

INFLUENCE OF STERIC SPECIFICITY ON THE RATES OF HYDROGEN EXCHANGE
BETWEEN SUBSTRATES OF NAD-COUPLED DEHYDROGENASES*

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Summary

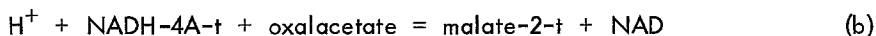
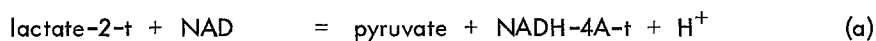
Enzyme-catalyzed isotope exchange rates between lactate-2-t and malate and between lactate-2-t and glycerol-3-P were measured using alternately high and low activities of each dehydrogenase. With the activity of lactate dehydrogenase setting the rate of the overall reaction, the lactate-malate exchange was found to be about 3 times faster than the lactate-glycerol-3-P exchange. This observation provides evidence that, with the activity of lactate dehydrogenase determining the reaction rate, lactate dehydrogenase-coenzyme association and dissociation, in one of two steps required to bring about the transposition of tritium from the A- to the B-side of NADH, slows the rate of the lactate-glycerol-3-P exchange.

In a previous report (1) a theoretical interpretation was offered to explain experimental data which suggested that, in the coupling of dehydrogenase systems in intact cells, the rate of hydrogen transfer from one substrate to the other was more rapid when the interacting enzymes had the same rather than opposite steric specificities for reduced coenzyme. It was suggested that this difference in kinetic behaviour between the two systems would be anticipated if, in the coupling of dehydrogenases having opposite steric specificity, enzyme-coenzyme associations and dissociations were obligatory steps in the reactions required to transfer a hydrogen atom from one side of the plane of the reduced nicotinamide moiety of the reduced coenzyme to the other. In an effort to throw further light on this question we have

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measured equilibrium velocities (exchange rates) between reduced substrates, of coupled dehydrogenase systems having the same and opposite steric specificities for NADH.

Exchange of tritium between lactate-2-t and malate occurs when the two reactions shown below are coupled:



At equilibrium the rate of the lactate-malate exchange, V_{L-M} , is related to V_{L-H} , the rate of the lactate-NADH exchange (reaction a) and to V_{M-H} , the rate of the malate-NADH exchange (reaction b) as shown in the following equation (1,2):

$$1/V_{L-M} = 1/V_{L-H} + 1/V_{M-H} \quad (1)$$

where the Vs are the exchange rate/unit of enzyme activity. In the presence of E_L units of lactate dehydrogenase and E_M units of malate dehydrogenase, the rate of the lactate-malate exchange, R_{L-M} , is related to V_{L-H} and V_{M-H} as shown below:

$$1/R_{L-M} = 1/E_L V_{L-H} + 1/E_M V_{M-H} \quad (2)$$

When $E_L = E_M = 1$, $R_{L-M} = V_{L-M}$.

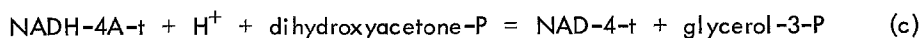
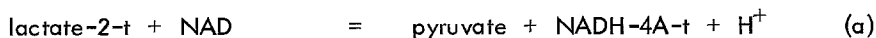
Equation 2 can be evaluated by separately measuring the rates of exchange between lactate and NADH and malate and NADH. However, if the magnitude of V_{L-H} and V_{M-H} do not differ widely, Equation 2 takes a simpler form by making $E_L > E_M$. In that case

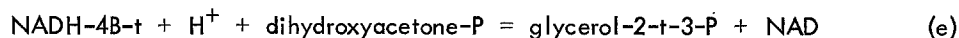
$$1/R_{L-M} = 1/E_M V_{M-H} \quad (3)$$

Similarly when $E_M > E_L$,

$$1/R_{L-M} = 1/E_L V_{L-H} \quad (4)$$

In the case of the exchange of isotopic hydrogen between lactate and glycerol-3-P, the steps required to accomplish the transfer of tritium from lactate-2-t to glycerol-3-P are:





The rate of exchange of tritium between lactate and glycerol-3-P, $V_{\text{L-G}}$, is related to the rates of exchange reactions a, c, d, and e as shown below:

$$1/V_{\text{L-G}} = 1/V_{\text{L-H}} + 1/V_{\text{H-D(L)}} + 1/V_{\text{G-H}} + 1/V_{\text{H-D(G)}} \quad (5)$$

where $V_{\text{L-H}}$ and $V_{\text{H-D(L)}}$ are the rates of the lactate dehydrogenase-catalyzed exchange reactions a and d, respectively, and $V_{\text{G-H}}$ and $V_{\text{H-D(G)}}$ are the rates of the glycerol-3-P dehydrogenase-catalyzed exchange reactions e and c, respectively.

