THE ENZYME–COENZYME–SUBSTRATE COMPLEXES OF PYRIDINE NUCLEOTIDE DEPENDENT DEHYDROGENASES*

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A number of reports have indicated that the combination of a dehydrogenase, DPN§ or an analogue of DPN, and a nucleophilic compound results in an addition complex, which exhibits a spectrum, similar to the spectrum exhibited by the enzyme-reduced coenzyme complex[1–6]. These nucleophilic substances, in all instances will give addition reactions with the nucleotides, in the absence of enzyme, resulting in complexes with spectra similar to reduced DPN. The enzymic addition complexes, however, invariably show a shift in the maximal absorption towards shorter wavelengths, when compared to the non-enzymic addition spectra. The previous reported examples of ternary complexes are those formed between horse liver alcohol dehydrogenase, hydroxylamine and DPN[1]; yeast alcohol dehydrogenase, hydroxylamine and pyridinealdehyde-DPN[2]; horse liver alcohol dehydrogenase, DPN and cyanide[1,3]; rat liver lactic dehydrogenase, sulfide and DPN[4]; and beef heart lactic dehydrogenase, bisulfite and DPN[5].

We have reported recently[6] that the mercaptan addition reactions forming ternary complexes appear to be general for all dehydrogenases of the group, which oxidizes primary and secondary alcohols to aldehydes and ketones, primary amines to imines, and hemiacetals to lactones.

The present paper will describe in detail a variety of ternary complexes, with special reference to the enzyme–coenzyme–mercaptan complexes[6]. The interest in the mercaptan group stems from the fact that their behavior seems to be different from most nucleophilic compounds, in that they show a discrepancy in stoichiometry with the number of DPN molecules bound per molecule of enzyme. A similar behavior was previously noted for the hydroxylamine complex of yeast alcohol dehydrogenase[2].

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§ Abbreviations used in this paper are: DPN and DPNH: oxidized and reduced diphosphopyridine dinucleotide resp.; acetylpyridine-DPN and pyridinealdehyde-DPN: the analogues of DPN, containing 3-acetyl-pyridine and pyridine-3-aldehyde in place of nicotinamide resp.; acetylpyridine-DPNH and pyridinealdehyde-DPNH: reduced acetyl pyridine-DPN and pyridinealdehyde-DPN resp.; and tris: trihydroxymethylaminomethane.

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MATERIALS AND METHODS

Enzymes

Pig heart malic dehydrogenase and crystalline yeast alcohol dehydrogenase were purchased from the Worthington Biochemicals Corporation. Crystalline lactic dehydrogenases from beef heart and rabbit skeletal muscle were either purchased from the same source or prepared by the methods of STRAUB[7] and BEISENHERZ *et al.*[8] respectively. Crystalline horse liver alcohol dehydrogenase was prepared by the method of BONNICHSEN[9]. Crystalline beef liver glutamic dehydrogenase was isolated by the method of STRECKER[10]. Crystalline rabbit skeletal muscle α-glycerol-phosphate dehydrogenase was prepared by the method of BEISENHERZ *et al.*[8]. Great care was taken to insure absence of cross contamination between this enzyme and the lactic dehydrogenase from the same source. In certain instances the latter enzyme required fractional recrystallization to insure complete absence of the glycerol phosphate dehydrogenase. The crystalline lactic dehydrogenases from beef muscle, pig muscle, and pig heart were obtained by procedures to be published elsewhere. Crystalline rat liver lactic dehydrogenase was prepared by the method of GIBSON *et al.*[11]. Glycerol dehydrogenase from *Aerobacter aerogenes* was obtained by the method of BURTON AND KAPLAN[12]. Propylene glycol dehydrogenase from the same organism was prepared by an unpublished method of LAMBORG AND KAPLAN*. A fraction precipitating between 50 and 75% saturation of ammonium sulfate from a sonicate of *Leuconostoc mesenteroides* contained a D-lactic acid specific dehydrogenase. The D- and L- specific lactic acid dehydrogenases from *Lactobacillus arabinosus* were prepared by an unpublished procedure of DENNIS AND KAPLAN**. Pig heart isocitric dehydrogenase was purified by the method of OCHOA[13]. Glucose-6-phosphate dehydrogenase was purchased from the Sigma Chemical Company.

