The Inhibition of β-Glucuronidase by Saccharic Acid and the Role of the Enzyme in Glucuronide Synthesis

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In view of the relationship shown to exist between the β-glucuronidase activity of a tissue and its state of growth (Levvy, Kerr & Campbell, 1948), it was considered important to find a specific inhibitor for this enzyme. It has been suggested (Fishman, 1940), without any direct evidence, that β-glucuronidase is responsible for the formation of glucuronides in the body. The use of an inhibitor for glucuronidase in testing this hypothesis forms an obvious first step towards elucidating the physiological function of the enzyme.

A variety of substances have been examined for their effect on the hydrolysis of phenylglucuronide by β-glucuronidase. Of those which caused inhibition, by far the most effective was D-glucosaccharic acid, and this compound was examined for its action on glucuronide synthesis by liver slices and on growth processes in the mouse.

EXPERIMENTS AND RESULTS

Determination of β-glucuronidase. The hydrolysis of phenylglucuronide by mouse-liver or kidney glucuronidase preparations was measured by the procedure of Kerr, Graham & Levvy (1948). In testing substances for a possible inhibitory action on the enzyme, incubation mixtures were made up as follows: 0·4 ml. enzyme preparation, 0·2 ml.

Table 1. *Inhibition of β-glucuronidase* in vitro (0·015 M-*phenylglucuronide*)

Compound	Concentration (10^{-4} M)	Phenol liberated: In controls (μg.)	Phenol liberated: In presence of inhibitor (μg.)	Inhibition (%)	Enzyme preparation
Saccharic acid	150	32·1	3·3	90	Liver
	150	25·4	4·2	84	,,
	150	17·1	3·2	81	,,
	50	39·1	8·0	80	,,
	50	32·7	6·5	80	,,
	50	27·0	6·5	76	,,
	50	25·5	7·9	69	,,
	50	21·2	3·2	85	,,
	50	16·5	5·8	65	,,
	50	20·5	4·6	78	Liver *A*
	50	22·8	3·4	85	Liver *B*
	50	22·5	0·5	98	Crude liver
	50	11·8	3·8	68	Kidney
	50	21·2	6·5	70	,,
	50	17·0	5·7	67	Crude kidney
Mucic acid	75	30·2	23·6	22	Liver
D-Gluconic acid	150	37·4	28·8	23	,,
D-Glucurone*	300	40·0	0	100	,,
	150	35·3	12·1	66	,,
	38	55·2	32·4	41	,,
	10	55·2	45·2	18	,,
	3·3	55·2	51·2	7	,,
L-Malic acid†	150	23·7	14·9	37	,,
DL-Malic acid	300	32·9	18·6	44	,,
	150	30·2	23·5	22	,,
Phlorrhizin‡	3	16·3	13·6	17	,,
	1·5	16·3	16·8	−3	,,
Vanillin‡	7·5	39·4	28·8	27	,,

* Interferes in colour reaction for phenol. Results are corrected for interference.
† The naturally occurring isomer, commonly called laevorotatory malic acid.
‡ Gives colour with phenol reagent. Results are corrected for this colour.

0·1 M-citrate buffer, 0·1 ml. 0·12 M-phenylglucuronide, 0·1 ml. inhibitor solution. In controls, water was substituted for the inhibitor solution. Buffer, substrate and inhibitor solutions were, as a rule, adjusted to pH 5·2 (glass electrode). In experiments in which the two glucuronidase fractions in mouse liver were separated (Kerr, Campbell & Levvy, 1949; Mills, 1948), however, hydrolysis with fraction *A* was carried out at pH 4·5 instead of 5·2. Occasionally, preliminary purification of the enzyme was omitted, and the crude liver or kidney homogenate was used for hydrolysis. Results are expressed as μg. of phenol liberated in 1 hr. at 37°.

Measurement of glucuronide synthesis. The conjugation of *o*-aminophenol with glucuronic acid was followed by the method of Levvy & Storey (1949). After removal of protein with a mixture of trichloroacetic acid and phosphate buffer, the glucuronide was diazotized and coupled with naphthylethylenediamine. At the pH selected for colour development, free *o*-aminophenol in comparatively large amounts did not interfere. To measure the synthetic activity of mouse-liver slices, they were shaken in sulphate-free bicarbonate Ringer solution, containing 0·02 M-lactate, 0·001 M-ascorbic acid and 0·0025% *o*-aminophenol, at 37° for 1 hr. in an atmosphere of 5% CO_2 in O_2. Results are expressed as μg. *o*-aminophenol conjugated/g. dry wt. of tissue in 1 hr.

