ENZYME SYSTEMS IN THE MYCOBACTERIA

VII. PURIFICATION, PROPERTIES AND MECHANISM OF ACTION OF THE ALANINE DEHYDROGENASE

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SUMMARY

An alanine dehydrogenase (AlD) has been purified from cell-free extracts of the H37Ra strain of *Mycobacterium tuberculosis* var. *hominis*. This enzyme catalyzes the reaction: L-alanine + DPN^+ ⇌ pyruvate + NH_4^+ + DPNH. Its sensitivity to certain inhibitors suggests that free sulfhydryl groups are necessary for enzymic activity. In the reductive amination reaction pyruvate and NH_4^+ are shown to each affect the K_s of the other. A kinetic analysis of the reductive amination reaction shows that the mechanism of action of AlD can be described as a modified THEORELL-CHANCE mechanism with substrate inhibition. Attempts to show the intermediation of spontaneously-formed imino-propionate were unsuccessful. It is proposed that reductive amination reactions proceed in two steps. The first step is the enzymic formation of enzyme-bound imino acid. This is followed by the enzymic reduction of the imino acid by DPNH to the amino acid.

INTRODUCTION

It has often been proposed that the biosynthesis of α-amino acids occurs through the reductive amination of α-keto acids (reaction 1). This reaction

$$RCOCOO^- + NH_4^+ + DPNH \rightleftharpoons RCH(NH_2)COO^- + DPN^+ + H_2O \qquad (1)$$

is catalyzed by the amino acid dehydrogenases. An example of this class of dehydrogenases is the alanine dehydrogenase (AlD) which catalyzes the reductive amination of pyruvate by DPNH to form alanine. Up to now the DPN-specific glutamic dehydrogenase[1] was the only member of this class of enzymes that had been purified and studied.

The reductive amination of pyruvate has been demonstrated in an unequivocal fashion in only two reports. WIAME AND PIÉRARD[2] obtained a soluble alanine dehydrogenase from cell-free extracts of *Bacillus subtilis*. The reaction was shown to

Abbreviations: AlD (alanine dehydrogenase) DPN and DPNH (oxidized and reduced forms of diphosphopyridine nucleotide), tris (tris(hydroxymethyl)aminomethane), diol (2,3-propanediol), TPN and TPNH (oxidized and reduced forms of triphosphopyridine nucleotide).

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be freely reversible. FAIRHURST *et al.*[3], also using a strain of *B. subtilis*, showed that washed whole-cell suspensions of this organism catalyze the reductive amination of pyruvate to DL-alanine. In a later report SHAH *et al.*[4] demonstrated that only the L-amino acids were formed by the reductive amination of α-ketoisovaleric and α-ketoisocaproic acids. They proposed that the formation of D-alanine in their system was due to the presence of a DL-alanine racemase.

KRITZMAN[5] has shown that, in crude systems, the reductive amination of pyruvate can result from the following linked system:

$$\text{pyruvate} + CO_2 \rightleftharpoons \text{oxalacetate} \quad (2)$$

$$\text{oxalacetate} + NH_3 \rightleftharpoons \text{aspartate} + H_2O \quad (3)$$

$$\text{aspartate} + \text{pyruvate} \rightleftharpoons \text{oxalacetate} + \text{alanine} \quad (4)$$

which is present in most animal tissues and in bacteria. FAIRHURST *et al.*[3] were able to rule out the possibility that, in their system, the formation of alanine was due to this reaction sequence.

Another system whose overall effect is that of an amino acid dehydrogenase has been suggested by BRAUNSTEIN[6] and NISMAN[7]. This linked system consists of an α-keto acid-glutamic acid transaminase (reaction 5) and glutamic dehydrogenase (reaction 6).

$$RCH(NH_2)COO^- + \alpha\text{-ketoglutarate} \rightleftharpoons RCOCOO^- + \text{glutamate} \quad (5)$$

$$\text{glutamate} + DPN^+ \rightleftharpoons \alpha\text{-ketoglutarate} + DPNH + NH_4^+ \quad (6)$$

$$RCH(NH_2)COO^- + DPN^+ \rightleftharpoons RCOCOO^- + DPNH + NH_4^+ \quad (\text{sum})$$

The presence of catalytic amounts of either glutamic or α-ketoglutaric acid would allow this overall reaction, ostensibly due to an "amino acid dehydrogenase", to proceed.

These two linked reaction sequences probably account for the results of POLLAK AND FAIRBAIRN[8] who reported that homogenates of ovaries of *Ascaris lumbricoides* catalyzed the reductive amination of pyruvate to alanine. In their system bicarbonate enhanced the formation of alanine.

