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Some Differences in the Conjugation of *o*-Aminophenol and *p*-Nitrophenol by the Uridine Diphosphate Transglucuronylase of Mouse-Liver Homogenates

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1. Glucuronide synthesis from uridine diphosphate glucuronate and *o*-aminophenol or *p*-nitrophenol in the presence of uridine diphosphate transglucuronylase of mouse-liver homogenates has been studied with respect to inhibition by compounds known to be conjugated under the experimental conditions, and also by thiophenol. 2. Raising the *o*-aminophenol concentration decreased the inhibition of *o*-aminophenyl glucuronide synthesis by the alternative glucuronyl acceptors phenol, menthol and benzoic acid, but was without effect on that caused by *p*-nitrophenol and thiophenol. 3. Raising the *p*-nitrophenol concentration decreased or abolished the inhibition of *p*-nitrophenyl glucuronide synthesis due to phenol, menthol, benzoic acid, anthranilic acid, *o*-aminophenol and thiophenol. 4. The *o*-aminophenol system was much more readily inhibited by all compounds than the *p*-nitrophenol system. 5. In tris buffer, pH 7·4, over 30% activation of the *o*-aminophenol system was achieved by 2mM-Mg^{2+}, but 10mM-Mg^{2+} was inhibitory. The *p*-nitrophenol system showed only inhibition from 2mM-Mg^{2+} upwards. 6. The results are discussed as suggesting that there are at least two uridine diphosphate transglucuronylases.

The considerable variety of glucuronyl acceptors utilized in the catalysis of glucuronide formation by UDP transglucuronylase has posed the question whether a single enzyme is involved, or a family of enzymes having different acceptor specificities. Although this problem has attracted much attention, the labile nature of the enzyme has greatly hindered progress, and, hitherto, most of the evidence has been related to variations in the rates of conjugation of different substrates by liver preparations of animals of various strains and species. Such efforts have been directed mainly to differentiating the systems responsible for the conjugation of hydroxyl, carboxyl and aromatic amino groups, and have resulted in various tentative conclusions. These results, and the difficulties attendant on their interpretation, have been discussed by others (Arias, 1960; Isselbacher, Chrabas & Quinn, 1962; Dutton, 1962, 1963; van Leusden, Bakkeren, Zilliken & Stolte, 1962).

Probably more informative and reliable are those findings based on competition between two acceptors. Dutton (1956) observed a competitive effect by benzoate and 2-ethylhexanoate against *o*-aminophenol in a microsome system from mouse liver, and Isselbacher *et al.* (1962) a similar effect with anthranilic acid on *p*-nitrophenol conjugation by a purified rabbit-liver enzyme. Axelrod, Inscoe & Tomkins (1958) found that aniline conjugation with microsomes from guinea-pig liver was likewise inhibited by phenol and benzoic acid. Grodsky & Carbone (1957) concluded that the inhibition of bilirubin conjugation by borneol was only partly competitive in nature. The enzyme of Isselbacher *et al.* (1962) was about equally active with *p*-nitrophenol and *o*-aminophenol, slightly less active with anthranilic acid, and only very slightly active with bilirubin and tetrahydrocortisone. All these results are consistent in suggesting that alcohols, phenols and carboxylic acids may be conjugated by the same enzyme system.

The conjugation of *o*-aminophenol and *p*-nitrophenol by UDP transglucuronylase of mouse-liver homogenates (which, for convenience, are referred to below as the *o*-aminophenol and *p*-nitrophenol systems, without necessarily implying that they are different) show certain differences in their inhibition by thiol reagents (Storey, 1965). Further studies on these systems, with respect to inhibition by a variety of acceptors known to form glucuronides under the experimental conditions, and of activation or inhibition by Mg^{2+}, suggest that two enzyme systems may be involved. A brief account of these experiments has already appeared (Storey, 1964).

MATERIALS AND METHODS

Competing substrates. Benzoic acid, anthranilic acid, phenol, (−)-menthol and thiophenol were all commercial specimens, the first two being recrystallized from water before use. Thiophenol was dissolved in NaOH, and HCl added until the appearance of a faint turbidity (pH about 7·5). The solution was made up immediately before use. The other compounds were dissolved in water and brought to pH 7·2–7·5 where necessary.

