The Binding of Dihyronicotinamide-Adenine Dinucleotide and Pyridine-3-Aldehyde-Adenine Dinucleotide by Yeast Alcohol Dehydrogenase

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1. Yeast alcohol dehydrogenase has been found to react with NADH in the presence of acetamide to form a highly fluorescent ternary complex. Titration of the enzyme to form this complex has provided a method for the estimation of the number of binding sites on the enzyme. 2. The binding of NADH by the enzyme has been studied independently, with a modified form of equilibrium dialysis, by using gel filtration. 3. A third method, depending upon the formation of a ternary complex of enzyme, hydroxylamine and pyridine-3-aldehyde-adenine dinucleotide, has also been used to titrate the enzyme. 4. Values obtained with all three methods are substantially in agreement that only three coenzyme-binding sites are available. This is in contrast with the established fact that the enzyme is composed of four identical subunits.

Many of the physical properties of yeast alcohol dehydrogenase (alcohol-NAD⁺ oxidoreductase, EC 1.1.1.1) were originally studied in detail by Hayes & Velick (1954). One important result obtained by these workers was that the enzyme appeared to possess four active sites, each of identical reactivity. Later work showed that the enzyme molecule contains four essential zinc atoms (Vallee & Hoch, 1955), four extra-reactive cysteine residues (Rabin, Ruiz Cruz, Watts & Whitehead, 1964) and four apparently identical subunits (Harris, 1964). However, Hayes & Velick (1954) did not in fact observe four coenzyme-binding sites on the enzyme. They found an extrapolated maximum of 3.6 binding sites, which they considered an approximation to a true value of 4.0. The technique used in these studies was the ultracentrifugal separation method first described by Velick, Hayes & Harting (1953). This technique involves centrifugation for prolonged periods of time, and is therefore subject to particular types of possible error. Thus, to maintain the stability of alcohol dehydrogenase and to reduce the blank destruction of NADH, the experiments were performed at 0–5°C in solutions containing 10 mM-semicarbazide. Because of the limitations involved in the experiments, and the somewhat ambiguous result of 3.6 binding sites/molecule obtained, it was thought important to re-examine the coenzyme-binding capacity of the enzyme with alternative techniques.

Initially it seemed that the formation of a highly

fluorescent ternary complex of the enzyme, with NADH and some third ligand, might provide a very effective alternative method. Theorell & McKinley-McKee (1961) have used such a method to assay horse liver alcohol dehydrogenase, with isobutyramide as the third partner. It was thought that this method could readily be applied to the yeast enzyme, by replacement of isobutyramide with an alternative amide better matched to the specificity requirements of this enzyme. Since ethanol is a much more effective substrate for the enzyme than isobutanol (Sund & Theorell, 1963), acetamide seemed a very likely candidate for the formation of a relatively strong complex with enzyme and NADH. In addition to the fluorescence method, the introduction of the gel-filtration technique by Hummel & Dreyer (1962) offered a new way to examine directly the binding of NADH to the enzyme. Again, the observation of van Eys, Ciotti & Kaplan (1957) that the enzyme combines with hydroxylamine and PAAD*, to form a ternary complex having a characteristic ultraviolet-absorption spectrum, appeared to offer a third method to attack the problem. It thus seemed possible that the methods outlined could be used to re-examine critically the coenzyme-binding behaviour of yeast alcohol dehydrogenase, and to assess the accuracy of the original studies and the validity of conclusions reached.

* Abbreviation: PAAD, pyridine-3-aldehyde-adenine dinucleotide.

MATERIALS AND METHODS

Water. All solutions were prepared with glass-distilled water.

Coenzymes. NAD⁺ was purchased from the Boehringer Corp. (London) Ltd., London W.5, U.K., and was used without further purification. Highly purified NADH was obtained by enzymic reduction of NAD⁺, by the method of Dalziel (1962a). PAAD was obtained from the Sigma (London) Chemical Co. Ltd., London S.W.6, U.K., and was used without further treatment.

Acetamide. This was purchased from British Drug Houses Ltd., Poole, Dorset, U.K., and was recrystallized from methanol-ether.

Hydroxylamine. This AnalaR solid was obtained as the hydrochloride from British Drug Houses Ltd. Solutions of the solid were neutralized to pH 7.0 before use.

Buffers. Solutions of sodium phosphate and sodium pyrophosphate buffers contained 0.3 mM-EDTA.

Method of enzyme assay. Alcohol dehydrogenase activity was measured spectrophotometrically at 25°C with a Zeiss PMQ II spectrophotometer. The initial rate of NADH formation was measured at 340 nm after the addition of enzyme to a 1 cm cuvette containing pyrophosphate buffer, pH 8.8, 30 μ mol, NAD⁺, 2.1 μ mol, ethanol, 3.1 mmol and water to a final volume of 3 ml. The unit of enzyme activity was defined as that amount of enzyme catalysing the formation of 1 μ mol of NADH/min in the above assay system.

Protein determinations. For measurements of protein concentrations during the enzyme purification the method of Warburg & Christian (1941) was used. For the measurement of the concentration of recrystallized alcohol dehydrogenase an extinction coefficient at 280 nm of $E_{1\%}^{1\text{cm}} = 12.6$ was used (Hayes & Velick, 1954).

