The Inhibition of the Uridine Diphosphate-Transglucuronylase Activity of Mouse-Liver Homogenates by Thiol Reagents

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1. A study of the catalysis of the formation of the glucuronides of *o*-aminophenol and *p*-nitrophenol by the uridine diphosphate transglucuronylase of homogenates of female mouse liver has been made, with reference to the effect of reagents reacting with thiol groups. 2. The synthesis of both glucuronides was completely inhibited by organic mercurials and *N*-ethylmaleimide. The inhibition was only partial with arsenite and the arsenoxides, iodoacetamide and *o*-iodosobenzoate. 3. The *o*-aminophenol system was much more sensitive than that for *p*-nitrophenol to all the thiol reagents, except *N*-ethylmaleimide, which was equally active in both systems. 4. At very low concentrations of the organic mercurials, the *o*-aminophenol system was activated. 5. With *o*-aminophenyl glucuronide formation, complete protection was given by glutathione and cysteine against the organic mercurials, *N*-ethylmaleimide and iodoacetamide, and partial protection against the arsenicals. Reversal was complete against the mercurials, and very limited against the arsenicals and iodoacetamide. The effects of *N*-ethylmaleimide and *o*-iodosobenzoate were irreversible. Results with *p*-nitrophenol were very similar. 6. Uridine diphosphate transglucuronylase was partially protected against *p*-chloromercuribenzoate and lewisite oxide by uridine diphosphate glucuronate, but not by *o*-aminophenol. 7. Glutathione did not prevent the decline in the rate of conjugation of *o*-aminophenol when homogenates were aged by incubation at 30°. Cysteine was unable to prevent or reverse inactivation by ultrasonic radiation.

The concept of glycosyl transfer mediated by nucleotide–sugar compounds is now firmly established, and further insight into the mechanism of such processes is a matter of considerable interest and importance. In glucuronide synthesis, a glucuronyl group is transferred from UDP-glucuronate under the influence of UDP transglucuronylase [UDP-glucuronate glucuronyltransferase (acceptor-unspecific), EC 2.4.1.17] to an acceptor, which may be one of a wide variety of relatively simple compounds (alcohols, phenols, carboxylic acids etc.). Consequently, the reaction might be considered as a model for those transglycosylations that take place with inversion of the stereochemical configuration about the glycosidic bond of the nucleotide–sugar. The overall mechanism of these inversions has been discussed previously (e.g. Axelrod, Inscoe & Tomkins, 1958; Hassid, 1962; Koshland, 1959; Storey, 1961).

Unfortunately, UDP transglucuronylase is unstable, the activity of tissue homogenates and microsomal preparations declining rapidly on aging (Dutton & Storey, 1954). The present work arose from attempts to activate or stabilize the enzyme with GSH and cysteine, and, although the experiments were unsuccessful, they led to the finding that reagents known to react with thiol groups were powerful inhibitors of the enzyme. With *o*-aminophenol and *p*-nitrophenol as acceptors, inhibition of the UDP transglucuronylase of mouse-liver homogenates was observed with all the inhibitors studied, and in some cases it was reversed or prevented by GSH and cysteine. Certain differences in the inhibition of the conjugation of *o*-aminophenol and *p*-nitrophenol were shown. A short account of some of these results has been published (Storey, 1964).

While the present work was in progress, Isselbacher, Chrabas & Quinn (1962) succeeded in solubilizing the enzyme from rabbit liver, in obtaining a relatively stable preparation and in purifying it 30-fold. Organic mercurials caused a partial inhibition, not reversible by GSH.

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METHODS

Materials

Inhibitors. Phenylmercuric acetate (a gift from Dr D. J. Manners), sodium *p*-chloromercuribenzoate, *o*-iodosobenzoic acid (both from L. Light and Co., Colnbrook, Bucks.) phenylarsenoxide, diethylaminophenylarsenoxide and arsenious oxide were all dissolved in NaOH, and HCl added to bring the pH to 7·2–7·5 (glass electrode, or indicator paper for small volumes of solution), or until a faint turbidity appeared. Lewisite oxide (2-chlorovinylarsenoxide), *N*-ethylmaleimide (L. Light and Co.) and iodoacetamide (prepared according to the method of Braun, 1908) were simply dissolved in water. The solution of *N*-ethylmaleimide was made up immediately before use, whereas the other reagents were made up less frequently. The quinquivalent arsenicals were dissolved in water and neutralized to pH 7·2–7·5.

