The Effect of Pressure on the NAD⁺/NADH System in the ADH Reaction

EDDIE MORILD and INGE TVEDT

Department of Chemistry, University of Bergen, N-5014 Bergen, Norway

The equilibrium of the alcohol dehydrogenase system has been studied in the pressure range 1 – 1500 bar and in the temperature range 15 – 35 °C.* The volume change of reaction has been found in tris-HCl and phosphate buffer to be − 5 and − 13 cm³ mol⁻¹, respectively. The reactants and products have been studied by densitometry and their molal volumes are found. From comparison of the results from both methods it is concluded that the volume change in the NAD⁺/NADH reduction is small and that the effect of pressure will depend on the volume change of substrate oxydation. Other thermodynamic quantities, like ΔH, $\partial \Delta H / \partial p$, $\partial \Delta V / \partial p$ and $\partial \Delta V / \partial T$ are also evaluated.

Very little is known about the response of the most vital processes of metabolism in living systems to high pressures, e.g. under deep sea diving conditions. Among the vital processes are the electron transfer steps in the respiration chain. In a number of these steps the NAD⁺/- NADH redox couple participates as coenzymes of various enzymes. One enzyme system using NAD⁺ as oxidant is the alcohol dehydrogenase (ADH) system, using alcohols as substrates. The products of the ADH reaction are NADH and an aldehyde, e.g., ethanal (acetaldehyde) from ethanol. The effect of pressure upon this reaction depends on the volume changes in going from NAD⁺ and ethanol to NADH and ethanal. As we shall see, the measurement of the molal volumes of NAD⁺ and NADH alone is not easy to do, although the molal volumes of ethanol and ethanal can easily be measured with great precision. This situation is rather characteristic; the volumes of the small substrates are likely to be found in the literature

or measured with ease, but the measurement of the volumes of coenzymes, vitamins and other macromolecules are more difficult. But once the volumes of the greater molecules are found, their occurrence in many similar reactions makes it possible to predict the pressure effect upon a reaction by looking only at the substrate-product volume change. To this end we have tried to measure the volume change of the reduction process NAD⁺→NADH in two independent ways.

The first method is by density measurements, after an ion exchange process. The second method is by study of the shift of the over-all equilibrium of the alcohol dehydrogenase reaction with pressure. The two methods are shown to agree well within the experimental uncertainty.

EXPERIMENTAL

Chemicals. Yeast alcohol dehydrogenase [alcohol-NAD⁺ oxido-reductase, E C 1.1.1.1] (90 % protein, activity: 300 units/mg), β-NAD [β-nicotinamide adenine dinucleotide, grade III], β-NADH disodium salt (grade III), cysteine-HCl, lyophilized bovine serum albumin and tris (Trizma) buffer were obtained from Sigma Chem. Co. Absolute ethanol, of high purity, was bought from A/S Vinmonopolet, Norway. For stabilizing purposes, 20 μM cys-HCl, 20 μM EDTA (Merck) and 1 g bovine serum albumin were added per litre of buffer. Two buffers were used, (1) Tris-HCl, pH 7.0, I = 0.05 (I is the ionic strength), and (2) Phosphate (Merck), pH 7.0, I = 0.1 and 0.4. All chemicals were used without further purification. For the ion exchange were used membranes of type Dowex-50W.

Spectrophotometric apparatus. All investigations were performed by spectrophotometry of
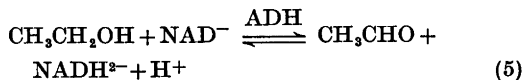
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* 1 bar= 10⁵ Pa= 10⁵ N m⁻².

the 340 nm peak of NADH. A Zeiss PMQ II spectrophotometer with an M4QIII mono-chromator was used. A logarithmic converter was inserted between the spectrophotometer and the Servograph recorder, so that optical density (OD) could be read directly.

High pressure apparatus. The reactions were run to equilibrium in an optical high pressure cell, constructed at our laboratory. It was equipped with saphire windows (3M), allowing the light beam to pass through the reaction chamber in the middle of the cell under pressures of up to 3000 bar. The reaction chamber was filled through a vertical passage from the top, and the pressure transmitting oil pressed a piston through a horisontal passage from the back of the cell. The pressure was generated by an Enerpac handpump, and a large pressure vessel served as a pressure reservoir. The pressure was measured by a Budenberg gauge and the accuracy was ± 10 bar. Thermostated oil was streaming through longitudinal holes in the cell, keeping the temperature within 0.1 K.

