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COMMON PROPERTIES OF D-GLUCURONOLACTONE AND ALDEHYDE DEHYDROGENASE FROM THE SOLUBLE FRACTION OF RAT LIVER

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SUMMARY

In the present investigation the properties of D-glucuronolactone dehydrogenase (D-glucurono-γ-lactone:NAD$^+$ oxidoreductase, EC 1.1.1.70) and aldehyde dehydrogenase (aldehyde:NAD$^+$ oxidoreductase, EC 1.2.1.3) from the soluble fraction of rat liver were compared. It was observed that:

1. After $(NH_4)_2SO_4$ fractionation and chromatography on Sephadex G-200 the peak of D-glucuronolactone dehydrogenase activity is indistinguishable from that of aldehyde dehydrogenase.

2. After agar gel electrophoresis of the Sephadex fraction one band of D-glucuronolactone dehydrogenase and one band of aldehyde dehydrogenase was observed. Both bands had the same mobility and appeared to migrate to the cathode.

3. The optimum of aldehyde and D-glucuronolactone dehydrogenase activity was found in the same area (around pH 9.3).

4. An apparent K_m value for NAD$^+$ of $5 \cdot 10^{-5}$ moles$\cdot$l^{-1} was measured with either D-glucuronolactone or acetaldehyde as substrate.

5. In the mixed substrate test the dehydrogenase activity at saturation concentrations of D-glucuronolactone and acetaldehyde was the same as the acetaldehyde dehydrogenase activity at saturation concentration.

6. Acetaldehyde inhibits the D-glucaric acid formation.

7. The heat inactivation at 57° of D-glucuronolactone dehydrogenase parallels that of aldehyde dehydrogenase.

8. The same holds true for the inactivation by *p*-chloromercuriphenylbenzoate.

The results indicate that D-glucuronolactone dehydrogenase is identical with aldehyde dehydrogenase obtained from the soluble fraction of rat liver.

INTRODUCTION

Marsh[1-5] described the identification in certain mammalian tissues of an enzyme system capable of converting D-glucuronolactone into D-glucaric acid. The

Abbreviation: PCMB, *p*-chloromercuribenzoate.

oxidative enzyme named D-glucuronolactone dehydrogenase (D-glucurono-γ-lactone: NAD^+ oxidoreductase, EC 1.1.1.70) is mainly present in the soluble fraction of tissue homogenates and requires 1 molecule of NAD^+ per molecule of D-glucuronolactone oxidized.

In solution, D-glucuronolactone is in equilibrium with the free aldehyde form[6]. The experimental results of Sadahiro *et al.*[7] indicate that the aldehyde form of D-glucuronolactone acts as direct precursor in its enzymatic dehydrogenation. The investigators observed that, with DEAE-cellulose column chromatography, D-glucuronolactone dehydrogenase of guinea pig liver is indistinguishable from aldehyde dehydrogenase (aldehyde:NAD^+ oxidoreductase, EC 1.2.1.3), which had been recognized to have a broad spectrum for aldehydic substrates[8]. The question was raised whether D-glucuronolactone dehydrogenase is identical with aldehyde dehydrogenase[9].

In order to get more information on this point, in the present investigation the properties of aldehyde and D-glucuronolactone dehydrogenase of the soluble fraction of rat liver are compared.

MATERIALS AND METHODS

Rat liver tissue was obtained from male albino rat of the Wistar strain (200–220 g). The animal was killed by stunning and decapitation. The liver was perfused with saline and stored in ice until further treatment. Sources of substrates and chemicals were as follows: D-glucuronolactone, phenazine methosulfate, nitro blue tetrazolium and 2-mercaptoethanol, Sigma Chemical Company; acetaldehyde and *p*-chloromercuriphenyl benzoate, British Drug Houses, acetaldehyde was distilled before use; NAD^+ and glutathione, Boehringer; Sephadex G-200, G-50 and blue dextran-200, Pharmacia Chemicals Inc.; Special agar-noble, Difco Laboratories.