Other reagents. Cysteine hydrochloride, GSH and GSSG (all from L. Light and Co.) were dissolved in water and neutralized with NaOH to pH 7·2–7·5 immediately before use. *p*-Nitrophenol, recrystallized from aqueous ethanol, was a gift from Dr D. J. Bell. UDP-glucuronic acid was supplied (as the ammonium salt) by the Sigma Chemical Co., St Louis, Mo., U.S.A., and (as the sodium salt) by the California Corp. for Biochemical Research, Los Angeles, Calif., U.S.A. Specimens from both sources gave a single spot under ultraviolet light after chromatography in M-ammonium acetate–ethanol (2:3, v/v), pH 5·5. The nucleotide was dissolved in water and stored at −20°.

Enzyme assay

Preparation of homogenate. Adult female mice were used throughout, except where stated otherwise, and were of the J stock of the Institute of Animal Genetics, University of Edinburgh. A 10% (w/v) suspension of mouse liver in 0·25 M-sucrose was centrifuged for 4 min. at about 2000*g* at 2°. The supernatant was used in all experiments, and is referred to below simply as 'homogenate'.

o-Aminophenol method. This procedure follows the method of Dutton & Storey (1962), with modifications as follows. Into 70 mm. × 9 mm. conical centrifuge tubes with wide tips were measured 0·02 ml. of 0·5 M-tris–HCl buffer, pH 7·4, 0·1 ml. of homogenate, 0·02 ml. of a stock solution containing 6 mg. of *o*-aminophenol and 50 mg. of ascorbic acid in 25 ml. of water (final concentrations: *o*-aminophenol, 0·14 mM; ascorbate, 0·76 mM), UDP-glucuronate and water to 0·3 ml. Lang–Levy pipettes (Bessey, Lowry & Brock, 1946) were convenient for measuring the smaller volumes of solution. The unstoppered tubes were inclined at a small angle from the vertical and shaken at 30°. After 20 min., the tubes were cooled in ice–water and protein was precipitated with 0·3 ml. of phosphate–trichloroacetate, pH 2·10. After centrifugation, 0·45 ml. of supernatant was taken and 0·05 ml. volumes of 0·15% $NaNO_2$, 0·5% ammonium sulphamate and 0·1% *N*-naphthylethylenediamine dihydrochloride added to each. The higher $NaNO_2$ concentration was necessary to avoid interference in the colour development (3 hr. at room temperature) by GSH and cysteine. None of the inhibitors at the highest concentration used interfered with colour development.

To economize in UDP-glucuronate, while giving a sufficiently high and constant rate of synthesis over 20 min. at 30°, a concentration of 0·049 mM was used. Though the rate was only some 75% of that at enzyme saturation (0·12–0·14 mM), the degree of inhibition by thiol reagents was not noticeably dependent on the concentration of the nucleotide (see below). Mg^{2+} ions were not added.

p-Nitrophenol method. This follows the method of Isselbacher (1956). The digest contained 0·02 ml. of 0·5 M-tris–HCl buffer, pH 7·4, 0·02 ml. of aq. 2·1 mM-*p*-nitrophenol, UDP-glucuronate, 0·1 ml. of homogenate and water to 0·3 ml. The tubes were incubated with shaking for 15 min. at 30°, then cooled in ice–water and 0·5 ml. of 0·2 N-trichloroacetic acid was added. To 0·45 ml. of the supernatant, 0·1 ml. of 2 N-NaOH and 1·0 ml. of water were added and the extinction was measured at 400 mμ against water as blank.

The concentration of UDP-glucuronate (0·098 mM) lies at or near saturation in this system; but, even so, the incubation was limited to 15 min. to preserve linearity of rate with time.

Inhibition experiments. The homogenate was mixed with buffer and inhibitor (0 min.), then, after keeping at 12–15°, acceptor and UDP-glucuronate were added and the incubation was commenced (5 min.). All the poisons with the exception of iodoacetamide reacted rapidly at 12–15°, maximum inhibition being reached within 5 min. With iodoacetamide, the degree of inhibition rose progressively for at least 15 min.

Protection experiments. The homogenate was mixed with the buffer and thiol. After 5 min. the inhibitor was added, and after a further 3 min. acceptor and UDP-glucuronate were also added. Incubation commenced at 10 min.

Reversal experiments. To the inhibitor and buffer, homogenate was added, then the thiol 5 min. later. After acceptor and UDP-glucuronate had been added (8 min.), the tubes were put in the bath at 10 min.

Units. Rates of conjugation are expressed as mμmoles of acceptor conjugated (or glucuronide formed)/mg. wet wt. of liver/hr. at 30°. All rates were obtained by deducting, from the values measured in presence of UDP-glucuronate, the 'endogenous' synthesis obtained in its absence (Dutton & Storey, 1954).