This article describes the isolation, purification and some properties of a soluble AlD from the H37Ra strain of *Mycobacterium tuberculosis* var. *hominis*. The AlD of H37Ra was described in a recent communication from this laboratory as one of several enzymes associated with a soluble pyruvic dehydrogenase[9]. The AlD was originally thought to be a lactic dehydrogenase since its presence was demonstrated solely by the reduction of pyruvate by DPNH. Subsequent analysis of this reaction showed a requirement for NH_4^+; the enzyme was then recognized as an AlD. A kinetic analysis of the reaction catalyzed by this enzyme has enabled us to gain some insight into the mechanism of reductive amination.

MATERIAL AND METHODS

DPN and DPNH were obtained from Sigma Chemical Company, St. Louis, Missouri and L-alanine from Mann Laboratories, New York. Li-pyruvate was prepared by the method of WENDEL[10]. Protein was determined by the biuret reaction[11].

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Assay of AlD

AlD is most conveniently assayed by following the reductive amination of pyruvate by DPNH and NH_3. Under the assay conditions described the reaction is about 10 times faster in this direction than in the reverse direction.

The assay mixture for the reductive amination reaction contains, in μmoles, tris–NH_3 buffer* of pH 8.6 (60), DPNH (0.080), Li-pyruvate (3), bovine serum albumin (0.9 mg) and AlD (2 to 80 μg). The final volume is 1.0 ml. The reaction is run at 21°. A recording spectrophotometer** is used to measure the rate of DPNH oxidation by following the change in optical density at 340 mμ. The reaction is followed for 2–3 min. No-substrate blanks are always used. One unit of AlD activity is defined as the amount of enzyme which catalyzes the oxidation of 1.0 μmole of DPNH per min under the above conditions. Specific activity is defined as units per mg of protein.

The assay mixture for the oxidative deamination reaction contains, in μmoles: diol buffer of pH 9.8 (100), DPN (1), L-alanine (10) and AlD (10 to 200 μg). The final volume is 1.0 ml; the reaction is carried out at 21°. One unit of AlD activity is defined as the amount of enzyme which catalyzes the reduction of 1.0 μmole of DPN per min under the above conditions.

The molar absorbancy index of DPN[12] is taken as $6.22 \cdot 10^3$ l/mole/cm.

RESULTS

Purification of AlD

All steps are carried out at 1 to 3°. The preparation of cell-free extracts of H37Ra has been described[13].

First ammonium sulfate step

The crude cell-free extract is fractionated at pH 7.5 with $(NH_4)_2SO_4$. The fraction (AS-1) precipitating between the saturation limits of 0.40 and 0.60 is separated by centrifugation at 5,000 $\times$ g and retained. AS-1 is dissolved in and dialyzed against 0.10 M phosphate buffer of pH 7.0.

Second ammonium sulfate step

The dialyzed solution of AS-1 is fractionated with alkaline $(NH_4)_2SO_4$***. The fraction obtained by raising the $(NH_4)_2SO_4$ concentration to 0.32 saturated is removed by centrifugation for 40 min at 5,000 $\times$ g and discarded. The $(NH_4)_2SO_4$ saturation of the supernatant is raised to 0.38 by the further addition of alkaline $(NH_4)_2SO_4$. This precipitate (AS-2) is removed by centrifugation for 60 min at 5,000 $\times$ g and retained. AS-2 is dissolved in and dialyzed against 0.10 M phosphate buffer of pH 7.0.

Gel adsorption and elution

An equal volume of water is added to the dialyzed solution of AS-2 and the pH is reduced to 6.0. Enough calcium phosphate gel is added to this solution to obtain a gel: protein ratio of 0.50. The gel is removed by centrifugation and discarded.

* The tris-NH_3 buffer is 0.30 M tris and 0.50 M $(NH_4)_2SO_4$, respectively.

** Process and Instruments Co., Brooklyn, N.Y., Model RS-3. This instrument was specially adapted for kinetic measurements.

*** Prepared by the addition of 5 ml of concentrated NH_4OH to 100 ml of saturated $(NH_4)_2SO_4$ at pH 7.5.

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Essentially all the AlD remains in the supernatant. Calcium phosphate gel is added in small increments and removed by centrifugation until between 50 and 55 % of the AS-2 protein has been removed. (The gel: protein ratio required for 50 % adsorption will depend, among other factors, on the age of the gel. We have found that relatively fresh gel gives better results in this step.) Two mg of gel are now added for each mg of protein remaining in solution. The gel is removed by centrifugation and retained. The AlD is recovered from the gel by elution with several small washes of 4 % $(NH_4)_2SO_4$ at pH 5.4. The eluates are assayed separately; those containing the highest activity are pooled (Gel El).

Third ammonium sulfate step

Finely powdered $(NH_4)_2SO_4$ (0.25 g/ml) is slowly added to the Gel El. The precipitate (AS-3) is permitted to form over a 3 h period and is recovered by centrifugation for 20 min at 20,000 × g.

The results of a typical fractionation for AlD are shown in Table I.