Enzymatic assays

Enzymatic activity was assayed by measuring spectrophotometrically the rate of NAD^+ reduction in the presence of D-glucuronolactone and acetaldehyde as substrates. A Beckman model DU spectrophotometer was used. The incubation mixture contained 1.25 μmoles of acetaldehyde or 27.5 μmoles D-glucuronolactone and 0.9 μmole NAD^+ in a final volume of 2.2 ml of 0.2 M glycine buffer, pH 9.2. The reaction was carried out in 1-ml silica cuvettes with a light path of 1 cm and the temperature was maintained at 37°. The increase in absorbance was recorded for 6 min. A 1:1 stoichiometric relationship between NADH appearance and D-glucaric acid appearance was shown. D-Glucaric acid was determined according to Marsh[2].

Purification of enzyme

The enzyme was purified as described by Hänninen[10] for D-glucuronolactone dehydrogenase. The liver was homogenized in Potter–Elvehjem tubes with 4 vol. of 0.15 M KCl. The homogenate was centrifuged for 1 h at 100 000 $\times$ *g* in a refrigerated Omega 2 preparative centrifuge (Martin Christ). While stirring 30 ml of the clear supernatant, 20 ml of saturated $(NH_4)_2SO_4$ solution was added (4°C). The mixture was placed in crushed ice for 10 min and the sediment (I) removed by centrifugation. A further 25 ml of saturated $(NH_4)_2SO_4$ was stirred into the supernatant fraction and

after 10 min a second sediment (Sediment II) was collected by centrifugation. The remaining solution was discarded. During salt fractionation the pH was maintained at 7. Sediment II was dissolved into 40 ml of 0.1 M sodium phosphate buffer, pH 6.5, and applied to a Sephadex G-200 column (2.5 cm internal diameter × 70 cm long) equilibrated with the same buffer. This buffer was also used for elution. Fractions of 3 ml were collected at a rate of 22.5 ml/h. The fractions with the highest enzyme activity were put together, divided into samples and stored at −15°C. The preparation appeared to be stable for at least 4 months. In order to obtain an aliquot the whole sample was thawed.

Agar gel electrophoresis

Agar gel electrophoresis on microscope slides according to the method of Wieme[11] and visualization of dehydrogenase activity according to the method of van der Helm[12] were performed. Slides of 26 mm × 76 mm were covered with 0.9% agar gel (thickness ±2 mm) in 0.05 M Tris–HCl buffer of pH 8.5. Moreover, 1 μmole of 2-mercaptoethanol was added per 1 ml of the gel. Two slit cuts of 6 mm were made on one slide. A sample of 5 μl of the enzyme preparation (activity, D-glucuronolactone: 1.3 $\mu moles \cdot h^{-1}$; acetaldehyde: 2.3 $\mu moles \cdot h^{-1}$) was placed in each slit cut. The electrophoresis of the two samples was carried out in one run. The circuit of electrophoresis consisted of a Vitatron d.c. power source generating 300 V, platinum electrodes and 0.05 M Tris–HCl buffer (pH 8.5) in the gel. The surfaces were covered with petroleum ether. During electrophoresis the petroleum ether was cooled with circulating ice water. A current of 20 mA was applied for about 20 min. After electrophoresis the gel was divided into two parts of 13 mm × 76 mm. One part was incubated with acetaldehyde and the other part with D-glucuronolactone. Solutions for the direct visualization of dehydrogenase activity in the agar gel were prepared shortly before use. The solution for visualization of D-glucuronolactone dehydrogenase activity was renewed every 5 min. The final concentrations of the solutions are given below: 0.06 M D-glucuronolactone and 0.01 M acetaldehyde; 0.15 M phosphate buffer, pH 7.0; 0.015 M NAD^+; 0.01 M KCN; 0.003% phenazine methosulphate; and 0.03% nitro blue tetrazolium. The bands were fixed with a mixture of 150 vol. ethylalcohol, 10 vol. glacial acetic acid and 40 vol. water.

Heat treatment

The enzyme samples were contained in a 0.1 M sodium phosphate buffer, pH 6.5. Portions of 0.5 ml were quickly heated to 57°C with rapid shaking in a 80°C bath, stirred at 57°C for a certain time, and then rapidly cooled in ice–water with continuous shaking. The solution was centrifuged for 10 min at 10 000 × g and the supernatant was used in the enzymatic assay.

