Organ Specificity and Lactate-Dehydrogenase Activity

2. SOME PROPERTIES OF HUMAN-HEART AND LIVER PREPARATIONS

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The preceding paper confirms the earlier finding of Rosalki & Wilkinson (1960) that the slow- and fast-migrating lactate-dehydrogenase isoenzymes of human serum differ considerably in their relative activities towards pyruvate and 2-oxobutyrate as substrate. Since in man and the rabbit the fastest isoenzymes (peak 1) are derived almost exclusively from the heart, and the slowest (peak 5) principally from the liver, diagnostic tests based upon the relative rates of reduction of these keto acids by serum have been developed for use in heart and liver disease (Elliott & Wilkinson, 1961, 1962; Elliott, Jepson & Wilkinson, 1962). The present paper is concerned with other means of differentiating between the lactate dehydrogenases of heart and liver preparations, including temperature coefficients and the effects of inhibitors.

Rosalki & Wilkinson (1960) observed that the temperature coefficients for the reduction of pyruvate and 2-oxobutyrate by normal human sera were approximately 8 and 6% respectively per degree over the range 21–28°. Recent experience with pathological specimens, however, suggests that these figures may not apply when the heart or liver is the principal contributor to a raised serum activity, and we have therefore re-assessed the effects of temperature changes on the velocities of these reactions with tissue extracts and pathological sera as enzyme sources.

It has recently been shown that oxamate inhibits lactate dehydrogenase by competing with pyruvate to form an inactive ternary complex with the enzyme and the coenzyme (NADH₂), and that oxalate similarly competes with lactate (Novoa, Winer, Glaid & Schwert, 1959). It seemed that the use of oxamate and oxalate might provide another means of relating an elevated serum lactate dehydrogenase to its tissue of origin. No information has hitherto been available on the effects of these inhibitors on the reduction of 2-oxobutyrate, but a brief account of our preliminary results was presented at a meeting of the Biochemical Society (Plummer & Wilkinson, 1961).

Most of the enzyme activities described in this paper have been determined with the keto acid as substrate and NADH₂ as coenzyme, but while the work was in progress it has been reported that a

potent inhibitor of liver alcohol dehydrogenase develops in solutions of NADH₂ and this substance might interfere with the action of other dehydrogenases (Dalziel, 1961; Fawcett, Ciotti & Kaplan, 1961). We have had no reason to suspect such a complication during our studies as we have invariably prepared fresh solutions of the reduced coenzyme immediately before each batch of enzyme determinations. Moreover, linear responses to increases in enzyme concentration were obtained with several different batches of NADH₂. However, we compared the reverse reactions, i.e. the oxidation of lactate and 2-hydroxybutyrate with NAD as coenzyme, with liver and heart preparations as the enzyme source.

MATERIALS AND METHODS

Enzymes. Crystalline rabbit-muscle lactate dehydrogenase and ox-heart lactate dehydrogenase were purchased from Worthington Biochemical Corp.

Tissue extracts. Extracts of human and rabbit heart, skeletal muscle and liver were prepared by the method of Plummer, Elliott, Cooke & Wilkinson (1963).

Determination of enzyme activity. (a) With NADH₂. A spectrophotometric method based upon that of Wróblewski & LaDue (1955) was used. Enzyme preparations were suitably diluted so that 0.1 ml. in 3 ml. of reaction mixture in a 1 cm. cuvette gave conveniently measurable absorption changes at 340 mμ. The reaction was carried out in 0.067 M-Sørensen phosphate buffer, pH 7.4, containing NADH₂ (0.35 μmole) with final substrate concentrations, unless otherwise stated, of 0.7 mM-sodium pyruvate or 3.3 mM-sodium 2-oxobutyrate in a total volume of 3 ml. During inhibitor studies 0.005–0.2 mM-potassium oxalate or 0.01–0.5 mM-sodium oxamate was incorporated into the reaction mixture.

