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PRESSURE VARIATION OF ENZYMATIC REACTION RATES: YEAST AND LIVER ALCOHOL DEHYDROGENASE

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The kinetics of yeast and liver alcohol dehydrogenase (YADH and LADH) have been investigated by spectrophotometry at pressures between 1 and 2000 bar. For YADH the common random two substrate mechanism has been used as a model for evaluation of the pressure variation of five kinetic constants in the ethanol–NAD reaction. The dissociation volume associated with each constant is estimated and it is found that the dissociation of binary complexes is followed by large volume decreases, while the dissociation of ternary complexes is followed by smaller volume increases. There is a volume increase following formation of the activated complex in the rate determining step, and the over-all reaction rate decreases with pressure, going to zero at 2000 bar. LADH shows a complicated behaviour at high pressure. This is believed to be due to the substrate inhibition phenomenon occurring at ethanol concentrations above 10 mM. At such concentrations the reaction rate increases with pressure, reaching a maximum at about 1200 bar and goes to zero at 2500 bar. At ethanol concentrations lower than 10 mM there is a small decrease of reaction rate with pressure. To relate the volume changes of the over-all process to those of the intermediate complexes, the partial molal volume of ethanol, acetaldehyde, NAD^+ and NADH are determined by density measurements.

1. Introduction

One of the many aspects of enzymatic mechanisms of action is the volume changes due to the formation of intermediate enzyme–substrate complexes. Investigations of pressure effects upon such systems may provide at least as much insight into mechanisms as do temperature studies, since the change in Gibbs free energy is dependent upon both these parameters. The combination of high pressure with variation in concentrations, temperature, pH, ionic strength, dielectric constants etc. may give information about valuable quantities such as volume changes, complex formations, aggregation, structural changes, charged complexes, rate and equilibrium constants, etc.

Although many workers are concerned with high pressure inactivation and protein denaturation [1], very few spectrophotometric studies have been made on enzymes at work. Andersen and Broe [2] have studied the fumarase enzyme. They found the Michaelis constant to be unaffected by pressure, and the maximum velocity being reduced by increasing pressure. Williams and Shen [3], in a study of pancreatic ribonuclease, found an increase of the hydrolytic rate constant and an increment in dipole moment during the hydrolytic step. Schmid et al. [4] have investigated the activity of the glycolytic enzymes glyceraldehyde-3-phosphate dehydrogenase and lactic dehydrogenase. They found volumes of activation of $\Delta V^{\ddagger}(\mathrm{LDH}) = 0 \pm 1$ cm^3 mole^{-1} and $\Delta V^{\ddagger}(\mathrm{GADPH}) = 60 \pm 4$ cm^3 mole^{-1}. Total deactivation of both enzymes was observed at $p \approx 2$ kbar. Lockyer et al. [5], for α-chymotrypsin, determined the rate of hydrolysis of some esters to be slightly accelerated by pressure, proposing the $\Delta V^{\ddagger}$ to correspond to the volume change for deacylation of the corresponding acyl-enzymes.

The reasons for investigating YADH and LADH in the present work are several. They are believed to be representatives of a group of enzymes, the dehydrogenases, and their kinetics are well known at ordinary pressure. Furthermore they are relatively stable and easy to study by spectrophotometry. It is shown that the combination of pressure measurement with standard biochemical procedures for treating kinetic data is able to present other important information on enzyme mechanisms *.

* A preliminary report on the YADH reaction was given at the 4th International Conference on Chemical Thermodynamics (IUPAC) at Montpellier, France in August 1975. The present work is an extension of that report and now also includes the LADH reaction.

2. Experimental

2.1. Pressure generator

A detailed description of the apparatus has been given by Grønlund and Andersen [7]. In a two-stage process a pre-set pressure up to 10^4 atm can be delivered by the generator in 30 s. The pressure transmitting medium was a mixture of 58% Esso petroleum and 42% Shell Diala D.

2.2. Measuring cell

A description of the cell has earlier been given by Andersen and Broe [2]. It has an upside down T-shape with liquid and pressure inlet at the top and light passage through the lower part. Each of the two sapphire windows is from the outside supported by a threaded cylinder. The surfaces towards the windows are polished to optical smoothness, and a steel spring is placed between the windows to ensure tightening at low pressures. The light path through the liquid is 10 mm long. At the top there is a rubber membrane to separate the pressure liquid from the reaction mixture. From the moment of mixing the substrates with enzyme, the time lapse to inject the mixture into the cell, replace the membrane, connect the pressure generator and raise the pressure was two minutes. During all runs the wavelength was held constant at 340 mm. This is a broad absorption maximum in the spectrum of the reduced coenzyme NADH. The increase in absorbance was measured by a EMI 9558 Q.C. photomoltiplier tube supplied by an Oltronix voltage source. The signals went through a logarithmic converter to a Radiometer Servograph REC 51 recorder, where the optical density increase (OD) was recorded.

2.3 Materials and methods

Both yeast and liver alcohol dehydrogenase (EC 1.1.1.1) were purchased from Boehringer, Mannheim. Coenzymes β-NAD and β-NADH di-Na salt came from Sigma Chem. Co. As second substrate was used commercial absolute alcohol, of high purity. Reactions were run at pH 7.5, both in 0.1 M phosphate and 0.1 M Tris-HCl buffers. To stabilize the enzyme, 30 mM Cys-HCl, 30 mM EDTA and 1 g Bovine serum albumin were added per litre buffer. These additives were found to keep the enzyme activity constant during the day.

