(\frac{k_h - k_a}{k_g} - \frac{k_{a_1}}{k_b}\right)(1 - e^{-k_g t}) + \frac{k_g k_{a_1}}{k_b(k_g - k_b)}(e^{-k_b t} - e^{-k_g t}) + k_a t. \tag{15}$$

This value for m may be substituted in

$$g = m - k_h t. \tag{16}$$

At time t_a when absorption is just complete, free benzoic acid in the body $= A_0 + B_0 - a - m$, and precursor in the body $= a$. g_T may be derived by application of the same calculation as under conditions of instantaneous absorption (see above). The correction for absorption leads to lower values as shown in Table 3, but the difference between corrected and uncorrected values is only considerable at low dose levels.

Table 3. *Effect (calculated) of absorption period on formation of benzoyl glucuronide*

(Values used for calculation were: $k_g = 0{\cdot}08$ hr.$^{-1}$; $k_h = 140$ mg. hr.$^{-1}$.)

Dose of benzoic acid (g.)	Calculated percentage of glucuronide formed assuming absorption: Instantaneous	Of 2 hr. duration
2·0	33	29
1·0	21	15
0·5	12	7

Fig. 6 shows the relation between dose level and the percentage of benzoic acid conjugated with glucuronic acid after the administration of benzoic acid or a precursor. The values are derived from the expression (9) above, without

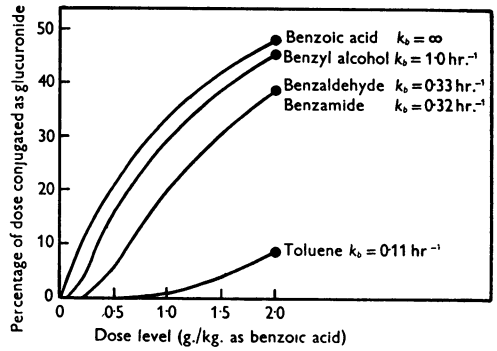

Fig. 6. Variation of glucuronide formation with dose level after administration of benzoic acid and some precursors.

correction for absorption and using the values $k_h = 140$ mg./kg./hr. and $k_g = 0{\cdot}08$ hr.$^{-1}$. In the case of toluene, allowance for excretion via the lungs of part of the dose was made in equations (1) and (2) as follows:

$$(1)\ a = Ae^{-Kt} \quad \text{and} \quad (2)\ b = B + \frac{k_b}{K}(A - a),$$

where K is a first-order reaction velocity constant for the total excretion of toluene by all routes.

SUMMARY

1. The rates of excretion of hippuric acid and benzoylglucuronide by the rabbit after administration of sodium benzoate, benzamide, toluene, benzyl alcohol and benzaldehyde have been studied.

2. The excretion of hippuric acid by rabbits which had received sodium benzoate took place at a constant rate ranging from 115 to 166 mg./hr. Administration of glycine increased this rate.

3. The formation of benzoylglucuronide follows the kinetics of a first-order reaction with velocity constant 0·08 hr.$^{-1}$. The processes of conversion of

benzamide, toluene, benzyl alcohol and benzaldehyde to benzoic acid also follow first-order reaction kinetics with velocity constants 0·32, 0·11, 1·00 and 0·33 hr.$^{-1}$ respectively.

4. Mathematical treatment of the observed kinetics gives expressions from which theoretical values can be derived which are in agreement with experimental observations.

5. The observed kinetics provide an explanation for the different proportions of benzoylglucuronide and hippuric acid excreted after administration of benzoic acid at different dose levels either as sodium benzoate or as precursors.

6. The well-known species differences in the degree of conjugation of benzoic acid with glucuronic acid is also explained.

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The Influence of 2:4-Dinitrophenol on the Oxidative Breakdown of Fatty Acids

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Certain substituted phenols have the property of increasing the oxygen uptake of tissue slices. Dodds & Greville (1933) and Elliott & Baker (1935) found that 4:6-dinitro-*o*-cresol in 10^{-5}M concentration accelerated the respiration of slices of several mammalian organs *in vitro*, but other workers found that similar compounds did not have this influence on tissue extracts (for references see Peiss & Field, 1948). Pickett & Clifton (1941) and Clifton (1946) indicated that 2:4-dinitrophenol (DNP) in suitable concentrations inhibited synthetic processes without influencing oxidation, and Hotchkiss (1944) reported that DNP prevented the uptake of inorganic phosphate by respiring yeast. Experiments carried out by Loomis & Lipmann (1948) led them to the conclusion that DNP uncoupled phosphorylation from oxidation in rabbit kidney homogenates. An increased breakdown of glycogen in muscle under the stimulus of DNP has been observed by several workers (for references see Pierce & Field, 1949). Since it is undecided whether phosphorylated compounds take part in the oxidative breakdown of fatty acids, the effect of DNP on this process has been investigated.

EXPERIMENTAL AND RESULTS

All experiments were carried out with liver slices taken from rats maintained on an adequate diet until required. The slices were incubated at 37° in Krebs-Ringer phosphate solution of pH 7·4 for 60 min., unless otherwise stated. The conventional Warburg technique was followed. In all experiments the gas phase was O_2.

Table 1 gives the average results of four experiments in which sodium octanoate was used as substrate. It will be observed that DNP increased the oxygen uptake in the absence and presence of octanoate. In order to establish whether the DNP