(b) With NAD. The initial rate of increase in the absorption at 340 mμ of reaction mixture (3 ml.; 1 cm. light-path) containing 0.1 ml. of enzyme preparation, NAD (1.5 μmoles) and the substrate in 0.1 M-sodium glycine buffer, pH 10.0 (Nielands, 1955), was determined. The substrate concentrations were 66 mM-sodium lactate and 83 mM-sodium DL-2-hydroxybutyrate.

Temperature coefficients of enzyme reactions. Dehydrogenase activities were determined by method (a) with a Unicam SP. 500 spectrophotometer fitted with a constant-temperature cell housing through which water was circulated to maintain various temperatures between 18° and 32°.

Table 1. Comparison of dehydrogenase activities of human-heart and liver preparations determined by both forward and reverse reactions

Reaction	Substrate	Preparation	Activity (μ moles of substrate reacted/min./ mg. of N)	Ratio (<i>b/a</i>)	Ratio (<i>c/d</i>)
Reduction	Pyruvate	Heart	31.2 (<i>a</i>)	0.91 (<i>c</i>)	3.2
Reduction	2-Oxobutyrate	Heart	28.4 (<i>b</i>)		
Reduction	Pyruvate	Liver	17.0 (<i>a</i>)	0.28 (<i>d</i>)	
Reduction	2-Oxobutyrate	Liver	4.8 (<i>b</i>)		
Oxidation	Lactate	Heart	7.7 (<i>a</i>)	0.34 (<i>c</i>)	2.6
Oxidation	2-Hydroxybutyrate	Heart	2.6 (<i>b</i>)		
Oxidation	Lactate	Liver	2.05 (<i>a</i>)	0.13 (<i>d</i>)	
Oxidation	2-Hydroxybutyrate	Liver	0.26 (<i>b</i>)		

RESULTS

The relative activities in reducing pyruvate and 2-oxobutyrate exhibited by heart and liver extracts with NADH_2 as coenzyme were paralleled by the relative rates observed during catalysis of the reverse reactions with NAD as coenzyme. The results are shown in Table 1, from which it will be seen that the ability of the heart preparation to reduce 2-oxobutyrate is about three times as great as that of the liver preparation, relative to their respective activities with pyruvate as substrate. A similar ratio was observed when the reverse reactions were studied. When the hydroxy acids were used as substrates the reaction proceeded at a much slower rate than when the keto acids were employed. When lactate was employed an appreciable deceleration, due to pyruvate inhibition (Quastel & Woolridge, 1938), occurred soon after the beginning of the reaction.

Effects of temperature variation on reaction rates

Effects of temperature changes on the lactate-dehydrogenase activity of normal human sera varied according to the substrate employed, since when pyruvate was used the reaction rate increased exponentially with rising temperature, but linearly with 2-oxobutyrate. The results obtained with normal human serum and serum from a patient with myocardial infarction are shown in Fig. 1. The slopes of the curves for the two substrates were approximately parallel in the range 20–25° with both sera, but at higher temperatures there was increasing divergence. With 2-oxobutyrate and the infarct serum the graph actually appears as a negative exponential curve.

When the logarithms of the activities against pyruvate were plotted against temperature in the range 20–30°, linear relationships were observed, and the slopes gave temperature coefficients of 5.7% per degree for the normal serum and 6.2 and 7.1% per degree for the serum from two cases of myocardial infarction. The effect of temperature

Fig. 1. Effect of temperature variations on the dehydrogenase activities of human serum. Substrates: 0.7 mM-pyruvate (O, Δ); 3.3 mM-2-oxobutyrate ($\bullet$, $\blacktriangle$). O, $\bullet$, Results with serum from a patient with myocardial infarction; Δ , $\blacktriangle$, results with normal serum.

changes on the reduction of 2-oxobutyrate are shown in Fig. 2. Unlike those for the pyruvate reaction, the coefficients decreased rapidly with increasing temperature, this effect being more pronounced with the infarct serum than with normal serum.