New dilute enzyme solution was made every day and kept at 0°C. NAD–EtOH mixtures were kept in deep freeze for several days. There was no possibility of thermostating the cell, and all studies were performed at room temperature, 24°C. Each run had a duration of about five minutes and the increase in optical activity was recorded. For each combination of enzyme concentration, substrate concentrations and pressure there were several parallel runs. Enzyme concentrations varied from 0.005 to 0.2 mg/ml. With YAHD, ethanol concentrations ranged from 5 to 200 mM, while NAD concentrations ranged from 0.02 to 0.75 mM. With LADH, NAD concentrations ranged from 0.01 to 0.75 mM. For all chosen combinations of concentrations, measurements were taken at 1, 500, 1000, 1500 and 2000 atm pressure.

The enzyme concentration was made to vary more or less inversely with the substrate concentrations for the reason of keeping the absorption curve as linear as possible. High concentrations of both enzyme and substrate caused too great deflection of the curve, and low concentrations made the slope of the curve too small for accurate determination at high pressure. Measurement of the specific enzyme activity after pressure exposure assured that irreversible denaturation of enzyme was negligible.

2.4. Partial molal volume measurements

The apparent molal volumes of reactants and products, NAD, ethanol, NADH and acetaldehyde were calculated from density measurements with a Paar density meter (DMA 02C). The temperature was measured with a Hewlett–Packard quartz thermometer and controlled to within ±0.008°C with an oil thermostat. The apparatus constant of the density meter was determined according to specification with air and water as standard substances. The densities were calculated relative to the density of water, 0.997075 g/cm^3, at 25.00°C.

Due to uncertainties in water content of the oxidized and reduced coenzyme, the densities were inaccurate by about 1%. The apparent molal volume was determined from

$$\phi_V = 1000(d_0 - d_s)/md_0d_s + M_s/d_s.$$

d_S is the density of the solution, d_0 the density of pure solvent (water), m the molality of the solution and M_S the molecular weight of the solute. From measurements of d_S at different molalities, the apparent molal volume of infinite dilution can be determined by extrapolation.

The extrapolation function was (8)

$$\phi_V = \phi_V^\infty + Am, \tag{1}$$

where A is an empirical constant.

2.5. pH dependence

Most investigations of the ADH's seem to have been undertaken in phosphate buffer. Initially, this buffer was also used in this work, but it rises difficulties due to its great pressure dependence of the dissociation equilibria. In the range about pH 7.5 the dissociation is

$$H_2PO_4^- \rightleftharpoons HPO_4^{2-} + H^+ \tag{2}$$

It may be shown that

$$\partial(\mathrm{pH})/\partial p \approx (1/2.303)\,\Delta V_a/RT, \tag{3}$$

where ΔV_a is the partial molar volume change of dissociation. The formation of a divalent anion clearly causes volume contraction due to increased electrostriction around the molecule, and this displaces the equilibrium to the right, making the solution more acidic. Neuman et al. [9] have found $\Delta V_a(H_2PO_4^-)$ to be -24 cm^3 mole^{-1}. This is greater than for most buffers and indicates that it should be omitted for pH-sensitive systems under pressure. The effect amounts to a pH reduction of 0.4 units per 10^3 bar. This was corrected for, by means of data from pH studies of YADH [10].

YADH was also investigated in 0.05 mM tris-HCl buffer, whose pH Neuman et al. [9] found to be nearly independent of pressure. LADH was only investigated in tris-HCl buffer. A disadvantage with the tris buffer is its temperature dependent dissociation equilibrium. This is discussed below.

2.6. Temperature dependence

Gierer [11] has investigated the temperature dependence of yeast alcohol dehydrogenase. He found an apparent energy of activation of about 58 kJ mole^{-1} and an apparent entropy of activation smaller than 0.8 J mole^{-1} deg^{-1}.

Related investigations for LADH have not been found, and we assume the energy and entropy changes to be of the same order as for YADH.

Activation energies of this order correspond to an increase of the reaction rate of about 8% deg^{-1} at 300 K. As is well known a sudden increase of the pressure may produce a temperature change in the reaction mixture, affecting rates and equilibria seriously. For an adiabatic reversible compression of water at room temperature and atmospheric pressure, the change in temperature is

$$(\partial T/\partial p)_S = -\frac{(\partial S/\partial p)_T}{(\partial S/\partial T)_p} = \alpha TV/C_p$$

$$= 1.86 \times 10^{-3} \text{ deg atm}^{-1}.$$

This is the maximum possible temperature increase, but in the solid metal cell the changes were believed to be less than 1 deg per 1000 atm. An effect was in fact observed at pressures above 1000 atm, manifested by a small peak in the curve of OD increase with time. The peak decayed after about a minute, but sometimes caused irregularities in the slope of the curve, especially at the highest pressures.

The pH of tris buffer decreases with increasing temperature, as much as 0.03 units deg^{-1}. Although the pressure cell was not thermostated, the temperature was assumed constant during 90% of each run. As the YADH reaction rate decreased with decreasing pH, this should partly compensate for the increase due to the increase of the rate constant with temperature. This was not corrected for, as the introduced error probably was within the experimental uncertainty.