Pig brain DPN-ase was prepared by the method of ZATMAN *et al.*[14]. This preparation was used to prepare analogues of DPN and TPN. For other experiments the solubilized preparation of DICKERMAN AND STOLZENBACH[15] was used. *Neurospora* DPN-ase was prepared from zinc-deficient mats as described previously[16]. Snake venom pyrophosphatase was used in a partially purified form[17,18].

Coenzymes and related compounds

DPN and TPN were obtained from the Pabst laboratories. The analogues of DPN and TPN were obtained by the action of pig brain DPN-ase in the presence of the appropriate pyridine base as described for the *iso*nicotinic acid hydrazide[14,18] and acetylpyridine[18,19] analogues of DPN. The α-isomer of DPN was isolated from commercial DPN as described previously[18,20]. The deaminated analogues were prepared from the appropriate coenzymes or coenzyme analogues as described for deamino-DPN[18,21]. The reduced forms of DPN, acetylpyridine-DPN and pyridine aldehyde-DPN were prepared enzymically, as described for DPNH[21]. The mono-nucleotides were obtained from the appropriate analogues by the action of snake venom pyrophosphatase[18].

Reagents

Most reagents used were commercial preparations. The mercaptans were purchased, with the exceptions noted, from the Eastman Kodak Company. Cysteine and glutathione were purchased from the Schwartz Laboratories, and 2,3-dithiopropanol was obtained from the Mann Research Laboratories. All reagents used in this study for enzyme catalyzed addition reactions have been shown to react with pyridine nucleotides non-enzymically. These include: cyanide[22,23], bisulfite[22,23], sulfide[24], mercaptans[24], and hydroxylamine[25]. It has been reported previously, that the analogues of DPN show the cyanide[19] and mercaptan[24] addition complexes. This is a general phenomenon; all addition reactions shown by DPN are shared by all analogues of DPN employed in this study. The reactions with analogues actually are more favorable[19,24,26].

The substrates for the different dehydrogenases were also commercial preparations. Glucose-6-phosphate was purchased from the Sigma Chemical Company. Calcium D- and calcium L-lactate were obtained from the California Foundation for Biochemical Research. DL-lactic acid was prepared by boiling commercial lactic acid in the presence of an equivalent amount of NaOH. This preparation of sodium lactate compared favorably with crystalline lithium lactate, as determined by enzymic assay.

When necessary, all reagents were adjusted to the pH required for the particular experiment.

Physical constants

The molecular weights for the dehydrogenases were obtained from the literature. The following were employed: for horse liver alcohol dehydrogenase, 73,000[27]; for yeast alcohol dehydrogenase, 150,000[28]; beef heart lactic dehydrogenase, 135,000[29]; and rat liver lactic dehydrogenase, 126,000[11,30]. The following extinction coefficients were employed: for horse liver alcohol dehydrogenase, $3.32 \cdot 10^4$/moles/l (calculated from the data of BONNICHSEN[9]); for yeast alcohol dehydro-

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genase, $1.98 \cdot 10^5$/moles/l[28]; for beef heart lactic dehydrogenase, $2.1 \cdot 10^5$/moles/l (calculated from the data of NEILANDS[29]); and for rat liver lactic dehydrogenase, $1.59 \cdot 10^5$/moles/l (calculated from the data of GIBSON *et al.*[11]).

For both glutamic dehydrogenase[31] and malic dehydrogenase[32] the molecular weight and extinction coefficients have been reported. Our preparations were, however, not pure enough to apply these criteria.

