SUBSTRATE SPECIFICITY OF MAMMALIAN D-GLUCURONOLACTONE DEHYDROGENASE

By P. G. TONKES* and C. A. MARSH*

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Abstract

The NAD^+-dependent D-glucuronolactone dehydrogenase from the soluble fraction of male rat liver homogenates was purified 350-fold with an overall recovery of 11%. The purified enzyme also catalysed the oxidation of D-mannuronolactone and a series of aliphatic aldehydes, and the activities with D-glucuronolactone, D-mannuronolactone, and acetaldehyde as substrates could not be separated during purification or on ultracentrifugation of the preparation. Kinetic properties with both D-glucuronolactone and acetaldehyde as substrate are compared. The NADH production from simultaneous D-glucuronolactone and acetaldehyde oxidation was not additive. Acetaldehyde was also found to be a competitive inhibitor of D-glucaric acid formation. From these results, and the effects of activating and inhibitory compounds on both activities, it is concluded that the enzyme oxidizing D-glucuronolactone is an aldehyde dehydrogenase.

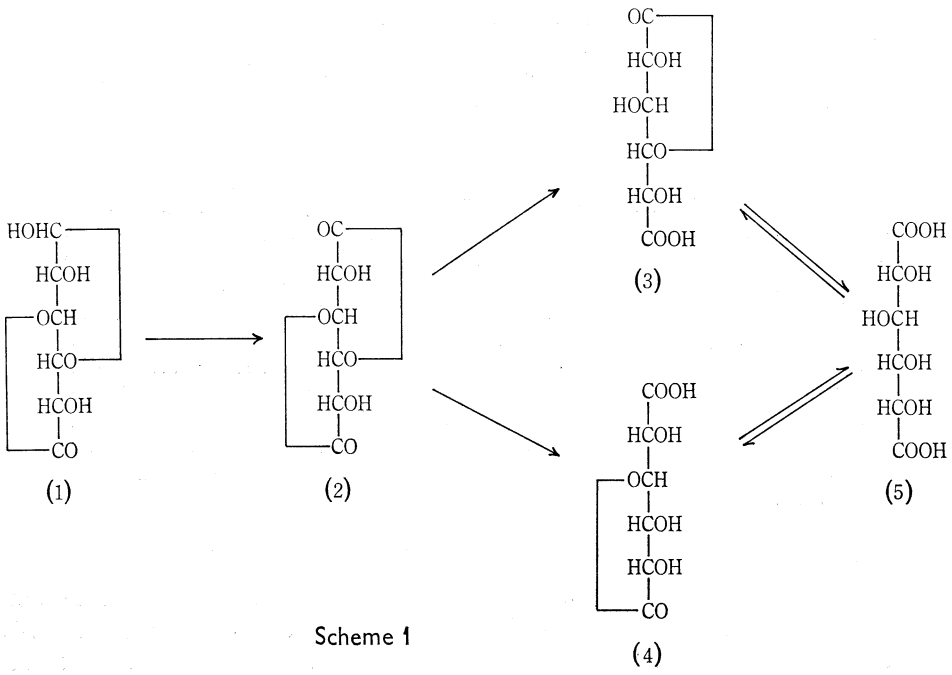
I. INTRODUCTION

The presence of D-glucaric acid in normal mammalian urine has been demonstrated (Marsh 1963*a*; Aarts 1965; Ishidate *et al.* 1965) and this appears to be partly responsible for the inhibitory effect of urine on β-glucuronidase [E.C. 3.2.1.31] (Marsh 1962). Large increases in D-glucaric acid excretion were observed in man, rat, and guinea pig after D-glucuronolactone ingestion (Marsh 1963*a*). An NAD^+-dependent enzyme was found in rat liver cytosol, designated D-glucuronolactone dehydrogenase [E.C. 1.1.1.70] which catalysed the oxidation of D-glucuronolactone to D-glucaric acid (Marsh 1963*b*). The proposed reaction mechanism of the dehydrogenation of D-glucuronolactone (1) is given in Scheme 1. The initial product of the postulated reaction sequence is D-glucaro(1→4),(6→3)dilactone (2) which breaks down to both D-glucaro(1→4)lactone (3) and D-glucaro(6→3)lactone (4) and then to D-glucaric acid (5). Sadahiro *et al.* (1966) purified the enzyme 15-fold from guinea pig liver and showed that it also oxidizes D-mannuronolactone to D-mannaric acid. The free aldehyde form of D-glucuronolactone is present in an aqueous solution of the substrate (Marsh 1966) and Sadahiro *et al.* (1966) suggested that the direct precursor in enzymic oxidation of D-glucuronolactone was the aldehydic form of the substrate.