Density and compressibility measurements. Densities were measured with a Paar DMA 02C digital densimeter. The accuracy is ordinarily of the order of 10^{-6} g cm^{-3}. All densities are measured relative to the density of water, 0.997075 g cm^{-3} at 25.00 °C. The molal volume can be determined from eqn.(1), where d_0 is

$$V = 1000 \ (d_0 - d_s)/md_0d_s + M_s/d_s \tag{1}$$

the density of water, d_s the density of the solution, m the molality of the solution and M_s the molecular weight of the solute. Measurements of d_s at different molalities allow extrapolation to infinite dilution, where V^∞ is determined.

Compressibilities were determined from sound velocity measurements according to the sing-around principle. A Systron Donner Datapulse generator (115) generates sound impulses through a transducer in the measuring cell. The frequency is measured by a Hewlett Packard 5326 A Time Counter. The adiabatic (isentropic) compressibility coefficient is calculated from eqn. (2), where v is the velocity of sound

$$\beta_s = \frac{10^6}{v^2d} \tag{2}$$

in a solution of density d. The adiabatic molal compressibility is then given by eqn. (3), where

$$\varkappa_s = \frac{1000}{m} \ (\beta_s - \beta_{0s}) + \beta_{0s}V \tag{3}$$

subscript s stands for constant entropy and subscript 0 for pure water. The temperature in both the density and the compressibility meas-urements was controlled to within 0.004 K with a Hewlwtt-Packard quartz thermometer.

THEORETICAL

Terminology. For this discussion the usual abbreviations NAD+ and NADH is insufficient, and may even be misleading. NAD+ is strictly not a positive ion, but at most a neutral zwitterion, or a negative ion, as is seen in Figure 1. There is a positive charge on the quaternary nicotinamide nitrogen and negative charge(s) on the dissociable phosphate group(s). It will be preferable to use the symbol NAD$^-$ for this molecule in this paper.

The NADH molecule, as an ion of a free acid, should be denoted NADH^{2-}, and the disodium salt should be denoted, Na$_2$NADH. The over-all reduction of NAD$^-$ would then look like

$$NAD^- + H_2 \rightleftharpoons NADH^{2-} + H^+$$

As will be seen later, substitution of H+ and Na+ ions in these molecules necessitates the use of a more adequate notation, like NaNAD and NaHNADH. The meaning of these terms should be evident.

The reaction. The over-all alcohol dehydrog-enase reaction is shown in eqn. 4.

$$CH_3CH_2OH + NAD^- \rightarrow CH_3CHO + NADH^{-2} + H^+ \tag{4}$$

The mechanism is complicated and depends on the catalyzing enzyme. The kinetics of both the yeast-(Y) and the liver-(L) ADH system have earlier been studied under pressure by Morild.[1,2] The rate was shown to depend on the volume changes following formation of intermediate complexes. The equilibrium, however, is only dependent on the volume difference between the initial and final state, as far as pressure is concerned. This volume change may be sepa-

Fig. 1. The NAD$^-$ molecule.

rated into two contributions, that of substrates and that of coenzymes:

(1) $\Delta V_1(s) = V(CH_3CHO) - V(CH_3CH_2OH)$

(2) $\Delta V_2(c) = V(NADH^{2-}) + V(H^+) - V(NAD^-)$

The effect of pressure. We now write the equilibrium

$$CH_3CH_2OH + NAD^- \underset{}{\overset{ADH}{\rightleftharpoons}} CH_3CHO +$$
$$NADH^{2-} + H^+ \tag{5}$$

with an equilibrium constant

$$K = \frac{[CH_3CHO][NADH^{2-}][H^+]}{[CH_3CH_2OH][NAD^-]} \tag{6}$$

This is a thermodynamic equilibrium constant, and therefore the square brackets in eqn. (6) should be taken to mean activities. But the spectrophotometry measures concentrations, and we have to include activity coefficients. We assume the activity coefficients to be similar for CH_3CHO and CH_3CH_2OH, and likewise for $NADH^{2-}$ and NAD^-. A resulting activity coefficient γ is then defined by eqn. (7), where

$$K = \gamma K_c \tag{7}$$

K is the thermodynamic equilibrium constant based on activities, and K_c is the corresponding expression based on eqn. (6) with [ ] meaning concentration. The molar volume change in the reaction (5) is given by eqn. (8), while the

$$\left(\frac{\partial \ln K}{\partial p}\right)_T = -\frac{\Delta \overline{V}}{RT} \tag{8}$$

volume change resulting from the observed concentration changes is given by eqn. (9).