Molar extinction coefficients of the reduced coenzymes were employed for DPNH: $6.24 \cdot 10^3$ at 340 mμ[33]; for acetylpyridine-DPNH: $7.8 \cdot 10^3$ at 365 mμ[19]; and for pyridinealdehyde-DPNH: $7.0 \cdot 10^3$ at 355 mμ[19,26]. The addition complexes of the coenzymes and coenzyme analogues have approximately the same extinction coefficients as do the reduced coenzymes (*cf.* [23,24]). When the complexes are bound to the dehydrogenase the extinction appears in general to be about 5% less. The deaminated nucleotides have, in the reduced form, the same extinction coefficient and absorption maximum as do the parent adenine nucleotides.

Spectra

For spectral determinations either a Beckman Spectrophotometer model DU or model DK-2 was used.

Rate determinations

The rate of enzymic reactions were always measured by the change in concentration of the reduced coenzyme at the appropriate absorption maximum. Specific reaction mixtures will be reported for the particular experiments cited.

RESULTS

The description of the ternary addition complexes

Criteria for complex formation

Usually conditions for complex formation were chosen under which the coenzyme and nucleophilic complexing agent did not show a significant extent of non-enzymic addition. In those instances the presence of the dehydrogenase induces the formation of the complex. These conditions could not always be achieved, however, since the bisulfite addition reactions in particular are very readily observed non-enzymically with DPN or DPN-analogues. In those cases the criterion for the complex formation on the enzyme is the presence of a shift in the position of maximal absorption of the bound complex when compared to the non-enzymic complex. This is similar to the shift observed when reduced coenzymes are bound to dehydrogenases[27,34]. The absence of a complex therefore does not indicate that such a complex does not exist, it rather means that the formation of the complex is so unfavorable that the non-enzymic complex obscures the enzymically formed one. This case has already been illustrated for the spectral shift observed when DPNH is bound to beef heart lactic dehydrogenase[34]. This shift is only apparent under special low temperature conditions. Yet, this spectral shift can be observed readily with acetylpyridine-DPNH[3]. This emphasizes, therefore, that the absence of a spectral shift is not to be construed as a qualitative difference with another enzyme, catalyzing the same reaction, but showing the addition complexes. The difference may only be a quantitative one.

Ternary complex formation

Figs. 1–3 illustrate the type of spectral shifts observed in this study for the three coenzymes mainly employed: Fig. 1 shows the mercapto-succinic acid–DPN–rat liver lactic dehydrogenase complex; Fig. 2, the ethanethiol–acetylpyridine–DPN–yeast alcohol dehydrogenase complex, and Fig. 3, the bisulfite–pyridinealdehyde–DPN–rabbit muscle lactic dehydrogenase complex. A summary of the observed spectral changes due to ternary complex formation is given in Table I. A short discussion of the individual enzymes is given below.

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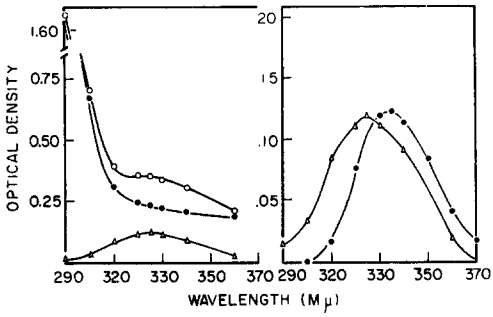
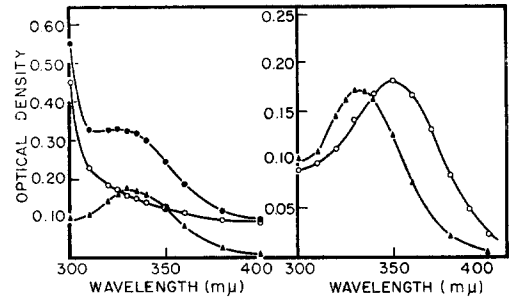


Fig. 1. Example of a ternary complex, employing DPN. The depicted spectrum is formed on rat liver lactic dehydrogenase, with mercaptosuccinic acid. Concentration of enzyme: $1.5 \cdot 10^{-5} M$, DL-mercaptosuccinic acid $3 \cdot 10^{-2} M$, DPN $3.3 \cdot 10^{-4} M$, in 0.1 M phosphate buffer, pH 7.5. Final volume 1.0 ml. ●—●: spectrum of enzyme and mercaptosuccinic acid *versus* mercaptosuccinic acid. ○—○: spectrum after addition of DPN to both blank and experimental cuvette. △—△: difference spectrum. The curves in the right graph compare this difference spectrum with the non-enzymically formed complex in the absence of enzyme. This latter spectrum was obtained at pH 11.0, in 0.5 M mercaptosuccinic acid.