While much information has been presented on the activity of the enzyme *in vivo* (Marsh 1963*b*; Marsh and Reid 1963; Marsh and Carr 1965), the enzyme has never been extensively purified and characterized, and this has now been attempted to enable the study of its specificity and kinetic properties. The results obtained indicate that D-glucuronolactone and D-mannuronolactone are dehydrogenated by the same enzyme that catalyses the oxidation of a number of aliphatic aldehydes.

* School of Biochemistry, University of New South Wales, Kensington, N.S.W. 2033.

A preliminary report on some of the results has already been published (Tonkes and Marsh 1971). Recently Aarts and Hinnen-Bouwmans (1972), using a preparation of the rat liver enzyme considerably less pure than ours, have arrived at similar conclusions.



Scheme 1

II. Materials and Methods

(*a*) *Substrates and Other Materials*

D-Glucuronolactone was obtained from Koch-Light Laboratories Ltd. D-Mannuronolactone was prepared by the hydrolysis of methyl α-D-mannosiduronic acid (Marsh 1952). Acetaldehyde and propionaldehyde (British Drug Houses Ltd.) and n-butyraldehyde, isobutyraldehyde, and n-valeraldehyde (Koch-Light Laboratories Ltd.) were redistilled at atmospheric pressure and stored at 0°C in air-tight containers. Phenolphthalein β-D-glucuronide was prepared biosynthetically (Levvy and Marsh 1959). Sources of other materials were as follows: NAD^+, NADH, and $NADP^+$ (sodium salts) and β-mercaptoethanol, Sigma Chemical Company; 8-hydroxy-5-quinolinesulphonic acid, Eastman Organic Chemicals; ammonium sulphate (special enzyme grade), Mann Research Laboratories; DEAE-Sephadex A50, Sephadex G200, and blue dextran-2000, Pharmacia Chemicals Inc.; CM22 cellulose (advanced fibrous grade with capacity of 0·6 m-equiv/g), Whatman, England. All other reagents were of the highest purity obtainable from commercial sources.

(*b*) *Enzyme Assay*

Enzyme activity was assayed by measuring the rate of increase in absorbance at 340 nm resulting from the reduction of NAD^+ in the presence of the enzyme and substrate using a Unicam SP800 recording spectrophotometer. The extinction coefficient of NADH at this wavelength was assumed to be $6{\cdot}25 \times 10^6$ cm²/mole (Rafter and Colowick 1957). The standard reaction mixture contained 12·5 μmoles of D-glucuronolactone, D-mannuronolactone, or acetaldehyde and 6·25 μmoles of NAD^+ in a final volume of 2·5 ml in silica cuvettes of light path 1 cm. Unless otherwise specified, the reaction mixture containing D-glucuronolactone or D-mannuronolactone was buffered

with 0·05M NaH_2PO_4–NaOH, pH 6·5, while 0·05M $Na_4P_2O_7$–HCl, pH 9·3, buffered the reaction mixture with acetaldehyde as substrate. The reaction was started by addition of the enzyme to the reaction mixture. The control cuvette contained all reagents except the substrate. The temperature was maintained at 37°C and the increase in absorbance was recorded for about 8 min. The measurement of D-glucuronolactone dehydrogenase activity by determination of D-glucaric acid production was performed according to Marsh (1963*b*). It was shown that the production of D-glucaric acid and NADH were in equimolar proportions.

One unit of dehydrogenase activity corresponds to the amount of enzyme necessary to catalyse the formation of 1 μmole of NADH per hour under the given conditions. Specific activity is expressed as units per milligram of protein assayed by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

(*c*) *Preparation of Chromatography Gels*

DEAE-Sephadex A50 was pretreated successively with 0·5N NaOH and 0·5N HCl followed by equilibration in the elution buffer. The suspension was used to pour a column which was washed with the elution buffer overnight at 4°C.

Fibrous CM-cellulose was pretreated with 0·2N NaOH and 0·2N HCl. The suspension was then equilibrated in 0·05M NaH_2PO_4–NaOH, pH 4·5, containing 0·005M 8-hydroxy-5-quinolinesulphonic acid, 0·02M β-mercaptoethanol, and 0·2M KCl; this was poured to give a column which was washed with the equilibrating buffer overnight at 4°C.