Inhibition of β-glucuronidase in vitro

Nearly fifty substances were examined for their effect on β-glucuronidase *in vitro*. Those which caused a fall in the activity of the enzyme are listed in Table 1. The most effective was D-glucosaccharic acid, and Fig. 1 shows the percentage inhibition produced by varying concentrations of this compound in three experiments with liver glucuronidase. It can be seen that 50% inhibition was obtained with 2×10^{-4} M-saccharate, and practically complete inhibition with less than 10^{-2} M (substrate concentration 0·015 M). From results given in Table 1, it appears that the inhibitory action of saccharate was independent of the following factors: the source of the enzyme, the activity of the preparation, the glucuronidase fraction present, and the degree of purity of the preparation.

Of other compounds listed in Table 1, three were closely related to saccharic acid (mucic, gluconic and glucuronic acids), but were much less efficient as inhibitors of β-glucuronidase. Glucuronic acid (D-glucurone) interfered in the determination of phenol liberated from phenylglucuronide. In the absence of phenol, glucuronic acid was without effect on the Folin-Ciocalteu reagent, but in the presence of phenol it apparently gave the colour reaction. This effect was independent of the phenol concentration. The figures shown in Table 1 for the inhibitory action of glucuronic acid on glucuronidase have been corrected for interference in the colour reaction, and

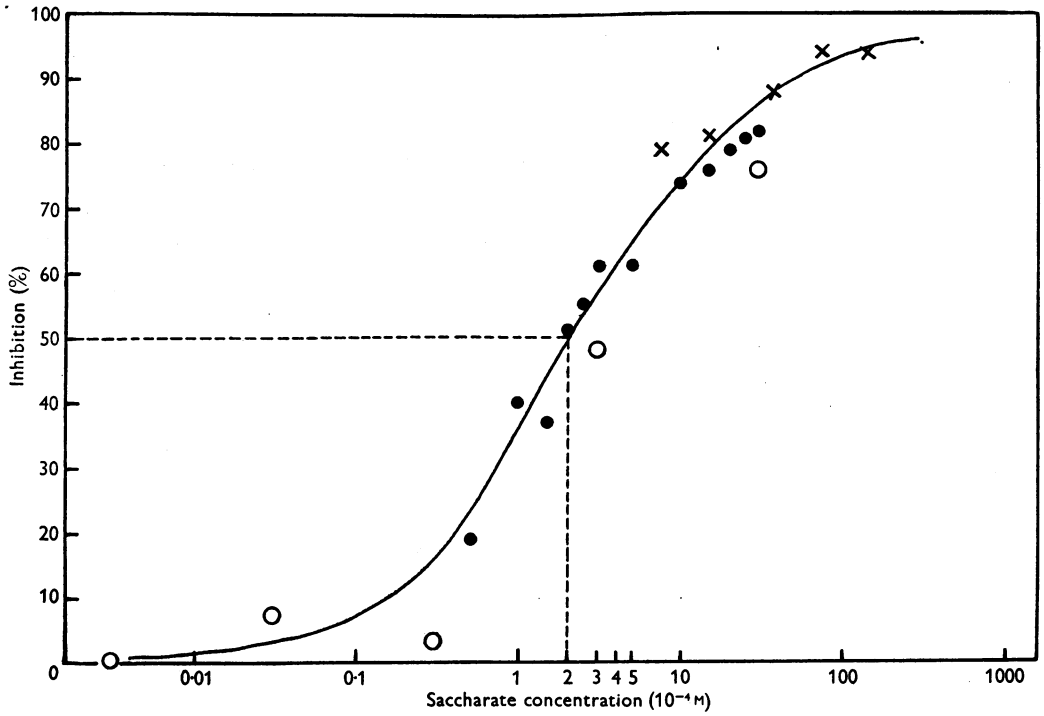

Fig. 1. Effect of varying concentrations of saccharic acid on the hydrolysis of phenylglucuronide (0·015 M) by mouse-liver glucuronidase (results for three separate experiments shown by ○, ● and ×).

are considered reliable. Correction of the hydrolysis figures was also necessary in the case of phlorrhizin and vanillin which gave the colour reaction directly. After correction, the results suggested that both compounds slightly inhibited β-glucuronidase. L-Malic acid in high concentration had an inhibitory action on the enzyme which entirely accounted for the effects produced by the racemic acid. A comparative study of the tartaric acids might provide interesting information regarding configurational requirements for glucuronidase inhibition. Unfortunately, only L-tartaric acid* was available, and this had no effect on the enzyme (see below).