RESULTS

Aldehyde dehydrogenase and D-glucuronolactone dehydrogenase activity do not elute in the void volume (previously established using blue dextran-200 as a standard). With both substrates the enzyme activity peak occurred in one area. The peak of acetaldehyde dehydrogenase activity was indistinguishable from that of D-glucuronolactone dehydrogenase (Fig. 1).

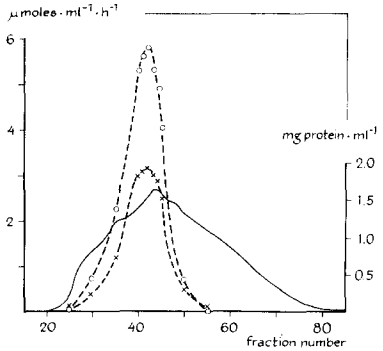


Fig. 1. Gel filtration of D-glucuronolactone-dehydrogenase (×) and aldehyde dehydrogenase (○) on Sephadex G-200. The fraction obtained after $(NH_4)_2SO_4$ fractionation of the 100 000 × *g* supernatant was loaded onto the column. 3-ml fractions were collected and assayed for enzyme activity (– – –) and protein concentration (———, absorbance at 280 nm).

Agar gel electrophoresis: The fractions with high activity were desalted by passage through a Sephadex G-50 column and freeze–dried. A part of the freeze–dried fraction was dissolved in buffer and a few μl of the clear solution was put on agar gel. One band was observed with either D-glucuronolactone or acetaldehyde. In both cases the band migrated to the negative pool. The two bands had the same mobility (Fig. 2).

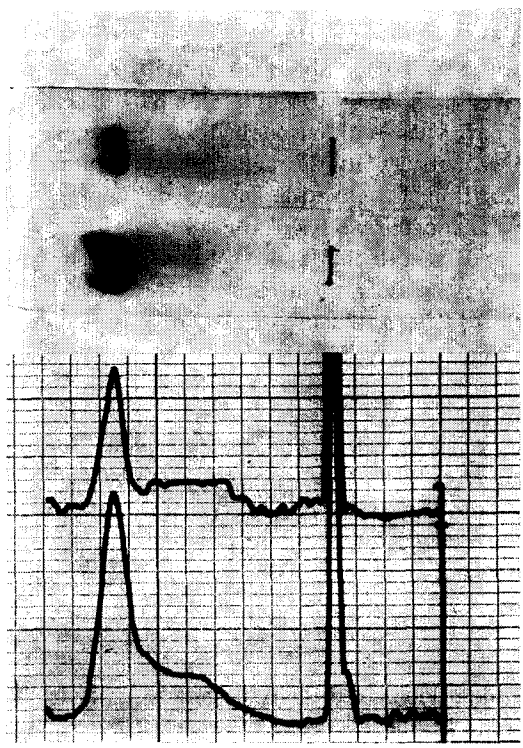


Fig. 2. Agar gel electrophoresis of the fraction obtained from Sephadex G-200. After electrophoresis the agar slide was divided in two parts, one part was incubated with D-glucuronolactone (band above), the other part with acetaldehyde (band below). After washing, fixing and drying, densitometry was carried out with an automatic scanning device.

Effect of pH on D-*glucuronolactone and aldehyde dehydrogenase activity*

Aldehyde dehydrogenase from rat liver has its pH optimum at alkaline pH[8,13]. For D-glucuronolactone dehydrogenase from rat liver a pH optimum of 6.5 has been described by Marsh[2]. In his experimental procedure the incubation time was 60 min. D-Glucuronolactone hydrolyses spontaneously at neutral and alkaline pH[6]. D-Glucuronic acid cannot act as substrate. For that reason the pH optimum of D-glucuronolactone dehydrogenase was re-examined with shorter incubation times and compared with the pH optimum of aldehyde dehydrogenase. A short incubation time (5 min) and a concentration of 27.5 μmoles of D-glucuronolactone were used. From pH changes of substrate–buffer solutions it could be concluded that the hydrolysis of D-glucuronolactone in the first 5 min is of minor importance. With respect to 3 buffer systems (phosphate, Tris–HCl, glycine) no optimum for D-glucuronolactone dehydrogenase activity at pH 6.5 was observed (Fig. 3). With glycine buffer an optimum about 9.5 was observed. The same holds true for aldehyde dehydrogenase. With glycine buffer and acetaldehyde as substrate a pH optimum around 9.3 was found.