Sephadex G200 was allowed to swell in excess 0·1M KCl solution on a boiling water-bath for 5 hr. The gel was then equilibrated in the eluting buffer and a column poured and further washed with the eluting buffer overnight at 4°C.

(*d*) *Enzyme Purification*

All operations were carried out between 0 and 4°C unless otherwise specified. Normal male Wistar rats (200–300 g) were killed by a blow on the skull. The livers were rapidly removed, washed with 0·17M KCl solution, and weighed. The chopped tissue was homogenized for 1 min (3 strokes, average speed 5000 rev/min) in a chilled glass and Teflon homogenizer (Arthur H. Thomas Co., Philadelphia, USA) with sufficient ice-cold 0·17M KCl to give a 30% (w/v) moist tissue concentration. The homogenate was centrifuged at 45,000 *g* for 120 min and the clear supernatant solution was decanted through a pad of glass wool to remove floating fat particles.

Solid $(NH_4)_2SO_4$ (240 g per litre of supernatant) was slowly added to the crude extract with mechanical stirring to give 40% salt saturation. After standing for 15 min the suspension was centrifuged at 3000 *g* for 10 min and the precipitate discarded. The supernatant was brought to 60% salt saturation by the addition of 130 g $(NH_4)_2SO_4$ per litre of supernatant. During both additions of $(NH_4)_2SO_4$ the pH of the solutions remained in the neutral region (pH 6–7). After standing for 15 min the precipitate was sedimented by centrifugation (10 min at 8000 *g*) then redissolved in 0·05M NaH_2PO_4–NaOH, pH 5·0, corresponding to one-tenth the volume of the 45,000-*g* supernatant. This buffer, and all those used in the later purification stages unless stated, also contained 0·005M 8-hydroxy-5-quinolinesulphonic acid and 0·02M β-mercaptoethanol. The resulting solution was dialysed against 2 litres of this buffer for 14 hr and the precipitate formed on dialysis was removed by centrifugation at 8000 *g* for 10 min.

The supernatant obtained from the dialysis step was applied to a column of DEAE-Sephadex (4·0 by 40 cm) equilibrated as described in Section II(*c*) and eluted with 0·05M NaH_2PO_4–NaOH, pH 8·0, containing β-mercaptoethanol but devoid of 8-hydroxy-5-quinolinesulphonic acid, at a flow rate of 1·0 ml/min. Fractions (10 ml) were collected and those of highest specific activity were pooled and dialysed against 0·05M NaH_2PO_4–NaOH, pH 4·5, and the undialysable residue was reduced to a volume of approximately 30 ml by vacuum dialysis.

This product was then added to a column of CM-cellulose (2·5 by 25 cm) equilibrated as described in Section II(*c*). The column was eluted with a linear gradient of KCl (0·2 to 0·4M, total 250 ml) containing 0·05M NaH_2PO_4–NaOH, pH 4·5; fractions (10 ml) were collected at a flow rate of approximately 2 ml/min. Fractions of highest specific activity were pooled and dialysed for about 14 hr against 0·05M NaH_2PO_4–NaOH, pH 6·5. The volume of this solution was reduced to approximately 5 ml (2·5 mg protein/ml) by vacuum dialysis.

The enzyme preparation (5 ml) was then added to a column of Sephadex G200 (3·5 by 50 cm) equilibrated as described in Section II(*c*) and was eluted with 0·05M NaH_2PO_4–NaOH, pH 6·5 (flow rate 13 ml/hr). Fractions (5 ml) of highest specific activity were pooled and stored at 4°C in the eluting buffer.

For protein determinations a sample of each fraction after chromatographic separation was dialysed against excess 0·05M NaH_2PO_4–NaOH buffer (pH 6·5) alone to remove interfering β-mercaptoethanol.

(*e*) *Sucrose Gradient and Ultracentrifugation*

Sucrose gradient centrifugation of purified hepatic D-glucuronolactone dehydrogenase was conducted as described by Martin and Ames (1961). A 0·25-ml sample of enzyme (0·75 mg protein) was layered onto a continuous sucrose gradient (5 to 20% w/v, 11 ml) containing 0·05M Tris–acetate buffer, pH 7·4, and 0·02M β-mercaptoethanol, and subjected to centrifugation at 70,000 *g* for 22 hr at 4°C in a Spinco model L-2 ultracentrifuge with an SW 41 rotor.