2.7. The effect of pressure on rate and equilibrium constants

Given a series of n reaction steps with corresponding equilibrium constants K_i,

$$\begin{aligned} &A + B \rightleftharpoons AB, && K_1, \\ &C + D \rightleftharpoons CD, && K_2, \\ &\quad\vdots && \quad\vdots \\ &P + QR \rightleftharpoons PQR, && K_n \end{aligned} \tag{4}$$

and a rate determining step

$$PQR \rightarrow XYZ, \qquad k, \tag{5}$$

where all products from the first n steps are removed via the rate determining step. It is then assumed that n equilibria result, and that the initial steady state velocity v of the rate step is given as a function of initial concentrations $c_j = (c_{Aj}, c_{Bj}, ...)$ and constants $K = (K_1, K_2, ..., K_n, k)$

$$v_j = v(c_j, K). \tag{6}$$

In principle a function

$$K = K(c, v) \tag{7}$$

is now given, where K can be found when a number of accurately measured pairs (c_j, v_j) is available. Assuming that the function (operator) K also holds for higher pressure p, one may in the same way find

$$K_p = K(c_p, v_p). \tag{8}$$

When K_p is continuous and differentiable with respect to p, one finds from

$$\Delta G^0 = -RT \ln K_p \tag{9}$$

that

$$(\partial \ln K_p/\partial p)_T = -\Delta v/RT. \tag{10}$$

From the transition state theory 12 one finds, analogously

$$(\partial \ln k/\partial p)_T = -\Delta V^{\ddagger}/RT. \tag{11}$$

The elements in $\Delta V = (\Delta V_1, \Delta V_2, ..., \Delta V_n)$ are the molal volume changes accompanying each of the steps 1–n in eq. (4). $\Delta V^{\ddagger}$ is the molal activation volume change in the process of reactants going into the transition state in eq. (5). From measurements of (c_j, v_j) one can find $K_p = K_p(p)$ and the corresponding ΔV.

3. Theoretical

3.1. Kinetic models

Yeast alcohol dehydrogenase (YADH) and liver alcohol dehydrogenase (LADH) catalyse the oxydation of ethanol (S_2) with the coenzyme nicotinamide adenine dinucleotide (NAD), (S_1) as oxidizing agent. The products are acetaldehyde (P_2) and NADH (P_1).

3.2. YADH

The type of mechanism considered is the following, where four binary and two ternary complexes are in rapid equilibrium with the free substrates:

$$\begin{aligned} E + S_1 &\rightleftharpoons ES_1 && (K_1),\\ E + S_2 &\rightleftharpoons ES_2 && (K_2),\\ ES_1 + S_2 &\rightleftharpoons ES_1S_2 && (K_3),\\ ES_2 + S_1 &\rightleftharpoons ES_1S_2 && (K_4), \end{aligned} \tag{12}$$

$$ES_1S_2 \overset{k}{\rightleftharpoons} EP_1P_2. \tag{13}$$

$$\begin{aligned} E + P_1 &\rightleftharpoons EP_1 && (K_5),\\ E + P_2 &\rightleftharpoons EP_2 && (K_6),\\ EP_1 + P_2 &\rightleftharpoons EP_1P_2 && (K_7),\\ EP_2 + P_1 &\rightleftharpoons EP_1P_2 && (K_8), \end{aligned}$$

According to Sund and Theorell [13], data from kinetic experiments are consistent with the following postulates:

(i) No compulsory order exists for binding of both coenzymes and substrates.

(ii) Both oxidized and reduced forms of reactants are bound at the same time.

(iii) The intramolecular hydrogen transfer of the ternary complex is the rate limiting step, eq. (13).

All K_i's are defined as dissociation constants, e.g.

$$K_i = k_i/k_{-i} = [E][C]/[EC]. \tag{14}$$

Due to postulate iii, the reaction rate may be deduced from

$$v = k[ES_1S_2]. \tag{15}$$

Considering only the initial velocity, not taking the steps 5–8 in eq. (12) into account, the rate expression for the forward reaction is found to be

$$v = \frac{V_e[S_1][S_2]}{K_1K_3 + K_3[S_1] + K_4[S_2] + [S_1][S_2]}, \tag{16}$$

where

$$V_e = k'e_0 \tag{17}$$

is the maximum initial velocity at enzyme concentration e_0.

3.3. LADH

From a kinetic point of view, the LADH mechanism is ordered, and the presence of at least one ternary

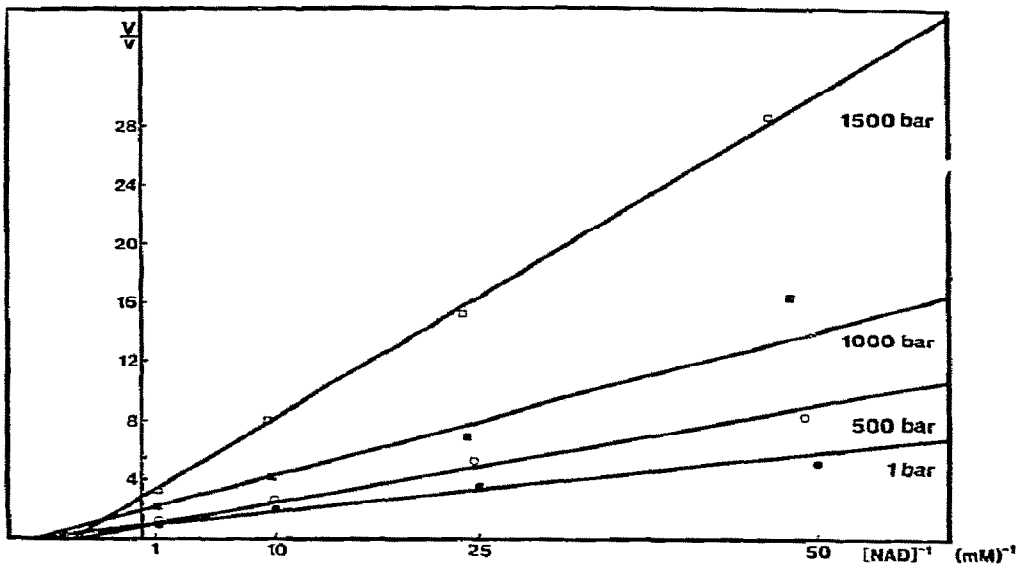

Fig. 1. Pressure inhibition of the YADH reaction in phosphate buffer with NAD as variable substrate. [EtOH] : 100 mM.