TABLE I

PURIFICATION OF ALANINE DEHYDROGENASE

Fraction no.	Volume (ml)	Protein			Units		
		Mg ml	Total mg	Percent recovered	Specific activity*	Total units	Percent recovered
Crude extract	500	16.9	8450	100	0.070	591	100
AS-1	91	30.4	2765	33	0.13	360	61
AS-2	8.5	30.2	257	3.0	0.44	113	19
Gel El	15.4	4.01	61.8	0.73	0.74	46	7.8
AS-3	2.0	14.4	28.8	0.34	1.32	38	6.5

* Reductive amination assay.

Characteristics of the assay systems

The effects of pyruvate and NH_4^+ concentrations and of pH on the rate of the reductive amination reaction are shown in Fig. 1.

The effects of L-alanine and DPN concentrations and of pH on the rate of the oxidative deamination reaction are shown in Fig. 2.

The rates of both the oxidative and reductive reactions are linear with respect to time and to enzyme concentration (Fig. 3).

Formation of pyruvate from L-alanine

The oxidative deamination of L-alanine to form pyruvate in stoichiometric amounts is shown in Table II. DPNH formation was determined by the change in optical density at 340 mμ; pyruvate was measured colorimetrically as the 2,4-dinitrophenylhydrazone[15]. The dinitrophenylhydrazone of the keto acid formed in the oxidative reaction was chromatographed on paper using water-saturated *n*-butanol as the solvent. A single spot was obtained with an R_F of 0.36, identical with that of a control spot of the 2,4-dinitrophenylhydrazone of authentic pyruvic acid.

Formation of alanine from pyruvate

The reductive amination reaction was carried out on a 20-fold scale in order to

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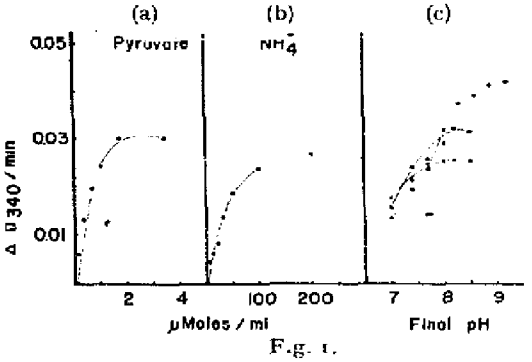

F.g. 1.

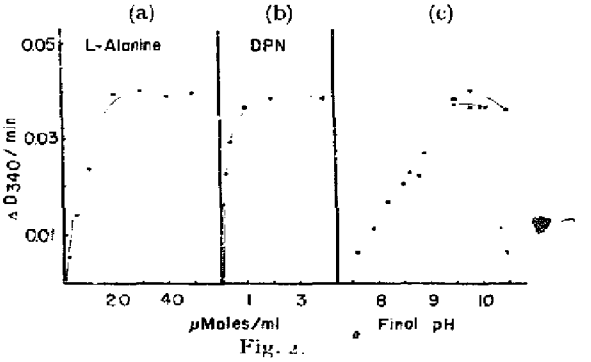

Fig. 2.

Fig. 1. Rate of reductive amination of pyruvate as a function of substrate concentration and pH. (1a) Pyruvate concentration curve; 2.8 μg AlD of specific activity 1.8 per test. (1b) NH_4^+ concentration curve; 4.2 μg AlD of specific activity 1.8 per test. (1c) pH curve; 11.2 μg AlD of specific activity 0.56 per test. O——O, 0.08 *M* phosphate, 0.20 *M* NH_4^+. ×——×, 0.04 *M* pyrophosphate, 0.20 *M* NH_4^+. ●——●, 0.08 *M* tris, 0.20 *M* NH_4^+.

Fig. 2. Rate of oxidative deamination of L-alanine as a function of the concentration of L-alanine (2a) and of DPN (2b) and of the reaction pH (2c). Conditions as described in the text; 11.2 μg of AlD of specific activity 0.7 per test. For the pH curve the following buffers were used: O—— O, 0.06 *M* tris; ×——×, 0.06 *M* diol; ● ——●, 0.06 *M* glycine.

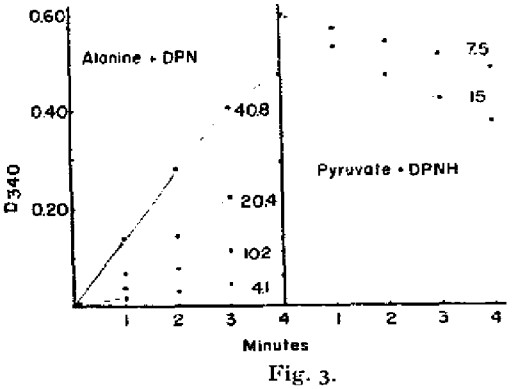

Fig. 3.

Fig. 3. Effect of time and of enzyme concentration on the AlD reaction. Enzyme concentrations (μg/ml) as shown. Rates have been corrected for no-enzyme blanks.