III. RESULTS

(*a*) *Purification*

The purification steps are summarized in Table 1. The D-glucuronolactone dehydrogenase was purified approximately 350-fold with an overall recovery of 11%.

TABLE 1

RESULTS OF THE PURIFICATION OF RAT LIVER D-GLUCURONOLACTONE DEHYDROGENASE

The data in this table are for a single preparation from approximately 80 g wet wt. of rat liver. An aliquot of the enzyme was incubated with 5 mM D-glucuronolactone and 2·5 mM NAD^+ in 0·05M orthophosphate buffer, pH 6·5, in a total volume of 2·5 ml. Protein concentrations were measured by the method of Lowry *et al.* (1951)

Fraction	Volume (ml)	Activity (units/ml)	Protein (mg/ml)	Specific activity (units/mg protein)	Total activity (units)	Yield (%)
High-speed supernatant	240	1·65	38·0	0·043	400	100
40–60% saturated $(NH_4)_2SO_4$ fraction, dialysed and concentrated	38	10·00	39·3	0·255	380	95·0
DEAE-Sephadex eluate, dialysed and concentrated	30	5·82	3·40	1·71	175	43·5
CM-cellulose eluate, dialysed and concentrated	5·0	14·00	2·35	5·96	70	17·5
Sephadex G200 eluate	20·0	2·20	0·14	15·7	44	11·0

It was found in preliminary experiments that the enzyme activity obtained after initial extraction and fractionation with ammonium sulphate was extremely unstable, decreasing by 50% overnight during dialysis against buffer alone. Dialysis trials against a number of different buffered solutions containing high and low salt concentrations, chelating agents, thiol protecting agents, D-glucuronolactone (20 mM), NAD^+ (1 mM), and at a variety of pH values and temperatures resulted in the use of orthophosphate buffer containing 8-hydroxy-5-quinolinesulphonic acid and β-mercaptoethanol throughout most of the purification, as this buffer stabilized the activity of both the crude and purified preparations.

In the column chromatography on DEAE-Sephadex D-glucuronolactone dehydrogenase activity appeared in the first protein peak which was not absorbed onto the resin (Fig. 1). The chelating agent (8-hydroxy-5-quinolinesulphonic acid) was not included in this elution buffer as it reacted with the charged groups on the resin, preventing protein–resin interactions. Maximal enzyme activity eluted from the column of CM-cellulose emerged in the fraction containing approximately 0·29M KCl (about 100 ml of eluate) (Fig. 2).

Fig. 1

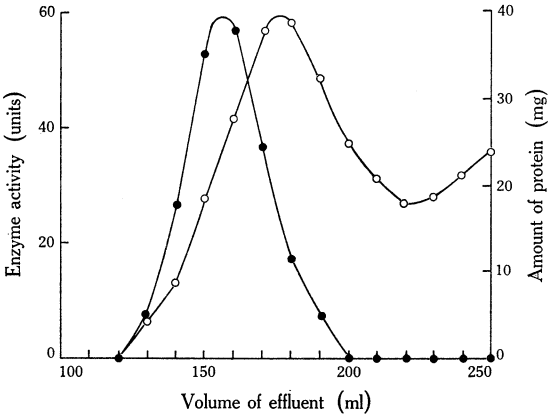


Fig. 2

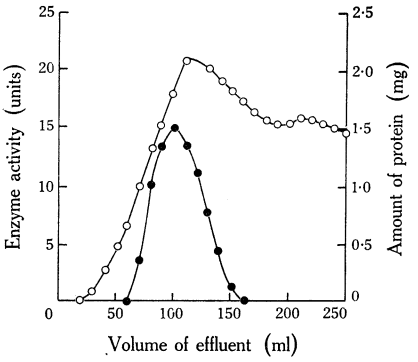


Fig. 1.—DEAE-Sephadex column chromatography of rat liver D-glucuronolactone dehydrogenase after $(NH_4)_2SO_4$ fractionation [see Section II(*d*)]. Elution from the column was with orthophosphate buffer, pH 8·0, containing 0·02M β-mercaptoethanol and the fractions (10 ml) were assayed for enzyme activity (●) and protein concentration (○).