complex has been established. The mechanism considered here is the following:

$$E + S_1 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES_1, \qquad ES_1 + S_2 \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EXY \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} EP_1 + P_2, \tag{18}$$

$$EP_1 \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} E + P_1.$$

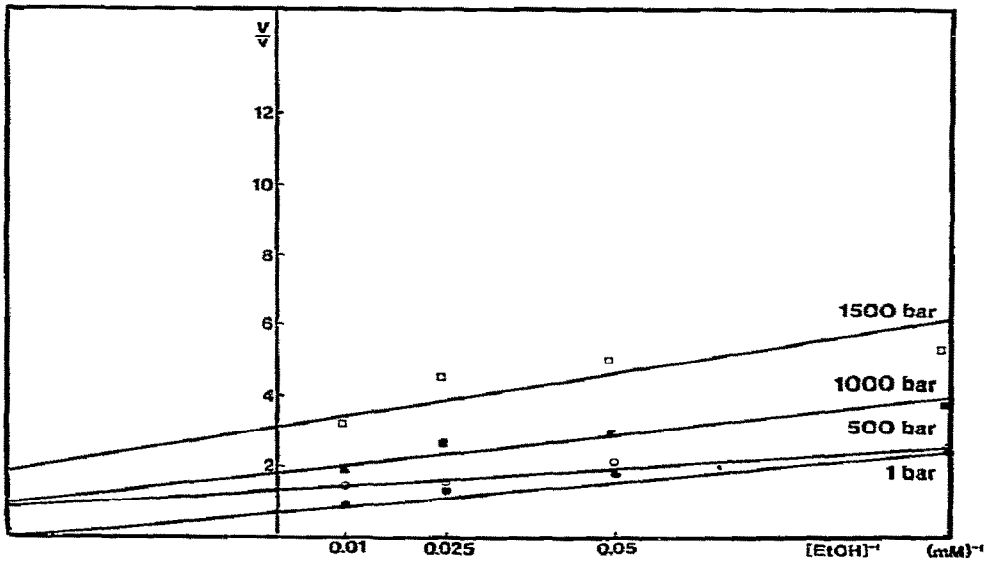

Fig. 2. Pressure inhibition of the YADH reaction in phosphate buffer with EtOH as variable substrate. [NAD] : 0.767 mM.

This is usually regarded as the reverse LADH reaction, so to keep the notation related to the YADH reaction the indices on the rate constants are as for the forward reaction

$$v = \frac{V_e[S_1][S_2]}{\phi_{12} + \phi_2[S_1] + \phi_1[S_2] + \phi_0[S_1][S_2]}, \qquad (19)$$

but without the symmetry in the YADH mechanism.

At high ethanol concentrations however, inhibition occurs, and additional equilibria must be introduced to account for the reaction rate. At ordinary pressure several lines of evidence favour the existence of a ternary complex as responsible for the substrate inhibition (14). This complex is EP_1S_2, formed in the step

$$EP_1 + S_2 \rightleftharpoons EP_1S_2, \qquad K_a, \qquad (20)$$

which, according to Dalziel [15] cause the coefficient of the fourth term in the denominator of eq. (18) to become

$$\phi_0' = \phi_0(1 + [S_2]/K_a). \qquad (21)$$

It is reasonable to expect the formation of this complex to be accompanied by a volume change. Then the equilibria (20) should be influenced by the pressure in such a way that a comparison of the combined eqs. (19) and (21) with the experimental results could determine the importance of (20) in the inhibition mechanism.

3.4. Treatment of the data

For experimental reasons each series of measurements (i.e. four runs with one substrate concentration and the pressure held constant, the other substrate concentration varying) had to be made with different enzyme concentrations. Afterwards all the observed values were multiplied by a factor corresponding to the ratio of these concentrations, according to eq. (17), bringing all observations to a common reference.

It was assured that this reference was in accordance with known data from measurements at atmospheric pressure [16]. The absolute value of the constants are nevertheless of minor importance, since we are primarily interested in their relative variation. For computational reasons, the rate constant k was put equal to unity at 1 bar.

The rate function

$$f = k(1 + K_3/[S_2] + K_4/[S_1] + K_1K_3/[S_1][S_2])^{-1} \qquad (22)$$

was expanded in terms of variations in each constant, using only terms including the first derivatives

$$f = f_0 + \sum_i (\partial f/\partial K_i)(K_i^0 - K_i), \qquad (22)$$

where f_0 is the value of f for a first choice of constants $K_1^0, K_2^0, \ldots$. By means of a FORTRAN program [17] all constants K_i were evaluated simultaneously in an iteration process. In a least square procedure the K_i's were fitted to the experimental v-plane in the $v-[S_1]-[S_2]$ space by minimizing

$$\sum (v/e_0 - f)^2. \qquad (23)$$

This minimization was performed for each pressure set of data, with the appropriate concentrations, rates and a set of trial constants as input.

4. Experimental results

4.1. YADH

Typical results of the pressure effect on the reaction rate of the ethanol–NAD reaction in phosphate buffer are shown in figs. 1 and 2. These are Lineweaver–Burk plots, i.e. of inverse rate versus inverse concentration, expressed by the linearization of eq. (16), simplified to

$$V_e/v = 1 + K_m/[S]. \qquad (24)$$

Here K_m is the Michaelis constant of the system. In fig. 1, the NAD concentration is varied, the EtOH concentration being constant at 100 mM. In fig. 2, the EtOH concentration is varied, the NAD concentration being constant at 0.767 mM. All lines are least square fits.