RESULTS

Comparison of rate of o-*aminophenol and* p-*nitrophenol conjugation*

The conjugation of *p*-nitrophenol in liver homogenates of female mice is a considerably more rapid process than that of *o*-aminophenol (Table 1). A small series with male mice is also included. Since the concentration of UDP-glucuronate is at or near the optimum for *p*-nitrophenol, whereas it is considerably below that (about 0·14 mM) for *o*-aminophenol, a number of rate measurements with the latter compound at 0·14 mM- and 0·049 mM-UDP-glucuronate were made, and a mean ratio approx. 1·3 was obtained. This gives a corrected rate for female mice of about 1·1 mμmoles of *o*-aminophenol conjugated/mg. wet wt. of liver/hr., or about one-third of the rate with *p*-nitrophenol. If the activation by Mg^{2+} of *o*-aminophenyl glucuronide formation of

about 33% is taken into account (Storey, 1965), the difference in rates is considerably smaller.

Table 1. *Comparison of rates of glucuronide synthesis by mouse-liver homogenates with* o-*aminophenol and* p-*nitrophenol as acceptors*

Digests contained tris–HCl buffer, pH 7·4 (33 mM), *o*-aminophenol or *p*-nitrophenol (0·14 mM), homogenate (10 mg. wet wt. of liver), UDP-glucuronate and water to 0·3 ml. Results are given as means ± S.D., with the numbers of animals used shown in parentheses.

Acceptor	Sex of mice	Concn. of UDP-glucuronate (mM)	Acceptor conjugated (mμmoles/mg. wet wt./hr. at 30°)
o-Aminophenol	Female	0·049	0·84 ± 0·24 (66)
o-Aminophenol	Male	0·049	0·80 ± 0·19 (10)
p-Nitrophenol	Female	0·098	3·34 ± 0·37 (43)

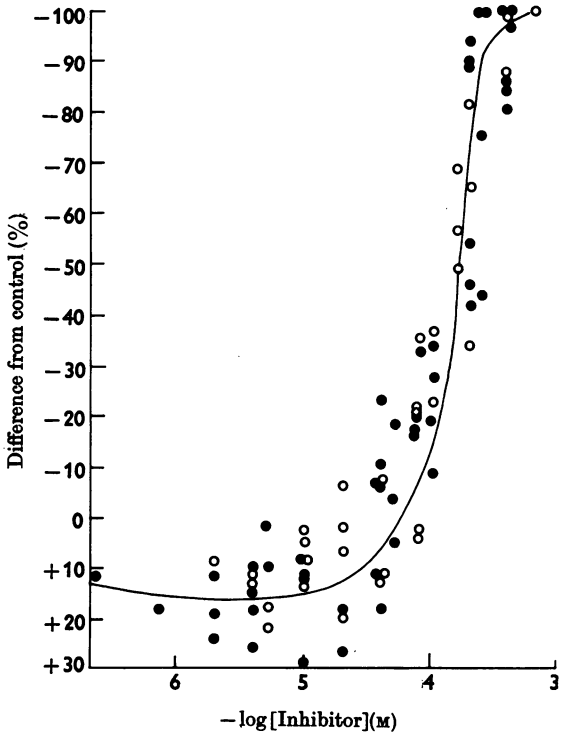

Fig. 1. Effect of *p*-chloromercuribenzoate (●) and phenylmercuric acetate (○) on *o*-aminophenyl glucuronide synthesis by homogenates of female mouse liver. Digests contained tris–HCl buffer, pH 7·4 (33 mM), *o*-aminophenol (0·14 mM), ascorbate (0·76 mM), UDP-glucuronate (0·049 mM), homogenate (10 mg. wet wt. of liver), inhibitor and water to 0·3 ml. Incubation was for 20 min. at 30°. Each point is the mean of duplicate determinations.

o-Aminophenyl glucuronide formation

Inhibition by organic mercurials (*Fig.* 1). *p*-Chloromercuribenzoate and phenylmercuric acetate inhibited synthesis completely at concentrations of 0·25–0·4 mM, but their effectiveness decreased rapidly to zero at a concentration of 0·04 mM. Below this, a considerable degree of activation was apparent, and was still considerable at a concentration of 1 μM. This activation was solely on the rate of the reaction, and did not affect the total amount of glucuronide synthesized, as measured at 20 min. and 45 min. respectively. Similar results were obtained in presence of saccharolactone, thus eliminating the possibility that activation was only apparent, through inhibiting hydrolysis of the glucuronide by β-glucuronidase (Fernley, 1962).

