Properties of Lubrol-Extracted Uridine Diphosphate Glucuronyltransferase

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1. A partially purified UDP-glucuronyltransferase was obtained by extracting rat liver microsomal preparations with Lubrol, a non-ionic detergent. 2. The soluble enzyme catalysed conjugation of both *o*-aminophenol and *p*-nitrophenol and was extremely stable when compared with untreated microsomal preparations. 3. The characteristics of the conjugation of the two phenols were found to differ with respect to pH optimum, bivalent cation requirement and Michaelis constants, suggesting that more than one enzyme is involved in the conjugation reaction.

Hepatic UDP-glucuronyltransferase (EC 2.4.1.17) catalyses the transfer of glucuronic acid from UDP-glucuronic acid to a variety of alcoholic, phenolic, carboxylic and amine acceptor substrates (Dutton & Storey, 1954; Dutton, 1956; Axelrod, Inscoe & Tomkins, 1957) with the concurrent formation of UDP and a glucuronide conjugate. Bilirubin (Lathé & Walker, 1958), thyroxine and some steroid hormones (Isselbacher, 1956), and several drugs (Smith & Williams, 1966), are conjugated by this enzyme.

Several lines of evidence suggest that there is more than one UDP-glucuronyltransferase. These include the inability of some species, such as the cat and the Gunn rat, to conjugate certain substrates (Dutton, 1966*b*; Arias, 1961), the apparently separate rates of development of activity towards *p*-nitrophenol and *o*-aminophenol in different species (Dutton, 1966*a*; Dutton, Langelaan & Ross, 1964) and the lack of mutual inhibition between different substrates (Dutton, 1956; Lathé & Walker, 1958; Isselbacher, Chrabas & Quinn, 1962; Storey, 1965; Temple, Clement & Done, 1968). The inability to solubilize activity towards certain substrates (Isselbacher *et al.* 1962) and the differences in the distribution of activity in rough- and smooth-surfaced microsomal fractions for different substrates (Gram, Hansen & Fouts, 1968; Howland & Burkhalter, 1970*a*) also support the idea that there are several UDP-glucuronyltransferases. However, these experiments were performed with crude homogenates, microsomal suspensions or

partially purified unstable preparations and thus only provide incomplete and indirect evidence for the existence of more than one enzyme.

The question of multiplicity is important. Not only would the answer be valuable in the comparison of UDP-glucuronyltransferase activities from different species and for different substrates, but it also would contribute to the understanding of the causes of hyperbilirubinaemia in the newborn human.

We have found a rapid and simple method for preparing hepatic microsomal fractions, which results in an apparently soluble, stable preparation of UDP-glucuronyltransferase that exhibits some differences in its ability to conjugate *o*-aminophenol and *p*-nitrophenol.

A preliminary report of this work was presented at the meeting of the Federation of American Societies for Experimental Biology in Atlantic City, N.J., U.S.A. (Howland & Burkhalter, 1970*b*).

METHODS

Chemicals. UDP-glucuronic acid was purchased from Sigma Chemical Co., St Louis, Mo., U.S.A. and *p*-nitrophenol (spectrophotometer grade) from Calbiochem, Los Angeles, Calif., U.S.A.; *o*-aminophenol and *o*-aminophenyl glucuronide were obtained from Aldrich Chemical Co., Milwaukee, Wis., U.S.A.; Lubrol WX, C₁₆H₃₃[OC₂H₄]₁₆OH, mol.wt. 947, was a gift of ICI (America) Inc., Stamford, Conn., U.S.A. All other chemicals were reagent grade and purchased from local suppliers.

