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EFFECT OF THE UREA–UREASE SYSTEM AND THE CARBONATE ION ON THE ACTIVITIES OF LACTATE DEHYDROGENASE ISOENZYMES

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SUMMARY

The inhibitory effect of urea on human and rabbit muscle M_4-lactate dehydrogenase (L-lactate:NAD^+ oxidoreductase, EC 1.1.1.27) is markedly increased in the presence of urease. Evidence is presented suggesting that the action is due to the formation of CO_3^{2-} which under certain conditions may be shown to be a powerful inhibitor of the M_4 enzyme. NAD^+ protects the enzyme against both inhibitory systems. The H_4 enzyme, however, is relatively insensitive to both the urea–urease system and to CO_3^{2-}.

INTRODUCTION

During the course of an investigation into the nature of the inhibitors of lactate dehydrogenase which occur in the sera of patients with uraemia, certain fractions of the diffusate obtained by therapeutic dialysis were found to inhibit the enzyme[1]. Some of these contained urea which is known to inhibit lactate dehydrogenase[2–4]. When the urea was removed by dialysis the fractions containing it lost their inhibitory effects but, rather unexpectedly, attempts to remove urea by hydrolysis with urease led to the almost complete inhibition of lactate dehydrogenase. We now report some experiments designed to elucidate the mechanism of this process.

The actions of intermediates in the urea–urease reaction such as $NH_4NH_2CO_2$ and NH_4HCO_3 (ref. 5) were studied, and evidence is presented suggesting that inhibition of lactate dehydrogenase by the urea–urease system is mediated *via* CO_3^{2-}.

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Nomenclature: The isoenzymes 1 and 5 of lactate dehydrogenase are denoted by LDH_1 (H_4) and LDH_5 (M_4), respectively.

MATERIALS AND METHODS

Lactate-dehydrogenase isoenzymes

Pure H_4-lactate dehydrogenase was isolated from the crystalline ox-heart enzyme (purchased from Worthington Biochemical Corp., Freehold, N.J.) by chromatography on DEAE-Sephadex A-50 according to the method of WACHSMUTH *et al.*[6]. The M_4 enzyme was similarly obtained from crystalline rabbit-muscle lactate dehydrogenase (purchased from Sigma Chemical Co., St. Louis, Mo.). Their purities were checked by acrylamide-gel electrophoresis[7].

Human LDH_5 (M_4)

Human LDH_5 was prepared by a modified version of the method of NISSELBAUM AND BODANSKY[8] from skeletal muscle obtained at autopsy performed within 24 h of death. After removing adherent blood as completely as possible by washing with ice-cold 0.067 M NaCl, the tissue (350 g) was homogenized with distilled water (1 l) in a Waring Blendor. All subsequent operations were carried out at 0–5°. The yields and recoveries at each step are listed in Table I. The $(NH_4)_2SO_4$ precipitate (Step C) was dissolved in distilled water (770 ml), 10 ml of which was applied to a DEAE-Sephadex A-50 column (Pharmacia, Uppsala, Sweden) (2.5 cm × 35 cm) equilibrated with 0.033 M sodium phosphate buffer (pH 7.4) and eluted with the same buffer. The eluate was collected in 3-ml fractions which were continuously monitored by measurement of the absorbance at 280 nm. LDH_5 (M_4) was eluted in Fractions 15–20 which were pooled to give an overall yield of 18%.

Human LDH_1 (H_4)

This isoenzyme was obtained from packed erythrocytes (500 ml) by the method of NISSELBAUM AND BODANSKY[9]. Final purification was effected by preparative acrylamide-gel electrophoresis in a RAYMOND[10] vertical cell (E.C. Apparatus Corp., Philadelphia, Pa., U.S.A.). 2 ml of sample was applied to an 8% gel and electrophoresis

TABLE I

PURIFICATION OF HUMAN LACTATE DEHYDROGENASE ISOENZYMES

Step	*Procedure*	*Total activity (μmolar units)*	*Specific activity (μmolar units/mg protein)*	*Yield (%)*
LDH_5 from skeletal muscle				
A	Extraction	585	4.6	100
B	Absorption on and elution from calcium phosphate gel	544	13.85	89
C	$(NH_4)_2SO_4$ precipitation	223	60.8	56
D	DEAE-Sephadex chromatography	106.5	905	18
LDH_1 from erythrocytes				
A	Haemolysate	121	0.38	100
B	Absorption on and elution from calcium phosphate gel	111	6.04	92
C	$(NH_4)_2SO_4$ precipitation	58.5	6.9	52
D	Dialysis and electrophoresis	5.8	386	5

was carried out in 0.09 M Tris–EDTA buffer at pH 9.2 for 5 h at 5°. A strip of gel was sliced off and LDH_1 was located by means of the tetrazolium staining technique. The corresponding portion of the unstained gel was homogenized with 5 ml 0.5 M sodium phosphate buffer (pH 7.4) then centrifuged at 10 000 × *g* for 30 min at 0°, after which the supernatant was dialysed overnight against distilled water at 5°. The overall yield was 5% of material with a specific activity of 386 μmolar units/mg (Table I).