Other materials. All other materials were identical with those described by Storey (1965). The UDP-glucuronate (sodium salt) was supplied by the California Corp. for Biochemical Research, Los Angeles, Calif., U.S.A. Adult female mice of the J stock of the Institute of Animal Genetics, University of Edinburgh, were used exclusively as source of enzyme.

Methods. The determination of the conjugation of *o*-aminophenol and *p*-nitrophenol was carried out as described by Storey (1965). Where a second acceptor was used, it was mixed with the buffer and *o*-aminophenol or *p*-nitrophenol before adding the homogenate. Anthranilic acid was not tested in the *o*-aminophenol system as it interfered in the colorimetric determination. None of the other acceptors, at the concentrations used, showed any interference with either method.

RESULTS

Inhibition of the o-*aminophenol and* p-*nitrophenol systems by other acceptors.* The effect of adding various acceptors known to be conjugated under the prevailing conditions was studied, the UDP-glucuronate concentration being sufficiently high to rule out competition for the nucleotide by the two systems as a limiting factor. The synthesis of both glucuronides was inhibited by all the compounds tested; but clearly the system with *o*-aminophenol was much the more sensitive. *p*-Nitrophenol, phenol and benzoic acid were highly inhibitory at a concentration equimolar with the *o*-aminophenol, whereas at this level the effect on the *p*-nitrophenol system was negligible (Fig. 1).

Effect of p-*nitrophenol concentration on inhibition by other glucuronyl acceptors.* To determine the nature of the above inhibitory effects, a fixed concentration of competing acceptor and two concentrations of *p*-nitrophenol, namely 0·28 mM and either 0·14 or 0·07 mM, were used (Table 1). Though the activation in some instances at the higher *p*-nitrophenol concentration is most probably only an apparent one, due to variations in the control blanks, in every experiment there was a decrease or complete abolition of inhibition at the higher concentration.

Effect of o-*aminophenol concentration on inhibition by other glucuronyl acceptors.* Similar experiments were carried out with *o*-aminophenyl glucuronide formation (Table 2). With phenol, menthol and benzoic acid a decrease in the inhibition was observed, although proportionately smaller in relation to the higher levels of inhibition in this system compared with the previous one. The inhibition by *p*-nitrophenol, however, was totally unaffected.

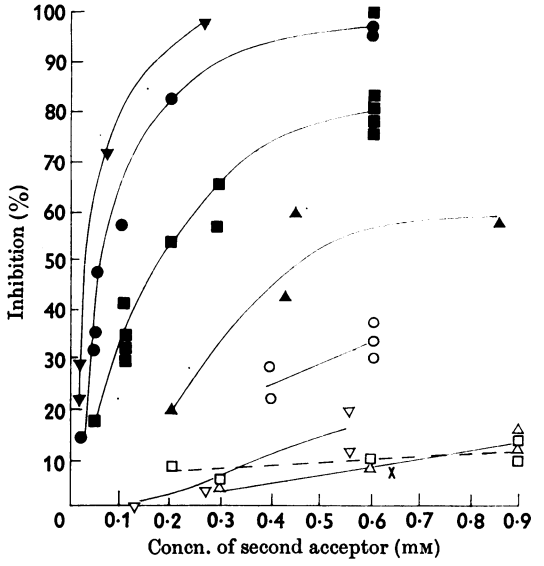

Fig. 1. Inhibition of the synthesis of *o*-aminophenyl glucuronide (●, ■, ▲, ▼) and *p*-nitrophenyl glucuronide (○, □, ×, △, ▽) in homogenates of female mouse liver by other acceptors: ●, ○, phenol; ■, □, benzoic acid; ▲, ×, (−)-menthol; △, anthranilic acid; ▼, *p*-nitrophenol; ▽, *o*-aminophenol. All digests contained tris buffer, pH 7·4 (33 mM), the second acceptor where appropriate and homogenate (10 mg. wet wt. of liver) in a total vol. of 0·3 ml. For conjugation of *o*-aminophenol (0·14 mM), they also contained ascorbate (0·76 mM) and UDP-glucuronate (0·138 or 0·184 mM). Incubation was for 20 min. at 30°. For conjugation of *p*-nitrophenol (0·14 mM), they contained UDP-glucuronate (0·184 mM). Incubation was for 15 min. at 30°.