Enzyme preparation. Male Wistar rats (150–200 g) were killed by decapitation and exsanguinated, and the livers

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removed and placed in ice-cold 0.32M-sucrose-1 mM-tris-HCl-1 mM-EDTA, pH 6.9 (buffered sucrose). The livers were then blotted dry, weighed, minced with scissors and homogenized in 3-4 vol. of buffered sucrose by using a Potter-Elvehjem homogenizer (Teflon pestle) with ten up-and-down strokes (2000 rev./min). All subsequent steps were performed at 0-4°C. The homogenate was centrifuged at 9000g_{av} for 20 min in a Lourdes Betafuge (rotor no. 9RA). Microsomal fractions were then isolated by centrifuging the 9000g supernatant at 105000g_{av} for 60 min in a Spinco model L preparative ultracentrifuge (rotor no. FA40). After the high-speed supernatant had been discarded, the microsomal pellet was resuspended by hand in buffered sucrose with a Dounce homogenizer; 1.0 ml of the suspension contained a microsomal fraction derived from 1 g of liver.

By using a slight modification of the method of Shirachi, Allard & Trevor (1970) the microsomal fractions were treated with 0.2% (w/v) Lubrol by adding an equal volume of 0.4% (w/v) Lubrol in buffered sucrose. After thorough mixing the Lubrol microsomal mixture was left on ice for 15 min and then centrifuged for 105000g_{av} for 60 min. The resulting supernatant was employed as the source of UDP-glucuronyltransferase. When recovery of enzyme activity was determined, the final pellet was resuspended in at least 2 ml of buffered sucrose.

Zonal centrifugation. After extraction with Lubrol, 30 ml (6 mg of protein/ml) of the extract of UDP-glucuronyltransferase (specific activity 80.2 nmol of *p*-nitrophenyl glucuronide and 37.4 nmol of *o*-aminophenyl glucuronide/15 min per mg of protein) was placed on a 200 ml linear 15-35% (w/w) sucrose gradient. Besides the gradient, the rotor also contained an underlay of 245 ml of 40% (w/w) sucrose and an overlay of 200 ml of 8% (w/w) sucrose. All sucrose solutions contained 0.5 mM-EDTA, pH 7.0. The gradient was loaded in a preparative zonal rotor (Beckman, rotor no. Ti 14; 5.3 cm path-length) and centrifuged in a Beckman model L2-65B ultracentrifuge at 48000 rev./min (171800g_{max}) for 20 h and subsequently collected in 10 ml fractions. Before protein determination, 3.0 ml samples were dialysed overnight against deionized water, since high sucrose concentrations interfered with the protein assay.

Assay of UDP-glucuronyltransferase. UDP-glucuronyltransferase activity was determined with either *o*-aminophenol or *p*-nitrophenol as the acceptor substrate. Typical incubation mixtures contained, in addition to microsomal preparation, 0.3-3.0 mg of protein, 25 μmol of tris-HCl buffer, pH 7.4, 7.5 μmol of MgCl₂, 1.0 μmol of UDP-glucuronic acid and either 0.5 μmol of *o*-aminophenol or 0.05 μmol of *p*-nitrophenol in a final volume of 0.5 ml. *o*-Aminophenol was sublimed before addition to the medium and was dissolved in 0.2% (w/v) ascorbic acid to prevent oxidation. The enzyme assays were performed in 12 ml conical centrifuge tubes. After a 3 min pre-incubation, UDP-glucuronic acid was added to start the reaction and the tubes were shaken for 15 min at 37°C in air in a Dubnoff metabolic shaking incubator. When *o*-aminophenol was the substrate, the reaction was stopped by addition of 0.5 ml of 0.625M-trichloroacetic acid in 1M-KH₂PO₄, pH 2.1; when *p*-nitrophenol was the substrate 0.5 ml of 0.5M-trichloroacetic acid was added to stop the reaction. Blanks containing all reactants except UDP-glucuronic acid were incubated with the

assay tubes and the reaction was terminated immediately after addition of enzyme. All determinations were made in duplicate.

o-Aminophenyl glucuronide was determined by the method of Dutton & Storey (1962). Formation of *p*-nitrophenyl glucuronide was measured by the disappearance of *p*-nitrophenol (Isselbacher, 1956). Protein was determined by the Lowry method (Lowry, Rosebrough, Farr & Randall, 1951) with bovine serum albumin (Calbiochem) as the standard.