Both human isoenzyme preparations gave single bands on acrylamide-gel electrophoresis[7]. In each case the band of enzyme activity coincided with the single protein band stained with Amido Black 10 B.

Enzyme assay

Lactate dehydrogenase solutions (0.3 ml containing approx. 0.07 μmolar unit) were mixed with 0.08 M Tris–EDTA buffer, pH 9.3 (2.5 ml) containing NAD^+ (10 mg). 1.2 M DL-lactate (0.2 ml) was added and the change in absorbance at 340 nm was measured in a Unicam SP 800 recording spectrophotometer at 25 ± 0.2°.

During inhibition studies urea (or $NH_4NH_2CO_3$ or NH_4HCO_3) and lactate dehydrogenase were incorporated into the buffer solution to produce the concentrations required. After pre-incubation for 2 h at 25° in 2.6 ml, NAD^+ (10 mg in 0.2 ml buffer) and lactate were added and the enzyme activity was determined as described above. In the experiments with urease, the lactate dehydrogenase solution was incubated at 25° with urease (1.25 mg) (obtained from the Hartman-Leddon Co., Philadelphia, Pa.) and the stated concentration of urea for 2 h before the addition of NAD^+ and lactate. Since the pH increases from about 7.5 to 9.3 during the reaction of urea with urease, all investigations were carried out at pH 9.3 which is close to the optimum for the oxidation of lactate.

RESULTS

In preliminary experiments with lactate as substrate urea was found to inhibit M_4-lactate dehydrogenase at much lower concentrations than the H_4 enzyme in much the same manner as with pyruvate[4]. At 25° 1.0 M urea completely inhibited purified human-muscle and rabbit-muscle M_4-lactate dehydrogenases, while the ox-heart H_4 enzyme was relatively unaffected and the human-erythrocytic H_4 enzyme was slightly activated as reported by EMERY[11] for the reduction of pyruvate. At 37°, however, rabbit muscle M_4-lactate dehydrogenase was completely inhibited by 0.3 M urea.

As shown in Table II, the urea–urease system inhibited the activities of rabbit-muscle (M_4), ox-heart (H_4), human-muscle (M_4) and human-erythrocytic (H_4) lactate dehydrogenases to a much greater extent than urea alone. The rabbit-muscle enzyme was almost completely inhibited in the presence of urease and urea concentrations exceeding 0.1 M at 25° and 0.05 M at 37°. The results shown in Table II were obtained after pre-incubating the lactate dehydrogenase with urea and urease for 2 h, a period originally chosen because the urea–urease reaction is substantially complete in this time, but similar inhibitory effects upon rabbit-muscle M_4-lactate dehydrogenase were observed after much shorter periods, *e.g.* 10 min (Fig. 1). During these experiments no change was observed in the pH of the reaction mixture.

The effects on the lactate dehydrogenase activity of human liver extracts were

TABLE II

THE EFFECTS OF UREA AND THE UREA–UREASE SYSTEM ON RABBIT-MUSCLE, OX-HEART, HUMAN-MUSCLE AND HUMAN-ERYTHROCYTE LACTATE DEHYDROGENASE

Measurements were made at 340 nm with lactate as substrate at pH 9.3 (25°). The lactate dehydrogenase was incubated with urea, urea–urease for 2 h before NAD^+ and lactate were added. Each value is the average of duplicate results.

Types of isoenzymes	*Urea concn. (M)*	*Lactate dehydrogenase activity (% of control) in presence of*	
		Urea	*Urease and urea*
LDH_5 (M_4)			
Human muscle	0.0	—	94
	0.01	99	78
	0.05	89	70
	0.10	72	33
	0.38	20	0
	0.50	10	0
	1.0	5	0
Rabbit muscle	0.0	—	92
	0.01	92	90
	0.05	90	75
	0.1	81	51
	0.38	22	0
	0.50	12	0
	1.0	2	0
LDH_1 (H_4)			
Human erythrocytes	0.0	—	91
	0.01	97	97
	0.05	100	101
	0.1	96	109
	0.38	118	109
	0.5	124	136
	1.0	132	139
	1.5	124	136
Ox heart	0.0	—	93
	0.1	103	110
	0.38	102	122
	0.5	111	135
	1.0	99	113
	1.5	52	87
	2.0	28	75

similar, inhibition being 80 and 95% complete in the presence of urease and urea concentrations of 0.05 M and 0.1 M, respectively.

