

The Binding of DPNH by Liver Alcohol Dehydrogenase

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1. Pure horse liver alcohol dehydrogenase (LADH) contains 28 —SH groups/mole enzyme as determined spectrophotometrically with PCMB. There is no decrease in —SH groups after addition of DPNH or of DPNH and *n*-butyramide, the reaction with PCMB, however, being somewhat retarded in the latter case.

2. Incubation of LADH and LADH-DPNH with sulfhydryl reagents leads to a protection against inactivation in case of the enzyme-coenzyme complex as compared with enzyme alone.

The number of reacted —SH groups seems to be the same in both cases, however.

3. With PCMB as the sulfhydryl reagent the decrease in enzymatic activity and the decrease in bound coenzyme is about linear with increasing PCMB concentrations.

Only after complete reaction of the LADH with PCMB, the enzymatic activity and the coenzyme binding capacity become zero.

4. The role of the —SH groups in the working mechanism of LADH is discussed. The role of helping in maintaining the requisite ternary structure seems more likely than one of direct binding of coenzyme alone, but a combination of these roles is possible.

5. N-(4-Dimethylamino-3,5-dinitrophenyl)maleimide reacts with only about 30 % of the —SH groups, leading to a corresponding decrease in activity. The partly reacted LADH is precipitated by *iso*-propanol (ca. 5 % v/v) as a deeply coloured product.

6. The inhibitory effect upon activity of *iso*propanol in higher concentration is probably due to oxidation of *iso*propanol by LADH and DPN.

7. Inactivation of LADH by Cd²⁺ is to a great extent prevented by DPNH and Zn²⁺. This leads to the conclusion that: a) DPNH is bound directly via Zn to LADH, and b) the inactivation by Cd²⁺ is primarily caused by replacement of Zn by Cd, leading to a less active enzyme and secondarily to reaction with —SH groups.

In 1951 Theorell and Bonnichsen¹ considered the possibility of direct interaction between the —SH groups of LADH** and its coenzyme. The absorb-

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** The following abbreviations will be used: LADH = Horse liver alcohol dehydrogenase; DPN respectively DPNH = oxidized and reduced form, respectively, of diphosphopyridine-nucleotide; PCMB = *p*-chloromercuribenzoic acid; YADH = yeast alcohol dehydrogenase; NEM = N-ethyl maleimide; DDPM = N-(4-dimethylamino-3,5-dinitrophenyl)maleimide; μ = ionic strength.

tion maximum of DPNH at $340\text{ m}\mu$ is replaced by a new absorption maximum at $325\text{ m}\mu$ when LADH was added, because of the formation of an enzyme-coenzyme complex. Addition of PCMB, which inhibits the enzyme, immediately caused the absorption maximum to shift back to $340\text{ m}\mu$, the maximum of free DPNH.

This, together with the fact that a dissociable group with $pK' = 10$ was found to be involved in the binding of DPNH by the enzyme, lead to the aforementioned possibility.

Although the $-\text{SH}$ groups in LADH definitely play a very important role in the working mechanism of this enzyme, there is no further evidence for its role being one of direct coenzyme binding. The finding that 2 Zn atoms are firmly bound by LADH and the fact that LADH is inhibited by metal binding agents such as 1,10-phenanthroline² indicate that Zn has a possible role in coenzyme binding³. This was stressed by the fact that both DPN and DPNH compete with 1,10-phenanthroline⁴.

Other parts of the protein molecule cannot *a priori* be excluded as possible binding sites for the coenzyme, but the known inhibition of LADH by PCMB and 1,10-phenanthroline draws attention first of all to $-\text{SH}$ groups and to Zn atoms.

Regarding the coenzyme, nearly all of the groups present in the coenzyme molecule (the carbonamide group, the pyridine nitrogen, the phosphate group, the adenine part) have been considered, as possible sites for binding to the protein molecule, especially in those papers dealing with speculative mechanisms for the action of the related enzyme YADH^{3,8,15}.

In this paper some experiments with LADH will be described which show the protective effect of coenzyme against some inhibitory $-\text{SH}$ reagents.