Inhibition by tervalent arsenicals (*Fig.* 2). Lewisite oxide differed from the mercurials in that the highest concentrations did not inhibit synthesis more than about 60%. At lower concentrations, however, it was much more potent than the mercurials, and activating effects were not observed. Arsenite, phenylarsenoxide and diethylaminophenylarsenoxide are also highly inhibitory.

The following compounds containing quinquivalent arsenic were without inhibitory effect at 1 mM concentration: methyl-, ethyl-, allyl- and 2-chlorovinyl-arsonic acid.

Addition and alkylating agents (*Fig.* 3). *N*-Ethylmaleimide (1 mM) completely suppressed glucuronide formation. With iodoacetamide, inhibition was incomplete at considerably higher concentra-

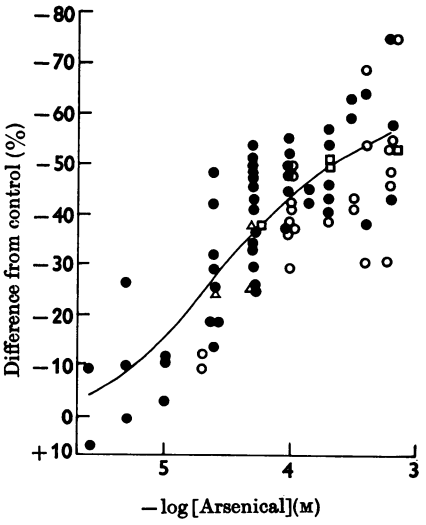

Fig. 2. Effect of lewisite oxide (●), arsenite (○), phenylarsenoxide (□) and diethylaminophenylarsenoxide (△) on *o*-aminophenyl glucuronide synthesis by mouse-liver homogenates. The conditions were as given in Fig. 1.

tions. The time of treatment was 10min., but, by analogy with the *p*-nitrophenol results, it is unlikely that maximum inhibition was reached.

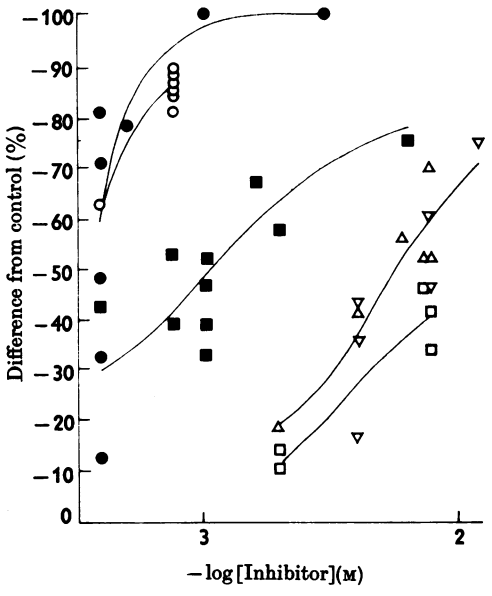

Fig. 3. Inhibition of glucuronide synthesis in mouse-liver homogenates by *N*-ethylmaleimide and iodoacetamide. (*a*) *o*-Aminophenol as acceptor: *N*-ethylmaleimide (●); iodoacetamide (■). The conditions were as given in Fig. 1. (*b*) *p*-Nitrophenol as acceptor: *N*-ethylmaleimide (○); iodoacetamide, preincubation period at 12–15° 10min. (□), 15min. (△), or 20min. (▽). Digests contained tris–HCl buffer, pH7·4 (33mM), *p*-nitrophenol (0·14mM), UDP-glucuronate (0·098mM), homogenate (10mg. wet wt. of liver), inhibitor and water to 0·3ml. Incubation was for 15min. at 30°.

o-Iodosobenzoate (*Fig.* 4). This reagent was about equal in inhibitory effect to *N*-ethylmaleimide, although complete inhibition was not attained at the highest concentration studied.

Protection and reversal by glutathione and cysteine. Owing to the form of the concentration–inhibition curves, the most suitable degree of inhibition for showing protection or reversal was about 50%. Another consideration was that, owing to interference in the colour reaction by both thiols, the highest practicable concentration of either was

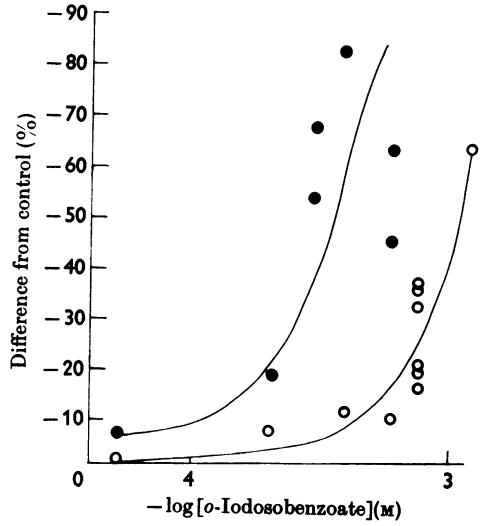

Fig. 4. Effect of *o*-iodosobenzoate on glucuronide synthesis in mouse-liver homogenates, with *o*-aminophenol (●) and with *p*-nitrophenol (○) as acceptor. The conditions were as given in Figs. 1 and 3.