Effect of thiophenol on the p-*nitrophenol and* o-*aminophenol systems.* Thiols, as nucleophilic reagents, might be expected to form glucuronides, and a metabolite believed to have such a constitution has been reported (Clapp, 1956). Parke (1952: quoted by Williams, 1959) has obtained some evidence that thiophenol is partly excreted as a glucuronide by the rabbit. The effect of thiophenol as a potential competitor was therefore examined in competition experiments (Table 3). Once more, the *p*-nitrophenol system was much the less readily inhibited, and the inhibition was relieved by raising the *p*-nitrophenol concentration. In the *o*-aminophenol system, thiophenol resembled *p*-nitrophenol in that the degree of inhibition was independent of *o*-aminophenol concentration.

Effect of Mg^{2+} *on the* p-*nitrophenol and* o-*aminophenol systems.* Dutton & Storey (1954) found that, in phosphate buffer, pH 7·4, optimum synthesis of

Table 1. *Effect of* p-*nitrophenol concentration on the inhibition of* p-*nitrophenyl glucuronide formation by other glucuronyl acceptors*

Digests contained tris buffer, pH 7·4 (33 mM), *p*-nitrophenol, the other acceptor, UDP-glucuronate (0·184 mM), sucrose homogenate (10 mg. wet wt. of female mouse liver) and water to 0·3 ml. In the controls, the only acceptor present was *p*-nitrophenol. Incubation was for 15 min. at 30°.

Concn. of *p*-nitrophenol (mM)	Conjugation in control (mμmoles/mg. wet wt./hr.)	Other acceptor	Concn. of other acceptor (mM)	Conjugation (% difference from control)
0·14	4·30	Phenol	0·6	−30
0·28	4·63		0·6	−10
0·14	3·03	Phenol	0·2	−28
0·28	3·38		0·2	−6
0·07	3·74	Phenol	0·4	−35
0·28	3·82		0·4	−7
0·07	3·09	Menthol	1·07	−17
0·28	3·22		1·07	+5
0·14	3·09	Anthranilic acid	0·9	−13
0·28	3·22		0·9	−2
0·07	3·48	Anthranilic acid	1·5	−26
0·28	5·17		1·5	−18
0·14	4·30	Benzoic acid	0·9	−14
0·28	4·63		0·9	−8
0·14	2·41	Benzoic acid	0·9	−10
0·28	2·02		0·9	+8
0·07	3·09	Benzoic acid	1·5	−40
0·28	3·22		1·5	−16
0·07	3·74	*o*-Aminophenol	0·56	−15
0·28	3·82		0·56	+13
0·07	2·50	*o*-Aminophenol	0·56	−17
0·28	2·92		0·56	+8
0·07	3·06	*o*-Aminophenol	0·56	−9
0·28	3·78		0·56	−3

o-aminophenyl glucuronide by mouse-liver homogenates was reached at 10 mM-magnesium chloride. A similar concentration in tris buffer is inhibitory, and many workers have therefore omitted Mg^{2+} entirely. In the above experiments and in other work (Storey, 1965) Mg^{2+} was also omitted; but further examination of the system showed there was an optimum Mg^{2+} concentration of 2 mM (Fig. 2), and above this inhibition occurred. In the *p*-nitrophenol system, however, Mg^{2+} was inhibitory at all concentrations greater than 2 mM.

DISCUSSION

The difficulties in working with microsomal enzyme preparations necessarily impose limitations in drawing conclusions, but the results suggest that there are at least two UDP transglucuronylases in mouse-liver homogenates. The *p*-nitrophenol system had a relatively low affinity for the potential acceptors phenol, menthol, benzoic acid, anthranilic acid and *o*-aminophenol, all of which behaved like competitive substrates. The formation of *o*-aminophenyl glucuronide was strongly inhibited by phenol, menthol and benzoic acid, which function as glucuronyl acceptors under similar experimental conditions. Increasing the *o*-aminophenol concentration decreased the inhibition considerably, suggesting that conjugation was taking place at the same enzyme centre. Attempts to analyse these results by the Lineweaver–Burk method were unsatisfactory owing to the low degree of accuracy possible with this type of system. By contrast, although *p*-nitrophenol was approximately as inhibitory as phenol itself, even small degrees of inhibition by *p*-nitrophenol were unaffected by the *o*-aminophenol concentration. It therefore seems unlikely that *p*-nitrophenol was undergoing conjugation by the *o*-aminophenol system. Were *p*-nitrophenol alone in this behaviour there might

Table 2. *Effect of* o-*aminophenol concentration on the inhibition of* o-*aminophenyl glucuronide formation by other glucuronyl acceptors*

Digests contained tris buffer, pH 7·4 (33 mM), various amounts of a stock solution containing 6 mg. of *o*-aminophenol and 25 mg. of ascorbic acid in 25 ml. of water, the other acceptor, UDP-glucuronate, sucrose homogenate (10 mg. wet wt. of female mouse liver) and water to 0·3 ml. In the controls, the only acceptor present was *o*-aminophenol. Incubation was for 20 min. at 30°.