Nucleic acid determinations. The concentration of nucleic acid in extracts was determined by the method of Warburg & Christian (1941).

Enzyme. Alcohol dehydrogenase was isolated directly from bakers' yeast by one of two procedures. The products of these procedures are referred to below as preparation I and preparation II.

Preparation I was made by a method based on that of Racker (1950). Dried yeast (400 g) was extracted with 66 mM-Na₂HPO₄ containing 0.3 mM-EDTA, and the extract was subjected to heat treatment, acetone fractionation and ammonium sulphate fractionation exactly as described by Racker (1950). After these steps the original procedure describes the crystallization of the enzyme. In the preparations made in this work, ion-exchange chromatography at 0–4°C was introduced at this stage. The enzyme preparation obtained after ammonium sulphate fractionation was dialysed exhaustively against 5 mM-phosphate buffer, pH 7.0, and then applied to a 5 cm $\times$ 50 cm column of DEAE-cellulose equilibrated with this buffer. The enzyme was adsorbed on to the column, which was then washed successively with 500 ml of 5 mM-phosphate buffer, pH 7.0, and 500 ml of 20 mM-phosphate buffer, pH 7.0. These washings served to elute some inactive protein. The enzyme was eluted from the column in greater than 85% yield by using a linear gradient made from 1.5 litres of 20 mM- and 1.5 litres of 0.15 M-phosphate buffer, pH 7.0. The main fractions contained enzyme which had been purified some fivefold, and 65% of the

total activity was in these fractions. The phosphate concentration in these fractions was about 35 mM and the concentration of protein was of the order of 1–2 mg/ml. The enzyme was precipitated by dialysis against 0.1 M-phosphate buffer, pH 7.0, containing sufficient solid ammonium sulphate (390 g/l) to bring the total volume of solution, both inside and outside the dialysis bags, to 60% saturation. Overnight, the volume inside the dialysis bags decreased to about one-third of the initial volume and the enzyme precipitated in 90% yield. The precipitate was dissolved in a minimum volume of 45 mM-phosphate buffer, pH 7.0, and the enzyme crystallized during the slow addition of saturated ammonium sulphate to bring the solution to 50% saturation. The crystallization procedure was repeated twice more to obtain the final product. Overall yields of enzyme from this procedure have been of the order of 25–35%.

Preparation II was made from wet yeast by a new procedure. Yeast (450 g) was suspended in 225 ml of 0.1 M-phosphate buffer, pH 7.6, and processed at 32 000 lb/in² in a Sorvall Ribi refrigerated cell fractionator. The temperature of the mixture was maintained at 18°C throughout the pressure shock treatment, and the effluent from the cell fractionator was collected and immediately cooled to 4°C. The cooled extract was centrifuged to remove cell debris and the supernatant was fractionated with ammonium sulphate. Solid ammonium sulphate was added (208 g for each litre of solution) and after 30 min the precipitate formed was centrifuged off and discarded. A further 142 g of ammonium sulphate/l was added and after 30 min the precipitate formed was collected by centrifugation and dissolved in 10 mM-phosphate buffer, pH 7.0. The solution was then dialysed against 2 $\times$ 3 litres of 10 mM-phosphate buffer, pH 7.0.

A 1% (w/v) solution of protamine sulphate in 10 mM-phosphate buffer, pH 7.0, was added to the dialysed enzyme, to the extent of 1 mg of protamine sulphate/60 mg of nucleic acid. A precipitate formed immediately and was centrifuged off after 5 min. Next, a suspension of calcium phosphate gel (30 mg/ml), equilibrated in 10 mM-phosphate buffer, pH 7.0, was added to the extent of 1 mg of calcium phosphate/2.8 mg of protein. The gel protein precipitate was removed by centrifugation after standing for 5 min and was rejected. The extract was now heated at 55°C for 15 min and the precipitate formed was discarded. After this the enzyme was precipitated by the addition of 306 g of solid ammonium sulphate/l of solution and the precipitate was redissolved in 5 mM-phosphate buffer, pH 7.0, and dialysed against 2 $\times$ 2 litres of this buffer.

The dialysed enzyme was applied to a 5 cm $\times$ 50 cm DEAE-cellulose column equilibrated with 5 mM-phosphate buffer, pH 7.0. Subsequently the column was washed and eluted and the enzyme was precipitated from column eluates and crystallized three times, in exactly the same way as for preparation I.

Fluorescence titrations. These were performed with a sensitive recording fluorimeter (Dalziel, 1962b). The original design of the instrument was modified slightly for the purpose of this work, in that the Corning 3389 filter and the M-sodium nitrite liquid filter were replaced by an interference filter. This filter was obtained from Grubb Parsons Ltd., Newcastle, U.K. and exhibited a maximum transmission at 430 nm with a 10 nm bandwidth measured

at half-maximal transmission. All titrations were performed at 25°C in phosphate buffer, pH 7.0 and *I* 0.1, by the addition of 10 μ l portions of NADH solutions to a 4 ml volume containing buffer with and without acetamide. As many as 12 portions of NADH were added in the course of a single titration, which could be completed within 15 min. The enzyme was found to be completely stable under the conditions of the titrations for at least 30 min.