RESULTS

Activation of UDP-glucuronyltransferase by Lubrol. Rat liver microsomal preparations were treated with various concentrations of Lubrol for 15 min. The ability of UDP-glucuronyltransferase to conjugate both *o*-aminophenol and *p*-nitrophenol was significantly enhanced by this treatment (Fig. 1). The greatest increase in activity for the two substrates (eightfold for *o*-aminophenol conjugation

Fig. 1. Effect of Lubrol on rat liver UDP-glucuronyltransferase. Microsomal fractions were treated with various concentrations of Lubrol and then assayed for UDP-glucuronyltransferase with either *p*-nitrophenol (●) or *o*-aminophenol (○) as the acceptor substrate.

and tenfold for *p*-nitrophenol conjugation) was observed when the final concentration of Lubrol was 0.2%. Other investigators have reported similar activations of UDP-glucuronyltransferase after treatment with detergents, such as Triton X-100 and deoxycholate (Winsnes, 1969; Mulder, 1970; Leuders & Kuff, 1967; Halac & Reff, 1967; Adlard & Lathe, 1970). When conventional microsomal fractions were used for assay of the enzyme, the addition of Lubrol directly to the reaction mixture also resulted in a marked activation.

Solubilization and recovery of UDP-glucuronyltransferase from Lubrol-treated microsomal fractions. Microsomal fractions were treated with 0.2% Lubrol and centrifuged at 105 000g_{av} for 60 min, which resulted in the three fractions: a top layer containing fat, which was discarded, a clear pale-yellow supernatant and a red pellet. Recovery of UDP-glucuronyltransferase in the supernatant and pellet was determined, the enzyme activity of the microsomal suspension in Lubrol being taken as 100% because of the activation of the enzyme by the detergent. Tables 1 and 2 summarize the re-

covery of protein and enzyme activity towards *p*-nitrophenol and *o*-aminophenol respectively. Approx. 25% of the total activity towards *p*-nitrophenol and 15% of the total activity towards *o*-aminophenol was recovered in a non-sedimentable form in the high-speed supernatant. The remaining activity was present in the pellet, which had a specific activity 7.4 and 13 times that in the supernatant for *p*-nitrophenol and *o*-aminophenol respectively.

The supernatant was subjected to further centrifugation at 105 000g for 3 h to assess the apparent solubility of the enzyme. Over 50% of the enzyme activity towards either substrate remained in the supernatant.

About 70% of the total microsomal protein was recovered in the 105 000g supernatant. Increasing the concentration of Lubrol (up to 0.8%) neither increased the solubilization of protein nor enhanced the recovery of enzyme activity for either substrate.

Several experiments were performed on the Lubrol-extracted enzyme to characterize it further.

Table 1. *Recovery of UDP-glucuronyltransferase activity towards p-nitrophenol after treatment with Lubrol*

Rat liver microsomal fractions were treated with 0.2% Lubrol (final concentration) and centrifuged at 105 000g for 60 min. UDP-glucuronyltransferase activity towards *p*-nitrophenol was determined in the high-speed supernatant and pellet and compared with the activity in untreated and Lubrol-treated microsomal preparations.

Fraction	Specific activity (nmol/15 min per mg of protein)	Total protein (mg)	$10^{-3} \times$ Total activity (nmol/15 min $\times$ total protein)	Percentage of total activity
Microsomal fraction	26.3	145	3.81	—
Microsomal fraction + Lubrol	269	145	39.0	100
Lubrol supernatant	92.0	107	9.84	25.2
Lubrol pellet	682	38	25.9	66.4
Total recovery				91.6

Table 2. *Recovery of UDP-glucuronyltransferase activity towards o-aminophenol after treatment with Lubrol*

Rat liver microsomal fractions were treated with 0.2% Lubrol (final concentration) for 15 min and then centrifuged at 105 000g for 60 min. UDP-glucuronyltransferase activity towards *o*-aminophenol was determined in the high-speed supernatant and pellet and compared with the activity in untreated and Lubrol-treated microsomal preparations.