Concn. of *o*-aminophenol (mM)	Conjugation in control (mμmoles/mg. wet wt./hr.)	Concn. of UDP-glucuronate (mM)	Other acceptor	Concn. of other acceptor (mM)	Conjugation (% difference from control)
0·14	0·92	0·138	Phenol	0·2	−83
0·28	0·88	0·138		0·2	−71
0·07	0·36	0·138	Phenol	0·05	−53
0·28	0·47	0·138		0·05	−43
0·07	0·53	0·138	Phenol	0·025	−18
0·28	0·68	0·138		0·025	−10
0·14	1·05	0·184	Menthol	0·45	−60
0·28	1·06	0·184		0·45	−54
0·07	0·49	0·138	Menthol	0·2	−19
0·28	0·59	0·138		0·2	0
0·14	0·92	0·138	Benzoic acid	0·2	−53
0·28	0·88	0·138		0·2	−44
0·07	0·57	0·184	Benzoic acid	0·2	−51
0·28	0·80	0·184		0·2	−29
0·07	0·80	0·138	Benzoic acid	0·1	−36
0·28	1·11	0·138		0·1	−12
0·07	0·87	0·138	*p*-Nitrophenol	0·07	−69
0·28	1·25	0·138		0·07	−77
0·07	0·57	0·184	*p*-Nitrophenol	0·023	−23
0·28	0·80	0·184		0·023	−35
0·07	0·53	0·184	*p*-Nitrophenol	0·023	−22
0·28	0·92	0·184		0·023	−22

Table 3. *Thiophenol as a potential competitor of glucuronide formation from* p-*nitrophenol and* o-*aminophenol*

Conditions of incubation were similar to those in Tables 1 and 2.

Concn. of *p*-nitrophenol (mM)	Concn. of *o*-aminophenol (mM)	Conjugation in control (mμmoles/mg. wet wt./hr.)	Concn. of UDP-glucuronate (mM)	Concn. of thiophenol (mM)	Conjugation (% difference from control)
0·07	—	3·35	0·184	0·5	−60
0·28	—	2·56	0·184	0·5	+15
0·07	—	2·25	0·184	0·5	−51
0·28	—	4·35	0·184	0·5	−9
—	0·07	0·62	0·092	0·05	−58
—	0·28	1·02	0·092	0·05	−54
—	0·07	0·40	0·184	0·05	−44
—	0·28	0·72	0·184	0·05	−44
—	0·07	0·80	0·184	0·05	−68
—	0·28	1·56	0·184	0·05	−61
—	0·07	0·32	0·184	0·05	−23
—	1·28	1·26	0·184	0·05	−46

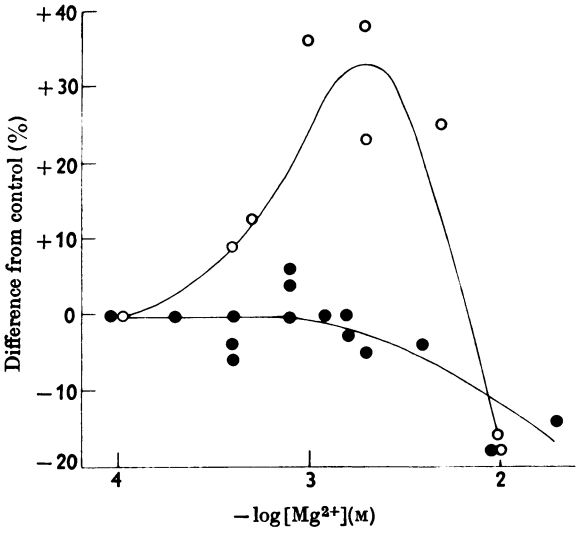

Fig. 2. Effect of Mg^{2+} on the synthesis of *o*-aminophenyl glucuronide (○) and *p*-nitrophenyl glucuronide (●) in mouse-liver homogenates, in tris buffer. Digests contained UDP-glucuronate (0·138 mM) and various amounts of a solution of $MgSO_4$. Other conditions were as in Fig. 1.