Lactate dehydrogenase proved to be sensitive to the products of the urea–urease reaction rather than to the reaction *per se*, since the same effect was observed when lactate dehydrogenase was added before, during or after incubation of urea with urease. This was confirmed by removing the products of the urea–urease reaction in a current of air. The resulting solution had no effect on lactate dehydrogenase.

$NH_4NH_2CO_3$ and NH_4HCO_3 strongly inactivated the rabbit-muscle lactate dehydrogenase at pH 9.3, but were almost without effect on the ox-heart enzyme,

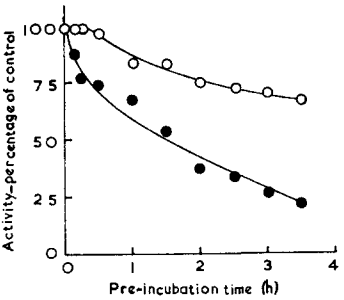

Fig. 1. Effect of pre-incubation with 0.1 M urea (○) or 0.1 M urea + urease (●) on human-skeletal-muscle lactate dehydrogenase activity with lactate as substrate. Enzyme activities were determined at 25° at pH 9.3.

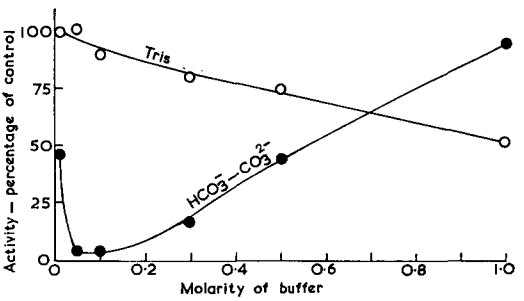

Fig. 2. Effect of variation of ionic strength on rabbit-muscle (M_4) lactate dehydrogenase at pH 9.3. Enzyme activities were determined after pre-incubation for 10 min in Tris–EDTA buffer (○) or CO_3^{2-}–HCO_3^- buffer (●) at 25°.

except at very high concentrations (Table III). Since the NH_4^+ is virtually without effect, it seemed that the CO_3^{2-} or HCO_3^- might be responsible for the inhibitory action of the urea–urease system.

$NaHCO_3$, added to the rabbit-muscle lactate dehydrogenase system in Tris–EDTA buffer at pH 9.3, markedly inactivated the enzyme. The effect was also demonstrated by carrying out the reaction in CO_3^{2-}–HCO_3^- buffers of different ionic strengths at pH 9.3 (Fig. 2). The rabbit-muscle enzyme was almost completely inactivated in 0.05 M and 0.1 M CO_3^{2-}–HCO_3^- buffers, but at both lower and higher concentrations

TABLE III

THE EFFECT OF $NH_4NH_2CO_3$ AND NH_4HCO_3 ON RABBIT-MUSCLE AND OX-HEART LACTATE DEHYDROGENASE

Measurements were made at 340 nm with lactate as substrate at pH 9.3. The enzyme was incubated with the inhibitors 2 h before NAD^+ and lactate were added. Each figure is the average of duplicate experiments.

	Inhibitor concn. (M)	*Lactate dehydrogenase activity (% of control) in presence of*		
		$NH_4NH_2CO_2$		NH_4HCO_3
		25°	*37°*	*25°*
Rabbit muscle	0.01	92	50	75
	0.05	66	3	46
	0.10	58	3	37
	0.38	59	0	10
	0.5	73	0	8
	1.0	73	0	7
Ox heart	0.01	100	98	95
	0.05	100	100	95
	0.10	103	100	95
	0.38	89	98	95
	0.5	82	100	94
	1.0	82	77	92

TABLE IV

EFFECT OF VARIATION OF pH AND CO_3^{2-} CONTENT ON THE INHIBITORY ACTION OF CO_3^{2-}–HCO_3^- BUFFERS ON LACTATE DEHYDROGENASE ISOENZYME ACTIVITIES

The lactate dehydrogenases were incubated in the Tris–EDTA (0.1 M) or CO_3^{2-}–HCO_3^- (0.1 M) buffer for 10 min at 25° before the addition of NAD^+ and lactate.

Isoenzymes	*Lactate dehydrogenase activity (% of activities in 0.1 M Tris–EDTA at the same pH)*					
pH:	*8.2*	*8.6*	*9.0*	*9.2*	*9.6*	*10.0*
CO_3^{2-} content (%):	*<1*	*3*	*10*	*14*	*33*	*59*
H_4						
Human erythrocyte	125	117	114	117	114	123
Ox heart	98	100	108	120	120	120
M_4						
Human muscle	80	89	55	15	0	0
Rabbit muscle	77	80	59	15	0	0

the effect was less pronounced. Purified human-skeletal-muscle lactate dehydrogenase behaved similarly but maximum inactivation was observed at 0.3 M. Neither the human-erythrocytic nor the ox-heart enzyme exhibited this effect, nor was any such inhibition observed with similar concentrations of NaCl.