$$\left(\frac{\partial \ln K_c}{\partial p}\right)_T = -\frac{\Delta V_c}{RT} \tag{9}$$

Then we may write eqn. (10), where

$$\Delta \overline{V} = \Delta V_c + \Delta V_\gamma \tag{10}$$

$$\left(\frac{\partial \ln \gamma}{\partial p}\right)_T = -\frac{\Delta V_\gamma}{RT} \tag{11}$$

according to eqn. (7).
The last quantity can be calculated from the theoretical expression of the Debye-Hückel theory, eqn. (12), where I is the ionic strength

$$\ln \gamma = \frac{z^2 e^2}{8\pi \varepsilon k T} \left(\frac{e^2 2000}{\varepsilon k T}\right)^{\frac{1}{2}} \sqrt{I} \tag{12}$$

and ε the dielectric constant. The derivative is

$$\left(\frac{\partial \ln \gamma}{\partial p}\right)_T = -\frac{3}{2} \ln \gamma \, \varepsilon^{-1} \left(\frac{\partial \varepsilon}{\partial p}\right)_T \tag{13}$$

and insertion of experimental values [3] yields the result

$$\Delta V_\gamma = 1.2 \text{ cm}^3 \text{ mol}^{-1}$$

which enters as a correction factor to the observed ΔV_c-values. From eqn. (5) we see that

$$\Delta \overline{V} = \Delta V_1 + \Delta V_2$$

RESULTS FROM THE EQUILIBRIUM MEASUREMENTS

The equilibrium constant of this system has earlier been investigated by Bäcklin [4] at one atmosphere. He found K to be 0.80×10^{-11} M at 20 °C and $I = 0.1$ in the pH range 7 − 10. Variation of log K with $\sqrt{I}$ showed a slope of 0.315 and an intercept corresponding to $K = 0.646 \times 10^{-11}$ at $I = 0$. From the change of equilibrium with temperature he calculated the thermodynamic parameters (at 20 °C) $\Delta G = 62.24$ kJ mol^{-1}, $\Delta H° = 29.80$ kJ mol^{-1} and $\Delta S° = -110.8$ J mol^{-1} K^{-1}.

In the present work, initial concentrations were [NAD$^-$]: 0.5 − 1.0 mM and [CH$_3$CH$_2$OH]: 20 − 40 mM. Control measurements showed that the pressure in the actual range (1 − 1500 bars) did not influence the absorption characteristics of NADH^{2-}. Earlier experiments [1] have shown that the enzyme is functionally active at pressures up to 2000 bars. The density increase of the reaction mixture with pressure was corrected for by division by the factors 1.022, 1.041 and 1.059 at the pressures 500, 1000 and 1500 bars, respectively.[5] Since the association constants of the oxidized and reduced form of the coenzyme with the enzyme are different, one can expect the amount of enzyme to slightly influence the equilibrium. In our studies, the enzyme concentrations have varied, and have been larger than in ordinary kinetic experiments. Some preliminary experiments suggested that NADH^{2-} undergoes decay when exposed to pressure for longer times, and it was
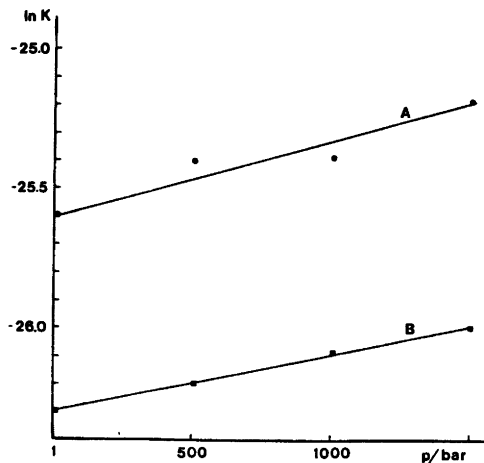
Fig. 2. ln k versus p of the equilibrium in tris-HCl buffer ($I = 0.05$) at $t = 25\,°C$ and pH 7.0. A: $[NAD^-]_0 = 1.0$ mM, $[CH_3CH_2OH]_0 = 40$ mM. B: $[NAD^-]_0 = 0.5$ mM, $[CH_3CH_3OH]_0 = 20$ mM.

therefore important to reach equilibrium quickly. For this reason the absolute values of K may vary from one experiment to another. This is unimportant, however, because we are mainly interested in the relative variation with pressure.