EXPERIMENTAL

The LADH was prepared as described by Dalziel⁵, and was stored at -15°C as a crystalline suspension in 30 % ethanol. After centrifugation, the crystals were dissolved in $\text{pH} = 9$ buffer⁶ and dialysed against phosphate buffer $\text{pH} = 7.05$, $\mu = 0.1$, and a solution about $4.5 \times 10^{-5}\text{ M}$ in this buffer was obtained. This solution, when stored at about 4°C , keeps its original activity for a few days. If not stated otherwise the purity of the preparation was about 65–70 % based on enzymatic activity⁶ and absorption at $280\text{ m}\mu$, assuming that all the material absorbing at this wavelength has the same extinction coefficient as LADH.

The DPNH and DPN used were preparations from Sigma Chemical Company. The purities were 90 % and 92 % for DPN and DPNH, respectively. Fresh solutions containing 1 mg/ml were used.

NEM was a Theodor Schuchardt (Munich) product.

$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ and $\text{Zn}(\text{OOCCH}_3)_2 \cdot 2\text{H}_2\text{O}$ were p.a. Merck products.

$3\text{CdSO}_4 \cdot 8\text{H}_2\text{O}$ was a Baker Analyzed Reagent, lot no. 3363.

PCMB c.p. was a product from the Amend Drug and Chemical Co., New York.

n-Butyramide was purchased from Eastman Kodak, while the isopropanol was an Analar product.

The $-\text{SH}$ groups were titrated spectrophotometrically with PCMB at $255\text{ m}\mu$ ⁷. Usually $20\text{ }\mu\text{l}$ quantities of a 10^{-3} M PCMB solution were added to a 2 to $2.5\text{ }\mu\text{M}$ solution of LADH in phosphate buffer $\text{pH} = 7.05$, $\mu = 0.1$, at 23.5°C .

In the presence of DPNH or DPN, however, smaller concentrations of LADH were used to permit the formation of the enzyme-coenzyme complex without having to add too much DPN(H).

Absorbance was measured with a Beckman DU Spectrophotometer and the end point was estimated graphically. A Beckman Energy Recording Adapter in connection with a Philips Recorder was used to follow the reactions kinetically.

RESULTS

The number of sulfhydryl reagents which also inhibit LADH is rather limited. Compounds such as iodoacetic acid or chloroacetophenone do not inhibit the enzyme and NEM reacts only very slowly. Heavy metal ions inhibit readily⁸, but their specificity is questionable. Amperometric titrations have not been carried out, but might give additional information.

I. Interactions with PCMB

98 % pure LADH has 28 SH groups/mole enzyme (mol.wt. 84 000) as was found independently at the Nobel Medical Institute⁹ and by the author at the State University's Laboratory for Organic Chemistry in Groningen. The enzyme preparation used in this investigation contained 25.5 ± 0.5 SH groups/mole of active enzyme. Active enzyme means that the enzyme concentration is calculated from the enzymatic activity test. In the case of pure LADH in the presence of enzymatic inactive, no —SH groups containing material, the number of —SH groups/mole of active enzyme, should be 28. The presence of enzymatic inactive, but —SH groups containing material should give a higher value, whereas enzymatic active, but no —SH groups containing material should give a lower value.

In the presence of DPNH or DPN the same number of —SH groups were found⁹. The dissociation constant for LADH-DPN at pH = 7.0 is

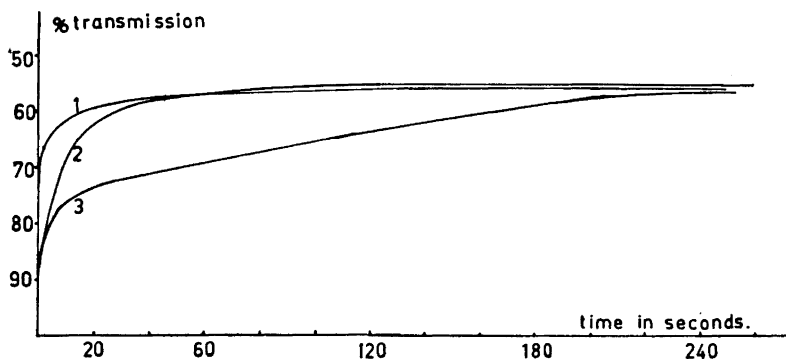


Fig. 1. The reaction of PCMB with LADH, LADH—DPNH—*n*-butyramide and LADH + imidazole *versus* time.

1: [LADH] * = 1 μ M, alone, or with [DPNH] = 20 μ M or [DPN] = 40 μ M.

2: [LADH] = 1 μ M, [DPNH] = 20 μ M, [*n*-butyramide] = 1 M.