Table 2. *Effect of glutathione and cysteine on the inhibition of o-aminophenol glucuronide formation in mouse-liver homogenates by thiol reagents*

Digests contained tris–HCl buffer, pH7·4, (33mM), UDP-glucuronate, *o*-aminophenol (0·14mM), ascorbate (0·76mM), homogenate (10mg. wet wt. of liver), inhibitor, GSH or cysteine as shown, and water to 0·3ml. Incubation was for 20min. at 30°. The procedure was as given in the Methods section.

Inhibitor	Concn. of inhibitor (mM)	Concn. of UDP-glucuronate (mM)	Inhibition: difference from control (%)	Thiol (4mM)	Protection: difference from control (%)	Reversal: difference from control (%)
p-Chloromercuribenzoate	0·2	0·049	−42	CySH	+2	+9
p-Chloromercuribenzoate	0·2	0·049	−54	GSH	0	−14
Phenylmercuric acetate	0·2	0·049	−81	CySH	−36	−24
Lewisite oxide	0·05	0·049	−43	GSH	−23	−33
Lewisite oxide	0·05	0·049	−46	CySH	−31	−41
Arsenite	0·1	0·049	−50	GSH	−34	−40
N-Ethylmaleimide	0·4	0·049	−81	GSH	−8	−67
N-Ethylmaleimide	0·4	0·049	−49	CySH	0	−44
Iodoacetamide	0·8	0·092	−50	GSH	−17	−40
Iodoacetamide	0·8	0·092	−39	GSH	−2	−15
o-Iodosobenzoate	0·3	0·092	−67	GSH	−61	−60
o-Iodosobenzoate	0·3	0·092	−54	CySH	−48	−55

Table 3. *Protection of UDP transglucuronylase by UDP-glucuronate against inactivation by thiol reagents*

Homogenate, buffer and UDP-glucuronate were mixed, and 5min. later were treated with *o*-aminophenol or *p*-nitrophenol. At 8min., inhibitor was added, and incubation begun at 10min. ('protection'). In 'reversal' experiments, the order of addition of UDP-glucuronate and inhibitor was interchanged.

Expt. no.	Inhibitor	Concn. of inhibitor (mM)	Concn. of UDP-glucuronate (mM)	Glucuronide formed in control (mμmoles/mg./hr.)	Reversal: difference from control (%)	Protection: difference from control (%)
(*a*) *o*-Aminophenol						
1	*p*-Chloromercuribenzoate	0·4	0·54	0·80	−81	—
		0·4	0·048	0·84	−86	—
2	*p*-Chloromercuribenzoate	0·4	0·576	1·21	—	−47
		0·4	0·048	0·94	—	−55
3	Lewisite oxide	0·2	0·54	0·80	−35	—
		0·2	0·048	0·84	−40	—
4	Lewisite oxide	0·2	0·196	0·92	—	−34
		0·2	0·049	0·85	—	−39
5	*p*-Chloromercuribenzoate	0·4	0·576	0·73	−50	−33
6	*p*-Chloromercuribenzoate	0·4	0·048	0·72	−86	−74
7	Lewisite oxide	0·2	0·576	1·20	−55	−40
8	Lewisite oxide	0·2	0·048	0·75	−43	−23
(*b*) *p*-Nitrophenol						
9	*p*-Chloromercuribenzoate	0·4	0·096	4·32	−58	−39
10	Lewisite oxide	0·4	0·096	4·32	−24	−14

4mM. At this concentration, they actually increased the intensity of the colour of the coupled dyestuff, and they were therefore added to the controls before incubation. Neither compound (4mM) of itself had an appreciable influence upon glucuronide synthesis.

The results (Table 2) show that complete protection against, and reversal of, the inhibition by the mercurials was obtained with GSH and cysteine. With the arsenicals, neither gave more than partial protection, and the reversal of inhibition was very small. Protection was complete against *N*-ethylmaleimide and iodoacetamide, but barely measurable against *o*-iodosobenzoate. With iodoacetamide, there was an apparent reversal, whereas the inhibition due to *N*-ethylmaleimide and *o*-iodosobenzoate was virtually irreversible.