TABLE II

FORMATION OF PYRUVATE FROM L-ALANINE BY REDUCTIVE AMINATION

Expt. no.	*Protein (mg)*	*Incubation time (min)*	*DPNH formed (μmoles)*	*Pyruvate formed (μmoles)*
1	0.055	45	0.460	0.429
2	0.055	30	0.286	0.323
3	0.055	60	0.425	0.430
4	0.110	30	0.436	0.475
5	0.110	60	0.609	0.605

isolate the product. After a 15 min incubation period the enzyme was inactivated by the addition of 0.5 ml of absolute alcohol. The tube contents were evaporated to dryness, the residues were extracted twice, each time with 0.50 ml of ethanol. The alcohol extracts were combined and evaporated to about 0.2 ml. The concentrate was spotted on paper; ascending chromatography was carried out using two different solvent systems. Solvent 1 consisted of isopropanol–formic acid–H_2O (75:13:12). Solvent 2 was methylethyl ketone–propionic acid–H_2O (75:25:30). The amino acid spots were developed by a ninhydrin spray followed by heating at 110° for 15 min. A single spot of R_F 0.62 (solvent 1) and R_F 0.22 (solvent 2) appeared in the track of the spot from each of the complete reaction tubes. Control tube samples (no-enzyme

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or no-substrate) yielded no ninhydrin-reacting spots. Under these conditions authentic L-alanine produced spots with R_F values identical with those of the unknown. Co-chromatography of a complete reaction mixture concentrate and L-alanine in a two-dimensional ascending system (water-saturated phenol followed by solvent 1, above, in the second dimension) yielded a single ninhydrin-reacting spot.

While the optical activity of the alanine formed by reductive amination of pyruvate has not been determined the substrate specificity (see below) of the AlD indicates that the L-form is produced.

Equilibrium of the alanine/pyruvate reaction

The equilibrium constant of the alanine/pyruvate system was determined. Typical results are shown in Fig. 4. The apparent equilibrium constant, K'_{eq}, is defined as follows:

$$K'_{eq} = \frac{[\text{pyruvate}][\text{DPNH}][NH_4^+]}{[\text{alanine}][\text{DPN}^+]}$$

At pH 9.8, and making no correction for ionization of reactants or products, $K'_{eq} = 5.5 \pm 1.8 \cdot 10^{-11}$.

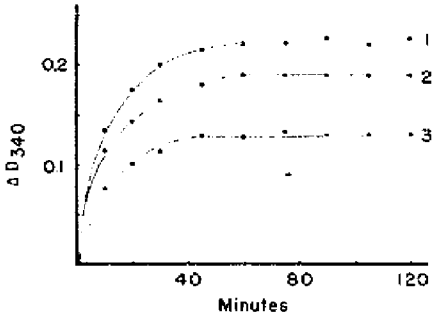

Fig. 4. Equilibrium of the oxidative deamination reaction. Each cuvette contained, in μmoles, diol buffer of pH 9.8 (100), L-alanine (2.0) and AlD (0.29 mg of specific activity 6.7). Initial DPN concentrations were, curve 1 (0.102 μmoles), curve 2 (0.204 μmoles) and curve 3 (0.306 μmoles), respectively. Final volume was 1.00 ml. The optical density at 340 mμ was followed until no further change was observed.

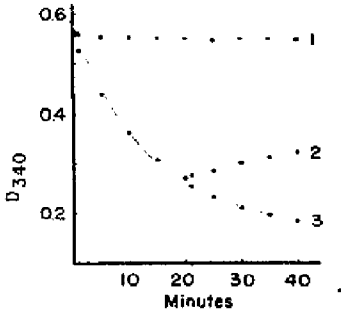

Fig. 5. Reversal by L-alanine of the reductive amination of pyruvate. Each cuvette contained, in μmoles, tris buffer of pH 8.5 (60), NH_4^+ (5), DPNH (0.08) and AlD (0.102 mg of specific activity 6.7). The final volume was 1.00 ml. The control cuvette (curve 1) contained no pyruvate, the other cuvettes each contained 0.08 μmoles of pyruvate. After 20 min 23 μmoles of L-alanine were added to cuvette 2.

Reversal of the reductive amination reaction

Fig. 5 shows the reversal by alanine of the reductive amination reaction carried out at pH 8.5. As expected from the equilibrium constant a large amount of L-alanine produces but a small reversal.

Specificity of the reactants

TPN or TPNH cannot replace DPN or DPNH, respectively, as the AlD coenzyme. The following α-keto- and α-amino-acids are not substrates for the AlD: oxalacetate, α-ketoglutarate, L-phenylalanine, β-alanine, DL-α-aminobutyric acid and L-serine. D-alanine is neither a substrate nor an inhibitor. The lack of activity of α-ketoglutarate in this system rules out BRAUNSTEIN's[6] mechanism of "amino acid dehydrogenase" action.