Fraction	Specific activity (nmol/15 min per mg of protein)	Total protein (mg)	$10^{-3} \times$ Total activity (nmol/15 min $\times$ total protein)	Percentage of total activity
Microsomal fraction	16.6	145	2.40	—
Microsomal fraction + Lubrol	120	145	17.4	100
Lubrol supernatant	25.3	107	2.71	15.5
Lubrol pellet	328	38	12.4	71.0
Total recovery				86.5

Fig. 2. Stability of Lubrol-extracted UDP-glucuronyltransferase activity. Both the Lubrol extract (○) and untreated microsomal fractions (●) were stored at 3–4°C. On subsequent days, the activity of UDP-glucuronyltransferase was determined with *p*-nitrophenol as the substrate and compared with the initial activity on the day of preparation, which was 26.3 and 103.6 nmol/15 min per mg of protein for untreated and Lubrol-extracted microsomal preparations respectively.

Stability of Lubrol-extracted UDP-glucuronyltransferase. The activity of non-sedimented UDP-glucuronyltransferase towards *p*-nitrophenol was stable for at least 3 days when stored at 0–4°C, whereas the untreated microsomal fractions rapidly lost activity when similarly stored (Fig. 2). When stored at –20°C, the solubilized preparation was stable for several weeks and could be thawed and re-frozen several times with little loss of activity. Similar results were obtained when UDP-glucuronyltransferase was assayed with *o*-aminophenol as the substrate.

Time-course of activation of UDP-glucuronyltransferase by Lubrol. The activation of UDP-glucuronyltransferase by Lubrol occurred immediately on addition of the detergent (Table 3). Guinea-pig microsomal fractions were prepared as described in the Methods section and the disappear-

ance of *p*-nitrophenol was determined by the decrease in E_{400} after the addition of UDP-glucuronic acid. This method was employed so that the initial rate of reaction could be measured. Within 1 min after addition of Lubrol there was a fivefold increase in the rate of disappearance of *p*-nitrophenol. When the activity was determined at approx. 2½ h after the addition of the detergent no increase was observed, indicating that the activation was not dependent on the duration of exposure to the detergent. Because of the duration of centrifugation, the time required for activation could not be compared with the appearance of enzyme activity in the high-speed supernatant.

Effect of pH on Lubrol-extracted UDP-glucuronyltransferase. The pH optimum of the Lubrol-extracted rat enzyme was pH 6.7 when *p*-nitrophenol was employed as the substrate (Fig. 3). When *o*-aminophenol was the substrate, the enzyme exhibited a broad pH optimum between pH 7.3 and 8.0 (Fig. 3). We also observed a decrease in the rate of *p*-nitrophenol conjugation in the presence of tris-maleate buffer, whereas the rate of *o*-aminophenol conjugation was not affected by this buffer.

Effect of bivalent cations on Lubrol-extracted UDP-glucuronyltransferase. The rate of glucuronidation of *p*-nitrophenol by the Lubrol-extracted enzyme was more than doubled when the concentration of either Ca^{2+} or Mg^{2+} in the medium was 1.0 mM or greater (Fig. 4a). When the concentration of either bivalent cation was 30 mM, the activity toward *p*-nitrophenol was only 60% greater than in the absence of bivalent cation.

In contrast to glucuronidation of *p*-nitrophenol, in which conjugation occurred without added Mg^{2+} or Ca^{2+} , glucuronidation of *o*-aminophenol required the presence of bivalent cations (Fig. 4b). Maximum enzyme activity was observed at a Mg^{2+} or Ca^{2+} concentration of 5 mM. UDP-glucuronyltransferase activity towards *o*-aminophenol was unchanged in the presence of increasing Ca^{2+} concentrations up to 30 mM. However, increasing Mg^{2+} concentrations slightly decreased the rate of *o*-aminophenyl glucuronide formation.

Kinetic constants of Lubrol-extracted UDP-glucuronyltransferase. In contrast with the enzyme in untreated microsomal fractions, which was not saturated with 4.0 mM-UDP-glucuronic acid, UDP-glucuronyltransferase in the Lubrol extract was saturated with UDP-glucuronic acid at a concentration of 2.0 mM.