Using E_{L} units of lactate dehydrogenase and E_{G} units of glycerol-3-P dehydrogenase to catalyze the reaction, the rate of the lactate-glycerol-3-P exchange, $R_{\text{L-G}}$, is related to the rates of the partial exchanges as follows:

$$1/R_{\text{L-G}} = 1/E_{\text{L}} (1/V_{\text{L-H}} + 1/V_{\text{H-D(L)}}) + 1/E_{\text{G}} (1/V_{\text{G-H}} + 1/V_{\text{H-D(G)}}) \quad (6)$$

If $V_{\text{L-H}}$ and $V_{\text{G-H}}$ are of comparable magnitude and if a similar relationship exists between values of $V_{\text{H-D(L)}}$ and $V_{\text{H-D(G)}}$, Equation 6 is simplified by making $E_{\text{L}} > E_{\text{G}}$:

$$1/R_{\text{L-G}} = 1/E_{\text{G}} (1/V_{\text{G-H}} + 1/V_{\text{H-D(G)}}) \quad (7)$$

Similarly if $E_{\text{G}} > E_{\text{L}}$,

$$1/R_{\text{L-G}} = 1/E_{\text{L}} (1/V_{\text{L-H}} + 1/V_{\text{H-D(L)}}) \quad (8)$$

Note that if the term in $1/V_{\text{H-D(L)}}$ vanishes, $R_{\text{L-G}}/E_{\text{L}}$ will be numerically equal to $R_{\text{L-M}}/E_{\text{L}}$ in Equation 4.

Not taken into account so far are possible results of the isotope effect on the reaction rates. Exchange reactions between NAD and NADH will not be slowed by a primary isotope effect since, in these reactions, a $\text{C-}^1\text{H}$ and not a $\text{C-}^3\text{H}$ bond is broken. When the rate of the isotope exchange between the reduced substrates is determined by the activity of one of the two dehydrogenases, the effect of isotope substitution on the rates of exchanges catalyzed by the enzyme in high concentration

will not influence the rate of the overall reaction. Accordingly any difference in the values of R_{L-M}/E_L (Equation 4) and R_{L-G}/E_G (Equation 8) must be ascribed to the influence of the lactate dehydrogenase-catalyzed exchange between NAD-4-t and NADH-4B-t on the overall rate.

Rates of the lactate-malate and lactate-glycerol-3-P exchange reactions were measured under the conditions indicated by the relationships outlined above. The experimental procedure was as follows: Reactions were carried out at 30° in 0.13 M Tris-HCl buffer, pH 8.00. The initial concentration of the reduced substrates was 1 mM and of NAD, 0.1 mM. No additions of oxidized substrates or of NADH were made; the unlabeled reactants were brought to equilibrium by adding the appropriate enzymes 5 min prior to the addition of a trace amount of L-lactate-2-t. The enzyme preparations employed were: Lactate dehydrogenase, Type II from rabbit muscle (Sigma); crystalline suspensions of malate dehydrogenase from pig heart and of glycerol-3-P dehydrogenase of rabbit skeletal muscle (Boehringer and Soehne). Before use the enzymes were sedimented by centrifugation and dissolved in buffer. Enzymatic activities in these solutions were immediately assayed by measuring the rate of oxidation of NADH by the appropriate oxidized substrate under conditions giving a constant initial rate. Enzyme units are expressed in μ moles of NADH oxidized/min at 25°. Methods of separation and measurements of radioactivity were similar to those previously published (2). Exchange rates were calculated by evaluating the McKay equation (3), $-Vt = mn / (m+n) \ln (1-f)$, where m and n are the concentrations of the reduced substrates and f is the extent of isotopic equilibration at time t.

Results of measurements of the rates of the lactate-malate exchange are shown in Table 1. It is of interest to note that, not unexpectedly, values of R_{L-M}/E_L and R_{L-M}/E_M are not identical, i.e. whereas the initial rates of the forward reaction (NADH oxidation) were in 1:1 correspondence, the equilibrium rates clearly were not. From the data of Table 1 V_{L-M} is found to be $2.27 \times 10^{-5} M/hr$.