The pressure effects on the same reaction undertaker in tris-HCl buffer are illustrated in figs. 3 and 4. In fig. 3, the NAD concentration is varied at an EtOH concentration of 10 mM. In fig. 4 the EtOH concentration is varied at an NAD concentration of 0.75 mM.

Measurements were taken in five series, first three with EtOH concentrations of 10, 50 and 100 mM respectively, thereafter two series with NAD concentrations of 0.1 and 0.75 mM respectively. All of the five series were run at the pressures 1, 500, 1000, 1500 and 2000 bars, and the corresponding reaction rates

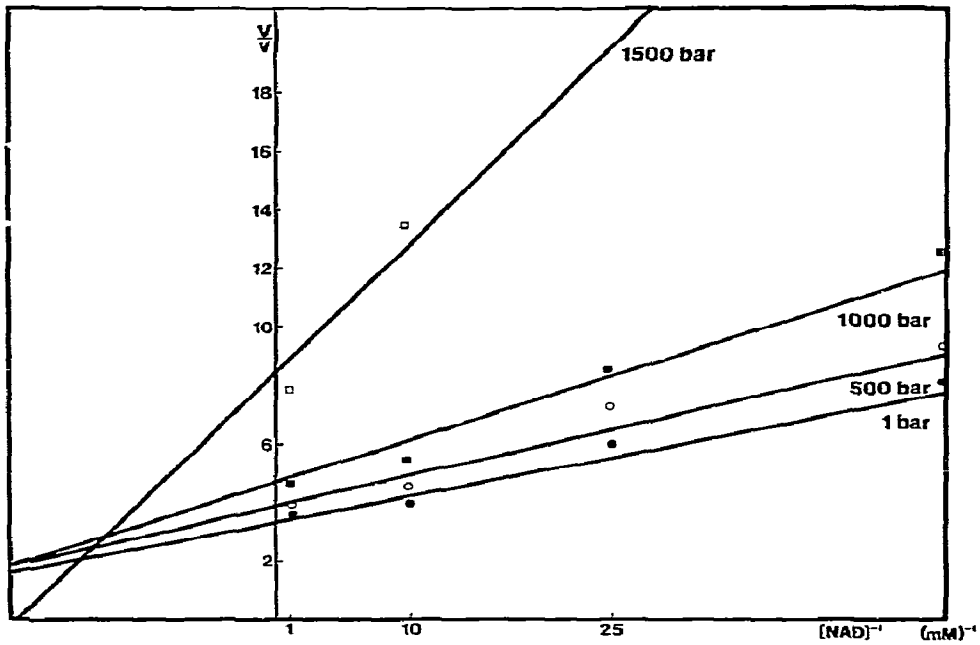

Fig. 3. Pressure inhibition of the YADH reaction in tris-HCl buffer with NAD as variable substrate. [EtOH] : 10 mM.

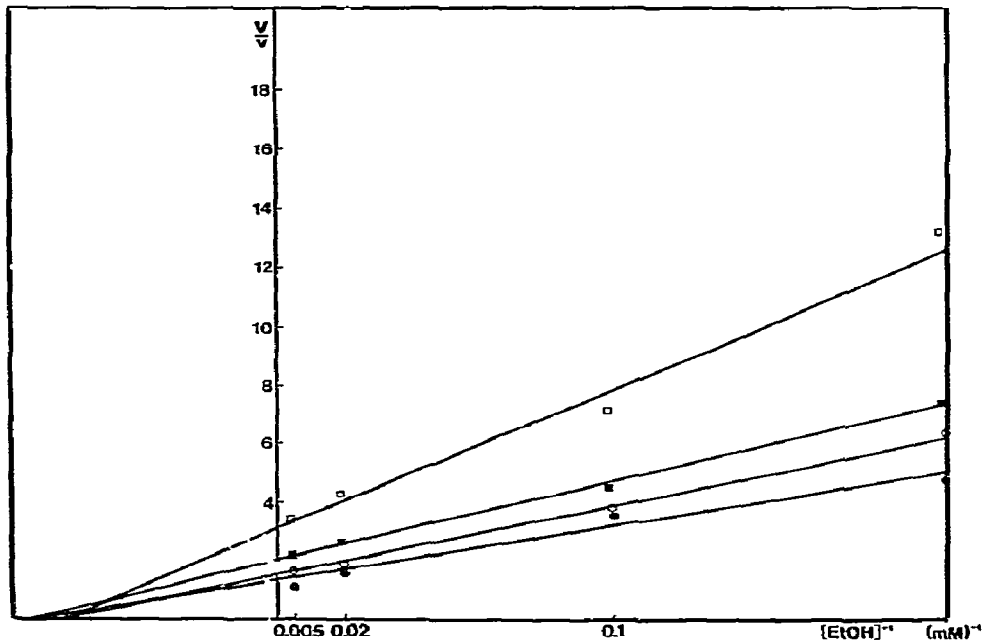

Fig. 4. Pressure inhibition of the YADH reaction in tris-HCl buffer with EtOH as variable substrate. [NAD] : 0.75 mM. ●: 1 bar, ○: 500 bar, ■: 1000 bar, □: 1500 bar.