Experiments with GSSG tended to give rather variable results. At 2mM, it did not protect against lewisite oxide, and the inhibition by the mercurials was considerably increased. GSSG (2mM) itself was not an inhibitor, when incubated with homogenate for periods up to 30min. before addition of *o*-aminophenol and UDP-glucuronate.

Protection by UDP-glucuronate. In the preceding experiments the enzyme was not completely saturated with UDP-glucuronate. The results in Table 3 (Expts. 1–4) show that merely increasing the concentration of UDP-glucuronate had little effect, irrespective of whether the nucleotide was added before ('protection') or after ('reversal') the homogenate. In further experiments (Expts. 5–8), the inhibition was much less when UDP-glucuronate was added to the homogenate before, as compared with after, the inhibitor, and was of the same order at both high and low substrate concentrations. This protective effect was not shown by the second substrate, *o*-aminophenol, if mixed with the homogenate before the inhibitor, and UDP-glucuronate added subsequently (Table 6, see footnote).

Table 4. *Effect of glutathione on the decline in UDP-transglucuronylase activity on aging mouse-liver homogenates*

Digests were aged by incubating for 30min. at 30° in the presence or absence of GSH (4mM); then *o*-aminophenol (0·14mM) and UDP-glucuronate (0·049mM) were added and there was a further 20 min. incubation. In the controls, the first incubation was omitted.

	Glucuronide formed (mμmoles/mg./hr.)		Decline in activity (%)	
Treatment	Expt. 1	Expt. 2	Expt. 1	Expt. 2
Control+GSH	1·05	0·82	—	—
Control−GSH	1·08	0·76	—	—
Aged+GSH	0·58	0·42	−45	−49
Aged−GSH	0·79	0·52	−27	−32

Attempted stabilization of enzyme activity with thiols. Experiments were performed to determine whether GSH was able to arrest the rapid decline in synthetic activity when homogenates are 'aged' by incubation at 30°. The results (Table 4) show

that the fall-off in activity was apparently accelerated by GSH.

Homogenates rapidly lose UDP-transglucuronylase activity when exposed to ultrasonic radiation. Cysteine was unable either to prevent or reverse the inactivation.

Homogenates in sucrose, contained in a glass tube cooled in ice–water, were irradiated for 20sec. in an MSE–Mullard ultrasonic generator (20kcyc./sec.) with a titanium probe, and the enzyme assay was afterwards carried out. Cysteine was added either before ('protection') or after ('reversal') irradiation. In a reversal experiment, the loss of activity caused by ultrasonic irradiation in the control was 24%, and in the presence of 2mM-cysteine it was 27%. In a second experiment with 3mM-cysteine, the corresponding values were 72 and 75%. In a protection experiment (cysteine concentration during irradiation, 6mM; in the digest, 2mM), the decline in the control was 33%, and in the presence of cysteine was 59%.

p-*Nitrophenyl glucuronide formation*

Inhibition by organic mercurials and tervalent arsenicals (*Fig.* 5). Complete inhibition of the formation of *p*-nitrophenyl glucuronide was attained by 0·8mM-*p*-chloromercuribenzoate. There was no inhibition below about 0·01mM, and no activation was detectable at lower concentrations. The maximum inhibition by the tervalent arsenicals was 25% at 0·4mM, below which value the curve resembled that of *p*-chloromercuribenzoate.

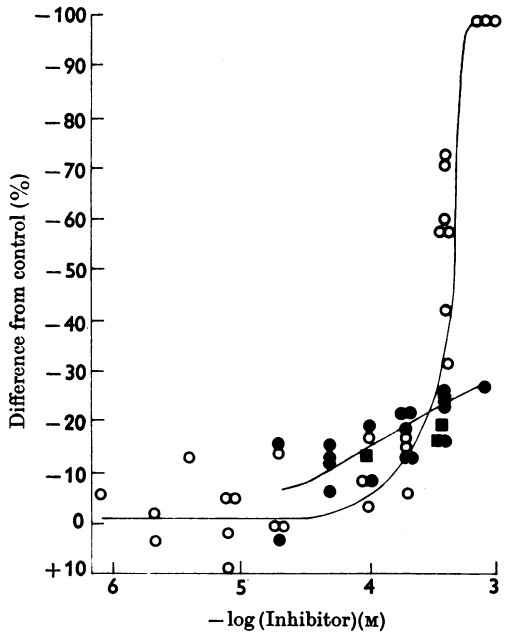

Fig. 5. Effect of *p*-chloromercuribenzoate (○), lewisite oxide (●) and arsenite (■) on the synthesis of *p*-nitrophenyl glucuronide in mouse-liver homogenates. The conditions were as given in Fig. 3.