3: [LADH] = 1 μ M, [imidazole] = 0.1 M.

In 3.0 ml μ = 0.1 phosphate buffer pH = 7.05. Temperature 23.5°C. In all cases PCMB was added at zero time to a final concentration of 30 μ M. λ = 255 $m\mu$.

* Concentrations of LADH in this article are given as molarities of active enzyme.

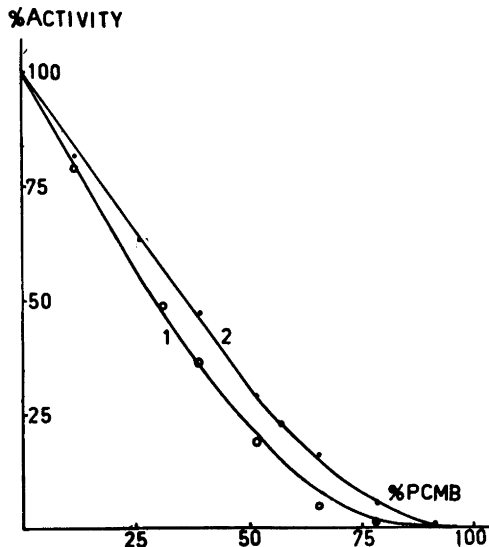


Fig. 2. The decrease in activity of LADH and LADH—DPNH, when increasing amounts of PCMB are added.

1: [LADH] = 2.03 μ M.

2: [LADH] = 2.03 μ M, [DPNH] = 40 μ M.

Total volume = 1.50 ml, μ = 0.1, phosphate buffer p_H = 7.05. Room temperature was used. All the enzyme or coenzyme solutions were incubated 15 min and 10^{-3} M PCMB was added, then, in increasing amounts and after 15 min the activities were measured. 100 % PCMB means conversion of all the —SH groups in LADH.

160×10^{-6} M;* the dissociation constant for LADH—DPNH at $p_H = 7.0$ is 0.31×10^{-6} M¹⁰. This makes it possible to have at least 95 % association in the case of DPNH, whereas in the case of DPN, under the experimental conditions used, it is difficult to get more than about 40 % association, without causing interference in the PCMB titration because of high absorption at 255 $m\mu$. In this case, a difference of 1 or 2 SH groups would be difficult to detect. When the reaction is followed with a recorder there is no detectable difference in the reaction velocities of the two complexes, but, in as much as the reaction is fast, a refinement in the measuring methods might possibly reveal differences (Fig. 1).

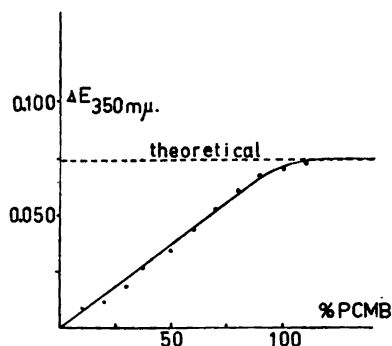
Recently Winer and Theorell¹¹ described very stable ternary complexes of the type LADH—DPNH—fatty acid-amide and LADH—DPN—fatty acid*. The ternary complex LADH—DPNH—*n*-butyramide was further investigated, but showed no difference from LADH alone in —SH groups titrable with PCMB. The —SH groups of the complex, however, react more slowly with PCMB (Fig. 1). There is no evidence from this figure, that some special —SH groups are involved in this retardation of the reaction. In this laboratory it was also found⁹ that imidazole forms a complex with LADH—DPNH; in this case the reaction with PCMB was strongly retarded (Fig. 1).

Bovine serum albumin (a commercial sample, titrating 0.42 SH/mole) reacted immediately with PCMB either with or without the presence of imidazole. In both cases the same amount of —SH groups was found, but, when plotting the $\Delta E_{255 \text{ m}\mu}$ against the amount of PCMB added, in the case when imidazole was present, it was found that the second part of the curve, representing the addition of PCMB to fully reacted serum albumin, was not straight, but bent downwards. This thus resulted in a smaller $\Delta E_{255 \text{ m}\mu}$ when excess

* See the following paper.

Fig. 3. The liberation of DPNH from LADH—DPNH by PCMB. [LADH] = 16.3 μ M, [DPNH] = 50 μ M in μ = 0.1 phosphate buffer pH = 7.05. Temperature 23.5°C.