Table 1
Variation with pressure of the kinetic constants in eq. (16)

Pressure	k	K_3 (mM)	K_4 (mM)	K_1K_3 (mM)^2
1	0.56 ± 0.14	7.6 ± 0.5	0.036 ± 0.022	0.09 ± 0.05
500	0.40 ± 0.10	6.2 ± 3.3	0.027 ± 0.015	0.07 ± 0.03
1000	0.30 ± 0.06	4.4 ± 1.9	0.025 ± 0.010	0.40 ± 0.30
1500	0.16 ± 0.03	2.7 ± 0.9	0.020 ± 0.05	1.10 ± 0.20
2000	0.07 ± 0.02	3.5 ± 0.5	0.043 ± 0.04	1.10 ± 0.10

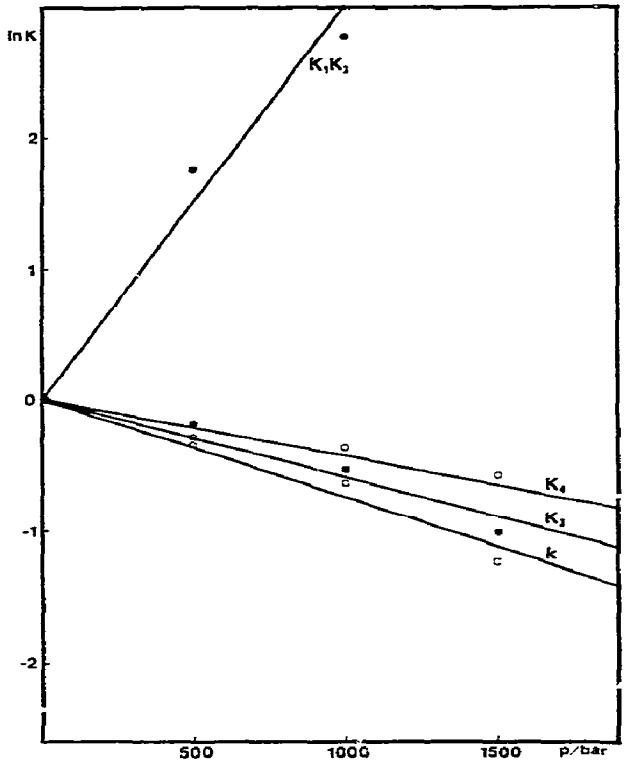

Fig. 5. Logarithm of relative kinetic constants K_i^p/K_i^p versus pressure. For K_1K_3 the reference $K_1^0K_3^0$ is the value at p = 500 bar.

were measured. The rate data were treated according to the least squares procedure based on eqs. (22) and (23). The direct constants found, are shown in table 1.

In fig. 5 a plot is made of the logarithm of the ratio K_i^p/K_i^0, where K_i^p is the value of the constant K_i at the pressure p and K_i^0 is the value of the constant at atmospheric pressure.

Subtracting the contribution of K_3 from the product K_1K_3, we find the pressure variation of K_1, K_3, K_4 and k. Also using the relation $K_2K_4 = K_1K_3$, due to the symmetry of the steps in eq. (12), we find variation of K_2. Using relation (10) the volume changes accompanying each of the steps 1–4 are found. From eq. (11) the activation volume in the rate step are found, and all volume changes are shown in table 2. Note that the constants in steps 1–4 have been treated as dissociation constants, eq. (14), and that the corresponding volume changes are related to dissociation of the enzyme–substrate complexes.

4.2. Density measurements

The apparent molal volume of the solutes EtOH, acetaldehyde, NAD, NADH were measured at different concentrations and extrapolated to infinite dilution using eq. (1). The partial molal volumes found are given in table 3.

As the NADH was purchased as disodium salt, a solution of this salt was cation exhanged to give the free acid. The resulting solution had a pH of 3.05 and therefore the dissociation state of the acid was probably

Table 2
Molal volume changes of the steps 1–4 in eqs. (12) and (13).

ΔV_1 (cm^3 mole^{-1})	ΔV_2 (cm^3 mole^{-1})	ΔV_3 (cm^3 mole^{-1})	ΔV_4 (cm^3 mole^{-1})	$\Delta V^{\ddagger}$ (cm^3 mole^{-1})
−75 ± 23	−69 ± 25	15 ± 10	9 ± 10	20 ± 5

Table 3
Molal volumes of reactants and products in the reactions. The greater uncertainty for NADH is due to the concentration uncertainty

	Solute			
	C_2H_5OH	CH_3CHO	NAD	NADH
ϕ_V^∞ (cm^3 $mole^{-1}$)	55 ± 1	42 ± 1	370 ± 8	380 ± 25

not the same as in the buffered reaction mixture. An estimated correction for this was made.

4.3. Experimental results, LADH

The LADH catalysis of the ethanol–NAD reaction has been undertaken in tris-HCl buffer only. The enzyme shows a rather complicated behaviour when exposed to pressure, as the overall activation volume is clearly pressure dependent, and even changes its sign. In addition the numerical value and sign also seems to be dependent on the ethanol concentration. It is assumed that this unexpected behaviour is due to the substrate inhibition effect of this enzyme. At atmospheric pressure the enzyme behaves normally for ethanol concentrations up to about 10 mM. Concen-