In the range of NADH concentrations used in titrations there was a small but significant deviation from linearity in the plot of NADH fluorescence versus concentration (see, for example, Fig. 1). Thus increasing the NADH concentration by 2.7 μ M from 21.1 μ M to 23.8 μ M gave a 15% smaller increase in fluorescence than was obtained on increasing the concentration from zero to 2.7 μ M. In an attempt to correct for this non-linear behaviour, observed fluorescence increases, produced by addition of NADH to enzyme solutions, have been multiplied by a correction factor. The factor was that which converts the fluorescence increment observed for the portion of NADH alone into the value it would have been with no deviations from linearity. Over a full titration curve the corrections produced an increase of 3% in the end-point value.

A more serious source of error than the above may arise from the fact that all preparations of alcohol dehydrogenase exhibit a slow blank reaction with NADH. Unlike Hayes & Velick (1954), who also observed this reaction with their enzyme, I have been quite unable to decrease the rate of the blank reaction by adding semicarbazide to the system. In my measurements the blank reaction takes the form of a decrease in fluorescence from the initially established value. In the course of titrations of small concentrations of enzyme (0.5 μ M) the blank reaction accounted for a loss of <5% of the total fluorescence change observed. There was little source of error here. On the other hand, when small concentrations of NADH (0.44 μ M) were titrated with large concentrations of enzyme (10 μ M) difficulties arose. In these cases, when the first portion of NADH was added to the enzyme, a rapid decrease in fluorescence was observed, starting from an initially high value. The rate of this reaction was so rapid ($t_{0.5} \approx 5$ s) that extrapolation to the zero time-value was not possible with any degree of accuracy. This initial blank reaction resulted in the destruction of about one-half of the estimated original fluorescence. At this stage another portion of NADH was added. Now there was no longer a rapid blank reaction and a value for the fluorescence increase was obtained. Up to another four portions of NADH could be added, and a series of four very closely agreeing values were taken. After this the addition of further portions of NADH produced a gradually diminishing response. One can have confidence that the values, obtained on the addition of the second to the sixth portions of NADH, were close to what would have been observed had there been no blank reaction on addition of the first portion. Nevertheless it must be recognized that the values obtained may be in error by as much as 10%. The error is probably biased towards giving estimates that are too high.

Spectrophotometric titrations. Titrations of alcohol dehydrogenase with PAAD in 20 mm-hydroxylamine and phosphate buffer, pH 7.0 and *I* 0.1, were monitored in a Zeiss PMQII spectrophotometer at 4°C. The low temperature was selected when it was found that, at 25°C, two side

reactions occurred involving the coenzyme analogue. It was found that PAAD and hydroxylamine reacted to form a species having an ultraviolet-absorption band with a maximum at 290 nm. Further it was found that the alcohol dehydrogenase reacted very slowly with the coenzyme analogue to form a compound (possibly the reduced form of PAAD) with an absorption maximum at 355 nm. At 4°C the effects of these reactions were eliminated.

For individual titrations the extinction at 315 nm of a cuvette containing buffer, enzyme and hydroxylamine was read against a similar cuvette containing only buffer and enzyme. Portions of PAAD were then added to each cuvette and the change in extinction was noted. 20 mm-Hydroxylamine had no detectable extinction at the wavelengths used. One unexpected finding was that the enzyme-PAAD-hydroxylamine complex formed quite slowly at 4°C. At the lowest PAAD concentrations, 15 min were required before the extinction at 315 nm in the test cuvette was complete. The value achieved at 15 min was stable for at least a further 40 min. At high concentrations of PAAD the reactions were complete within 4 min. The enzyme was completely stable to the conditions used in the titrations for at least 3 h.

Gel-filtration studies. The binding of NADH by alcohol dehydrogenase, in the absence of any third ligand, was studied by the gel-filtration technique of Hummel & Dreyer (1962). Samples (1 ml containing 50–99 nmol of enzyme and NADH) were applied to a 1.2 cm $\times$ 65 cm column of Sephadex G-50. The column, equipped with a constant-temperature water jacket, was maintained at 25°C and was equilibrated with phosphate buffer, pH 7.0 and *I* 0.1, containing the required concentration of NADH. NADH concentrations of 9–340 μ M were used in this series of experiments. When the sample had entered the column bed, it was washed in with 0.2 ml of the equilibrating buffer before the buffer reservoir was connected to develop the column. The eluate was collected at the rate of about 1 ml/min in 1.5–2.0 ml fractions. The concentration of NADH in the fractions was determined spectrophotometrically at 340 nm, in a Zeiss PMQII spectrophotometer, by using a molar extinction coefficient of $6.22 \times 10^3 \text{ mol}^{-1} \text{ cm}^{-1}$, which applied equally for free NADH (Horecker & Kornberg, 1948) and for NADH bound to yeast alcohol dehydrogenase (van Eys & Kaplan, 1957). The extinction of eluted fractions was measured immediately, and the activity of the enzyme-bearing fractions was measured within 1 h. In all cases, 95–105% of the initial enzyme activity was detected in eluted fractions. The extinction at 340 nm of eluted fractions, including those containing enzyme, was stable for at least 20 min.