PCMB added every 2 min and measurements made 1 min after each PCMB addition. Corrected for protein absorption and oxidation of DPNH in the presence of LADH.



PCMB was added to serum albumin. The same was also found to occur in the case of LADH. Furthermore, it was found that when PCMB was added to imidazole, a small absorption maximum occurred at around 245 $m\mu$.

When the activity of LADH is plotted against increasing amounts of PCMB, a curve as in Fig. 2 is obtained, as was found in this laboratory⁹.

The first part of the curves is practically straight and only towards the end is the fall in activity somewhat less than observed at the beginning. An incubation time of 15 min with PCMB ensures practically complete reaction at a temperature of 23°C. It can be seen that with or without DPNH all the —SH groups are necessary for activity. I am much indebted to Dr. J. S. McKinley McKee for letting me know the results of these experiments.

Another interesting feature is the small, but consistent protective effect of DPNH against PCMB inactivation. Although the titration curve is the same with or without DPNH, the inactivation curve is clearly distinct in the two cases.

In order to get somewhat more information about the breakdown of the LADH—DPNH complex by PCMB, it seemed useful to add the PCMB stepwise and to measure the breakdown at 350 $m\mu$ ¹. Accurate titration of the LADH—DPNH complex with PCMB was rather difficult, however.

Rather high concentrations of LADH and DPNH must be used in order to follow the breakdown of the complex spectrophotometrically, and at these high concentrations, there is an as yet unexplained oxidation of DPNH, which can be easily followed by the decrease of absorption at 340 $m\mu$ with time. This effect had earlier been observed with water purified on ion exchangers and was then explained by the presence of aldehyde which had dissolved from the resin¹⁰. Here, however, ordinary distilled water was used.

As was described by Theorell and Bonnichsen¹, the greatest difference in extinction between free and enzyme bound DPNH occurs at 350 $m\mu$. The increase in extinction at 350 $m\mu$ versus added PCMB, corrected for protein absorption and DPNH oxidation, and thus representing the breakdown of the complex by PCMB, is shown in Fig. 3.

The curve obtained resembles that of Fig. 2. Again, complete breakdown of the complex seems to be reached only after complete reaction of LADH with PCMB. This breakdown is at first directly proportional to the amount

of PCMB added and deviates from linearity, at higher PCMB concentrations. The most likely conclusion is that most, if not all, of the —SH groups are important for the coenzyme binding.

II. Interactions with NEM

Direct titration of the —SH groups of LADH or LADH—DPNH with NEM at $300 \text{ m}\mu^{12}$ is practically made impossible by turbidity and even the formation of a precipitate after about 8 h incubation. Furthermore, the reaction is very slow. It was observed, however, that in the case of LADH—DPNH the turbidity was much less pronounced than in the case of LADH alone.

LADH and LADH—DPNH were incubated with NEM, and the decrease in activity and —SH groups was followed. The determination of remaining —SH groups was carried out by titrating back with excess PCMB as follows. The incubation mixtures were centrifuged, and 3.0 ml of the supernatant solution was immediately pipetted out, the extinction at $255 \text{ m}\mu$ measured, and a small excess of PCMB added. The increase in extinction at $\lambda = 255 \text{ m}\mu$ was measured after 1, 10, 20 and 30 min and the solution was again centrifuged. The $\Delta E_{255 \text{ m}\mu}$ was measured immediately and the percentage of remaining —SH groups was calculated from the last values. The discrepancies between the percentages obtained at the different time intervals indicated that the SH titration was only approximate. The results are given in Fig. 4.

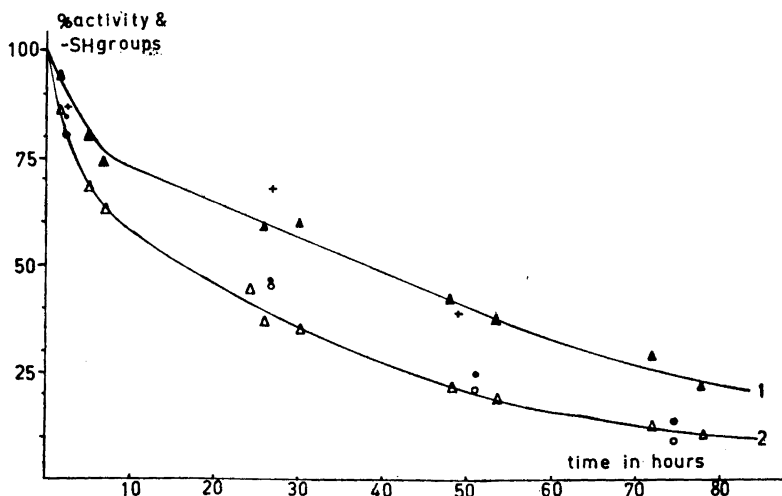


Fig. 4. The effect of excess NEM on activity and —SH titer of LADH and LADH—DPNH with time.