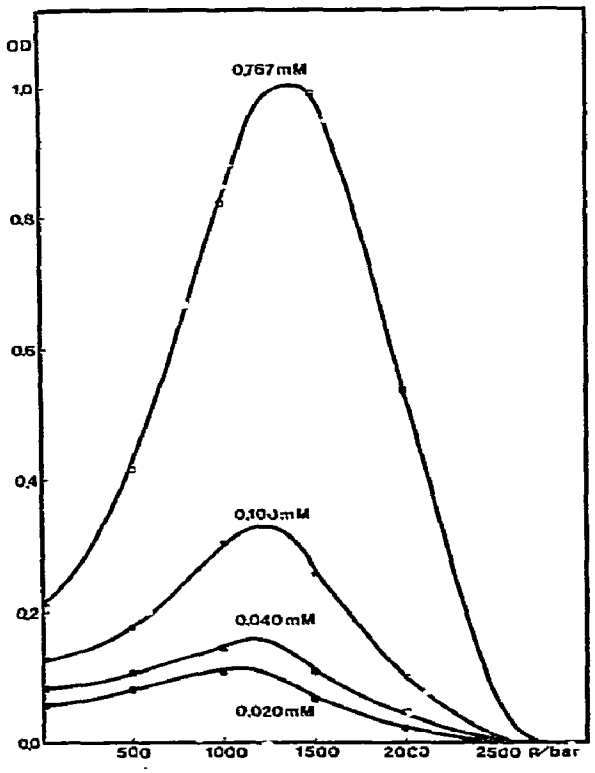

Fig. 6. Reaction rate of the LADH reaction represented by optical density increase versus pressure at four NAD concentrations. [EtOH] : 100 mM.

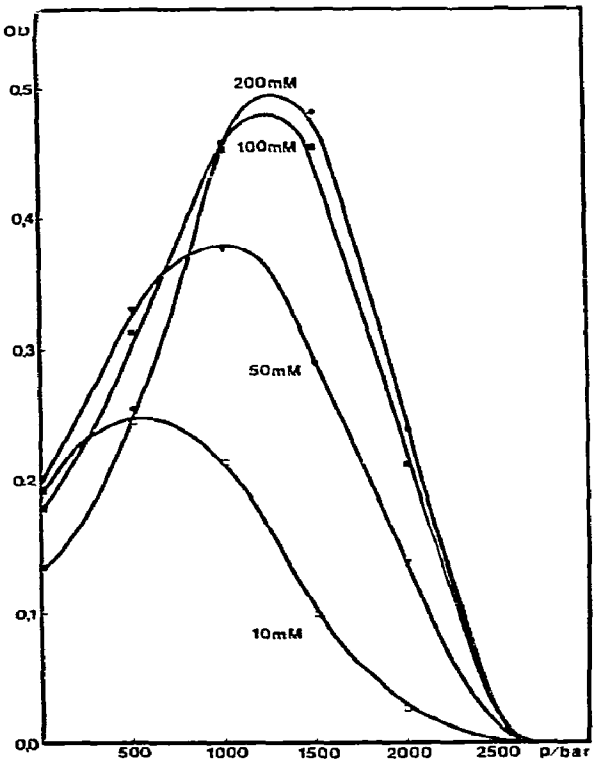

Fig. 7. Reaction rate of the LADH reaction represented by optical density increase versus pressure at four EtOH concentrations. [NAD] : 0.767 mM.

trations higher than this inhibit the reaction. At [EtOH] = 10 mM the effect of moderate pressure (<1000 bar) on the reaction rate seems to be small or negligible. At lower [EtOH] the reaction rate seems to decrease slowly with pressure, and at [EtOH] higher than 10 mM the rate shows an increase with pressure, which is larger the higher the ethanol concentration. But as the pressure increases beyond 1000–1200 bar, the rate starts to decrease with pressure and goes to zero at about 2500 bar.

In fig. 6 is shown the reaction rate represented by the optical density, as a function of pressure. Curves are drawn for four NAD concentrations, the EtOH concentration being held constant at 100 mM. In fig. 7 is also shown the rate versus pressure, with curves drawn for four EtOH concentrations, the NAD concentration being held constant at 0.767 mM.

Attempts have been made to evaluate the kinetic constant for the chosen model [eq. (18)] but with the present data this has not yet been successful. Within this model and with these data the equations are not sensitive enough towards variation of the constants for their accurate determination.

5. Discussion

The result from figs. 1 and 2 might be interpreted as if the pressure induced inhibition increases with increasing EtOH concentration and is unaffected by increasing NAD concentration. In this case of a two substrate mechanism, however, at least ten different cases of inhibition result when one or two steps are inhibited. Then there is no simple correlation between observed behaviour and the question of competition for enzyme sites. Neither is the extrapolation of the inhibitor analogy into the molecular domain by any means justified.

One thing that can be done is to perform an analysis of inhibition mechanisms leading to different enzyme behaviour, and look for effects similar to those shown in figs. 1 and 2. This has been done, and it may be inferred that it is the concentration of the complexes ES_2 and ES_1S_2 which are diminished by pressure exposition. Consequently, the formation of these complexes are accompanied by volume increases. The data from the measurements in phosphate buffer did not permit a more detailed discussion.

From the YADH results in tris-HCl buffer it has been possible to establish volume changes for each single step in the mechanism considered. These are not very accurate, but the large difference in dissociation volume of binary (ΔV_1, ΔV_2) and of ternary (ΔV_3, ΔV_4) complexes seems to be real. Now EtOH has a molecular weight of 46, while NAD has a molecular weight of 663, so they are very different in size. Despite this difference, the dissociation volumes both of binary and of ternary complexes are nearly symmetrical with respect to both substrates.

One explanation of this may be that the enzyme itself is present in different conformations in binary and in ternary complexes.

This difference may be due to structural changes followed by rearrangements in the water structure around the protein molecule. Usually, such changes in the solvation shell make the largest contributions to the observed volume differences. Another explanation could be that the active site is situated in a narrow cleft in the enzyme. Then whichever substrate molecule entered first would squeeze out watermolecules increasing the volume. Entrance of the second substrate could then perhaps only introduce minor changes.