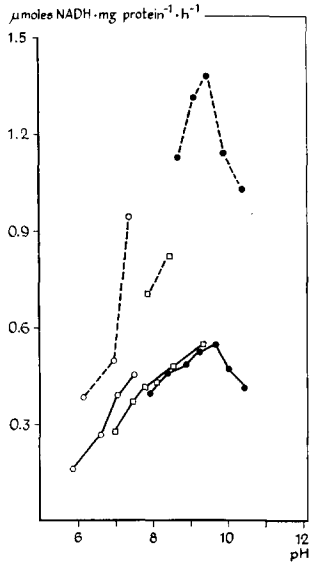


Fig. 3. Effect of pH on D-glucuronolactone dehydrogenase (———) and aldehyde dehydrogenase activity (– – –). 0.2 M phosphate buffer (○), 0.2 M Tris–HCl buffer (□) and 0.2 M glycine buffer (●) were employed. All reaction mixtures contained 1.25 μmoles acetaldehyde or 27.5 μmoles D-glucuronolactone and 0.9 μmole NAD^+. Assay mixtures contained 0.9 mg protein in a volume of 2.2 ml.

Kinetic studies

Activity measurements were carried out with enzyme concentrations and incubation periods established on the basis of preliminary experiments, so that initial reaction velocities were determined. The apparent K_m values were determined with Lineweaver–Burk plots. 0.2 M glycine buffer, pH 9.2, was employed; the results are shown in Table I. With either D-glucuronolactone or acetaldehyde as substrate the apparent K_m value for NAD^+ is $5 \cdot 10^{-5}$ moles$\cdot l^{-1}$. The measurement of the apparent K_m value for acetaldehyde required the use of very low substrate concentra-

TABLE I

APPARENT K_m VALUES OF NAD$^+$ AND D-GLUCURONOLACTONE FOR DEHYDROGENATION

0.2 M Glycine, pH 9.2 was employed. The assay mixtures contained 0.70 mg protein and substrates in 2.2 ml. V for D-glucuronolactone 1.22 μmoles·mg protein^{-1}·h^{-1}. V for acetaldehyde 2.25 μmoles·mg protein^{-1}·h^{-1}.

Substrate	*Cosubstrate (μmoles·ml^{-1})*		*K_m (moles·l^{-1})*
NAD$^+$	D-Glucuronolactone	27.5	$5 \cdot 10^{-5}$
NAD$^+$	Acetaldehyde	2.5	$5 \cdot 10^{-5}$
D-Glucuronolactone	NAD$^+$	0.9	$5.5 \cdot 10^{-3}$
Acetaldehyde	NAD$^+$	0.9	$< 10^{-5}$

tions. Under these conditions an accurate measurement of the rate of formation of NAD was beyond the sensitivity of the spectrophotometer. For D-glucuronolactone a K_m value of $5.5 \cdot 10^{-3}$ was found. The V for acetaldehyde was about two times higher as the V for D-glucuronolactone.

Test with combined substrates: The test was performed with the buffer systems 0.2 M glycine, pH 9.2, and Tris–HCl, pH 8.6. Saturation concentrations of D-glucuronolactone and acetaldehyde were applied. The formation of NADH was measured. In Table II the NADH productions with D-glucuronolactone and acetaldehyde were given both separately and in combination. The NADH formation in the medium with the substrates mixed is not the sum of the activities as measured with the substrates separately but equals the value obtained with acetaldehyde.

TABLE II

TEST WITH COMBINED SUBSTRATES

Assay mixtures contained 2 μmoles NAD, substrates and 0.75 mg protein in 2.2 ml buffer. The incubation time was 5 min.