Since the degree of inhibition is less at lower pH values, CO_3^{2-} rather than HCO_3^- is responsible. Table IV shows the relation between the inhibitory action and the CO_3^{2-} content of the system calculated from the Henderson–Hasselbalch equation.

Previous treatment with NAD^+ protected the enzyme against the CO_3^{2-} but not if added after CO_3^{2-} (Table V). NAD^+ was also found to protect human M_4-lactate dehydrogenase against inhibition by urea and by the urea–urease system. For example, 0.38 M urea caused 76% inhibition, but preincubation of the enzyme with NAD^+

TABLE V

EFFECT OF PRE-TREATMENT WITH NAD^+ ON THE INHIBITION OF RABBIT-MUSCLE LACTATE DEHYDROGENASE BY THE CO_3^{2-}

The enzyme was incubated at 25° in carbonate–bicarbonate buffer, pH 9.3 (2.7 ml), or Tris–EDTA buffer, pH 9.3 (2.7 ml), for 10 min, NAD^+ (10 mg) being added before or after the incubation period. Lactate (0.2 ml) was added and enzyme activity measured spectrophotometrically at 340 nm.

Buffer	*Buffer concn. (M)*	*Enzyme activity (μmoles/min per ml)*	
		Pre-incubated without NAD^+	*Pre-incubated with NAD^+*
Tris–EDTA	0.1	0.179	0.106*
CO_3^{2-}–HCO_3^-	0.05	0.01	0.102
	0.1	0.019	0.135
	0.5	0.157	0.116

* Unpublished work has shown that under the conditions employed excess NAD^+ causes substrate inhibition.

reduced this to 19%. Similarly while complete inhibition was produced by urease-treated 0.38 M urea, 58% of the control activity was retained when NAD^+ was added first.

DISCUSSION

Urea has proved to be a clinically useful differential inhibitor of lactate dehydrogenase isoenzymes[2,3,12–15]. The cationic M_4 enzyme of rabbit or human skeletal muscle and liver is much more sensitive to inhibition by urea than the H_4 enzyme of ox-heart or human erythrocytes.

Removal of urea by treatment with urease abolishes its capacity to inhibit M_4-lactate dehydrogenase, but if the products of the urease reaction are allowed to accumulate, the inhibitory effect is markedly increased, even when adequate controls are employed to allow for the change in pH. In the present work all determinations were performed with lactate as substrate at pH 9.3.

Under these conditions it has been shown that the CO_3^{2-} is mainly responsible for the urea–urease effect on lactate dehydrogenase. This is likely since (a) the intermediate products of the urease reaction, $NH_4NH_2CO_2$, and NH_4HCO_3 are also inhibitors of M_4-lactate dehydrogenase at pH 9.3, (b) the NH_4^+ is without significant effect, (c) because CO_3^{2-}–HCO_3^- buffers strongly inhibit this isoenzyme, and (d) because the enzyme is protected against inhibition of both systems by pre-treatment with NAD^+.

Incorporation of CO_3^{2-} or HCO_3^- into the M_4-lactate dehydrogenase system prepared in Tris–EDTA buffer also causes inactivation, but under these conditions the effect is somewhat less than with the CO_3^{2-}–HCO_3^- buffer alone. Inhibition may be partly due to changes in the ionic strength of the medium, since maximum inhibitory effects occur at concentrations about 0.1 M, but other factors are also concerned because varying concentrations of NaCl have no effect on the enzyme activity.

It is tempting to ascribe physiological significance to the effect of CO_3^{2-}–HCO_3^- buffers on M_4-lactate dehydrogenase since inhibition is very sensitive to changes in the physiological range between 10 and 50 mM, but the effect appears to be at least partly due to the failure of the weak buffers to maintain pH 9.3.

Since M_4-lactate dehydrogenase is protected against the action of CO_3^{2-} by pre-treatment with NAD^+, it seems likely that the CO_3^{2-} affects the active centre of the enzyme. The results obtained during the present work suggest that the CO_3^{2-} may replace coenzyme in the ternary enzyme–coenzyme–substrate complex[16], though it is difficult to explain why such an effect should be so pronounced at 0.1 M and scarcely evident at 1.0 M. Possibly some allosteric phenomenon resulting from the high ionic strength may produce sufficient alteration in the active centre to permit substrate or NAD^+ to displace CO_3^{2-}.

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