Fig. 2.—CM-cellulose chromatography of rat liver D-glucuronolactone dehydrogenase. The dialysed and concentrated eluate from the DEAE-Sephadex column was applied to the column which had been equilibrated as described in Section II(*c*). Fractions (10 ml) were assayed for enzyme activity (●) and protein concentration (○) after elution with a linear gradient of KCl (0·2 to 0·4M, total 250 ml).

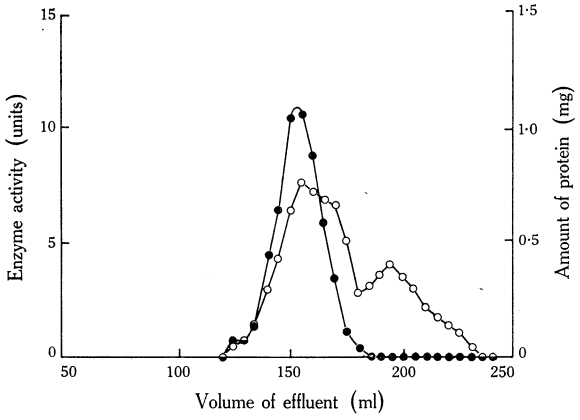


Fig. 3.—Sephadex G200 gel filtration of rat liver D-glucuronolactone dehydrogenase. The enzyme solution obtained after CM-cellulose column chromatography was dialysed and concentrated before loading onto the previously equilibrated column, then eluted with 0·05M orthophosphate buffer (pH 6·5) containing 0·005M 8-hydroxy-5-quinolinesulphonic acid and 0·02M β-mercaptoethanol. Eluted fractions (5 ml) were assayed for enzyme activity (●) and protein concentration (○).

As shown in Figure 3, D-glucuronolactone dehydrogenase activity eluted from the column of Sephadex G200 in the void volume which had been previously

established using blue dextran-2000 as a standard. The activity eluted from this column then was found to be stable for at least 2 months when stored at 4°C.

(*b*) *Substrate Specificity*

In addition to D-glucuronolactone, D-mannuronolactone and a series of aliphatic aldehydes were oxidized by the purified enzyme preparation as shown in Table 2.

TABLE 2

ALDEHYDE DEHYDROGENASE ACTIVITY OF PURIFIED RAT LIVER D-GLUCURONOLACTONE DEHYDROGENASE

The enzyme was incubated at 37°C with the various substrates and 2·5 mM NAD^+ in ortho-phosphate buffer, pH 6·5 and in pyrophosphate buffer, pH 9·3, and the activity measured by the initial reaction velocity

Substrate (5 mM)	Enzyme activity (units/ml)		Substrate (5 mM)	Enzyme activity (units/ml)	
	pH 6·5	pH 9·3		pH 6·5	pH 9·3
D-Glucuronolactone	1·6	—	n-Butyraldehyde	6·4	11·2
D-Mannuronolactone	3·1	—	Isobutyraldehyde	5·7	32·6
Acetaldehyde	7·9	16·2	n-Valeraldehyde	0	11·2
Propionaldehyde	7·4	22·0			

Initial rates of oxidation of the sugar lactones could not be measured at pH 9·3 due to their rapid hydrolysis. Replacement of NAD^+ with $NADP^+$ gave no detectable

TABLE 3

COMPARISON OF D-GLUCURONOLACTONE, D-MANNURONOLACTONE, AND ACETALDEHYDE DEHYDROGENASE ACTIVITIES DURING THE PURIFICATION PROCEDURE

Assay mixtures contained 5 mM substrate, 2·5 mM NAD^+, and buffer (pH 6·5 or 9·3) [see Section II(*b*)]

Stage of purification	Ratio of activities		
	D-Glucurono-lactone	D-Mannurono-lactone	Acetaldehyde
High-speed supernatant	1·0	1·9	—
40–60% satd. $(NH_4)_2SO_4$ fraction, dialysed	1·0	2·2	10·5
DEAE-Sephadex eluate, dialysed	1·0	1·8	10·4
CM-cellulose eluate, dialysed	1·0	1·9	10·6
Sephadex G200 eluate	1·0	1·9	10·5

activity with any of the above substrates. D-Glucuronolactone, D-mannuronolactone, and acetaldehyde dehydrogenase activities were measured at each stage of the purification and the ratio of activities was found to be constant at 1 : 2 : 10 (Table 3).

The three activities could not be separated when a sample of the purified preparation was subjected to sucrose gradient centrifugation (Fig. 4).