The Michaelis constants for *o*-aminophenol, *p*-nitrophenol and for UDP-glucuronic acid, when *o*-aminophenol was the acceptor substrate, were of the same order of magnitude when determined for the extracted enzyme (Table 4). On the other hand, the K_m for UDP-glucuronic acid when *p*-nitro-

Table 3. *Time-course of activation of UDP-glucuronyltransferase by Lubrol*

Guinea-pig liver microsomal fractions were prepared and their ability to conjugate *p*-nitrophenol was determined by recording the decrease in E_{400} . Both the non-treated and Lubrol-treated microsomal preparations had equivalent amounts of protein. The final concentration of Lubrol was 0.2%. In addition to microsomal preparations, the cuvettes contained 25 μ mol of tris-HCl buffer, pH 8.0, 0.05 μ mol of *p*-nitrophenol and 1.0 μ mol of UDP-glucuronic acid, which was added last to start the reaction. The total volume was 0.5 ml.

Time after addition of Lubrol	<i>p</i> -Nitrophenyl glucuronide (nmol/min)	
	Non-treated microsomal fraction	Lubrol-treated microsomal fraction
1 min 25s	0.11	0.57
6 min	0.11	0.52
10 min	0.11	0.55
167 min	0.11	0.46

Fig. 3. Effect of pH on Lubrol-extracted UDP-glucuronyltransferase. The pH optima for *p*-nitrophenol (Δ , $\blacktriangle$) and *o*-aminophenol ($\circ$, $\bullet$) were determined. The buffers employed were tris-maleate (Δ , $\circ$) and tris-HCl ($\blacktriangle$, $\bullet$) at a final concentration of 50 mM.

phenol was the acceptor substrate was an order of magnitude less. The K_m that we obtained for *p*-nitrophenol (6.0×10^{-4} M) was in good agreement with other reported values of 5.5×10^{-4} M, 6.2×10^{-4} M and 6.7×10^{-4} M in partially purified preparations from rat (Halac & Reff, 1967), rabbit (Isselbacher *et al.* 1962) and mouse (Winsnes, 1969) liver respectively.

The V_{max} for *o*-aminophenol was 2.7 nmol/min per mg of protein and for *p*-nitrophenol was 41.7 nmol/min per mg of protein.

Zonal centrifugation of Lubrol-extracted UDP-glucuronyltransferase on linear sucrose gradient. We were unable to obtain a complete separation of

Fig. 4. Effect of Mg^{2+} and Ca^{2+} on Lubrol-extracted UDP-glucuronyltransferase. Enzyme activity with *p*-nitrophenol (a) or *o*-aminophenol (b) was assayed in the presence of various concentrations of Mg^{2+} ($\circ$) or Ca^{2+} ($\bullet$). The chloride salts of the cations were used.

activities towards *o*-aminophenol and *p*-nitrophenol, most of the activity being found in those fractions containing the most protein (Fig. 5). However, UDP-glucuronyltransferase activity towards *o*-aminophenol was detected in fractions of density less than 1.056, whereas enzyme activity towards *p*-nitrophenol was found only in fractions of density

Table 4. *Michaelis constants of UDP-glucuronyl-transferase in Lubrol extracts of rat liver microsomal fractions*

All incubation media contained the following: 50 μ mol of tris-HCl buffer, pH 7.4, 15 μ mol of $MgCl_2$ and 0.1 ml of Lubrol extract containing 1.05 mg of protein, in a final volume of 1.0 ml. When the K_m for *o*-aminophenol or *p*-nitrophenol was determined the final concentration of UDP-glucuronic acid was 2.0 mM and the final concentration of *o*-aminophenol ranged between 0.1 and 1.0 mM and that of *p*-nitrophenol ranged between 0.025 and 0.20 mM. When the K_m for UDP-glucuronic acid (*o*-aminophenol as acceptor) was determined, the final concentration of *o*-aminophenol was 1.0 mM, and final concentrations of UDP-glucuronic acid ranged between 0.25 and 1.5 mM. When the K_m for UDP-glucuronic acid (*p*-nitrophenol as acceptor) was determined, the final concentration of *p*-nitrophenol was 0.1 mM and UDP-glucuronic acid final concentrations ranged between 0.1 and 1.2 mM. The results were analysed by fitting the best straight line obtained with linear regression analysis to a $1/v$ versus $1/s$ plot of the results.