Similar temperature-rate curves were obtained when heart, liver and muscle extracts were employed, though the relative activities against the two substrates varied considerably with different tissues (Fig. 3). The response to temperature changes of the rates of reduction of pyruvate by

all three tissues was also exponential, and the coefficients calculated for 25° are shown in Table 2. As with the sera, increasing temperature led to a fall in the temperature coefficients for the reduction of 2-oxobutyrate. The values observed at 25° are included in Table 2; with each substrate, the highest figures were found for the heart extract and the lowest for the liver.

That the results summarized in Figs. 1-3 represent the true variations of the reaction rates with temperature was shown by comparing the effects of preincubating heart and liver extracts at various temperatures for 30 min. before determining enzyme activity at 25°. As shown in Fig. 4, preincubation at temperatures below 50° had little or no deleterious effect on either enzyme preparation whichever substrate was employed. At higher temperatures the liver extract was appreciably less stable than the heart extract and at 60° the activity of the former was completely destroyed whereas the latter retained about 75 % of its activity. With each preparation, however, ability to reduce pyruvate was destroyed at the same temperature as activity with 2-oxobutyrate.

Effect of variations in substrate concentration

Effects of variation in the substrate concentration on the rate of reduction of pyruvate are shown as Lineweaver-Burk (1934) reciprocal plots of the initial reaction velocity in Fig. 5. Preparations of heart, skeletal muscle and liver gave the same value for the velocity at infinite substrate concentration,

Fig. 2. Effect of temperature changes on the temperature coefficients of the enzymic reduction of 2-oxobutyrate (3.3 mM) by normal serum (○) and by serum from a patient with myocardial infarction (●).

Table 2. *Temperature coefficients of human-tissue lactate dehydrogenases at 25° (percentage increase/degree)*

Tissue	Substrate	
	0.7 mM-Pyruvate	3.3 mM-2-Oxobutyrate
Normal serum	5.7	5.6
Serum (myocardial infarction) { (a)	6.2	6.0
{ (b)	7.1	5.0
Heart	8.3	5.6
Skeletal muscle	6.3	4.6
Liver	3.9	3.5

Fig. 3. Effect of temperature changes on the enzymic reduction of pyruvate (○, △, □) and 2-oxobutyrate (●, ▲, ■) by preparations of human heart (○, ●), liver (△, ▲) and skeletal muscle (□, ■).

Fig. 4. Effect of preincubation for 30 min. on the rates of reduction of pyruvate (○, △) and 2-oxobutyrate (●, ▲) by extracts of human heart (○, ●) and liver (△, ▲). Enzyme activities were determined at 25°.

but the apparent enzyme-substrate constants (K_s) were $0.055 \times 10^{-3} \text{ M}$ for heart, $0.116 \times 10^{-3} \text{ M}$ for skeletal muscle and $0.26 \times 10^{-3} \text{ M}$ for liver. With all three tissues high concentrations of pyruvate inhibited the reaction.

Similar results were obtained with 2-oxobutyrate as substrate (Fig. 6), but the apparent enzyme-substrate constants (K_s) were appreciably higher (heart, $1.0 \times 10^{-3} \text{ M}$; skeletal muscle, $2.0 \times 10^{-3} \text{ M}$; liver, $6.2 \times 10^{-3} \text{ M}$) than the corresponding figures for pyruvate. The velocity at infinite substrate concentration was again the same for all three preparations, and the numerical value ($12.5 \mu\text{moles/min./mg. of nitrogen}$) was identical with that observed with pyruvate. High concentrations of 2-oxobutyrate also inhibited the activities of all three preparations.

Optimum substrate concentrations for human serum were evaluated in a similar manner and the results confirmed the earlier findings of Rosalki &

Wilkinson (1960), who plotted Michaelis curves. For heart and liver extracts and serum the optimum concentration of pyruvate was 0.7 mM , but though the optimum 2-oxobutyrate concentrations for heart extract and serum were 3.3 mM , that for liver extract was 10 mM .