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Effect of inhibitors on the alanine dehydrogenase

Table III shows the effects of several inhibitors on the reductive amination assay. The necessity of –SH groups for enzymic activity is suggested by the extreme sensitivity of the enzyme to PCMBA. This may also explain the stimulation of activity noted when the enzyme is treated with EDTA which could remove metals causing the oxidation of enzyme-bound –SH groups. When the enzyme is diluted in 0.02 *M* tris buffer of pH 7.5 it rapidly loses activity. When the dilution is made at pH 7.0 in either 0.10 *M* tris buffer or in 0.02 *M* tris buffer with albumin (3 mg/ml) present, the inactivation is not observed. Similarly, glutamic dehydrogenase has been shown to contain –SH groups[16–18] which have been shown to participate in the binding of substrates and products[19].

TABLE III

EFFECT OF INHIBITORS ON THE ALANINE DEHYDROGENASE REACTION

Inhibitor	*Concentration (μmoles/ml)*	*Percent of control*
None		100
Fluoride	20	100
Arsenate	10	100
EDTA	4	140
PCMBA	0.01	0
	0.001	48
	0.0003	87
2-amino-fluorene*	0.28	72
2-acetylamino-fluorene*	0.23	72
BEP**	0.05	100
Propionic acid	1.0	100
Malonic acid	1.0	100

* The generous gift of Dr. JAMES A. MILLER, University of Wisconsin Medical School.

** Bis-1,3-β-ethylhexyl-5-methyl-5-amino-hexahydropyrimidine, the generous gift of Dr. HARLYN HALVORSON, Department of Bacteriology, University of Wisconsin.

KIELLEY[20] reported the inhibitory effect of fluorene carcinogens on both mitochondrial and crystalline glutamic dehydrogenase. Two fluorene derivatives inhibit the alanine dehydrogenase.

Bis-1,3-β-ethylhexyl-5-methyl-5-amino-hexahydropyrimidine is a potent inhibitor of spore germination[21]. The central role of pyruvate in spore germination[21] suggested that this inhibitor might act in the alanine dehydrogenase reaction. This hypothesis was not borne out.

IWATSUBO *et al.*[18] have shown that glutaric acid inhibits glutamic dehydrogenase. The mono- and dicarboxylic acid analogs of alanine (propionic and malonic acids) are without effect in the AlD system.

Mechanism of the reductive amination reaction

The mechanism proposed by VON EULER[1] for the reductive amination of α-ketoglutaric acid to glutamic acid, as catalyzed by the glutamic dehydrogenase, involves a two-step reaction. In the first step α-ketoglutaric acid reacts non-enzymically with NH_3 to form imino-glutaric acid (9a). This is followed (9b) by the enzymic reduction of the imino acid to glutamic acid by DPNH.

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$$^{-}OOC(CH_2)_2COCOO^{-} + NH_3 \rightleftharpoons {}^{-}OOC(CH_2)_2C(NH)COO^{-} + H_2O \quad (9a)$$

$$^{-}OOC(CH_2)_2C(NH)COO^{-} + DPNH + H^{+} \rightleftharpoons OOC(CH_2)_2CH(NH_2)COO^{-} + DPN^{+} \quad (9b)$$

No direct evidence for this proposed mechanism has been available[22]. When our investigation of the AlD indicated the basic similarity of this enzyme to the glutamic dehydrogenase this mechanism was studied.

According to the mechanism shown in (9a, b) above, the imino acid is the actual substrate for the dehydrogenase. The concentration of the imino acid should be a function of the product of the concentrations of the keto acid and NH_3. It was predicted, therefore, that if the product of the concentrations of the reactants is held constant the rate of DPNH oxidation by a given amount of enzyme will be constant. Experiments, however, failed to confirm this relationship. At any given concentration product the rate of DPNH oxidation fell off as the pyruvate concentration was reduced and NH_4^+ concentration increased. This was true for concentration products of 13 to 110 ([μmoles/ml]^2). The data showed that the rate of DPNH oxidation at a given enzyme concentration was a function of both the product and the ratio of the substrate concentrations. These data are shown in Fig. 6. It was concluded that the simple mechanism proposed by VON EULER could not adequately explain this reaction. Any mechanism proposed for the reductive amination reaction must account for the complex relationship between the substrates[22].

ALBERTY[23], in a recent discussion of coenzyme mechanisms, based, in part, on the work of THEORELL AND CHANCE[24], described a series of reaction mechanisms applicable to the overall enzyme-catalyzed reaction.

$$A + B \rightleftharpoons C + D \quad (10)$$

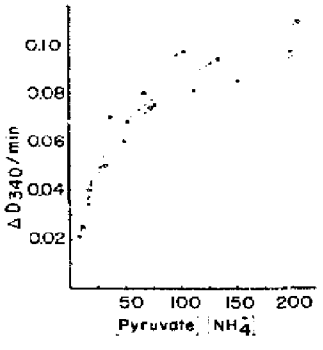

Fig. 6. Rate of DPNH oxidation as a function of both the product and the ratio of substrate concentrations. O——O, NH_4^+/Pyr = 97; O——O, NH_4^+/Pyr = 196; ×——×, NH_4^+/Pyr = 292.