Molecular-weight determinations. These were performed with the Spinco model E analytical ultracentrifuge. Sedimentation-equilibrium experiments were performed at 9°C in 0.1 M-phosphate buffer, pH 7.0, with the short-column method of Van Holde & Baldwin (1958). The partial specific volume of alcohol dehydrogenase was determined with the use of a pycnometer in the manner described by Schachman (1957). The absolute concentration of protein, in the samples used for determination of partial specific volume, was determined by drying to constant weight at 105°C.

Electrophoresis. Enzyme preparations were analysed on

0.5 cm $\times$ 7.5 cm polyacrylamide-gel columns at 4°C in the manner of Clarke (1964). Electrophoresis was continued for 2 h with a potential of 500V. The gels were stained for protein with 0.1% Naphthalene Black in 7% acetic acid, or for alcohol dehydrogenase with an activity stain. The enzyme activity-staining mixture contained NAD⁺, 40mg, Nitroblue Tetrazolium, 10mg, ethanol, 85 μ mol, and phenazine methosulphate, 0.40mg, dissolved in 40ml of 30mM-sodium pyrophosphate buffer, pH 8.8.

RESULTS

Purification and some properties of the enzyme. The purification of alcohol dehydrogenase, preparation II, from wet yeast is summarized in Table 1. The specific activity of the product compares very favourably with that of the enzyme obtained by using a modification of the original method (Racker, 1950). In numerous preparations of enzyme made by that method the specific activity of the final product has varied between 380 and 410. Three times as much activity was extracted from the yeast by pressure shock treatment than by autolysis of dried yeast in the manner of Racker (1950). Both preparations I and II were found to be homogeneous in the analytical ultracentrifuge. On electrophoresis in polyacrylamide gels at 4°C, with 50–100 μ g quantities of either preparation, two very close-running bands were observed, both of which possessed substantial enzymic activity. The bands were separated by a distance of about 1mm after moving through a distance of about 40mm.

With preparation I the extinction coefficient of the enzyme at 280nm was found to be $E_{1\text{cm}}^{1\%} = 12.4$ and the partial specific volume was determined at 0.744 ± 0.003 ml/g. From the results of sedimentation-equilibrium experiments, the molecular weight of the enzyme was estimated to be 149000.

Studies of coenzyme binding. The fluorescence titration of preparation I and preparation II with NADH, in the presence of 0.5M-acetamide, is shown in Fig. 1. Also shown here is the variation of NADH fluorescence with concentration in the presence or

the absence of 0.5M-acetamide, but without added enzyme, and the titration of preparation I in the absence of acetamide. It should be noticed that under these experimental conditions there was no appreciable change in the fluorescence of NADH on the addition of 0.5M-acetamide. It is clear that the increases in the fluorescence in Fig. 1 are due to the formation of enzyme-coenzyme complexes that have a greater fluorescence than free NADH. In the absence of acetamide the binary enzyme-NADH complex is formed, whereas in the presence of this reagent a ternary complex of the type enzyme-NADH-acetamide is formed. The detailed course of the titration shown in Fig. 1 is best seen in Fig. 2, where the fluorescence of equivalent concentrations of NADH have been subtracted from the total fluorescence values. It is evident that, in 0.5M-acetamide, a concentration of 25 μ M-NADH was sufficient to saturate the available binding sites on the enzyme. In the absence of acetamide, on the other hand, a concentration of 40 μ M-NADH was insufficient to reach an end point. Fig. 3 shows the titration of 0.44 μ M-NADH with preparations I and II in the presence of 0.5M-acetamide. A concentration of about 9 μ M-enzyme was sufficient to achieve the end point.

Figs. 1–3 contain sufficient information to allow an estimate of the number of available binding sites on the enzyme. It is to be noted that the results shown in Figs. 1–3 are corrected to exactly the same conditions of instrumental sensitivity. Fig. 3 shows that 0.44 μ M-ternary complex exhibited an increase in fluorescence of 49 units in the presence of preparation I and 44 units with preparation II over that shown by an equivalent concentration of NADH. Fig. 2 shows a total increase of 250 units in the fluorescence of NADH when 0.92 μ M-preparation I was saturated with complex and of 152 units when 0.54 μ M-preparation II was saturated with complex. Simple division of the two quantities provides an estimate of 2.45 binding sites on preparation I and 2.8 binding sites on preparation II

Table 1. *Purification of yeast alcohol dehydrogenase, preparation II*

Step	Volume (ml)	Total units	Total protein (mg)	Specific activity (units/mg)	Yield (%)
Extract	966	207000	29000	7.2	100
Ammonium sulphate, dialysis	230	120000	9800	12.3	58
Protamine sulphate	298	102000	5800	16.6	49
Calcium phosphate	340	87000	3850	23.0	42
Heat, ammonium sulphate, dialysis	42	70000	830	84	34
Chromatography	193	50000	180	278	24
Crystallize and twice recrystallize	4.3	32000	77	420	16

Fig. 1. Fluorescence titration of 0.92 μM -preparation I, specific activity 390 ($\square$), and 0.54 μM -preparation II, specific activity 410 ($\blacktriangle$), in the presence of 0.5 M-acetamide, and of 0.82 μM -preparation I, specific activity 390 (Δ), in the absence of acetamide, with NADH at 25°C and pH 7.0. Also shown is the fluorescence of NADH in the presence ($\bullet$) and the absence ($\circ$) of 0.5 M-acetamide.