1: [LADH] = $1.00 \mu\text{M}$, [DPNH] = $40 \mu\text{M}$, [NEM] = $267 \mu\text{M}$ (\blacktriangle).

2: [LADH] = $1.00 \mu\text{M}$, [NEM] = $267 \mu\text{M}$ (\triangle).

Total volume 15.0 ml in $\mu = 0.1$ phosphate buffer pH = 7.05. Room temperature.

● = percentage of —SH groups for 1, ○ = *idem* for 2.

+ = *idem* for LADH—DPNH without NEM.

Determined by addition of $200 \mu\text{l}$ $5.15 \times 10^{-4} \text{ M}$ PCMB to 3.0 ml solutions, after centrifugation, at $255 \text{ m}\mu$.

The activity decreases of LADH and LADH—DPNH without the addition of NEM were comparable.

The loss in activity of the LADH—DPNH without added NEM is similar to that for curve 1. However, in this case the decrease in free —SH groups runs parallel with the decrease in activity. There is a very strong protective effect of DPNH against inactivation, but there seems to be no protection against the decrease in free —SH groups. There is, however, a small difference in —SH groups titrated between LADH—DPNH and LADH at the end of the experiment, where it is most difficult to get clear-cut results with PCMB. Whether this difference is real or not has yet to be ascertained.

In the presence of DPN, a protection against inactivation is also present; no —SH determinations were carried out as relatively high DPN concentrations were used. On the other hand, in the case of the ternary complex LADH—DPNH—*n*-butyramide no activities could be measured because *n*-butyramide completely inhibits the enzyme. The decrease in —SH titre, however, was rather slow, about 60 % of the —SH groups still being present after 75 h.

III. Interaction with DDPM

DDPM, a coloured maleimide derivative used for the identification of cysteinyl peptides (Witter and Tuppy, unpublished results), behaves somewhat differently from NEM. Because of its rather poor solubility in water, the reaction was performed in alcohol-water mixtures.

Reaction in isopropanol. Solutions were made up in the following way: in a total volume of 3.0 ml (phosphate, $\mu = 0.1$, pH = 7.05) containing [LADH] = 0.71 μM , the concentrations of isopropanol were, respectively, 0.22 M, 0.44 M, 0.66 M, 0.88 M, 1.10 M, 2.20 M, and the corresponding concentrations of DDPM were, respectively, 4 μM , 8 μM , 12 μM , 16 μM , 20 μM , 40 μM , made by the addition of a 2.42×10^{-4} M solution of DDPM in isopropanol. The enzymatic activities of these solutions, and of the corresponding solutions with only

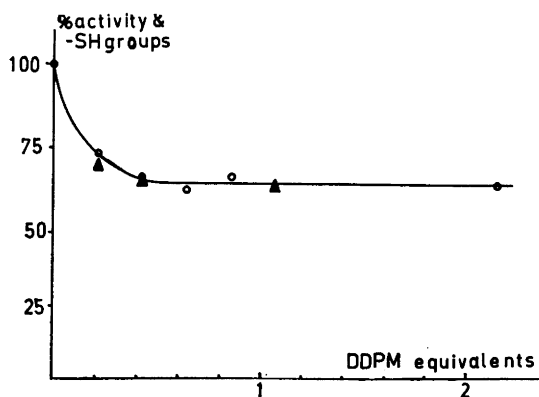


Fig. 5. The effect of DDPM on activity and —SH titer of LADH, corrected for the influence of the solvent, isopropanol.

○ = % activity, ▲ = % free —SH groups.

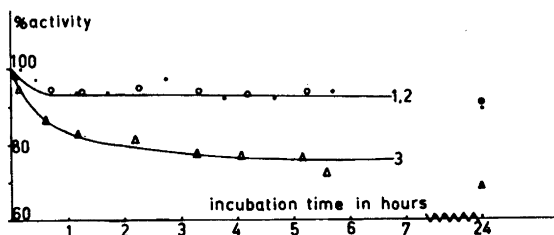


Fig. 6. The effect of excess DDPM on activity of LADH and LADH- DPNH with time.