The THEORELL-CHANCE (T-C) mechanism of coenzyme function, derived from their investigations of the mechanism of action of the liver alcohol dehydrogenase, may be represented as

$$\begin{aligned} E + A &\rightleftharpoons EA \\ EA + B &\rightleftharpoons EC + D \\ EC &\rightleftharpoons E + C \end{aligned} \quad (11)$$

High substrate concentrations may cause inhibition of certain dehydrogenases as was described, for example, by THEORELL *et al.*[25]. The basic T-C mechanism was expanded by DALZIEL[26] and ALBERTY[23] to a form accounting for the substrate inhibition

$$\begin{aligned} E + A &\rightleftharpoons EA && (12\text{-}1) \\ E + B &\rightleftharpoons EB && (12\text{-}2) \\ EA + B &\rightleftharpoons EC + D && (12\text{-}3) \\ EC &\rightleftharpoons E + C && (12\text{-}4) \end{aligned} \quad (12)$$

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The relation between the initial concentration of enzymic sites, $(E)_0$, the initial steady state velocity, V, and the rate constants, k, is given in equation (13)[23]

$$\frac{(E)_0}{V} = \frac{1}{k_1} + \frac{1}{k_1 a}\left[1 + \frac{k_{-1}k_2}{k_{-2}k_3}\right] + \frac{1}{k_3 b} + \frac{k_{-1}}{k_1 k_3 ab} + \frac{k_2 b}{k_1 k_{-2} a} \qquad (13)$$

The positive subscripts refer to the individual forward reactions and the negative subscripts indicate the corresponding reverse reactions; a and b are the concentrations, respectively, of the two substrates. This is the simplest mechanism in which V is a function of a, b, ab, and a/b. Certain relationships can be anticipated from mechanism (12) and equation (13). First, V will be a function of the concentration of the two substrates, the product of the concentrations and the ratio of the concentrations. Second, the K_s for one substrate is a function of the concentration of the other substrate. Third, the slope of the LINEWEAVER-BURK[27] plot for each substrate is a linear function of the reciprocal of the concentration of the other substrate. Fourth, the y-intercept of the LINEWEAVER-BURK plot is a linear function of the reciprocal of the concentration of the other substrate. If equation (13) is used to predict $(E)_0/V$ at constant a it is seen that $(E)_0/V$ will be a function of $[1/b + b]$. The linearity of the LINEWEAVER-BURK plot slopes and y-intercepts as a function of $1/b$ will be observed at those low concentrations of b which do not show substrate inhibition ($b \ll 1/b$). As the b term increases and becomes increasingly important in the determination of $(E)_0/V$ then substrate inhibition would be predicted since the b term will become significant. In the experiments described below, substrate concentrations were held below inhibitory levels and, accordingly, linearity for both the slope and intercept plots was obtained.

Data for pyruvate

Fig. 7a shows the effect of pyruvate concentration on V at different NH_4^+ concentrations. The inhibition of V by high pyruvate concentrations will be discussed below. LINEWEAVER-BURK plots for these data are shown in Fig. 7b. The relationships between the LINEWEAVER-BURK plot slopes and the y-intercepts as functions of the reciprocal of NH_4^+ concentration are shown in Fig. 8a,b. The linearity is apparent.

Data for ammonia

In Figs. 9a,b and 10a,b data similar to those above are shown for ammonia at several pyruvate concentrations. Again the expected relationships are realized. In all instances the method of least squares was used to obtain the best straight line.

Form of the ammonia added to the keto acid

Mechanisms for the reductive amination of pyruvate can be written in such a way that either NH_4^+ or NH_3 can be added to the keto acid to form the imino acid. At a constant NH_4^+ concentration the concentration of NH_3 is a function of pH. In the pH range of 8.3 to 8.9 only a small amount of NH_3 will be present in relation to the NH_4^+ concentration. Since the pH optimum of the AlD is not sharp any change in the reaction velocity at constant enzyme and NH_4^+ concentration may be referable to the change in NH_3 concentration. Experiments were carried out in which, at pH 8.3, 8.6 and 8.9, the NH_4^+ concentrations were varied from 25 to 100 μmoles/ml. The

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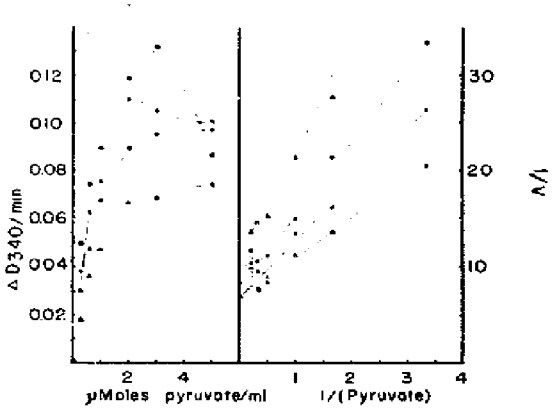

Fig. 7. Effect of NH_4^+ on the K_s value for pyruvate. Each cuvette contained, in μmoles, tris buffer of pH 8.5 (60), DPNH (0.08), beef serum albumin (0.9 mg), AlD of specific activity 12 (1.8 μg), and pyruvate as shown. NH_4^+ concentrations, in μmoles, were ○——○, 160; ×——×, 80; ●——●, 40 and △——△, 20. Final volume was 1.00 ml. LINEWEAVER-BURK plots shown on the right.