The following substances had no apparent effect on the hydrolysis of phenylglucuronide by β-glucuronidase in the concentrations shown: β-phenyl-D-glucoside (0·015 M), α-methyl-D-glucoside (0·015 M), α-methyl-D-mannoside (0·003 M), β-methyl-D-glucoheptoside (0·003 M), β-methyl-D-xyloside (0·003 M), α-methyl-D-galactoside (0·003 M), gum arabic (0·15 %), degraded egg-plum gum (0·05 %), pyromucic acid (0·015 M), sorbic acid (0·02 M), oxalic acid (0·015 M), malonic acid (0·015 M), succinic acid (0·015 M), glutaric acid (0·015 M), maleic acid (0·015 M), L-tartaric acid (0·015 M), valeric acid (0·015 M), ouabain (0·0015 M), digitonin (0·0015 M), urethane (0·015 M), phenylurethane (0·015 M), nitroso-*N*-methylurethane (0·015 M), heparin (6·6 Toronto units/ml.), sulphapyridine (0·00015 M), inositol (0·015 M), piperonal (0·015 M), *n*-hexyl alcohol (0·015 M), *NN*-di-(2-chloroethyl)-aniline (0·02 M), 2′-methyl-4-dimethylaminostilbene (0·01 M), NaF (0·015 M), Na_2SO_4 (0·03 M).

The following compounds gave colours with the phenol reagent, but, when correction was made for this, they were apparently without effect on β-glucuronidase in the concentrations shown: salicin (0·015 M), thiourea (0·0015 M), eserine (0·0001 M), ascorbic acid (0·00075 M), oestrone (0·0005 M), colchicine (0·0001 M).

The following compounds interfered too badly in the colour reaction to be tested with β-glucuronidase: sodium azide, ω-bromoacetophenone, phenylarsenoxide, ethyl cyanoacetate, ethane-1:2-dithiol.

The action of saccharic acid on β-glucuronidase

From its similarity in structure to glucuronic acid, one would expect saccharic acid to act competitively in inhibiting glucuronidase. That the inhibition was reversible was shown by precipitating the enzyme from 0·03 M-saccharate solution with an equal volume of saturated $(NH_4)_2SO_4$ solution. After one precipitation, the activity was 70 % of that shown by a control sample of the enzyme. After dissolving in water and reprecipitating, the activity was as great as in the control.

The normal substrate-activity curve for the hydrolysis of phenylglucuronide by mouse-liver glucuronidase has been studied by Kerr *et al.* (1948; see also Kerr *et. al.* 1949). An approximate value of 0·0035 M was obtained for K_m, the concentration giving half the maximum velocity of hydrolysis. The maximum was usually reached with 0·015 M-substrate. Inhibition by excess substrate was pronounced.

Fig. 2 shows the effect of increasing the concentration of phenylglucuronide on its initial rate of hydrolysis in presence of 2×10^{-4} M-saccharate. Results are expressed in terms of the relative activity, where hydrolysis of 0·015 M-phenylglucuronide in absence of inhibitor is taken as unity. The points show averages for two representative experiments, one with liver glucuronidase fraction *A* and the other with fraction *B*. There was no appreciable difference in the results for the two fractions. It is clear that

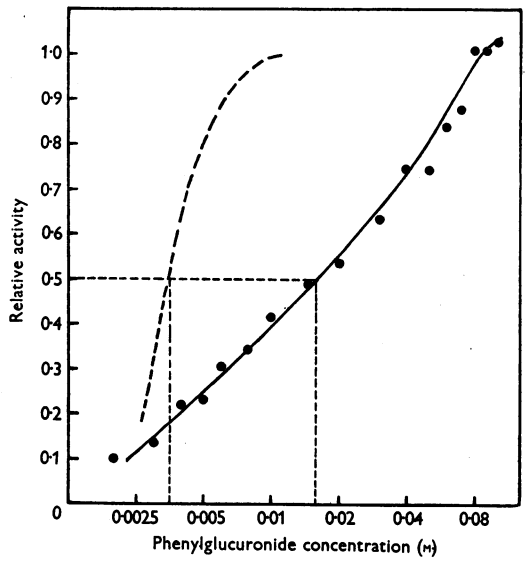

Fig. 2. Effect of varying concentrations of phenylglucuronide on its hydrolysis by mouse-liver glucuronidase in presence of 2×10^{-4} M-saccharate (●——●). Results expressed as fractions of the maximum activity observed in absence of inhibitor. Substrate-activity curve in absence of inhibitor (Kerr *et al.* 1948) shown by broken line.

saccharate acted competitively since the effect decreased with increasing substrate concentration till at 0·08 M-phenylglucuronide the activity of the enzyme was fully restored.