Effect of inhibitors

The observation by Novoa *et al.* (1959) that with pyruvate as substrate crystalline lactate dehydrogenase is competitively inhibited by oxamate but non-competitively by oxalate was confirmed with a commercial sample of the purified ox-heart

Fig. 5. Reciprocal plots showing effect of variation in pyruvate concentration on lactate-dehydrogenase activities of preparations of human heart (O), liver (Δ) and skeletal muscle ($\square$).

Fig. 6. Reciprocal plots showing effect of variation in 2-oxobutyrate concentration on the dehydrogenase activities of preparations of human heart (O), liver (Δ) and skeletal muscle ($\square$).

Fig. 7. Effect of oxamate on dehydrogenase activities of preparations of human heart (O, $\bullet$) and liver (Δ , $\blacktriangle$) with 0.7 mM -pyruvate (O, Δ) and 3.3 mM -2-oxobutyrate ($\bullet$, $\blacktriangle$) as substrates. v/v_i , Activity in the absence of oxamate/activity in the presence of oxamate.

Fig. 8. Effect of oxalate on reduction of pyruvate (O, Δ) and 2-oxobutyrate ($\bullet$, $\blacktriangle$) by extracts of human heart (O, $\bullet$) and liver (Δ , $\blacktriangle$). v/v_i , Activity in the absence of oxalate/activity in the presence of oxalate.

Fig. 9. Effect of oxalate on reduction of pyruvate (○, □) and 2-oxobutyrate (●, ■) by extracts of human serum (○, ●) and skeletal muscle (□, ■). v/v_i , Activity in the absence of oxalate/activity in the presence of oxalate.

enzyme. These studies were extended to human-heart and -liver preparations with 2-oxobutyrate as well as pyruvate as substrate. In Figs. 7 and 8 the inhibitory effects are expressed as the ratio v/v_i , where v represents the activity in the absence of an inhibitor and v_i the activity in its presence. 0.7 mM-Pyruvate and 3.3 mM-2-oxobutyrate were used as substrates throughout these experiments.

Oxamate competitively inhibited the reduction of 2-oxobutyrate by both heart and liver preparations to a much greater extent than that of pyruvate (Fig. 7). By contrast, with a particular enzyme preparation, oxalate inhibited the reduction of both substrates equally (Fig. 8). The same effect was observed with oxalate when human sera and human skeletal-muscle extracts were used as enzyme sources (Fig. 9).

DISCUSSION

In the preceding paper it has been shown that the relative rates of reduction of 2-oxobutyrate and pyruvate (ratio 2-oxobutyrate activity/pyruvate activity) may be used to differentiate between the heart and the liver as the tissue of origin of a raised serum lactate-dehydrogenase activity. The validity of this conclusion has now been confirmed by the observation that the relative rates of oxidation of lactate and 2-hydroxybutyrate by heart and liver preparations are in similar proportions to those of the reverse reactions. The difference in the temperature coefficients of the reduction reactions indi-

cates, however, that a particular set of activity ratios is reliable for diagnostic purposes only over a relatively narrow temperature range, and increasing the temperature above 25° leads to appreciably more rapid reduction of pyruvate than of 2-oxobutyrate.

The fact that similar differences in relative reaction rates for the heart and liver preparations were observed for both forward and reverse reactions is consistent with the view that the lactate dehydrogenases of these tissues are separate entities. Furthermore, these results show that the precautions adopted to avoid difficulties due to enzyme inhibitors, possibly present in solutions of NADH_2 (Dalziel, 1961; Fawcett *et al.* 1961), were successful.

Using pyruvate as substrate, Plagemann, Gregory & Wróblewski (1961) demonstrated differences between the thermal stabilities of the anionic lactate-dehydrogenase isoenzymes, characteristic of rabbit-heart muscle, and the cationic isoenzymes typical of the rabbit-liver isoenzymes. They observed that the principal heart isoenzyme was unaffected by exposure for 40 min. at 55° whereas the main liver component was completely inactivated after 6 min. at 53°. These studies have now been extended to human tissues with pyruvate or 2-oxobutyrate as substrate. Since the liver preparation was inactivated by preincubation at 60° whereas under similar conditions the heart extract was but little affected, the difference in thermal stabilities may be used as another means of distinguishing the tissue of origin of the enzyme. That activity against both substrates is destroyed at the same temperature (Fig. 5) is consistent with the view that both substrates are acted upon by the same enzyme protein.