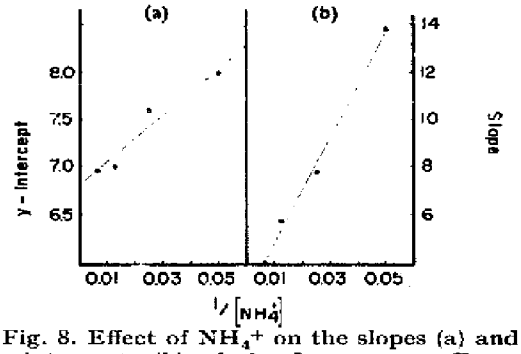

Fig. 8. Effect of NH_4^+ on the slopes (a) and y-intercepts (b) of the LINEWEAVER-BURK plots for pyruvate. Data taken from Fig. 7.

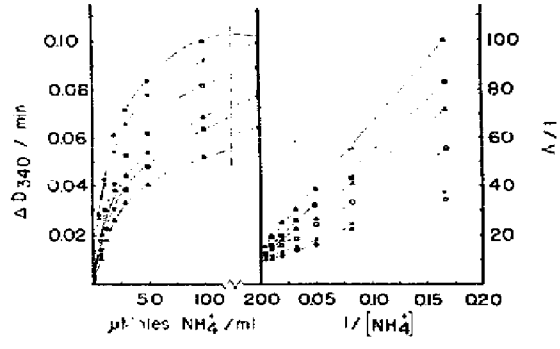

Fig. 9. Effect of pyruvate on the K_s values for NH_4^+. Conditions were the same as in Fig. 7. Pyruvate concentrations, in μmoles, were ○——○, 2.0; ×——×, 1.2; □——□, 0.80; △——△, 0.65; ■——■, 0.50 and ▲——▲, 0.40.

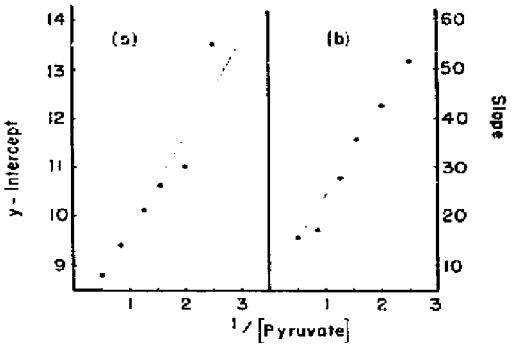

Fig. 10. Effect of pyruvate on the slopes (a) and y-intercepts (b) of the LINEWEAVER-BURK plots for NH_4^+. Data taken from Fig. 9.

NH_3 concentration at pH 8.9 is about four times that at pH 8.3. At each pH tested the reaction velocity was a function of NH_4^+ concentration. However, at a constant NH_4^+ concentration there was little or no change in the reaction velocity as the pH was varied over the range described. While this lack of change of reaction rate with pH under these conditions lends support to the idea that NH_4^+ is the actual substrate in the reductive amination reaction, a final conclusion cannot be reached until the ionization of the AlD itself can be taken into consideration.

DISCUSSION

The mechanism proposed in (12) above is the simplest mechanism which fits our data. ALBERTY[23] has expanded reaction (12) to encompass more complex mechanisms, *viz.* (a) a ternary complex mechanism with substrate inhibition, (b) the T-C mechanism

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with substrate and product inhibition and (c) a ternary complex mechanism with substrate and product inhibition. In the absence of a more extensive kinetic analysis of the AlD reaction it is not possible to specify which of these mechanisms is applicable to this enzyme. At the present time we will base our interpretation on the simplest form, that shown in (12). In the event a more complex mechanism is shown for the AlD the basic conclusions drawn here will still be valid.

A mechanism involving substrate inhibition implies that both substrates compete for the active site of the enzyme. Since we predict that

$$EA + B \rightleftharpoons EC + D \qquad (11)$$

it follows that one substrate must bind to the enzyme before the second substrate can be accepted by EA to form the product. The data shown in Figs. 9a and 7a indicate that at a given pyruvate concentration increasing amounts of NH_4^+ lead to little or no inhibition of the rate of reductive amination. At a given NH_4^+ concentration, however, pyruvate acts as a potent inhibitor of the AlD. We may therefore designate NH_4^+ as "A" and pyruvate as "B" in mechanism (12).