The experiments in tris buffer were performed by first equilibrating the reaction mixture at atmospheric pressure and measure K^1. Then

Fig. 3. ln K versus p of the equilibrium in tris-HCl buffer ($I = 0.05$) at $t = 30\,°C$ and pH 7.0. A: $[NAD^-]_0 = 1.0$ mM, $[CH_3CH_2OH]_0 = 40$ mM. B: $[NAD^-]_0 = 0.5$ mM, $(CH_3CH_2OH]_0 = 20$ mM.

the pressure was increased to a specified value p, and K^p was measured at the new equilibrium. Thereafter the reaction mixture was discarded. To eliminate individual differences within a series of experiments of increasing pressure, all K^1 s was averaged to $\overline{K}$; and each final value of $K(p)$ was calculated from

$$K(p) = \overline{K} + (K^p - K^1) \qquad (14)$$

In this way we obtained the curves shown in Figs. 2 and 3. The curvature observed at 30 °C probably results from an increased compressibility difference between products and reactants, due to different expansibilities. The volume changes calculated from the observed K-values in the tris buffer are given in Table 1.

The experiments in phosphate buffer were arranged a little differently; for two reasons: The reaction mixture seemed to be more stable in this buffer at high pressure. The pH of the phosphate buffer changes appreciably with pressure, contrary to the tris buffer.[6] The reaction mixture was now pressurized to 500 bar, equilibrated and pressurized further to 1000 bar and so on. For every pressure, we had to correct the H^+-activity in eqn. (6) according to the initial pH at 1 bar and to the variation of pH with pressure,

$$\left(\frac{\partial(pH)}{\partial p}\right)_T \approx \frac{1}{2.303}\frac{\Delta V_a}{RT} \qquad (15)$$

where ΔV_a is the dissociation volume of the buffer.[6] With this buffer measurements were also made at two different ionic strengths, $I = 0.1$ and $I = 0.4$.

Table 1. The volume changes in tris-HCl buffer ($I = 0.05$).

| Concentrations (mM)	ΔV (cm³ mol⁻¹) $t = 25.0\,°C$	ΔV (cm³ mol⁻¹) $t = 30.0\,°C$
$[NAD^-]_0 = 0.5$ $[CH_3CH_2OH]_0 = 20.0$	-5 ± 2	-7 ± 2
$[NAD^-]_0 = 1.0$ $[CH^3CH_2OH]_0 = 40.0$	-4 ± 2	-6 ± 2
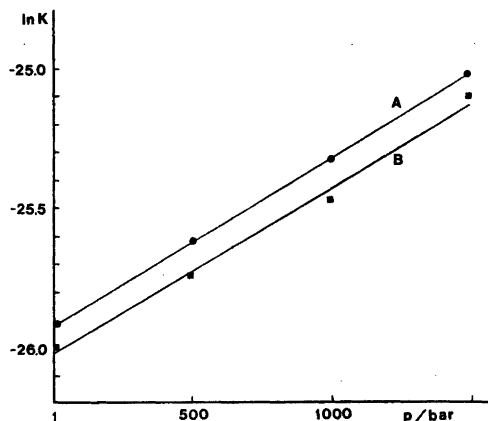
Fig 4. ln K versus p of the equilibrium in phosphate buffer. $(I = 0.1)$ at $t = 15\,°C$ and pH (1 bar) = 7.08. A: $[NAD^-]_0 = 1.0$ mM, $[CH_3CH_2OH]_0 = 40$ mM. B: $[NAD^-]_0 = 0.5$ mM, $[CH_3CH_2OH]_0 = 20$ mM.