From Figs. 1 and 2, it can be seen that, in presence of a saccharate concentration [I] of 2×10^{-4} M, half the maximum enzyme activity was reached with a substrate concentration of 0·015 M. The latter figure can be designated K'_m, and K_i, the dissociation constant of the enzyme-inhibitor complex, can be calculated from the equation $K_i = [I] . K_m/(K'_m - K_m)$ (Lineweaver & Burk, 1934). Using the value for K_m given by Kerr *et al.* (1948), $K_i = 6 \times 10^{-5}$ M. It should be stressed that, presumably owing to the presence of impurities, K_m can vary from enzyme preparation to preparation by as much as 50 % in absence

* The naturally occurring isomer, commonly called dextrorotatory tartaric acid.

of added inhibitor. This is associated with variations in the substrate concentrations at which maximum activity is reached and inhibition by excess substrate becomes marked. The value for K_i is not materially altered since K'_m varies with K_m. Attempts to determine K_i by the method of Hunter & Downs (1945), in which knowledge of K_m is not required, were unsuccessful.

The effect of saccharic acid on glucuronide synthesis by mouse-liver slices

Table 2 shows the effect of saccharic acid in varying concentration on the formation of *o*-aminophenylglucuronide by mouse-liver slices. Each figure is an average for a determination done in quadruplicate, as recommended by Levvy & Storey (1949). These authors found the standard deviation of a single observation from the mean to amount to 17 %

Table 2. *Action of dicarboxylic acids on glucuronide synthesis by mouse-liver slices*

Exp. no.	Compound	Concentration (10^{-4}M)	*o*-Aminophenol conjugated/g. dry wt. (μg.)	Difference from control (%)
1	Control	—	210	
	Saccharate	8	190	-10
2	Control	—	200	
	Saccharate	8	170	-15
3	Control	—	460	
	Saccharate	50	500	+ 9
4	Control	—	390	
	Saccharate	50	300	-23
5	Control	—	620	
	Saccharate	50	540	-13
	Saccharate	100	540	-13
6	Control	—	840	
	Saccharate	100	580	-31
	Saccharate	170	550	-34
7	Control	—	340	
	Saccharate	100	270	-20
	Maleate	100	270	-20
8	Control	—	780	
	Saccharate	100	720	- 8
	Succinate	100	660	-15

in their procedure. The standard error for a figure based on four results is thus 10 %. Differences in synthetic activity between saccharate-treated slices and control slices from the same animal approached significance in only one experiment (no. 6). Taking the results as a whole, however, synthesis in presence of saccharate tended to be slightly less than in its absence. This effect was non-specific since it was also seen in experiments with succinic and maleic acids. These acids have no action on β-glucuronidase (see p. 601). All three dicarboxylic acids studied were added as solutions brought to neutrality with potassium hydroxide (glass electrode).

Experiments were done to show that saccharic acid can inhibit hydrolysis of *o*-aminophenylglucuronide by β-glucuronidase. The final substrate concentration was arbitrarily fixed at 0·13 % and the pH at 4·5 (citrate buffer). After 2 hr. incubation at 38° with mouse-liver glucuronidase, 11 % hydrolysis of the glucuronide was observed in absence of saccharate. In presence of 10^{-3}M-saccharate, the hydrolysis was 0·7 %. When the experiment was repeated with another enzyme preparation, the hydrolysis in absence of saccharate was 17 % and in its presence 3·6 %.

Penetration of saccharic acid into the cell

Attempts were made to show inhibition of β-glucuronidase in the intact cell by saccharic acid. In the first experiment, mouse-liver slices of known weight from two animals were shaken in sulphate-free bicarbonate Ringer solution containing 0·01M-saccharate for 90 min. at 37°. At the end of this period, the slices were removed, washed in three changes of distilled water, and homogenized. Inactive protein was precipitated by incubation of the homogenate for 30 min. at pH 5·2. Without further purification, the supernatant was examined for β-glucuronidase activity. The activity in terms of μg. phenol liberated at 37° in 1 hr. by 1 g. liver was 220, compared with 334 for control slices from the same two animals put through the procedure in absence of saccharate. Unless saccharate was strongly adsorbed on the surface of the slices, it would appear that an appreciable amount penetrated the cells. On the assumption that the saccharate concentration within the slices rose to 0·01M, the inhibition expected was of the order of 75 %.