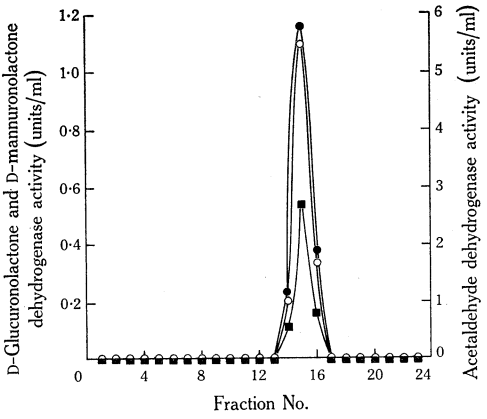


Fig. 4.—A sample of purified enzyme (0·75 mg, 0·25 ml) was subjected to centrifugation at 70,000 *g* in a continuous sucrose gradient as described in Section II(*e*). Fractions (approximately 0·5 ml) collected from the tubes were diluted (1 : 1) with buffer (0·05M Tris–acetate) and assayed for D-glucuronolactone (■), D-mannuronolactone (○), and acetaldehyde dehydrogenase (●) activities.

(*c*) *pH Optima*

D-Glucuronolactone dehydrogenase activity displayed a pH optimum of 7·3 in orthophosphate buffer (Fig. 5). The rates expressed in Figure 5 are initial rates which remained constant for at least 15 min at the pH values studied. The sharp fall in activity seen at higher pH values can possibly be attributed to the spontaneous hydrolysis of D-glucuronolactone at alkaline pH (Dowben 1959). Acetaldehyde dehydrogenase activity displayed a pH optimum of 9·3 in pyrophosphate buffer (Fig. 5).

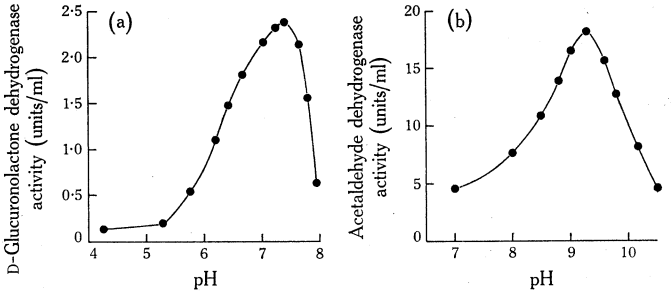


Fig. 5.—Effect of pH on activity of purified D-glucuronolactone dehydrogenase activity with (*a*) 5 mM D-glucuronolactone, 2·5 mM NAD^+, and 0·05M orthophosphate and (*b*) 5 mM acetaldehyde, 2·5 mM NAD^+, and 0·05M pyrophosphate.

(*d*) *Kinetic Studies*

Preliminary experiments showed that the initial velocities of the reactions were linear with both time and enzyme concentration at the initial stage of the reactions when optimal pH conditions were employed. By employing the method of Lineweaver and Burk (1934), the apparent Michaelis constant for D-glucuronolactone was found to be 23 mM with a fixed concentration of 2·5 mM NAD^+ and the corresponding constant for NAD^+ was 0·15 mM with a concentration of 5 mM D-glucuronolactone.

The determination of the apparent Michaelis constant (K_m) for acetaldehyde required the use of very low concentrations of acetaldehyde, for which absorbance changes due to NAD^+ reduction were too small to be read accurately. Results, however, indicated an apparent K_m of less than 10^{-5}M for acetaldehyde with a concentration of 2·5 mM NAD^+.

NADH was found to inhibit the oxidation of both D-glucuronolactone and acetaldehyde by the enzyme. Examination of the kinetics of inhibition (Lineweaver and Burk 1934) showed that NADH acted competitively with NAD^+ when either D-glucuronolactone or acetaldehyde was the other substrate.

(*e*) *Activities with Combined Substrates*

Activities were measured by determining total NADH production in 0·05M orthophosphate buffer, pH 6·5, with 2·5 mM NAD^+ and substrate concentrations of 5·0 mM acetaldehyde and 20 mM D-glucuronolactone. Activities with acetaldehyde and D-glucuronolactone as substrates both separately and in combination were as follows:

Substrate	Enzyme activity (units/ml)
5 mM Acetaldehyde	2·70
20 mM D-Glucuronolactone	1·60
5 mM Acetaldehyde + 20 mM D-glucuronolactone	2·68

The activity when both acetaldehyde and D-glucuronolactone were included in the reaction mixture was not the sum of the activities measured with the substrates separately, but equalled the value obtained with acetaldehyde alone. This result might be expected if a single enzyme were responsible in view of the large difference in K_m values for the two substrates and the fact that acetaldehyde was present at a saturating concentration.