The results are shown in Figs. 4, 5 and 6. The volume changes calculated from data in Figs. 4 and 5 are given in Table 2. There seems to be no significant difference between the volume changes calculated for the two choices of substrate concentrations. The measurements in increased ionic strength were therefore made with only one combination of substrate concentrations. It is seen from Fig. 6 that the slopes of the curves decrease with increasing temperature. This leads to a temperature dependent
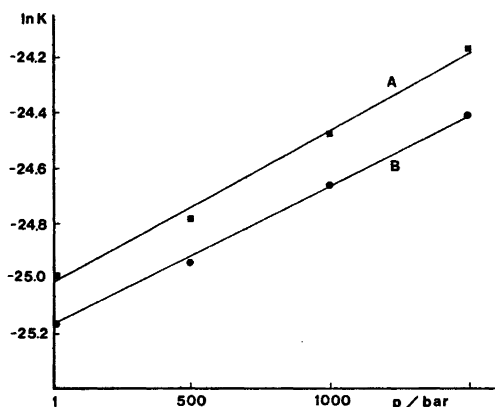
Fig. 5. ln K versus p of the equilibrium in phosphate buffer. $(I = 0.1)$ at $t = 35\,°C$ and pH (1 bar) = 7.08. A: $[NAD^-]_0 = 1.0$ mM, $(CH_3CH_2OH]_0 = 40$ mM. B: $[NAD^-]_0 = 0.5$ mM, $[CH_3CH_2OH]_0 = 20$ mM.
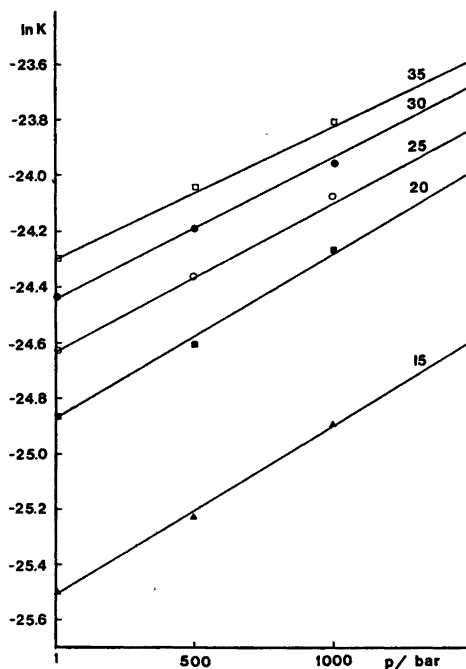
Fig. 6. ln K versus p of the equilibrium in phosphate buffer $(I = 0.4)$ at different temperatures (in °C) at pH (1 bar) = 7.08. $[NAD^-]_0 = 0.5$ mM, $[CH_3CH_2OH]_0 = 20$ mM.

volume change of the reaction, shown in Fig. 7. From the slope of this curve one obtains the expansibility

$$\left(\frac{\partial \Delta \overline{V}}{\partial T}\right)_p = (0.13 \pm 0.02) \text{ cm}^3 \text{ mol}^{-1} \text{ K}^{-1} \qquad (16)$$

From the relation

$$\ln K = \frac{\Delta S}{R} - \frac{\Delta H}{R}\frac{1}{T} \qquad (17)$$

Table 2. The volume changes in phosphate buffer $(I = 0.1)$.

Concentrations (mM)	ΔV (cm³ mol⁻¹) $t = 15.0\,°C$	ΔV (cm³ mol⁻¹) $t = 35.0\,°C$
$[NAD^-]_0 = 0.5$ $[CH_3CH_2OH]_0 = 20.0$	-13 ± 1	-12 ± 1
$[NAD^-]_0 = 1.0$ $[CH_3CH_2OH]_0 = 40.0$	-13 ± 1	-12 ± 1

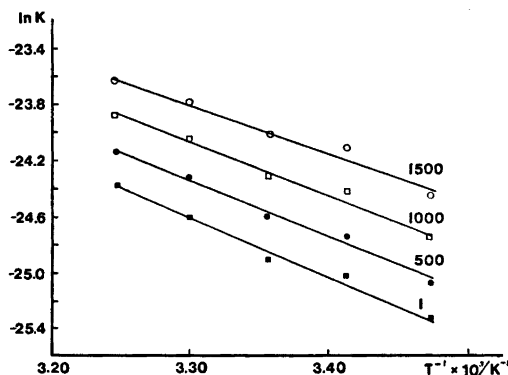*Fig. 7.* ΔV of the equilibrium in phosphate buffer as a function of temperature ($I = 0.4$).