Substrate	$10^4 K_m$ (M)
<i>o</i> -Aminophenol	3.4
UDP-glucuronic acid	4.3
<i>p</i> -Nitrophenol	6.0
UDP-glucuronic acid	0.58

greater than 1.056. Fraction 13 contained peak specific activity towards *o*-aminophenol (191 nmol/15 min per mg of protein) that was fivefold the activity in the original extract. No increase in specific activity towards *p*-nitrophenol was observed.

DISCUSSION

The partial purification of UDP-glucuronyl-transferase by Lubrol extraction has several advantages in that it is rapid and simple, it results in increased enzyme activity and the preparation is stable.

Other methods available for the solubilization of UDP-glucuronyltransferase have several disadvantages. These include the length of time for preparation, during which there is loss of activity (Halac & Reff, 1967; Mowat & Arias, 1970), the instability of the final preparation (Isselbacher *et al.* 1962; Halac & Reff, 1967; Adlard & Lathe, 1970) and, for deoxycholate-solubilized UDP-glucuronyltransferase, the need to remove the detergent before assay, since it inhibits enzyme activity (Halac & Reff, 1967). In contrast with these methods, the Lubrol extraction can be completed within 1 day, including assay of the extract, and the preparation is stable, losing negligible activity when stored at 0–4°C for 3 days or at –20°C for several weeks.

Fig. 5. Sucrose-density-gradient centrifugation of Lubrol-extracted UDP-glucuronyltransferase. O, *o*-Aminophenol conjugation; ●, *p*-nitrophenol conjugation; Δ, protein. The conditions of centrifugation are given in the Methods section.

A prominent feature of microsomal fractions treated with Lubrol was the eight- to ten-fold increase in activity. Increases in UDP-glucuronyl-transferase activity of a similar nature have been observed with other detergents, such as digitonin (Winsnes, 1969; Heirwegh & Meuwissen, 1968), deoxycholate (Leuders & Kuff, 1967; Halac & Reff, 1967; Adlard & Lathe, 1970) and Triton X-100 (Winsnes, 1969; Mulder, 1970; Leuders & Kuff, 1967). One may speculate that these substances increase enzyme activity either by enhancing access of the substrate to the enzyme or by uncovering more active sites on the enzyme.

Although we were unable physically completely to separate UDP-glucuronyltransferase activity towards *o*-aminophenol and *p*-nitrophenol by sucrose-density-gradient centrifugation, the partially purified enzyme exhibited different characteristics with regard to the conjugation of *o*-aminophenol and *p*-nitrophenol. These differences, although not conclusive, suggest that the two phenols are substrates for distinctive UDP-glucuronyltransferases. The pH optimum for the two substrates differed; the pH optimum for *o*-aminophenol conjugation was higher and broader than that for *p*-nitrophenol conjugation. The substrates also differed with respect to the effect of Mg^{2+} and Ca^{2+} . Activity towards *o*-aminophenol exhibited an absolute requirement for bivalent cations, whereas significant

activity towards *p*-nitrophenol was found in the absence of bivalent cations. Conjugation of neither *o*-aminophenol nor *p*-nitrophenol was inhibited by Ca^{2+} . Other authors have observed differences in the effects of bivalent cations on the conjugation of *o*-aminophenol (Dutton & Storey, 1954; Stevenson & Dutton, 1962) and bilirubin (Tomlinson & Yaffe, 1966; Stevenson & Dutton, 1962; Van Roy & Heirwegh, 1968; Lage & Spratt, 1968), suggesting that more than one enzyme conjugates these substrates.

The Lubrol-extracted UDP-glucuronyltransferase exhibited different kinetics with respect to *o*-aminophenol and *p*-nitrophenol. The ratio of K_m values for *o*-aminophenol and UDP-glucuronic acid was approx. 1:1, whereas the ratio of K_m values for *p*-nitrophenol and UDP-glucuronic acid was 10:1. The differences in ratio of Michaelis constant suggest separate enzymes for conjugation of *o*-aminophenol and *p*-nitrophenol. If a single enzyme was responsible for conjugation of both phenols, it would seem unlikely that the substrate common to both reactions (UDP-glucuronic acid) would have K_m values separated by an order of magnitude ($4.3 \times 10^{-4} \text{ M}$ compared with $0.58 \times 10^{-4} \text{ M}$), depending on whether *o*-aminophenol or *p*-nitrophenol was the acceptor substrate.