Another mechanism accounting for substrate inhibition should be considered. It is possible that, rather than competing for the same site on the enzyme surface, the substrates bind to closely adjacent enzyme sites. In some manner the binding of pyruvate by AlD on the one site prevents the binding of NH_4^+ on the adjacent site, but not *vice versa*. No evidence as yet exists relative to this possibility.

A probable mechanism of reductive amination may be written as follows:

$$E + NH_4^+ \rightleftharpoons [E - NH_4^+]$$

$$E + CH_3COCOO^- \rightleftharpoons [E - CH_3COCOO^-]$$

$$[E - NH_4^+] + CH_3COCOO^- \rightleftharpoons [E - CH_3C(NH)COO^-] + H_2O + H^+$$

$$[E - CH_3C(NH)COO^-] + DPNH + H^+ \rightleftharpoons [E - CH_3CH(NH_2)COO^-] + DPN^+$$

$$[E - CH_3CH(NH_2)COO^-] \rightleftharpoons E + CH_3CH(NH_2)COO^-$$

This mechanism requires the intermediate formation of imino-propionate which is then reduced to alanine by DPNH. However, as distinguished from the mechanism proposed by VON EULER[1] the imino acid is not formed spontaneously but enzymically. STRECKER[22], from an analysis of the kinetics of the glutamic dehydrogenase reaction, concluded that there was no evidence supporting the non-enzymic formation of imino-glutaric acid from α-ketoglutaric acid and NH_4^+. We cannot yet determine if one enzyme carries out the formation of the imino-acid and the reduction of the imino to the amino acid or if two enzymes are involved. Our data so far gives no evidence of a dissociation of the alanine dehydrogenase into two enzymes. Similarly, electrophoretically-pure glutamic dehydrogenase appears to be a single protein species[17,18]. If the reductive amination of pyruvate is, indeed, catalyzed by only one enzyme then it is reasonable to assume that imino-propionate is an enzyme-bound intermediate during the reaction.

An alternate mechanism for reductive amination[28,29] involves the formation of dehydroalanine rather than imino-propionate. The reduction of dehydroalanine ($>C = C(NH_2)-$) would form alanine. We have no evidence bearing on this possibility. Evidence has recently been presented by HOBERMAN *et al.*[30] and by FISHER[31] that dehydroglutamate is not an intermediate in the reductive amination of α-ketoglutarate as catalyzed by crystalline glutamic dehydrogenase. If the analogy may be

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drawn between glutamic and alanine dehydrogenases then the intermediary role of dehydroalanine may be discounted.

Thus far in this discussion of the mechanism of reductive amination we have not dealt with the effect of the DPNH concentration on V. The justification of this omission is that zero-time reaction velocities were measured in all experiments. Accordingly, the concentration of DPNH, for all practical purposes, did not vary during an experiment and may be taken as a constant. All changes in V may, therefore, be attributed directly to the variations in concentrations of pyruvate and/or NH_4^+. An expression containing the kinetic constants for the overall three-substrate reaction will be extremely complex. The effect on V of variations in the DPNH concentration and the position of DPNH in the reaction mechanism will be the subject of a future study.

It is implicit in this discussion that we assume a basic similarity of mechanism of action between the glutamic and alanine dehydrogenases. A kinetic analysis of the glutamic dehydrogenase similar to that described above for the AlD has not yet been carried out. STRECKER, however, has reported that, in the glutamic dehydrogenase reaction, one substrate has an effect on the K_s of the other substrate[22]. SINGER AND KEARNEY[32] have drawn attention to the large discrepancies between the reported K_s values for the substrates of glutamic dehydrogenase. Since similar observations were made showing the effect of one substrate on the kinetic constants of the other substrate in the AlD reaction it appears reasonable to assume that a kinetic analysis of the glutamic dehydrogenase reaction will yield data similar to those obtained from the AlD reaction.

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BREAKDOWN OF ADENOSINE AND INOSINE NUCLEOTIDES IN BONE AT PHYSIOLOGICAL pH

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SUMMARY

The breakdown of adenosine and inosine nucleotides and nucleosides have been studied in articular and epiphyseal cartilage, epiphyseal and metaphyseal cancellous bone, diaphyseal compact bone and periosteum. Dephosphorylating, deaminating, aminating and adenylate-kinase activities have been demonstrated.

INTRODUCTION

Much attention has recently been given to the role of ATP in bone formation. The enzyme mechanism responsible for this phenomenon, however, is not clear. Suggested

Abbreviations: ATP, adenosinetriphosphate; ADP, adenosinediphosphate; AMP, muscle adenylic acid; AS, adenosine; Ad, adenine; ITP, inosinetriphosphate; IDP, inosinediphosphate; IMP, inosinemonophosphate; IS, inosine.

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