Phenylarsonic acid and 2-chlorovinylarsonic acid (3·2mM) were not inhibitory.

Addition and alkylating agents (*Fig.* 3). *N*-Ethylmaleimide was approximately as inhibitory with *p*-nitrophenol as with *o*-aminophenol. Iodoacetamide was much less inhibitory. High concentrations were required and the development of inhibition was relatively slow, the points for 15min. and 20min. treatment with iodoacetamide before incubation lying on the same curve. The exact significance of these results is doubtful, since it is accepted that this reagent may react with groups in proteins other than thiol (Boyer, 1959).

o-Iodosobenzoate (*Fig.* 4). Glucuronide formation with *p*-nitrophenol was considerably less sensitive to *o*-iodosobenzoate than with *o*-aminophenol.

Protection and reversal by glutathione. With some of the inhibitors these experiments were less satisfactory than with *o*-aminophenol, since higher concentrations of inhibitor were required and the degree of inhibition was smaller. Higher GSH concentrations were possible, but corrections were necessary since above 4mM inhibition of *p*-nitrophenyl glucuronide synthesis became evident (with 12mM-GSH, −10%; with 18mM-GSH, −15%). In general, the results (Table 5) resembled those with *o*-aminophenol, the inhibitions with *N*-ethylmaleimide and *o*-iodosobenzoate being irreversible, those with lewisite oxide and iodoacetamide partly reversible and that with *p*-chloromercuribenzoate possibly completely so. GSSG did not reverse the inhibition by lewisite oxide or *p*-chloromercuribenzoate.

Protection by UDP-glucuronate (*Expts.* 9 *and* 10 *in Table* 3). UDP-glucuronate gave partial protection to the enzyme against lewisite oxide and *p*-chloromercuribenzoate.

Possibility of competitive inhibition by reagents containing carboxyl groups

Since UDP-transglucuronylase can use carboxyl groups as acceptors (Dutton, 1956), it seemed possible that the inhibitory effects of *p*-chloromercuribenzoate and *o*-iodosobenzoate might be, at least partially, competitive in character, rather than entirely by reaction with thiol groups. Frisell & Hellerman (1957) have shown this is true of the inhibition of D-amino acid oxidase by these two reagents. The results in Table 6 do not support this possibility, for when the acceptor concentra-

Table 5. *Effect of glutathione on the inhibition of* p-*nitrophenyl glucuronide formation by thiol reagents*

Digests contained tris–HCl buffer, pH 7·4 (33 mM), UDP-glucuronate (0·096 mM), *p*-nitrophenol (0·14 mM), homogenate (10 mg. wt wet.), inhibitor, GSH and water to 0·3 ml. Incubation was for 15 min. at 30°. The procedure was as given in the Methods section.

Inhibitor	Concn. of inhibitor (mM)	Inhibition: difference from control (%)	Concn. of GSH (mM)	Protection: difference from control (%)	Reversal: difference from control (%)
p-Chloromercuribenzoate	0·4	−58	4·0	—	−12
Lewisite oxide	0·4	−26	4·0	—	−9
N-Ethylmaleimide	0·8	−85	12·0	−4	−88
Iodoacetamide	4·0	−43	18·0	−12	−20
o-Iodosobenzoate	0·8	−20	8·0	—	−19

Table 6. *Effect of acceptor concentration on the inhibition of UDP-transglucuronylase by* p-*chloromercuribenzoate and* o-*iodosobenzoate*

To acceptor, inhibitor and buffer, homogenate was added 6 min. before incubation in (*a*), and 10 min. in (*b*). UDP-glucuronate (final concn. 0·184 mM) was added just before beginning of incubation. Other conditions were as given in the Methods section.

Inhibitor	Concn. of inhibitor (mM)	Acceptor	Concn. of acceptor (mM)	Glucuronide formed in control (mμmoles/mg./hr.)	Inhibition: difference from control (%)
(*a*) *p*-Chloromercuribenzoate	0·2	*o*-Aminophenol	0·14	0·85	−48
			0·56	0·78	−63
			0·56	0·78	−71*
(*b*) *o*-Iodosobenzoate	0·8	*p*-Nitrophenol	0·07	2·98	−19
			0·28	1·21	−16

* The acceptor, homogenate and buffer were first mixed, and then the inhibitor was added, followed later by UDP-glucuronate.

tion was increased the degree of inhibition was not diminished.