Fig. 2. Increases in fluorescence on the addition of NADH to 0.92 μM -preparation I (Δ) and 0.54 μM -preparation II ($\bullet$) in the presence of 0.5 M-acetamide, and to 0.82 μM -preparation I in the absence ($\circ$) of acetamide. Values shown are calculated from Fig. 1.

that are capable of forming the enzyme-NADH-acetamide complex.

The titration of preparation I has been repeated a further six times with as many different preparations of the enzyme. Values obtained were 2.3, 2.3, 2.2, 2.5, 2.4 and 2.6 sites/molecule of enzyme. The values are obviously in close agreement and an average of 2.4 sites is calculated.

It may be noted here that the greater fluorescence of the enzyme-bound forms of NADH does not result from a changed absorption spectrum of the coenzyme. It has been shown that the absorption spectrum of NADH is unaffected on binding to the enzyme (van Eys & Kaplan, 1957). This result has been confirmed here. Further, it was also found that the absorption spectrum of NADH in the enzyme-NADH-acetamide complex is identical with that of free NADH in the wavelength range 310–370 nm.

The results of studies on the binding of NADH by alcohol dehydrogenase, obtained with the gel-filtration technique of Hummel & Dreyer (1962), are summarized in Table 2. A typical elution profile, obtained in one of these experiments, is shown in Fig. 4. When the data of Table 2 are

Fig. 3. Fluorescence titration of 0.44 μM -NADH at pH 7.0 and 25°C with preparation I ($\bullet$) and preparation II ($\circ$) in the presence of 0.5 M-acetamide.

treated in the manner of Hayes & Velick (1954), Fig. 5 is obtained. Here the average number of mol of NADH bound/mol of enzyme ($\bar{n}$) is

Table 2. *Binding of NADH by alcohol dehydrogenase*

Experiments were performed at 25°C in phosphate buffer, pH 7.0 and *I* 0.1.

Concn. of NADH (μM)	Enzyme added to column (μmol)	NADH bound	
		calc. from peak fractions (μmol)	calc. from trough fractions (μmol)
340	0.099	0.26	0.27
320	0.09	0.24	0.25
105	0.05	0.122	0.131
55	0.05	0.103	0.110
53	0.046	0.125	0.117
30.5	0.052	0.104	0.109
8.8	0.052	0.063	0.056

Table 3. *Binding of NADH by alcohol dehydrogenase*

Values were calculated from fractions constituting the peak in the elution profile shown in Fig. 4.

Tube no.	Concn. of enzyme (μM)	Excess of NADH (μM)	$\bar{r}$
8	2.0	5.7	2.8
9	9.3	24	2.6
10	18.8	47.5	2.5
11	17.4	48.5	2.8
12	7.4	19.4	2.6

plotted against this quantity divided by the concentration of unbound NADH in the mixture ($\bar{r}/[\text{NADH}]$). Only the results obtained from the peak fractions of elution profiles were used to produce Fig. 5. The intercept at the ordinate of Fig. 5 provides an estimate of a maximum of 2.7 NADH binding sites/molecule of enzyme. The slope of Fig. 5 is equal in magnitude to the dissociation constant of the enzyme-NADH complex (Hayes & Velick, 1954) and its value is estimated to be $11 \mu\text{M}$.

It will be apparent that the individual enzyme-bearing fractions from the gel-filtration experiments can also be used to estimate $\bar{r}$. Each fraction was assayed for enzyme activity and the concentration of enzyme then determined. This information, together with that from extinction measurements at 340 nm, allowed an estimate of the saturation of the enzyme in individual fractions across the protein peak. Such calculations have been performed for all the gel-filtration experiments. The individual results from the elution profile shown in Fig. 4 are summarized in Table 3. The value of $\bar{r}$ obtained for each fraction is, within the limit of experimental error, independent of the protein concentration over a tenfold range. Similar results

Fig. 4. Elution profile from gel-filtration studies of the binding of NADH by preparation I, specific activity 400, at pH 7.0 and 25°C. Preparation I (90 nmol) was applied to the column equilibrated with $320 \mu\text{M}$ -NADH. Extinction measurements were performed in cuvettes of 2 mm path length.

Fig. 5. Binding of NADH by preparation I, specific activity 400, at pH 7.0 and 25°C, calculated from gel-filtration studies.

were obtained from experiments at different NADH concentrations.