Substrate (μmoles·ml^{-1})		*μmoles NADH·mg protein^{-1}·h^{-1}*	
		0.2 M Tris–HCl (pH 8.6)	*0.2 M glycine (pH 9.2)*
D-Glucuronolactone	27.5		0.94
Acetaldehyde	1.25		1.74
Combined			1.71
D-Glucuronolactone	80	0.74	
Acetaldehyde	5	1.57	
Combined		1.60	

Inhibition of the D-glucuronolactone dehydrogenase activity by acetaldehyde: In Tris–HCl buffer the D-glucaric acid formation was inhibited for about 35% by an acetaldehyde concentration 22 times lower. See Table III. A possible influence of acetaldehyde on the D-glucuronolactone dehydrogenase activity in glycine buffer could not be established because of the interference of glycine with the enzymatic D-glucaric acid determination.

TABLE III

INHIBITION OF THE ENZYMATIC FORMATION OF D-GLUCARIC ACID BY ACETALDEHYDE

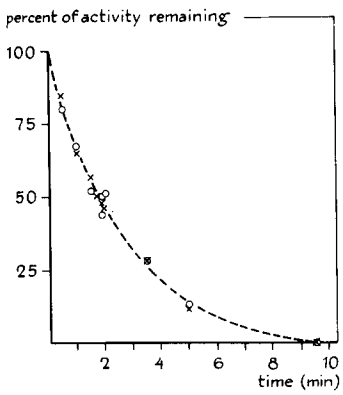
0.2 M Tris–HCl (pH 8.6) was employed. The assay mixtures contained 2 μmoles NAD^+ and 0.70 mg protein in 2.2 ml (activity 0.52 μmole D-glucaric acid·mg protein^{-1}·h^{-1}.)

Substrate	*NAD^+ used (μmoles)*	*D-Glucaric acid produced (μmoles)*	*Average inhibition*
D-Glucuronolactone (27.5 μmoles)	0.064	0.059	
	0.066	0.061	
D-Glucuronolactone (27.5 μmoles)	0.101	0.037	
and acetaldehyde (1.25 μmoles)	0.106	0.041	35%

Inactivation studies

The effect of temperature and of *p*-chloromercuriphenyl benzoate (PCMB) on D-glucuronolactone and acetaldehyde dehydrogenase acivity was studied. In Fig. 4 the percentage of remaining activity after heat treatment is given. The aldehyde and D-glucuronolactone dehydrogenase activity were inhibited to the same degree during the heating. After 2 min at 57°C both activities were reduced to 50%.

The sensitivity of aldehyde dehydrogenase to PCMB was also exhibited by D-glucuronolactone dehydrogenase. See Fig. 5. The two activities were inhibited to the same extent by this compound at concentrations from 10^{-4} to $16 \cdot 10^{-4}$ M PCMB.



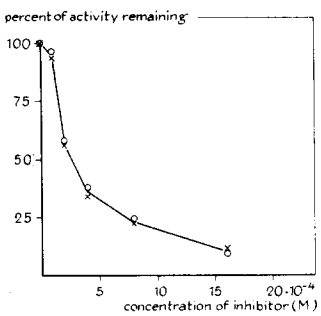


Fig. 4. Thermal inactivation of dehydrogenase activity at 57 °C with either acetaldehyde (○) or D-glucuronolactone (×) as substrates. Enzyme preparations obtained from Sephadex G-200, containing 0.9 mg protein were used.

Fig. 5. Inactivation of aldehyde dehydrogenase activity (○) and D-glucuronolactone dehydrogenase activity (×) by *p*-chloromercuriphenyl benzoate. The inhibitor was incubated with the dehydrogenase (3.5 mg of protein) in 0.1 M phosphate buffer, pH 6.5, in a final volume of 2 ml for 30 min at 37 °C. Glutathione (8 μmoles) was added and the solution was rapidly chilled to 0 °C. Aliquots of 0.4 ml were assayed in the same manner as described under Materials and Methods.

DISCUSSION

In solution there is an equilibrium between the free aldehyde group and the pyranose ring of D-glucuronolactone. The polarographic experiments of Sadahiro *et*

al.[7] indicate that the free aldehyde group is involved in the enzymatic conversion. Aldehyde dehydrogenase has a low specificity[8]. Both D-glucuronolactone dehydrogenase[2] and aldehyde dehydrogenase[8] catalyse in one direction and are NAD^+ dependent. So a comparison of the two activities was indicated.