Fig. 8. ln K of the equilibrium in phosphate buffer as a function of inverse temperature ($I = 0.4$). The curves are based upon least squares.
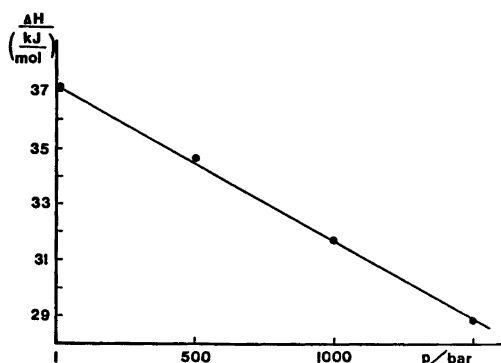

Fig. 9. ΔH of the equilibrium as a function of pressure. The goodness of fit is fortuitous.

one can make a van't Hoff plot of ln K *versus* T^{-1}. This is done in Fig. 8 at four pressures. The slopes of the curves show a slight decrease with pressure, indicating a pressure dependent

Table 3. The numerical values of the equilibrium constant at various pressures and temperatures in phosphate buffer ($I = 0.4$).

| T (K) | $K \times 10^{11}$ (M) | | | |
	1 bar	500 bar	1000 bar	1500 bar
288.0	0.908	1.234	1.681	2.296
293.0	1.340	1.803	2.460	3.282
298.0	1.509	2.016	2.696	3.610
303.0	2.048	2.687	3.513	4.596
308.0	2.557	3.279	4.198	5.376

ΔH. This pressure dependency is shown in Fig. 9, and the slope is

$$\left(\frac{\partial \Delta H}{\partial p}\right)_T = -(5.2 \pm 0.1) \times 10^{-3} \text{ kJ mol}^{-1} \text{ bar}^{-1} \tag{18}$$

RESULTS FROM THE DENSITY AND COMPRESSIBILITY MEASUREMENTS

The volume difference between HNAD and H₂NADH could have been found easily if both molecules were stable in water. However, the acid H₂NADH is rather unstable and is commercially available only as disodium salt, Na₂NADH. It was therefore necessary to convert the volume of the disodium salt to that of the free acid by means of other known volume quantities. The partial molal volumes have been measured for HNAD, Na₂NADH and CH₃CHO in pure water. The experimental curves are shown in Figs. 10 and 11. For
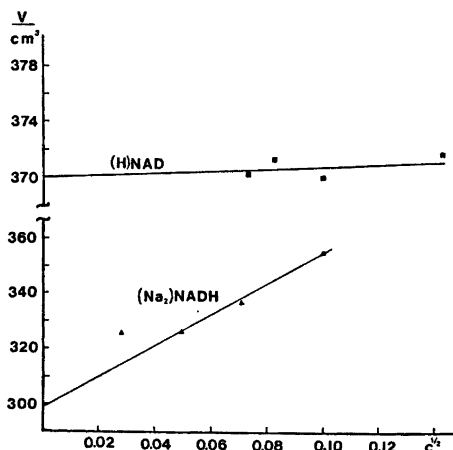

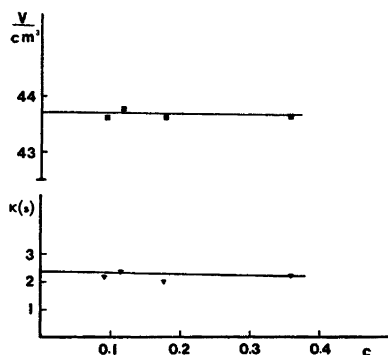*Fig. 10.* The molal volumes of HNAD and Na₂NADH as functions of concentration.

Fig. 11. The molal volume and compressibility of acetaldehyde as functions of concentration. $\varkappa$ in 10^4 cm^3 bar^{-1} mol^{-1}.

ethanol, values have been taken from the literature.[7,8] The results are shown in Table 4. At infinite dilution, we define the dissociation volumes of the protons from the two phosphate groups in H_2NADH by

$$\Delta V_a = V(H^+) + V(HNADH^-) - V(H_2NADH) \quad (19)$$

$$\Delta V_b = V(H^+) + V(NADH^{2-}) - V(HNADH^-) \quad (20)$$

Unfortunately, these volumes are not known experimentally, because of the difficulty of measuring $V(H_2NADH)$. It is, however, known [5] that the corresponding dissociations from ordinary phosphoric acid are followed by the volume changes $\Delta V_a = -16.5$ and $\Delta V_b = -24.0$ cm^3 mol^{-1}. By using the fact that the sodium salt is completely dissociated, we write

$$V(Na_2NADH) = 2V(Na^+) + V(NADH^{2-}) \quad (21)$$

Insertion of eqns. (20) and (21) in (19) gives

$$V(H_2NADH) = V(Na_2NADH) + 2[V(H^+) - V(Na^+)] - (\Delta V_a + \Delta V_b) \quad (22)$$

The volume of conversion from Na$^+$ to H$^+$, $[V(H^+) - V(Na^+)]$, is of the order 1 cm^3 mol^{-1} and can be neglected compared to the uncertainty of the term $(\Delta V_a + \Delta V_b)$.