A characteristic substrate constant (K_s) for pyruvate and 2-oxobutyrate was observed for each of the tissues studied, but the affinity of each preparation for pyruvate was much greater than that for 2-oxobutyrate. The observation that the limiting velocity at infinite substrate concentration was the same for both substrates and for preparations of human heart, liver and skeletal muscle is a remarkable coincidence, which might possibly be interpreted that both substrates are reduced at similar active centres by all three tissue lactate dehydrogenases. Plagemann, Gregory & Wróblewski (1960) found that different human lactate-dehydrogenase isoenzymes may be distinguished by comparing activities at two different pyruvate concentrations.

As expected, the more weakly bound substrate, 2-oxobutyrate, was more easily displaced from the enzymes in all the preparations investigated by the competitive inhibitor oxamate. The effect of oxamate is therefore to differentiate between the

substrates. The non-competitive inhibitor, oxalate, however, inhibited the reduction of both substrates equally, but different effects were observed with enzyme preparations from different tissues.

For a non-competitive inhibitor it may be deduced that $v/v_i = 1 + (i/K_i)$, where i is the inhibitor concentration and K_i the enzyme-inhibitor constant. Accordingly the slopes of the lines in Figs. 8 and 9 give the reciprocals of the inhibitor constants of the various preparations. These values may be regarded as indices of the enzyme-inhibitor affinities, and, when the values observed for different tissues are plotted against the corresponding 2-oxobutyrate activity/pyruvate activity ratios (Plummer *et al.* 1963), an almost linear relationship was observed (Fig. 10). The parallelism between the values for the ratio and the electrophoretic mobility of the various lactate-dehydrogenase isoenzymes has been discussed in the preceding paper, and it now seems that the inhibitor constants for oxalate provide an alternative means of distinguishing these isoenzymes. The intermediate value found for serum was to be expected since the serum enzyme consists of a mixture of isoenzymes derived from a variety of tissues.

Inhibitor studies with oxalate might form the basis of a new diagnostic test and preliminary unpublished results with pathological sera accord well

with this possibility. Serum from a patient with myocardial infarction was found to have a reciprocal inhibitor constant ($10^3/K_i$) of 12.0 for oxalate intermediate between the values for normal sera (10.8) and for a heart extract (12.8). The corresponding figure for the serum of a patient with infective hepatitis was 3.8, which closely approximates to the value of 3.5 found in a liver extract.

Since the same value for K_i for a given enzyme preparation was found when either pyruvate or 2-oxobutyrate was used as substrate, it seems probable that the same enzyme protein is combining with oxalate in both cases, and that the two substrates normally form similar complexes with the enzyme.

SUMMARY

1. The lactate-dehydrogenase activities of human-heart and -liver preparations have been determined by measuring the rates of reduction of pyruvate and of 2-oxobutyrate with NADH_2 as coenzyme, and also by means of the reverse reaction. For both reactions the 2-oxobutyrate activity/pyruvate activity ratio for heart is about three times as great as that for liver.

2. Temperature coefficients, thermal stabilities and apparent enzyme-substrate constants (K_s) have been determined for various human-tissue preparations with pyruvate and 2-oxobutyrate.

3. Oxamate competitively inhibits the reduction of 2-oxobutyrate by both heart and liver preparations to a much greater extent than that of pyruvate.

4. With a given tissue preparation, oxalate inhibits the reduction of both substrates equally. A relationship has been established between the reciprocal inhibitor constant ($1/K_i$) and the activity ratios for human heart, serum, skeletal muscle and liver.

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Fig. 10. Relation between the reciprocal inhibitor constants for oxalate and the 2-oxobutyrate activity/pyruvate activity ratios of some human tissues. H, Heart muscle; S, serum; M, skeletal muscle; L, liver.