DISCUSSION

The high rate of conjugation of *p*-nitrophenol, and the greater sensitivity and simplicity of the determination as compared with *o*-aminophenol, have some attractions as a routine method for determining glucuronide conjugation. The colour reaction was relatively free from interference, and controls incubated without UDP-glucuronate showed that other likely metabolic reactions catalysed by tissue enzymes, such as reduction, were probably of small account. Nevertheless, since this is a subtractive method, in that conjugation is measured merely by the decrease in the extinction at 400 mμ, more attention to blanks was required than with *o*-aminophenol. Duplicates were less concordant and considerable care in pipetting the very small volumes of reagents was essential. The *o*-aminophenol method has the great advantage of measuring glucuronide formation directly, and for most purposes is considered the more reliable and the results easier to interpret.

The UDP-transglucuronylase systems for both *o*-aminophenol and *p*-nitrophenol were inhibited by all the types of reagent known to react with thiol groups, namely, mercaptide-forming, addition, alkylating and oxidizing. Further, with the first three types of reagent, some degree of protection was afforded by GSH and cysteine, and in certain cases varying degrees of reversal could be brought about. Even allowing for the rather low specificity of some of these reagents for thiol groups (Boyer, 1959), these results appear to justify classifying the UDP transglucuronylase of mouse liver with other thiol enzymes.

Isselbacher *et al.* (1962) remarked on the high concentrations (1 mM) of organic mercurials required for maximum effect (60% inhibition) on the purified rabbit-liver enzyme. The inconclusive nature of these results was further borne out by the inability of GSH to restore the activity, and the lack of evidence from other types of inhibitor. In the present work, the sensitivity to the mercurials was remarkably high, considering the large amount of protein in the homogenates, whereas activation of the *o*-aminophenol system was measurable below

1 μM. Similar activating effects by organic mercurials have been noticed with some other thiol enzymes, e.g. myosin (Kielley & Bradley, 1956) and the glutamate dehydrogenase of calf and chicken liver (Hellerman, Schellenberg & Reiss, 1958).

The tervalent arsenoxides and arsenite, perhaps the most specific of all reagents for thiol groups, require two such groups close enough together to enable a ring structure to be formed. The potency of the arsenicals at low concentrations, and the poor ability of the monothiols to reactivate or protect, are in agreement with this mechanism. It may not be altogether coincidental that *o*-iodosobenzoate, another reagent that requires two neighbouring thiol groups, inactivated the *o*-aminophenol and *p*-nitrophenol systems to about the same extent as the arsenicals, though much higher concentrations were required.

The protective effect of the substrate against the toxicity of thiol reagents has been observed with a considerable number of thiol enzymes. The difficulties in interpreting such results, by the binding of substrates by thiol groups, or by alterations in the spatial configuration of the enzyme protein, have been discussed by Boyer (1959). In the present case one would have to consider such findings as the high reactivity, yet incomplete inhibition, shown by tervalent arsenicals, and the lack of protection by the acceptor. Whatever the nature of the enzyme–substrate binding, it must be highly specific for the donor. In the briefly reported work of Storey & Dutton (1955), a concentration of UDP-glucose ten times as great as that of UDP-glucuronate had no inhibitory or activating effect; whereas, in other experiments (I. D. E. Storey, unpublished work) and in the extensive results of Pogell & Leloir (1961) UDP-*N*-acetylglucosamine was an activator.

Though the effects of thiol inhibitors with *o*-aminophenol and *p*-nitrophenol were qualitatively rather similar, some differences were apparent. *o*-Aminophenol conjugation was much more sensitive to all the inhibitors with the exception of *N*-ethylmaleimide, which was virtually equally active with both acceptors. The activation of *o*-aminophenol conjugation by *p*-chloromercuribenzoate was absent from that of *p*-nitrophenol, and the greater activity against *o*-aminophenol of low concentrations of lewisite oxide compared with *p*-chloromercuribenzoate was barely appreciable with *p*-nitrophenol. Such differences, though suggesting that different enzymes may be concerned, must be interpreted cautiously (Singer, 1948). The suggestion is, nevertheless, reinforced by the observation that *p*-nitrophenol was not a competitive inhibitor of the *o*-aminophenol system (Storey, 1965). On the other hand, *o*-aminophenol was a competitive inhibitor of *p*-nitrophenyl glucuronide formation; but, even if it were a substrate in this system, the high concentrations required make it unlikely that enough conjugation would have taken place to affect the interpretation of the *p*-nitrophenol results appreciably.

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