(*f*) *Inhibition of* D-*Glucuronolactone Dehydrogenase Activity by Acetaldehyde*

Varying concentrations of acetaldehyde were included in determinations of glucaric acid production from D-glucuronolactone and NAD^+ in orthophosphate buffer, pH 6·5, using dialysed enzyme preparations after the ammonium sulphate fractionation. Examination of the kinetics of inhibition of D-glucaric acid production by acetaldehyde showed that acetaldehyde acts competitively with D-glucuronolactone with a K_i value of 0·48 mM.

(*g*) *Activation by β-Mercaptoethanol*

Removal of β-mercaptoethanol from the buffer in which the purified enzyme was stored resulted in considerable reduction in activity with either D-glucuronolactone or acetaldehyde as substrate (Table 4). Both activities could be restored, however, to the original levels by including β-mercaptoethanol in the reaction mixture. The extent of reactivation was dependent on the concentration of β-mercaptoethanol in the assay mixture; thus 10 mM β-mercaptoethanol fully restored both activities, while lower concentrations produced the same degree of reactivation using either substrate. Inclusion of 20 mM β-mercaptoethanol in the reaction mixture

caused no further activation of either activity if the enzyme was previously stored in buffer containing 20 mM β-mercaptoethanol.

TABLE 4

ACTIVATION BY β-MERCAPTOETHANOL

Purified enzyme was dialysed against excess 0·05M orthophosphate buffer, pH 6·5, for 12 hr at 4°C and activities of dialysed and non-dialysed enzyme determined in various concentrations of β-mercaptoethanol. Assay mixtures contained 5·0 mM D-glucuronolactone or acetaldehyde, 2·5 mM NAD^+, and 0·05M orthophosphate buffer, pH 6·5. 0·1 Unit of enzyme was included in a total reaction volume of 2·5 ml. All values are relative to the activity of non-dialysed purified enzyme activity of 1·0 with 5 mM D-glucuronolactone as substrate

β-Mercaptoethanol concn. (mM)		Relative activity	
In enzyme storage buffer	In assay mixture	D-Glucuronolactone	Acetaldehyde
20	0·8	1·0	5·2
	20·0	1·0	5·2
0	0	0·1	0·3
	1·0	0·4	1·8
	2·0	0·5	2·4
	5·0	0·7	3·8
	10·0	1·0	5·0
	15·0	1·0	5·1
	20·0	1·0	5·2

TABLE 5

INHIBITION BY MERCURIC IONS AND REVERSAL BY EDTA

Purified enzyme (0·1 unit) was added to the reaction mixture (2·5 ml) containing a final concentration of 5·0 mM D-glucuronolactone or acetaldehyde, 2·5 mM NAD^+, 0·05M Tris–HCl buffer, pH 7·4, and inhibitor at the stated concentrations. EDTA was added 5 min after mixing the enzyme with the reaction mixture containing the mercuric ion

Additions to reaction mixture:	Nil	10^{-6}M Hg^{2+}	10^{-5}M Hg^{2+}	10^{-4}M Hg^{2+}	10^{-6}M Hg^{2+}, 10^{-4}M EDTA
Activity remaining (%):					
D-Glucuronolactone	100	40·5	15·2	9·3	100
Acetaldehyde	100	41·0	15·0	9·1	100

(*h*) *Inhibition by Mercuric Ion and Reversal by EDTA*

Low levels of mercuric ion inhibited both activities of the purified preparation when assayed in Tris–HCl buffer, pH 7·4 (Table 5). This inhibition could be reversed by addition of EDTA to the reaction mixtures.

IV. DISCUSSION

Some properties of mammalian liver D-glucuronolactone dehydrogenase have been studied (Marsh 1963*c*; Sadahiro *et al.* 1966), but using very crude enzyme

preparations. Investigations into the substrate specificity of the purified enzyme was necessary following the evidence presented by Sadahiro *et al.* (1966) suggesting that the free aldehyde form of D-glucuronolactone was involved in the enzymic oxidation to D-glucaric acid. The enzyme is concentrated in the cytoplasmic fraction of liver homogenates (Marsh 1963*b*), whereas non-specific aldehyde dehydrogenase activity has been demonstrated in both the cytosol and mitochondrial fractions of rat liver by a number of workers. Deitrich (1966) compared the supernatant and mitochondrial aldehyde-oxidizing enzymes and concluded that two different enzyme systems existed.