If we assume that $(\Delta V_a + \Delta V_b)$ of NADH^{2-} is of the same order as that of phosphoric acid, we obtain

$$V(H_2NADH) \approx 340 \text{ cm}^3 \text{ mol}^{-1}$$

At infinite dilution in pure water then, the volume difference between HNAD and H_2NADH amounts to

$$\Delta V' = V(H_2NADH) - V(HNAD) \approx -30 \text{ cm}^3 \text{ mol}^{-1}$$

This result conflicts with the result from the equilibrium measurements and the ethanol-ethanal volume difference. An explanation of this is that the two volumes are not strictly comparable. A solution of HNAD in water yields a pH of 3.5 at a concentration of 8×10^{-4} M, while a corresponding solution of Na$_2$NADH is slightly alkaline. Then there is reason to believe that in their respective solutions these molecules are present in different dissociation states. The term $(\Delta V_a + \Delta V_b)$ will therefore include contributions from this difference. In order to avoid this, one must ensure that the two species are studied under equal conditions. This is not so easy, because the H$^+$-form of NADH^{2-} is unstable and so is the Na$^+$-form of NAD^{2-}. A possible way to circumvent this, is to do the density measurements on both molecules in a semineutralized state. We thereby obtain an intermediate state which we believe is stable to both molecules, and the contributions from these ions will then cancel out by subtraction. When the diluent used in the volume determinations is a buffer, keeping constant pH, comparable dissociation states should be quaranteed.

Ion exchange of the Na$_2$NADH molecules was performed with a DOWEX-50W membrane ion exchanger. The ion exchange capacity of the exchanger was determined both from titration with NaOH and from back titration of eluted Na$^+$ as chloride, with AgNO$_3$. The

Table 4. Molal volumes of reactants and products in pure water.

Molecule	HNAD	Na$_2$NADH	CH$_3$CH$_2$OH	CH$_3$CHO
V(cm^3 mol^{-1})	370 ± 2	299 ± 2	55.1 ± 0.2	43.7 ± 0.3
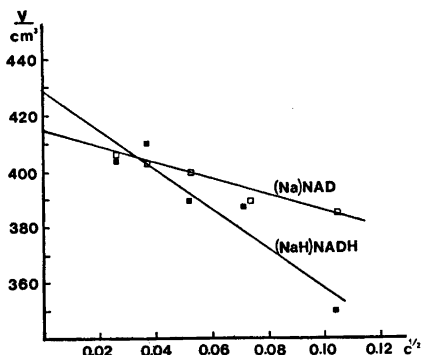
Fig. 12. The molal volumes of NaNAD and NaHNADH as functions of concentration.

Fig. 13. The molal compressibilities of HNAD and Na$_2$NADH as functions of concentration. $\varkappa$ in 10^4 cm^3 bar^{-1} mol^{-1}.

resulting capacity was found to be 2.5 ± 0.1 meq. g^{-1}. In 2 h 99 % of the ion exchanger was converted from H$^+$ to Na$^+$ form. Equivalent amounts of H$^+$-form ion exchanger and Na$_2$NADH were transferred to 30 ml distilled water and allowed to stand with stirring for more than 2 h. Then buffer solution was added, making a solution of NaHNADH with pH 7.02 and $I = 0.05$. The molal volumes were determined as usual with buffer as diluent.

An HNAD solution was titrated with NaOH until neutral reaction, so that equivalent amounts of HNAD and NaOH were present. Then buffer was added, making a solution of NaNAD with pH 7.02 and $I = 0.05$. Also here the molal volumes were determined with buffer as diluent. The experimental curves are shown in Fig. 12. These yield the limiting volumes

V(NaNAD) $= 414 \pm 10$ cm^3 mol^{-1} and V(NaHNADH) $= 428 \pm 20$ cm^3 mol^{-1}.

Taking the uncertainties into consideration, it is possible that this volume difference is small.