Aldehyde dehydrogenase is present to a large degree in the 100 000 $\times$ *g* supernatant of mammalian liver[13,15]. Also the mitochondria have an aldehyde dehydrogenase activity. However, this aldehyde dehydrogenase activity has a pH optimum at pH 7.5 (ref. 13). The purified aldehyde dehydrogenase described by Racker[8] has its optimum at pH 9.3. The same holds true for the activity originating from the soluble fraction studied by us. The enzyme of the mitochondria displays a different behaviour on Sephadex G-200 (ref. 16). The data indicate that the enzymes in the mitochondria and in the soluble fraction are distinct proteins. Marsh[1,2] characterized the D-glucuronolactone dehydrogenase activity originating from the 100 000 $\times$ *g* supernatant of rat liver. Our study is also concerned with the soluble enzyme(s).

Aldehyde dehydrogenase[16] as well as D-glucuronolactone dehydrogenase[10] can be fractionated from other proteins with Sephadex G-200. From our comparative experiments with Sephadex G-200 followed by electrophoresis it may be concluded that, for both D-glucuronolactone dehydrogenase and aldehyde dehydrogenase, a homogeneous fraction is studied. The dehydrogenases have common properties with respect to chromatography and electrophoresis. So both activities are related to a protein of the same molecular weight and the same electrophoretic mobility.

The affinity of aldehyde dehydrogenase for acetaldehyde is very high[8]. The affinity for D-glucuronolactone dehydrogenase was found to be low (apparent K_m: $5.5 \cdot 10^{-3}$), Hänninen[10] found the same affinity (phosphate buffer, pH 7.4). The affinity of D-glucuronolactone dehydrogenase and of aldehyde dehydrogenase for NAD^+ was found to be the same (apparent K_m NAD^+: $5 \cdot 10^{-5}$).

Racker[8] observed a sharp pH optimum for aldehyde dehydrogenase activity around pH 9.3 in pyrophosphate buffer. Using glycine buffer, we observed also a sharp optimum for aldehyde dehydrogenase activity around that value. Moreover, we found that the pH optimum of D-glucuronolactone dehydrogenase is in the same area. Marsh[2] observed a pH optimum around 6.5 for D-glucuronolactone dehydrogenase. The long incubation time (1 h) used by Marsh in combination with hydrolysis of D-glucuronolactone may be responsible for this discrepancy.

In the mixed substrate tests (glycine buffer and Tris–HCl buffer) it was observed that a combination of D-glucuronolactone and acetaldehyde does not lead to a higher NADH production as compared with acetaldehyde. This can be easily explained if it is assumed that D-glucuronolactone and acetaldehyde are oxidized by the same enzyme. In that case acetaldehyde also occupies the action sites on the enzyme in the presence of D-glucuronolactone because of its high affinity as compared with D-glucuronolactone (apparent K_m value in glycine buffer: K_m D-glucuronolactone $5.5 \cdot 10^{-3}$; apparent K_m acetaldehyde $< 10^{-5}$).

Acetaldehyde inhibits the formation of D-glucaric acid. The method used leads to a rough estimate of the inhibition.

The aldehyde dehydrogenase is a heat-labile enzyme[8]. In our investigation it was observed that the D-glucuronolactone dehydrogenase activity parallels the aldehyde dehydrogenase activity during the heat inactivation process. This means that either two proteins, each responsible for one activity have the same susceptibility

to heat treatment, or one single protein is responsible for the two activities. The same holds true for susceptibility to PCMB. Pre-incubation with various concentrations of PCMB results in the same inactivation curve for D-glucuronolactone and acetaldehyde.

The results of each of the experiments described, taken individually, can be explained on a basis different from that of the involvement of a single enzyme. It is possible that two enzymes show common characteristics with chromatography followed by electrophoresis, have identical susceptibility to thermal inactivation and to chemical inactivation with PCMB, have the same affinity for the cosubstrate NAD^+ and so on. However, the combined results of the variety of experiments described constitute evidence for the catalysis of D-glucuronolactone and acetaldehyde by one single protein. The probability of obtaining by chance such a combination of consistent results for two different enzymes is exceedingly small. So the present investigation indicates that the formation of D-glucaric acid is the result of a catalysis by the unspecific aldehyde dehydrogenase (EC 1.2.1.3). The name D-glucuronolactone dehydrogenase with an EC number of its own seems superfluous.

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