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The Intracellular Teichoic Acid from *Staphylococcus aureus* H

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Staphylococcus aureus H contains in its wall a large amount of a ribitol teichoic acid in which each of the eight D-ribitol 5-phosphate units possesses a α - or β -N-acetyl-D-glucosaminyl residue at position 4, and the usual D-alanine ester residues at positions 2 or 3 (Baddiley, Buchanan, Raj-Bhandary & Sanderson, 1962*b*; Baddiley, Buchanan, Martin & Raj-Bhandary, 1962*a*). In earlier work trichloroacetic acid extracts of whole cells of this organism were shown to contain, in addition to the ribitol compound, a small amount of a glycerol phosphate polymer (Armstrong, Baddiley, Buchanan, Carss & Greenberg, 1958). The demonstration that intracellular glycerol teichoic acids occur almost universally in Gram-positive bacteria (Baddiley & Davison, 1961; Davison, 1962) supports the view that the glycerol compound from the staphylococcal extracts is an intracellular teichoic acid.

In view of the serological importance of teichoic acids, several of which are now known to be group antigens (Baddiley & Davison, 1961; Haukenes, Ellwood, Baddiley & Oeding, 1961; Sanderson, Juergens & Strominger, 1961; Morse, 1962; Haukenes, 1962; Wicken, Elliott & Baddiley, 1963), an examination of the structure of the intracellular representative from the *Staphylococcus* was undertaken. Although the ribitol teichoic acid in walls of *S. aureus* is associated with the group antigenic reactions, intracellular teichoic acids from other bacteria are serologically active (Wicken *et al.* 1963); consequently, studies on this intracellular compound were desirable. Moreover, in the two cases examined so far, these so-called intracellular compounds are probably located between the wall and the protoplast membrane (Hay, Wicken & Baddiley, 1963), and thus may be

concerned in transport and similar important metabolic activities.

It is shown here that the compound from *S. aureus* H is a glycerol 1,3-phosphate polymer with D-alanine ester residues and occasional β -gentiobiosyl [β -D-glucopyranosyl-(1 $\rightarrow$ 6)-O-D-glucose] or N-acetylglucosamine residues at position 2 on glycerol. It is not known whether the gentiobiose and glucosamine substituents occur in the same molecule or whether the preparation is a mixture.

EXPERIMENTAL

Materials. *S. aureus* H was grown by Dr A. L. Davison from a culture provided by Dr J. T. Park, calf intestinal phosphatase was obtained from the Sigma Chemical Co., St Louis, Mo., U.S.A., β -glucosidase (from almond emulsin) from L. Light and Co. Ltd., Colnbrook, Bucks. and Sephadex G-75 from Pharmacia, Uppsala, Sweden.

Analytical methods. Phosphate was determined as described by Chen, Toribara & Warner (1956), alanine as described by Rosen (1957), periodate as described by Dixon & Lipkin (1954), formaldehyde as described by Hough, Powell & Woods (1956) and Hanahan & Olley (1958), and hexoses as described by Dubois, Gilles, Hamilton, Rebers & Smith (1956).

Paper chromatography. Paper chromatography was carried out with Whatman no. 1 or no. 4 paper previously washed with 2*N*-acetic acid and then water. The following solvent systems were used: A, propan-1-ol-aq. ammonia (sp.gr. 0.88)-water (6:3:1, by vol.) (Hanes & Isherwood, 1949); B, butan-1-ol-ethanol-water-aq. ammonia (sp.gr. 0.88) (40:10:49:1, by vol.; organic phase) (Foster, Horton & Stacey, 1957); C, pyridine-ethyl acetate-water-acetic acid (5:5:3:1, by vol.) (Fischer & Nebel, 1955). Solvent A was used for ascending chromatography on Whatman no. 4, solvent B for descending chromatography on Whatman no. 4 and solvent C for descending chromatography on Whatman no. 1 papers. The products were detected by the