- 1: $[\text{LADH}] = 0.9 \mu\text{M}$, $[\text{DPNH}] = 14 \mu\text{M}$, $[\text{DDPM}] = 52.5 \mu\text{M}$ (O).
- 2: $[\text{LADH}] = 0.9 \mu\text{M}$ (●).
- 3: $[\text{LADH}] = 0.9 \mu\text{M}$, $[\text{DDPM}] = 52.5 \mu\text{M}$ (Δ).

All solutions in $\mu = 0.1$ phosphate buffer $\text{pH} = 7.05$, containing 1 % ethanol, which gives the optimal ethanol concentration in the test, as 150 μl of the incubation mixture was used for activity tests. Incubation at room temperature.

isopropanol (and thus no DDPM) were tested after 5 h incubation; after 7 h of incubation, the —SH groups were titrated with PCMB, after centrifugation. The differences in activities and —SH groups for the solutions containing DDPM and those containing only *isopropanol* (*vide infra*) are shown in Fig. 5.

As can be seen from this figure the decrease in activity and —SH titer are about parallel and seem to reach a constant value after about a 30 % decrease.

As in the case of NEM, an excess of DDPM is necessary to get complete reaction of the —SH groups, but a further increase in reagent concentration than used here will lead to difficulties. The enzyme will precipitate at high *isopropanol* concentrations, the absorption of DDPM at 255 $\text{m}\mu$ will make the —SH titrations doubtful, and also the DDPM itself may precipitate. However, as the decrease is constant from about 0.5 to 2 equivalents DDPM, it seems that with the rather weak reagent DDPM, only about 30 % of the —SH groups can react. Also in the case of NEM (Fig. 4), and to a lesser extent in the case of PCMB (Figs. 1 and 2), there seem to be faster and slower-reacting —SH groups.

Two other interesting observations were made. In the solutions containing DDPM, the reacted LADH forms a deep-yellow precipitate (from the solution containing 12 μM DDPM and 0.66 M *isopropanol* onward), which can be easily removed by centrifugation. In the solution containing only *isopropanol*, a small precipitate was observed in the test tube containing 2.20 M *isopropanol* (that is about 13 %), but in all the other solutions no precipitate was formed during the time the experiment lasted.

The second point is the effect of *isopropanol*. This secondary alcohol is reported not to be oxidized^{13,14}. However, an inhibitory effect of *isopropanol* on LADH activity was found under the conditions described here (in the case of the solution containing 2.20 M *isopropanol*, there is present in the test solution 73 mM *isopropanol* and 8 mM ethanol). The inhibition might be due to impurities in the *isopropanol* used, but as other secondary alcohols (second-

ary butanol for example) are reported to be oxidized¹⁴, the oxidation of *isopropanol* itself cannot be excluded.

In a separate experiment the K_m for this *isopropanol* at pH = 10.05 and 23.5°C was found to be 9.2 mM, using [LADH] = 13.3 μ g/ml, [DPN] = 0.42 mM and [*isopropanol*] = 1.75 to 44.0 mM.

Acetone (Analar) in a concentration of 900 mM with [DPNH] = 0.2 mM and [LADH] = 13.3 μ g/ml at pH = 7.05 (μ = 0.1, phosphate) was found to be very slowly reduced.

It thus seems possible that *isopropanol* also is a substrate for LADH, but highly purified material will be necessary to ascertain this.

The effect of *isopropanol* on the —SH titer is very small, so that the corrections for the solvent made in Fig. 5 are mainly those caused by the reduced enzymatic activity, being about 10, 15 and 45 % for *isopropanol* concentrations of, respectively, 0.88, 1.10 and 2.20 M.

Reaction in ethanol. In this case an excess of DDPM in ethanol was added to LADH and LADH—DPNH and the fall in activity was measured with time. No —SH titrations were carried out. The addition of DDPM (5.25×10^{-3} M, dissolved by heating) is best carried out with the help of a stirring rod, as otherwise the DDPM will precipitate. In the enzymatic activity test, minor corrections were made for the absorbance of the DDPM at 340 $m\mu$. The results and the experimental conditions can be seen in Fig. 6.