Attempts were made to repeat the study of NADH binding by alcohol dehydrogenase by using the ultracentrifugal separation method as described by Hayes & Velick (1954). It was found, however, that with the buffer systems used, either phosphate buffer, pH 7.0 and *I* 0.1, or the sodium pyrophos-

Fig. 6. Titration of alcohol dehydrogenase preparation I, specific activity 380, at pH 7.0 and 4°C with PAAD in the presence of 20mM-hydroxylamine. Enzyme concentrations (μM): ○, 10.6; ●, 15.6.

phate-glycine buffer of Hayes & Velick (1954), significant blank destruction of NADH occurred. Contrary to the findings of the earlier workers, no decrease in the rate of the blank reaction was observed as a result of including 10mM-semicarbazide in the mixtures. The blank reaction was similarly unaffected by prolonged dialysis of the enzyme against buffers made up in water distilled from alkaline permanganate, and by use of this water for the preparation of all reagents. With the SW 39 L rotor of the Spinco model L preparative ultracentrifuge used at 40 000 rev./min about 4 h was required to achieve adequate sedimentation of the enzyme from the meniscus. In that time, at 20°C with a comparatively low enzyme concentration of 7 μM , sufficient blank destruction of the NADH occurred to decrease the concentration of 75 μM -NADH by 15%. In view of the uncertainties introduced by this large blank reaction the experiments were not continued.

Fig. 6 shows the increases in extinction at 315 nm when 10.6 μM - and 15.6 μM -preparation I were titrated with PAAD in 20mM-hydroxylamine. The enzyme was evidently fully titrated when the total concentration of the coenzyme analogue reached about 400 μM . van Eys *et al.* (1957) give a value of $\epsilon = 6 \times 10^3 \text{ mol}^{-1} \text{ cm}^{-1}$ for the molar extinction coefficient of the enzyme-PAAD-hydroxylamine complex. By using this value in conjunction with the results shown in Fig. 6 we obtain estimates of a maximum of 2.6 and 3.0 sites/molecule of enzyme capable of forming the ternary complex.

DISCUSSION

Alcohol dehydrogenase prepared from dried yeast, by a modification of Racker's (1950) procedure, and from wet yeast by a new method, appeared to have identical properties. The specific

activities of the materials were very similar, and analysis by sedimentation in the ultracentrifuge and by electrophoresis on polyacrylamide gels showed both to be of high purity. There was no evidence that either preparation contained any significant amount of inactive protein. Both materials did show two very close-running protein bands on electrophoresis, but both bands were found to contain substantial enzyme activity. The significance of this finding is not understood, but it may well be that one of the bands arises from the loss of a very small number of amide groups from the native enzyme. The appearance of multiple bands in the electrophoresis of ox heart cytochrome *c* is known to result from the loss of amide groups from the protein (Flatmark, 1964).

With preparation I, a specific extinction coefficient at 280 nm of $E_{1\text{cm}}^{1\%} = 12.4$ agrees very closely with the value obtained by Hayes & Velick (1954). Further, the value of 0.744 ml/g for the partial specific volume agrees very closely with a value of 0.743 ml/g determined by Bühner & Sund (1969), and with the value of 0.736 ml/g obtained from amino acid analysis (Butler & Harris, 1968). The molecular weight of 149 000 obtained from sedimentation-equilibrium experiments agrees well with a value of 150 000 obtained by Hayes & Velick (1954), in spite of the fact that these authors used a value of 0.769 ml/g for the partial specific volume. Bühner & Sund (1969) obtained an estimate of molecular weight of 141 000 from the separate determination of sedimentation and diffusion coefficients. Titration results in this paper were calculated by using a molecular weight of 149 000.

The results shown in Figs. 1-3 provide definite evidence for the existence of a ternary complex of yeast alcohol dehydrogenase of the form enzyme-NADH-acetamide. A similar complex has already been demonstrated for liver alcohol dehydrogenase (Winer & Theorell, 1959). These complexes, formed by the alcohol dehydrogenases, are distinguished by a high fluorescence compared with that shown by an equivalent concentration of NADH. With the yeast enzyme the high relative fluorescence of the ternary complex is due solely to a higher quantum yield of the complex. Results of this sort are normally interpreted as arising from a restriction of the vibrational and rotational movements of the NADH within the complex (see, e.g., Velick, 1958). Fig. 1 shows clearly that the binary enzyme-NADH complex also has an enhanced fluorescence, which is, however, considerably less than that shown by the ternary complex. At 430 nm the fluorescence of the ternary complex is some 13 times that of an equivalent concentration of NADH. It is not possible to be accurate in estimations of the fluorescence of the binary complex, but at 430 nm it is estimated to be about fivefold that of NADH.

The results in Figs. 1–3 show that yeast alcohol dehydrogenase can be saturated with the acetamide-containing ternary complex. Consequently, a direct estimate of the number of binding sites available on the enzyme can be obtained. Since the ternary complex formed exhibits a very high relative fluorescence, the titrations are potentially of high accuracy. As one approaches the end of a titration, less and less of the added coenzyme is bound. In the results presented here, even if only one-twentieth of a concentration increment were bound there would be a 1.6-fold increase in the observed fluorescence. The apparatus used here could quite accurately detect fluorescence increases of 1.2-fold. Since the titrations of Figs. 1–3 indicate no detectable increase in fluorescence at NADH concentrations of greater than $25\mu\text{M}$, we may be sure that the enzyme was fully saturated. For titrations in the absence of acetamide, the reverse is true. The fluorescence of the binary complex is much smaller and the end point of the titration would be more difficult to detect accurately. However, it is clear from Fig. 1 that, under the conditions used, the enzyme was not fully saturated with respect to NADH at concentrations that were acceptable. High NADH concentrations ($>30\mu\text{M}$) were unacceptable because of the considerably diminished fluorescence of the NADH at high concentrations, and the uncertainties that would be introduced in trying to correct for this.