Now, Gierer [11] has also studied energies and entropies of association of substrates to the enzyme. Most probably, Gierer had no knowledge of the actual mechanism, which was published the same year as his own article. Anyhow, he did not consider a particular model of the mechanism, relating the entropies to single steps. Instead he introduces the empirical equation,

$$v^{-1} = V^{-1}(1 + K_{1m}/[S_1])(1 + K_{2m}/[S_2]), \qquad (25)$$

where K_{1m} and K_{2m} are supposed to be Michaelis constants, not recognizing that these are dependent on substrate concentrations.

A recourse is made by the sentence "... the correspondence between dissociation and Michaelis constants may not be quantitative so that other steps in the reaction contribute to a smaller degree to V ...".

On the basis of the temperature variation of these Michaelis constants he finds association entropies of the substrates NAD and EtOH. As these entropies are very low, and since conformational changes should be accompanied by large entropy changes, he concludes firmly that the enzyme remains rather rigid in course of the reaction. He considers it very unlikely that

within one step large entropy changes cancel each other. For mathematical equivalence between eq. (25) and eq. (16), using primed constants in the latter, it is necessary that

$$K_{1m} = \frac{K_4'(1 + K_2'/[S_2])}{1 + K_3'/[S_2]} \tag{26}$$

and

$$K_{2m} = \frac{K_3'(1 + K_1'/[S_1])}{1 + K_4'/[S_1]}. \tag{27}$$

From this it is clear that Gierer's Michaelis constants includes contributions to their Gibbs free energy from those of three single steps in the mechanism. The possibility that large entropy changes cancel each other is then not so small. This is confirmed by the fact that for [EtOH] > 50 mM, K_{1m} is nearly invariant with pressure increase even though the constants K_2', K_3' and K_4' are not.

The conclusions of Gierer are therefore not relevant to a discussion in terms of the model chosen in this work.

Concerning the validity of the present results it should be mentioned that the error sources are several. Primarily, the velocities measured are not initial velocities, because there was a certain time lapse prior to the measurements. The slopes of the hyperbolic absorption curves show a complicated relation between measurements at $t = 0$ and at $t = 2$ min., for different substrate concentrations. It was estimated that at the relatively low enzyme activities, the amount of substrate destroyed during the two-minute deadtime was a small fraction of the total. pH and temperature effects have earlier been mentioned. In addition comes also the importance of compressibilities of the different species participating in the reaction. These have not been considered, because the number of parameters then would be doubled, increasing the uncertainties of the observed values. Nevertheless, compressibilities are of some importance, as can be seen from the curvature of plots of the logarithm of the overall velocity versus pressure. The activation volume and activation compressibility of the overall reaction calculated from

$$-RT \ln (k_p/k_0) = \Delta V^{\ddagger} p - \tfrac{1}{2}\Delta\kappa^{\ddagger} p^2, \tag{28}$$

was found to be $\Delta V^{\ddagger} \approx 1 \pm 3$ cm^3, $\Delta\kappa^{\ddagger} \approx -0.03$ cm^3 bar^{-1}.

Comparison of the kinetic data with those from the density measurements allows the conclusion to be drawn that the effect of pressure on the reaction rate is solely dependent on the dissociation and activation volumes of the enzyme–substrate complexes, and not on the difference in volume of reactants and products if this is not too large.

As for the difference in behaviour of YADH in the two buffers, it may be mentioned that this is a common phenomenon. This may in part be due to different ionic strengths, which was the case here ($\mu_{PO_4} = 0.27$, $\mu_{tr} = 0.1$). The ratio of the rate constants resulting from these two ionic strengths, assuming unit charges involved, is about 1.6. The importance of μ may also be enchanced at higher pressures, because of electrostriction effects on partly developed charges on the different complexes in transition states. A further complicating factor may have been the change of pH in the phosphate buffer with pressure increase.

The results from the studies of LADH are largely qualitative, and are at present under refinement. From the special role played by the ethanol concentration it is believed that these studies will reveal important features concerning the inhibition mechanism. The strange phenomenon of a pressure dependent activation volume indicate that two counteracting effects are present, each dominating different parts of the pressure range.

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References

[1] A.M. Zimmermann, ed., High pressure effects on cellular processes (Academic Press, New York, 1970).
[2] B. Andersen and E. Broe, Acta Chem. Scand. 26 (1972) 3691.
[3] R.K. Williams and C. Shen, Arch. Biochem. Biophys. 152 (1972) 606.

[4] G. Schmid, H.D. Lüdemann and R. Jaenicke, Biophys. Chem. 3 (1973) 90.
[5] G.D. Lockyer et al., J. Am. Chem. Soc. 96 (1974) 7303.
[6] E. Morild, 4th ICCT (IUPAC), Vol. 4 (1975) p. 81.
[7] F. Grønlund and B. Andersen, Acta Chem. Scand. 23 (1969) 2452.
[8] O.D. Masson, Phil. Mag. 8 (1929) 21?.
[9] R.C. Neuman, W. Kauzmann and A. Zipp, J. Phys. Chem. 77 (1973) 2687.
[10] S. Bayne, Thesis, St. Andrews (1974).
[11] A. Gierer, Biochem. Biophys. Acta 17 (1955) 111.
[12] M. Polanyi and M.G. Evans, Trans. Faraday Soc. 31 (1935) 875.
[13] H. Sund and H. Theorell, in: The enzymes, Vol. 7, 2nd Ed., eds. P.D. Boyer, H. Lardy and K. Myrback (Academic Press, New York, 1963) p. 25.
[14] J.D. Shore and H. Theorell, Arch. Biochem. Biophys. 117 (1966) 375.
[15] K. Dalziel, Acta Chem. Scand. 11 (1959) 1706.
[16] C.C. Wratten and W.W. Cleland, Biochemistry 2 (1963) 935.
[17] O. Holte, KIRA, Norway (1966).