Because of the low alcohol concentration (1 %), all solutions remained clear throughout the experiment. Again a decrease of about 30 % in activity was observed. In the presence of DPNH, there was complete protection against this fall in activity.

IV. Interaction with heavy-metal ions

Heavy metals inhibit LADH and this was explained by their interaction with the —SH groups of the enzyme⁸. In order to get more information about the interaction of LADH with heavy metals, the fall in activity of LADH and LADH—DPNH upon incubation with heavy metals was followed, as was the —SH titer. The addition of Ag^+ ions results in a rapid precipitation and inactivation of both LADH and LADH—DPNH. In the case of Cu^{2+} , precipitation started after complete inactivation had occurred, and increased gradually with time. LADH was completely inactivated within 3 min, whereas LADH—DPNH was completely inactivated after 45 min. With Zn^{2+} on the other hand, a very slow inactivation occurred, the enzyme still having about 50 % of its original activity after 48 h. There was a definite protective effect against inactivation in the case of LADH—DPNH. In both cases no precipitation occurs.

The most interesting effects were obtained with Cd^{2+} , and were studied in more detail. The concentrations, which were the same as used for the experiments with Ag^+ , Cu^{2+} and Zn^{2+} , were: [LADH] = 2.0 μ M, [DPNH] = 40 μ M, [$CdSO_4$] = 80 μ M. All solutions were made up in μ = 0.1 phosphate buffer pH = 7.05, and incubated at room temperature. The result can be seen in Fig. 7. The protective effect of DPNH against inactivation of LADH

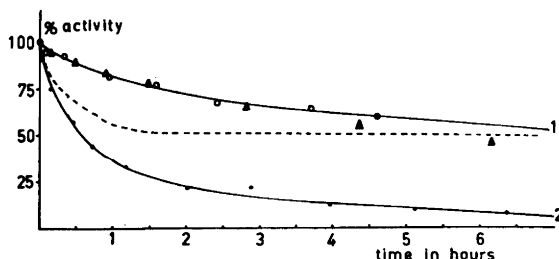


Fig. 7. Inactivation of LADH (2) and LADH—DPNH (1) by Cd^{2+} ions. \blacktriangle represents the fall in activity in the case: $[\text{LADH}] = 2 \mu\text{M} + [\text{Zn acetate}] = 80 \mu\text{M} +$ after 10 min $[\text{CdSO}_4] = 80 \mu\text{M}$. ----- = (1) + 100 % - (2), (see text).

by Cd^{2+} is very large. The same effect was observed when LADH alone was pre-incubated with Zn^{2+} before the addition of Cd^{2+} .

Addition of Zn^{2+} to LADH + Cd^{2+} after 15 min incubation does not lead to reactivation, but the slope of the inactivation curve decreases to become similar to that of the LADH—DPNH + Cd^{2+} curve. The concentration of the added Zn was $400 \mu\text{M}$. The results show that the inactivation of LADH by Cd^{2+} is not primarily due to a reaction with —SH groups, but probably to replacement of the Zn by Cd in the active centre, leading to a less active enzyme. As DPNH has the same effect as Zn^{2+} , the coenzyme seems to be bound via Zn^{2+} to the enzyme, thus protecting the Zn atom against the replacement reaction. The very slow inactivation by Zn^{2+} could thus be due entirely to reaction with —SH groups. In the latter case, the DPNH has a smaller protective effect.

Assuming that in the case of LADH—DPNH the inactivation by Cd^{2+} is due entirely to interaction with —SH groups, the sum of curve 1 and 100 % minus curve 2 in Fig. 7 should roughly give the effect due to replacement alone. Equilibrium is reached within 2 h and remains constant for the 6 h of the experiment; the inactivation due to replacement is thus roughly 50 %.

Once replaced by Cd in the concentration used, Zn is unable to replace the Cd again, but can prevent further replacement when added in excess. Further evidence that not the reaction of —SH groups, but rather the replacement of Zn^{2+} plays the primary role in inactivation was obtained by titrating the —SH groups. Solutions containing $[\text{LADH}] = 2.3 \mu\text{M}$ with or without $[\text{DPNH}] = 45 \mu\text{M}$ were incubated with $[\text{CdSO}_4] = 150 \mu\text{M}$ at room temperature and in $\mu = 0.1$ phosphate buffer pH 7.05. Three ml samples, immediately after centrifugation, were titrated with excess PCMB ($50 \mu\text{l}$ of a 5×10^{-4} M solution), the $\Delta E_{255 \text{ m}\mu}$ measured after 1 min and 30 min and the percentage of titratable —SH groups (which were within 2 % for the two time intervals) calculated. The decrease in —SH titer was roughly parallel to the inactivation curve of LADH—DPNH (curve 1 in Fig. 7), but was about 5–10 % lower, indicating that in the case of LADH—DPNH, the replacement reaction takes place to a very small extent. The decrease of —SH groups was the same for both LADH and for LADH—DPNH, but as in the case of NEM, at the end of the incubation time, the fall was somewhat less in the case of LADH—DPNH.