The results of fluorescence titrations with yeast alcohol dehydrogenase show good agreement. All laboratory preparations of the enzyme have shown about 2.4 ternary-complex-forming sites/molecule. The errors affecting this value are small, probably of the order of 10%, and it is expected that the errors probably have resulted in estimated values that are too low.

In addition to the small error of 10% possibly affecting the results of fluorescence titrations, there was another potentially very serious error that required consideration. The results of fluorescence-titration experiments were calculated with the assumption that the intrinsic fluorescence of the enzyme–NADH–acetamide complex is not dependent on the extent of saturation of the enzyme. Variation in the optical properties of enzyme–coenzyme compounds with change in fractional saturation are not, however, unknown. Thus Conway & Koshland (1968) observed that in the binding of NAD^+ by rabbit muscle glyceraldehyde 3-phosphate dehydrogenase the binary complex, formed at the fourth site of the enzyme, does not exhibit the characteristic ultraviolet-absorption spectrum shown by complexes formed at the other three sites at earlier stages of the titration.

To check the validity of values deduced from our fluorescence titrations, it was considered important

to obtain estimates of the number of NADH-binding sites on the enzyme by an independent method. The method chosen for this purpose was the gel-filtration technique of Hummel & Dreyer (1962). This method did not require the use of a third ligand such as acetamide, did not involve fluorescence measurements and was not as sensitive to the blank reaction. Of course, the blank reaction would occur on mixing enzyme and NADH in the same way as before. However, in the course of the gel filtration the enzyme would move into areas where the NADH concentration was unaffected and the enzyme was expected to emerge in fractions that showed no appreciable blank reaction. This expectation was borne out, and therefore results obtained from the peak regions of the gel-filtration experiments should be free from errors arising from the blank reaction. On the other hand, the trough regions might be expected to show errors arising from this source. This argument may account in part for the generally larger values for binding obtained in the trough regions. Nevertheless it is clear from Table 2 that there were no great differences in the results obtained from both regions of elution profiles.

The main conclusion to be drawn from the gel-filtration experiments is that the results obtained are entirely consistent with those obtained in the fluorescence titrations with acetamide. For preparation I, a value of 2.7 binding sites/molecule is, within the experimental error, the same as the value of 2.4 binding sites obtained by the fluorescence method. It is clear that the presence of acetamide, necessary to perform the fluorescence titrations, does not alter the general availability of NADH-binding sites at the surface of the enzyme. It is also evident that the estimate of a 10% error affecting the fluorescence titrations is of the right order, and that the assumption that the intrinsic fluorescence of the enzyme–NADH–acetamide complex is independent of the fractional saturation of the enzyme is justified.

It was estimated from the slope of Fig. 5 that the dissociation constant of the enzyme–NADH binary complex is $11\mu\text{M}$ at 25°C in phosphate buffer, pH 7.0 and I 0.1. This value shows that concentrations of NADH as high as 1 mM are required to saturate small concentrations of enzyme in the absence of acetamide. This is consistent with the fact that it was not possible to perform fluorescence titrations of the enzyme in the absence of acetamide (see, e.g., Fig. 1). The situation here is quite different from that observed with horse liver alcohol dehydrogenase. For that enzyme the dissociation constant of the enzyme–NADH complex at pH 7.0 and 23.5°C is $0.3\mu\text{M}$ (Theorell & McKinley-McKee, 1961), and fluorescence titrations of the enzyme, in the absence of any third ligand, are readily performed. The value for the dissociation constant of

the enzyme-NADH complex found in this work is in remarkable agreement with the value of $10\mu\text{M}$, at 23°C in phosphate buffer, pH 7.15 and I 0.1, estimated from kinetic studies by Nygaard & Theorell (1955b).

The results already discussed are concerned only with the binding of NADH into either a binary or a ternary complex with the enzyme. To establish whether observed titration values were dependent on the nature of the coenzyme, an attempt was made to study the binding of PAAD. Since PAAD is an analogue of NAD^+ , the enzyme-PAAD-hydroxylamine complex can presumably be used to determine the number of NAD^+ -binding sites on the enzyme in the same way that the enzyme-NADH-acetamide complex can be used to find the number of NADH-binding sites. The titrations of preparation I shown in Fig. 6 give estimates of 2.6 and 3.0 binding sites/molecule of enzyme. These values are obviously in reasonable agreement with the results obtained in binding studies with NADH. It should be noted that in similar experiments with PAAD and hydroxylamine, van Eys *et al.* (1957) estimated a maximum of 0.8 binding site on the enzyme. The reason for the discrepancy between the two sets of results is not clear, but certain points should be noted. The earlier experiments were done at fairly low concentrations of PAAD and the estimate of the maximum number of binding sites available was obtained by a large extrapolation. There is the possibility for the introduction of a large error here. Further, the enzyme used by van Eys *et al.* (1957) was of commercial origin, and no indication of specific activity was given. It is possible that the enzyme preparation was of low activity and that this also contributed to the low value for the number of available binding sites.