In other experiments, the enzyme preparation in the incubation mixture used in the assay of glucuronidase activity was replaced by mouse-liver slices of known weight. The results had little quantitative value as the 'enzyme blank' was variable and high, but they suggested that some hydrolysis of phenylglucuronide took place and that this process was strongly inhibited by 0·015M-saccharate.

The effect of saccharate on the oxygen uptake and anaerobic glycolysis of mouse-liver slices

Saccharate in a concentration of 0·014M had no effect either on the oxygen uptake or on the anaerobic glycolysis of mouse-liver slices as measured in the Warburg apparatus. The Ringer solutions of Krebs & Henseleit (1932) were used, the O_2 consumption being determined in phosphate Ringer and an atmosphere of O_2, and the CO_2, output in bicarbonate Ringer and an atmosphere of 5 % CO_2 in N_2.

The action of saccharic acid on growth processes in the mouse

A compound that inhibits β-glucuronidase *in vitro* can be hardly expected to arrest whatever mechanism is responsible for the increase in the activity of the enzyme normally observed *in vivo* when a tissue is stimulated to rapid growth. If, however, glucuronidase plays an essential part at some stage in the growth process, administration of an inhibitor might modify the process at that stage. This possibility was examined with saccharic acid. When large doses were administered to mice, saccharic acid was apparently without effect on liver regeneration following damage and on growth in infant mice. Figures for glucuronidase activity, the weights of single organs or of the whole animal, and the histological picture were invariably identical with those observed in appropriate controls. It should be pointed out that during the preparation of the enzyme for assay it would be freed from any saccharic acid which might have been present in the original tissue.

Saccharic acid given at frequent intervals by subcutaneous injection of neutralized solutions of the potassium hydrogen salt in doses totalling up to 5 g./kg. daily for periods up to 8 days had no action on liver repair after administration of CCl_4 or partial hepatectomy (Levvy *et al.* 1948). It failed to modify the increase in uterine weight and glucuronidase activity observed in ovariectomized mice during liver regeneration (Kerr *et. al.* 1949). In infant mice, intraperitoneal injection of 2 g. saccharic acid/kg. daily or the addition of 3% potassium hydrogen saccharate to the solid diet had no effect on normal growth after as long as 3 weeks.

Attempted synthesis of o-aminophenyl-glucuronide by β-glucuronidase

Florkin, Crismer, Duchateau & Houet (1942) claim to have demonstrated conjugation of borneol (saturated solution) with glucuronic acid (0·01 M) in presence of ox-spleen glucuronidase. At the end of the incubation period, free glucuronic acid was removed with copper sulphate and calcium hydroxide and glucuronic acid in combination was estimated by the Tollens colour reaction. Only a small fraction of the total glucuronic acid present was in the combined form, even after incubation for several days.

The use of *o*-aminophenol as the aglycone in demonstrating glucuronide synthesis (Levvy & Storey, 1949) has the advantage that in the final reaction traces of the conjugate give a pink colour which is never seen in controls. No formation of *o*-aminophenylglucuronide was detected in experiments in which the free phenol was incubated with glucuronic acid in the presence of concentrated preparations of mouse liver glucuronidase.

D-Glucurone was present in final concentrations varying from 0·4 to 0·0125 M in 0·05 M-citrate buffer at pH 5·2, or 0·05 M-phosphate buffer at pH 7·4, containing 0·0025% *o*-aminophenol, 0·001 M-ascorbic acid, and the enzyme. The mixture was shaken for periods of 2 and 22 hr. at 37°. In the longer-term experiments, the incubation flasks were filled with N_2 to prevent oxidation of the free phenol.

When liver slices were replaced in the procedure of Levvy & Storey (1949) by crude liver homogenate, no glucuronide synthesis was detected.