Table 1

E_L	E_M	R_{L-M}	R_{L-M}/E_L	R_{L-M}/E_M
units		$M/hr \times 10^4$	$M/hr/unit \times 10^5$	
11.6	233	$4.37 \pm 0.37^*$	3.76 ± 0.32	
306	22.5	12.9 ± 0.22		5.74 ± 0.10

* Values of R were calculated from values of f at 3 time intervals. Each sample was counted 2-4 times. The standard error is calculated from the data so obtained.

The validity of the method was tested by carrying out the lactate-malate exchange using enzyme activities of comparable magnitude. In this way Equation 2 could be evaluated by substitution with the values of V_{L-H} and V_{M-H} , i.e. R_{L-M}/E_L and R_{L-M}/E_M respectively, (Table 1) and those of E_L and E_M used in the test experiment. When this was done using 53.0 units of lactate dehydrogenase and 52.5 units of malate dehydrogenase, R_{L-M} was observed to be $11.8 \times 10^{-4} M/hr$. The rate calculated by evaluating Equation 2 as indicated was $12.1 \times 10^{-4} M/hr$.

Table 2 gives the results of measurements of the lactate-glycerol-3-P exchange. Repeating the experiment using 100 units of lactate dehydrogenase and 104.5 units of glycerol-3-P dehydrogenase gave $10.0 \times 10^{-4} M/hr$ as the observed rate of the exchange.

Table 2

E_L	E_G	R_{L-G}	R_{L-G}/E_L	R_{L-G}/E_G
units		$M/hr \times 10^4$	$M/hr \times 10^5$	
18.0	276	2.35 ± 0.15	1.30 ± 0.08	
360	27.6	11.2 ± 0.09		4.05 ± 0.04

Substituting the tabulated values of R_{L-G}/E_L and R_{L-G}/E_G and those of E_L and E_G used in the test experiment in Equation 8 gave $9.8 \times 10^{-4} M/hr$ as the expected rate.

Attention is particularly directed toward the values of R_{L-M}/E_L and R_{L-G}/E_L given in Tables 1 and 2, respectively, showing that, with lactate dehydrogenase limiting the rate of the exchange, the rate of the lactate-glycerol-3-P exchange is slowed by the step which involved the interconversion of NAD⁺ and NADH⁺. The step which doubtlessly limits the rate of the latter reaction is the dissociation of NADH⁺ from the enzyme. In considering the kinetic aspects of the overall reaction resulting from the coupling of two dehydrogenase systems, it becomes evident that the rate of hydrogen transfer from the reduced substrate of one dehydrogenase to the oxidized substrate of the other would occur more rapidly if enzyme-coenzyme association and dissociation were not obligatory steps in the reaction. Taking into account the steric specificities of the interacting dehydrogenases the possibility of facilitated hydrogen transfer would seem to be limited to the case of coupling of dehydrogenases having the opposite steric specificities for the reduced coenzyme. In that situation the coenzyme can be pictured as lying between the two dehydrogenases in such a way that the hydrogen atom donated to the oxidized coenzyme by the reduced substrate would project from the reduced coenzyme toward the dehydrogenase catalyzing the reduction while the hydrogen atom in the opposite plane would be oriented toward the second dehydrogenase and removed in the oxidative step. If, in the coupling of lactate and glycerol-3-P dehydrogenases, lactate dehydrogenase were capable of catalyzing the reduction by lactate of NAD bound to glycerol-3-P dehydrogenase, the term in $1/V_{H-D(L)}$ in Equation 8 would vanish and R_{L-M}/E_L would be numerically equal to R_{L-G}/E_L . The present results indicate that, under the conditions employed, reduction of enzyme-bound NAD does not occur.

It will be noted that, with glycerol-3-P dehydrogenase limiting the rate of the lactate-glycerol-3-P exchange, R_{L-G}/E_G is 3 times R_{L-G}/E_L . In the absence of

knowledge of the values of V_{G-H} and $V_{H-D(G)}$ and of a possible difference in the isotope effect on the reactions catalyzed by the two dehydrogenases, the relatively fast rate of the lactate-glycerol-3-P exchange with limiting glycerol-3-P dehydrogenase cannot be interpreted at this time.

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