have been room for doubt as to the validity of the results, since nitrophenols have in some respects unusual properties. As is well known, many nitrophenols can dissociate phosphorylative from oxidative processes. Esters of *p*-nitrophenol, such as the acetate, are enzyme inhibitors; and there are grounds for believing that the introduction of nitro groups into substrates may influence enzyme–substrate interaction (Levvy, McAllan & Marsh, 1958). Any such effects seem to be absent or minimal here, however, since thiophenol behaved in a very similar fashion to *p*-nitrophenol in the *o*-aminophenol system. In the *p*-nitrophenol system, thiophenol resembled the other compounds in acting in the manner expected of a competing substrate.

In addition to the differential effects of thiol inhibitors (Storey, 1965), there are other lines of evidence for more than one UDP transglucuronylase in mouse-liver homogenates. When whole liver was frozen, there was a more rapid decline in the ability to conjugate *o*-aminophenol than *p*-nitrophenol (Myles, 1964). Late foetal and neonatal liver showed very much lower rates of formation of *o*-aminophenyl glucuronide than adults; but with *p*-nitrophenol the perinatal conjugating ability approached that of the adults (Dr G. J. Dutton, personal communication). In a different species a similar situation may exist, as suggested by studies on the Gunn strain of jaundiced rats. Homozygous animals show abnormally low rates of glucuronide formation, not only from bilirubin, but also from *o*-aminophenol, anthranilic acid and menthol (Arias, 1961; Schmid, Axelrod, Hammaker & Swarm, 1958). By contrast, van Leusden *et al.* (1962) found no difference between homozygous and heterozygous animals with regard to *p*-nitrophenyl glucuronide.

Although *o*-aminophenol and *p*-nitrophenol are both conjugated through their phenolic groups, they have markedly different p*K* values. Nevertheless, the results suggest, in agreement with previous workers, that alcohols, phenols and carboxylic acids may be conjugated by the same enzyme; but the high concentrations of menthol, *o*-aminophenol, benzoic acid and anthranilic acid required for appreciable inhibition of *p*-nitrophenyl glucuronide synthesis make it somewhat debatable whether the conjugation of any of these acceptors by this system, assuming it could take place, would be of much quantitative importance. Phenol, and especially thiophenol, might have to be regarded differently, as they were considerably more inhibitory than the other compounds. On the other hand, it seems rather probable, though not certain, that phenol, menthol and benzoic acid were conjugated by the *o*-aminophenol system, and unlikely that *p*-nitrophenol and thiophenol were so conjugated.

With these considerations and limitations in mind, the question arises as to the probable significance of two enzyme systems of differing, yet overlapping, specificities. A possible explanation may relate to the fact that neither *o*-aminophenol nor *p*-nitrophenol could be regarded as the normal substrates. The ability of the liver to conjugate these two compounds, as well as the vast range of other, purely synthetic, organic compounds recorded as forming glucuronides, is a reflection of the activity of enzymes concerned with more 'physiological' substrates. Clearly, purification of the enzymes concerned will be necessary to resolve these problems and is an urgent task, and some progress has already been made. Isselbacher *et al.* (1962) have reported that their partly-purified soluble enzyme from rabbit liver, though active towards *o*-aminophenol and *p*-nitrophenol, was unable to conjugate aniline, the activity for which was entirely in the insoluble fraction. Though this represents the first separation of any UDP-transglucuronylase activity, the insoluble residue still contained some ability to conjugate *p*-nitrophenol, though this may have been due merely to residual contamination. *N*-Glucuronides, however, differ from *O*-glucuronides in that, under certain conditions, they may be formed non-enzymically by the spontaneous interaction of glucuronic acid with the amine (Bridges & Williams, 1962); and some caution may be needed in interpreting the results of experiments with this class of acceptor (Shuster, 1964).

The differing requirements for Mg^{2+} are in agree-

ment with the suggestion that two enzymes are involved, although other interpretations are possible. They do, however, point to the necessity for examining the requirements of individual substrates. Species differences may also have to be taken into account, but the results of Isselbacher *et al.* (1962) on the inhibition by Mg^{2+} of *p*-nitrophenyl glucuronide synthesis with the rabbit-liver enzyme resemble those found in the present work.

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