DISCUSSION

These results, together with the previous results of other investigators, mainly Vallee, Hoch and coworkers^{2,4}, make it seem highly probable that DPNH is bound to LADH by way of Zn atoms. The way this binding takes place is as yet unknown; the carbonamide group³, the phosphate groups¹⁵ and the adenine part⁸ have all been proposed. Also the mode of binding of the Zn to the enzyme is unknown; it has not been possible to demonstrate the role of sulfhydryl groups in this respect.

It is difficult to understand what role the —SH groups play in the working mechanism. The following possibilities seem to be the most likely ones:

1) *Help in maintaining a requisite ternary structure.* From the experiments, specially those with PCMB, it can be concluded that both activity and DPNH binding depend on the presence of all the —SH groups, about equally for the first 70 % and to a lesser extent for the last 30 % of the —SH groups. To this comes the fact that the protective effect of DPNH against inactivation is very marked, and in the first period of inactivation with NEM or Cd^{2+} or Zn^{2+} this protective effect is not caused by a protection of —SH groups. In the case of Cd^{2+} , this is very probably due to prevention of the Zn-Cd replacement, but this is not so in the case of NEM or Zn^{2+} , nor in the case of PCMB, where the protective effect is small, but the amount of reacted —SH groups the same throughout the PCMB concentration range used. The necessity of all —SH groups for both activity and DPNH binding, and the lack of a convincing demonstration of direct coenzyme binding through —SH groups, could thus indicate that the role of the —SH groups lies in their help in maintaining a requisite ternary structure. This would explain the necessity for all the —SH groups, and the fact that the last remaining —SH groups have a smaller effect. In the case of LADH—DPNH it is possible that bound DPNH, to a certain extent, takes over the role of the —SH groups in the maintenance of the ternary structure, leading to a partial protection against inactivation by sulfhydryl reagents.

2) *Binding of coenzyme.* When some —SH groups are involved in coenzyme binding, the explanation of the experiments would not be that both activity and DPNH binding depend on the presence of all the —SH groups, but that all —SH groups have the same reactivity towards the sulfhydryl reagents used and that the binding of coenzyme, as far as —SH groups are involved, is rather weak.

Because of the equal reactivity of the —SH groups towards sulfhydryl reagents, the decrease in activity with increasing concentration of sulfhydryl reagent will be linear, independent of the value of the coenzyme and not coenzyme binding —SH groups for enzymatic activity.

Because of the weak bond between coenzyme and binding —SH groups, the relative reactivity of these —SH groups towards sulfhydryl reagents will only be somewhat reduced, the degree depending upon the efficiency of the sulfhydryl reagent.

With an effective reagent for sulfhydryl groups, as for example PCMB, the effect of bound coenzyme will be small. The same number of —SH groups will be found, and only the somewhat higher enzymatic activity of the enzyme-

coenzyme complex as compared with enzyme alone (Fig. 2) results from the bound DPNH because of the suppressed possibility of reaction of the coenzyme binding —SH groups with PCMB.

With NEM, which only reacts very slowly with the —SH groups of LADH, the effect of DPNH in preventing the reaction of the coenzyme binding —SH groups with the sulfhydryl reagent will be more efficient and the resulting protection against enzymatic inactivation is much larger.

When the reaction of these essential —SH groups with NEM would be prevented completely, a difference in remaining —SH groups between LADH and LADH—DPNH after complete reaction with NEM would be found. This was indeed the case, but as outlined, these results were rather unreliable and the reaction with NEM was not complete at the end of the experiment (Fig. 4). It would also implicate that the —SH groups, which don't play a role in coenzyme binding do play a role in enzymatic activity.

It is of course possible that the role of the —SH groups involves both possibilities, but further work will be necessary to ascertain the role of —SH groups in LADH.

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