At present, we do not know whether the non-sedimentable enzyme is in true solution or not, since only gravitational methods were employed as criteria for solubility. However, even if the enzyme(s) were in particle form, the ability to purify further and to characterize the enzyme(s) is not excluded.

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REFERENCES

- Adlard, B. P. F. & Lathe, G. H. (1970). *Biochem. J.* **119**, 437.
 Arias, I. M. (1961). *Biochem. biophys. Res. Commun.* **6**, 81.
 Axelrod, J., Inscoc, J. K. & Tomkins, G. M. (1957). *Nature, Lond.*, **179**, 538.
 Dutton, G. J. (1956). *Biochem. J.* **64**, 693.
 Dutton, G. J. (1966a). *Biochem. Pharmac.* **15**, 947.
 Dutton, G. J. (1966b). In *Glucuronic Acid, Free and Combined*, p. 185. Ed. by Dutton, G. J. New York and London: Academic Press.
 Dutton, G. J., Langelan, D. E. & Ross, P. E. (1964). *Biochem. J.* **93**, 4p.
 Dutton, G. J. & Storey, I. D. E. (1954). *Biochem. J.* **57**, 275.
 Dutton, G. J. & Storey, I. D. E. (1962). In *Methods in Enzymology*, vol. 5, p. 159. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
 Gram, T. E., Hansen, A. R. & Fouts, J. R. (1968). *Biochem. J.* **106**, 587.
 Halac, E., jun. & Reff, A. (1967). *Biochim. biophys. Acta*, **139**, 328.
 Heirwegh, K. P. M. & Meuwissen, J. A. T. P. (1968). *Biochem. J.* **110**, 31p.
 Howland, R. D. & Burkhalter, A. (1970a). *Proc. Western Pharmac. Soc.* **13**, 8.
 Howland, R. D. & Burkhalter, A. (1970b). *Fedn Proc. Fedn Am. Soc. exp. Biol.* **29**, 412.
 Isselbacher, K. J. (1956). *Recent Prog. Horm. Res.* **12**, 134.
 Isselbacher, K. J., Chrabas, M. F. & Quinn, R. C. (1962). *J. biol. Chem.* **237**, 3033.
 Lage, G. L. & Spratt, J. L. (1968). *Archs Biochem. Biophys.* **126**, 175.
 Lathe, G. H. & Walker, M. (1958). *Biochem. J.* **70**, 705.
 Leuders, K. K. & Kuff, E. L. (1967). *Archs Biochem. Biophys.* **120**, 198.
 Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
 Mowat, A. P. & Arias, I. M. (1970). *Biochim. biophys. Acta*, **212**, 65.
 Mulder, G. J. (1970). *Biochem. J.* **117**, 319.
 Shirachi, D. Y., Allard, A. A. & Trevor, A. J. (1970). *Biochem. Pharmac.* **19**, 2893.
 Smith, R. L. & Williams, R. T. (1966). In *Glucuronic Acid, Free and Combined*, p. 457. Ed. by Dutton, G. J. New York and London: Academic Press.
 Stevenson, I. M. & Dutton, G. J. (1962). *Biochem. J.* **82**, 330.
 Storey, I. D. E. (1965). *Biochem. J.* **95**, 209.
 Temple, A. R., Clement, M. S. & Done, A. K. (1968). *Proc. Soc. exp. Biol. Med.* **128**, 307.
 Tomlinson, G. A. & Yaffe, S. J. (1966). *Biochem. J.* **99**, 507.
 Van Roy, F. P. & Heirwegh, K. P. M. (1968). *Biochem. J.* **107**, 507.
 Winsnes, A. (1969). *Biochim. biophys. Acta*, **191**, 279.