DISCUSSION

Considerable difficulties were encountered in determining K_i, the dissociation constant for the inhibitor-enzyme complex, in the case of saccharic acid and β-glucuronidase, but it is considered that the value of 6×10^{-5} M finally arrived at is at least as reliable as values quoted for K_m, the dissociation constant of the substrate-enzyme complex, in the hydrolysis of biosynthetic glucuronides by glucuronidase. Figures available for K_m are as follows: phenylglucuronide, 0·0035 M (Kerr *et al.* 1948); bornylglucuronide, 0·01 M, methylglucuronide, 0·004 M, and oestriolglucuronide, 0·0005 M (Fishman, 1939); phenolphthaleinglucuronide, 0·00005 M (Talalay, Fishman & Huggins, 1946). Saccharic acid has a higher affinity for glucuronidase than all except one of these glucuronides. Changing the carboxyl at $C_{(6)}$ in saccharic acid to a primary alcohol group to give gluconic acid, or changing the configuration to give mucic acid, resulted in considerable diminution of the inhibitory power. The effect of glucuronic acid on the hydrolysis of phenylglucuronide by the enzyme may have been inhibition in the usual sense or a mass action effect. Hydrolysis of a glucuronide by glucuronidase is known to result in formation of free glucuronic acid (Levvy, 1948).

The failure of saccharic acid in large doses to modify liver regeneration after damage, or growth in infant mice may indicate that the enzyme is not directly concerned in cell division, but the results are capable of explanation in other ways. Saccharic acid may be too rapidly metabolized or excreted to produce any perceptible changes *in vivo*. Alternatively, normal cell division may involve hydrolysis of a naturally occurring glucuronide with a much greater affinity for the enzyme than that of saccharic acid. Experiments designed to exclude the possibility that saccharic acid does not penetrate the intact cell were unsatisfactory on technical grounds, but the results, for what they were worth, suggested that penetration did occur. Preliminary results obtained by Dr J. G. Campbell (private communication) suggest that saccharic acid considerably retards hydrolysis of the glucuronide of '1-*ortho*-hydroxyphenylazo-2-naphthol' by frozen mouse kidney sections in the histochemical test of Friedenwald & Becker (1948).

As a result of the work of Levvy *et al.* (1948), it is no longer necessary to postulate a synthetic role for β-glucuronidase in the body (Fishman, 1940) in order to explain the changes in the activity of the enzyme which can be produced in various organs. The view that β-glucuronidase is not involved in glucuronide synthesis (Levvy, 1948) receives support from the failure of saccharic acid to influence formation of *o*-aminophenylglucuronide by mouse-liver slices, and of β-glucuronidase preparations to effect condensation of glucuronic acid with *o*-aminophenol.

Certain sex hormones are known to be excreted as glucuronides. The effect of administering saccharic acid on the metabolism of these compounds might repay investigation.

SUMMARY

1. Hydrolysis of phenylglucuronide by β-glucuronidase was strongly inhibited by saccharic acid. Closely related compounds were much less effective. Inhibition by saccharic acid was competitive, and a value of 6×10^{-5} M was obtained for K_i, the dissociation constant of the enzyme-inhibitor complex.

2. Saccharic acid had no marked effect on synthesis of *o*-aminophenylglucuronide by mouse-liver slices.

3. Administration of large doses of saccharic acid to mice did not influence liver regeneration after damage or growth in infant animals.

4. No conjugation of *o*-aminophenol with glucuronic acid was observed after incubation in the presence of β-glucuronidase.

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The Metabolism of Chrysene: the Isolation of 3-Methoxychrysene by Methylation of the Phenolic Metabolite of Chrysene from Rat Faeces*

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Attention has been drawn to the fact that in mice and rats there is a remarkable similarity in the position of metabolic oxidation in the molecule for the three hydrocarbons 1:2-benzanthracene, 1:2:5:6-dibenzanthracene and 3:4-benzpyrene (Berenblum & Schoental, 1943). It was pointed out that these positions are not the ones which are chemically the most reactive. A preliminary report by Dickens (1945), that 9:10-dimethyl-1:2-benzanthracene is metabolized into its 4′-hydroxy derivative, brings this hydrocarbon into line with those mentioned above, and provides additional support for the similarity in metabolic pattern.

It was thought that useful information might be derived from a study of the metabolism of chrysene, in which the anthracenoid ring structure is lacking. The following investigation was, therefore, undertaken.

* A preliminary report on this work was communicated to the Biochemical Society (*Biochem. J.* 1945, **39**, lxiv).

EXPERIMENTAL

The procedure adopted was essentially the same as that used by the authors in their previous metabolic studies. Thirty rats were injected intraperitoneally with 2 ml. of a warm,