The compressibilities of reactants and products are given in Table 5 and Fig. 13. These are limiting values in pure water and for the same reason as for the volumes, not strictly compar-
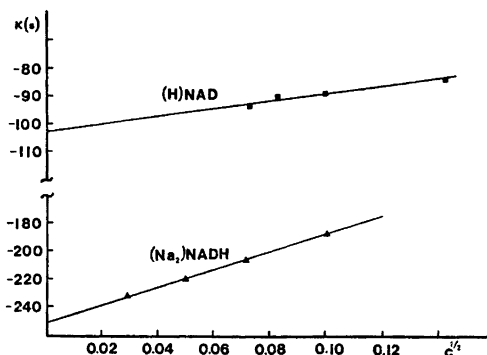
able. The resulting compressibility change of reaction is

$$\Delta\varkappa = \varkappa(H_2NADH) + \varkappa(CH_3CHO) - \varkappa(HNAD) - \varkappa(CH_3CH_2OH) \qquad (23)$$

where

$$\varkappa(H_2NADH) = \varkappa Na_2NADH + 2[\varkappa(H^+) - \varkappa(Na^+)] - (\Delta\varkappa_a + \Delta\varkappa_b) \qquad (24)$$

(The derivative of eqn. (22) with respect to pressure).

If we use the literature values [9,10]

$[\varkappa(H^+) - \varkappa(Na^+)] = 44 \times 10^{-4}$ cm^3 bar^{-1} mol^{-1} and $\Delta\varkappa_a = \Delta\varkappa_b = -25 \times 10^{-4}$ cm^3 bar^{-1} mol^{-1}, we obtain

$\Delta\varkappa = -116 + 2 + 103 - 1 = -12 \times 10^{-4}$ cm^3 bar^{-1} mol^{-1}

The uncertainty is probably of the order 5×10^{-4} bar^{-1} mol^{-1}. From the curves in Fig. 3, a polynomial regression gives a compressibility $\Delta\varkappa = -5 \times 10^{-4}$ cm^3 bar^{-1} mol^{-1} with the equation

$$\ln K_p = \ln K_1 - \frac{\Delta V}{RT} p + \frac{1}{2} \frac{\Delta\varkappa}{RT} p^2 \qquad (25)$$

This is in agreement with the single compressibilities.

Table 5. Molal compressibilities of reactants and products in pure water.

Molecule	HNAD	Na$_2$NADH	CH$_3$CH$_2$OH	CH$_3$CHO
$\varkappa \times 10^4$ (cm^3 mol^{-1} bar^{-1})	-103 ± 2	-254 ± 2	1.1 ± 0.1	2.3 ± 0.2

DISCUSSION

From the results of the two methods used, the volumes can now be compared. The density method has given the following volume difference:

$$\Delta V = V(\text{NaHNADH}) + V(\text{CH}_3\text{CHO}) - (V(\text{NaNAD}) + V(\text{CH}_3\text{CH}_2\text{OH})) = 428 + 44 - (414 + 55) = 3 \text{ cm}^3 \text{ mol}^{-1}$$

with an uncertainty about 20 cm³ mol⁻¹. The equilibrium method has given two values of this volume change, dependent on the choice of buffer. Tris buffer gives $\Delta V(t) = (-5 \pm 2)$ cm³ mol⁻¹ and phosphate buffer gives $\Delta V(p) = (-13 \pm 1)$ cm³ mol⁻¹. The volume difference between CH_3CHO and $\text{CH}_3\text{CH}_2\text{OH}$ is accurately determined to -11.4 cm³ mol⁻¹. The resulting volume changes deduced for the transition from HNAD to H₂NADH should then be

$$\Delta V_2 = V(\text{H}_2\text{NADH}) - V(\text{HNAD}) = \Delta V - (-11.4)$$

$$\Delta V_2(t) = -5 + 11.4 \approx 6.5 \text{ cm}^3 \text{ mol}^{-1}$$

$$\Delta V_2(p) = -13 + 11.4 \approx -1.5 \text{ cm}^3 \text{ mol}^{-1}$$

with uncertainties about 2 cm³ mol⁻¹. These volumes are relatively small, considering the molal volumes of order 400 cm³ mol⁻¹. The difference between the values in different buffers may be due to some kind of complex formation between buffer and reacting molecules. One can conclude that a shift of the overall equilibrium with pressure of an NAD⁻ coupled redox process will depend mainly on the volume change of the associated substrate/-product.

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