Previous preparations of D-glucuronolactone dehydrogenase from mammalian liver have been shown to be unstable (Marsh 1963*b*; Sadahiro *et al.* 1966; Hänninen 1968). At all stages of its purification by the procedure used in this paper, the rat liver enzyme was particularly unstable unless stored in the buffer described. Under conditions of instability the activity decreased equally with either D-glucuronolactone or acetaldehyde as substrate. The stability of mammalian liver aldehyde dehydrogenase appears to be species-dependent, for the enzyme from human liver has been reported as being stable (Kraemer and Deitrich 1968; Blair and Bodley 1969) while the bovine liver enzyme is unstable (Racker 1949).

Substrate specificity studies revealed that the purified enzyme catalysed the NAD^+-dependent oxidation of a series of aliphatic aldehydes in addition to D-glucuronolactone and D-mannuronolactone. These three activities could not be separated by any of the procedures employed to purify the protein and the ratio of activities remained constant at each stage of the purification. The coincidence of the three activities on sucrose density gradient centrifugation and the inability to separate them during purification suggest that these reactions are catalysed by proteins of the same molecular weight and chromatographic properties. Kinetic studies undertaken to determine if these activities were due to a single protein species were performed with D-glucuronolactone and acetaldehyde as substrates. The apparent Michaelis constant for acetaldehyde was less than 10^{-5}M, indicating the high affinity of the enzyme for acetaldehyde. The K_m value obtained for D-glucuronolactone agreed with that presented by Marsh (1963*b*). The apparent Michaelis constants for NAD^+ were found to be the same when either D-glucuronolactone or acetaldehyde was the substrate.

The pH optimum for the oxidation of acetaldehyde of 9·3 is in agreement with the values reported for the hepatic enzyme of a number of species (Racker 1949; Maxwell and Topper 1961; Kraemer and Deitrich 1968; Blair and Bodley 1969). Marsh (1963*c*) observed a pH optimum of 6·5 for D-glucuronolactone oxidation, which is rather lower than the value of 7·3 obtained with the purified enzyme. The rapid decrease in the activities at pH values above this optimum may possibly be explained by the instability of the lactone ring of D-glucuronolactone in alkaline conditions as demonstrated by Dowben (1959). There appears, however, still to be some uncertainty concerning the pH optimum of rat liver D-glucuronolactone dehydrogenase. A value of pH 7·4 was obtained by Hänninen (1968) for this enzyme at each stage of the purification procedure employed, whereas Aarts and Hinnen-Bouwmans (1972) reported a pH optimum of about 9·5 for the final product of the same purification procedure. These differences may be partly due to differing stabilities of the enzyme preparations, for whereas our product at the same degree of purification was very unstable except in the presence of the chelating agent, that

of Aarts and Hinnen-Bouwmans (1972) could be stored for at least 4 months at −15°C.

The concept that acetaldehyde and D-glucuronolactone were being oxidized at a single catalytic site was substantiated by the results showing that acetaldehyde was a competitive inhibitor of D-glucaric acid production from D-glucuronolactone by acting as a competing substrate for the oxidation of D-glucuronolactone by the enzyme. The production of NADH when measured with a combination of both acetaldehyde at a saturating concentration and D-glucuronolactone as substrates was not equal to the sum of the activities when measured with each substrate alone. The activities were not additive as would be expected if two independent enzymes were involved. Further, both activities were activated to the same extent by β-mercaptoethanol and low levels of mercuric ion caused inhibition of both activities. This latter effect could be relieved by addition of EDTA.

The evidence of a number of common properties for the protein catalysing these reactions strongly suggests that a single enzyme of wide specificity can oxidize both D-glucuronolactone and aliphatic aldehydes. Since the excretion of D-glucaric acid is a physiological process, it now seems of some importance to establish whether D-glucaro(1→4)lactone, the powerful β-glucuronidase inhibitor, is formed as an intermediate and can control β-glucuronidase activity *in vivo* as suggested by Marsh (1963*b*). If this were the case, substrate competition by the immediate oxidation products of exogenous alcohols could be a significant factor.

V. References

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