Results presented in this work are entirely consistent. They indicate that there are 2.4–3.0 coenzyme-binding sites/149 000 g of enzyme protein. The data do not, however, seem to fit well with what is known of the structure of yeast alcohol dehydrogenase. At first there appeared to be three possible explanations for the discrepancy. The most obvious of these was that possibly the enzyme preparations contained inactive protein. This explanation now seems very unlikely, since electrophoresis on polyacrylamide gels revealed no inactive protein in the preparations. A second possibility was that enzyme prepared by the Racker (1950) procedure, or its modified form used here, contained much enzyme of a partly active, partly inactive type. This explanation can probably be ruled out also. Enzyme prepared by a new method, which dispenses with air-drying of the yeast and autolysis, steps most likely to cause partial destruction of the enzyme, apparently had the same properties as enzyme prepared in the alternative manner. A

third possible explanation is that yeast alcohol dehydrogenase exhibits complex coenzyme-binding behaviour, a fact not previously appreciated. The last-named possibility now appears the most likely.

Close examination of the original NADH-binding experiments of Hayes & Velick (1954) indicates that the present results are not, perhaps, as different from those of the earlier workers as at first appears. Observations of the insensitivity of the blank oxidation of NADH by enzyme preparations to 10 mM-semicarbazide suggest that the values for NADH binding obtained by Hayes & Velick (1954) may be too high. Such errors would most likely affect values obtained at low NADH concentrations to a greater extent than those obtained at high NADH concentrations, and the value of the dissociation constant of the enzyme-NADH complex would be affected to a greater extent than the determination of the maximum number of binding sites.

Whatever the truth of the above statements there is one correction that must be applied to at least some of the data given by Hayes & Velick (1954), and which does produce a substantial decrease in the estimate of the total number of available binding sites. In Table II of Hayes & Velick (1954), the authors present a selection of data from a number of experiments in which the binding of NADH was studied. Calculations of the average number of molecules of NADH bound/molecule of enzyme ($\bar{r}$), and of this quantity divided by the free NADH concentration ($\bar{r}/[\text{NADH}]$), yield values that are substantially less than those given in the table. If the calculated values are plotted in the manner described by Hayes & Velick (1954) a maximum value for $\bar{r}$ of 3.0 is obtained. From the slope of the plot a value for the dissociation constant of the enzyme-NADH complex of $11.7\mu\text{M}$ is obtained.

It is clear that a figure of 3.0 as a maximum value for $\bar{r}$ agrees well with results presented in this paper. Further, if a small error of 5% is allowed for possible effects of the blank reaction, the agreement is remarkable. Agreement between values for the dissociation constant of the enzyme-NADH complex cannot be taken as being very meaningful since our value of $11\mu\text{M}$ was determined in phosphate buffer, pH 7.0 and I 0.1, at 25°C whereas Hayes & Velick performed their experiments at 0 – 5°C in pyrophosphate-glycine buffer, pH 7.8.

It is certain that the results presented in Table II of Hayes & Velick (1954) do not represent all the data obtained by these workers and it is not clear how representative are the results in that table. However, it appears that there are no substantial differences between results presented here and at least some of those originally obtained.

This work indicates that, despite its tetrameric structure, yeast alcohol dehydrogenase exhibits a

maximum of three coenzyme-binding sites within accessible ranges of coenzyme concentration. This finding is perhaps best explained in terms of negative co-operativity, so that the affinity for coenzyme at unfilled sites is lessened by increased saturation of the enzyme. If the interactions are of sufficient strength, the fourth site may not bind detectable amounts of coenzyme at accessible ranges of coenzyme concentration. Yeast glyceraldehyde 3-phosphate dehydrogenase, another enzyme comprising four apparently identical subunits, shows negative co-operativity of this type (Koshland, Cook & Cornish-Bowden, 1969). The saturation of this enzyme does not appear to have been increased beyond 3 mol of NAD^+ bound/mol of enzyme, despite the use of very large (0.5 mm) NAD^+ concentrations.

The existence of negative, homotropic interactions between the subunits of yeast alcohol dehydrogenase will explain the apparent discrepancy between the known number of four identical subunits and the ability in this work to demonstrate only a maximum of three coenzyme-binding sites. Normally one would expect an enzyme exhibiting negative interactions to show non-linear behaviour in the plot of $\bar{r}$ versus $\bar{r}/[\text{NADH}]$, such as is shown in Fig. 5. This I have not observed. However, the deviations from linearity may not be easy to detect, particularly with only a limited amount of data covering the range 30–68% of the total possible titration curve. The gel-filtration technique is not one that lends itself to the performing of large numbers of experiments over wide ranges of concentration.

There is one additional point of evidence which may support the above arguments. Enzymes exhibiting negative homotropic interactions would be expected to show deviations from linearity when initial-rate data of the overall catalytic reaction are presented in Lineweaver–Burk plots. The reciprocal plots should be concave towards the abscissa. The very striking behaviour of glutamate dehydrogenase in this respect has been interpreted as resulting from negative homotropic interactions between the subunits of the enzyme (Dalziel & Engel, 1968). With yeast alcohol dehydrogenase, deviations of this general type were observed in the oxidation of ethanol with NAD^+ by Nygaard & Theorell (1955a), the deviations being well marked only at very high concentrations of NAD^+ and ethanol.

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