Fig. 2. Example of a ternary complex, employing acetylpyridine–DPN. The enzyme is yeast alcohol dehydrogenase ($1.5 \cdot 10^{-5} M$), and the complexing agent ethyl mercaptan (0.013 M). The buffer employed is phosphate buffer, 0.1 M, pH 7.5. ○—○: spectrum of the dehydrogenase and mercaptan *versus* mercaptan alone; ●—●: spectrum after addition of acetylpyridine–DPN (final concentration $1.4 \cdot 10^{-3} M$) to both blank and experimental cuvette. ▲—▲: difference spectrum. The curves on the right compare the difference spectrum with the equivalent non-enzymic spectrum, observed in phosphate buffer, pH 10.0, 0.1 M, with an ethyl mercaptan concentration of 0.013 M.



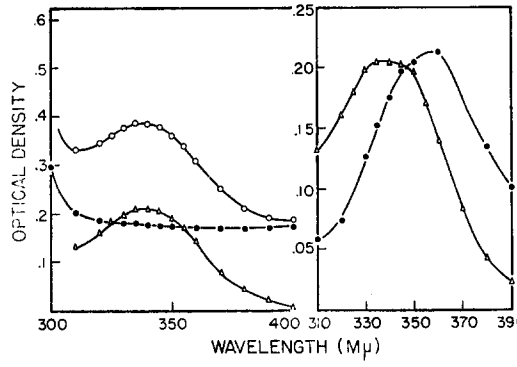


Fig. 3. Example of a ternary complex, employing pyridinealdehyde–DPN. The enzyme is rabbit skeletal muscle lactic dehydrogenase (concentration 2.5 mg/ml), and the complexing agent bisulfite ions (final concentration $1 \cdot 10^{-3} M$), in tris buffer, 0.05 M, pH 7.5. Pyridinealdehyde–DPN $1.35 \cdot 10^{-3} M$. ●—●: enzyme in the presence of bisulfite *versus* bisulfite; ○—○: spectrum after the addition of analogue to both blank and experimental cuvette. △—△: difference spectrum. The curves on the right compare the difference spectrum with the spectrum obtained non-enzymically (in phosphate buffer, pH 7.5, 1 molar bisulfite ions).

Horse liver alcohol dehydrogenase

Since the initial observation by THEORELL AND BONNICHSEN[27] that bound DPNH shows a shift in maximal absorption from 340 mμ to 325 mμ, this enzyme has proven to be very fruitful in the study of complex formation. Previously, similar absorption shifts have been reported for the DPN-hydroxylamine complex and the cyanide complex, when bound to the enzyme[1,3]. To these complexes one must add now the complexes formed between enzyme, DPN and either sulfide, bisulfite or mercaptan derivatives*. In all cases the maximum of the bound complex is shifted 15 mμ towards the ultraviolet. The alkyl mercaptan complex is not limited to the ethyl

* The horse liver alcohol dehydrogenase also appears to show a hydrazine–DPN-enzyme complex. The non-enzymic addition of hydrazine to DPN shows, however, peculiarities, not germane to other addition reactions. For this reason, the data on this complex are not included in this communication.

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mercaptan but rather all normal alkyl mercaptans through C_8 will give the reaction. The reaction is not general, however, for all mercaptan derivatives. Glutathione and cysteine do not form enzymic complexes, even though the non-enzymic addition complexes are formed as readily as is the ethyl mercaptan addition product[24]. Thus there is shown specificity for the enzymic complexes, which is not shown by the non-enzymic counterparts. All complexes, shown by DPN, are evident when DPN is replaced by acetyl pyridine-DPN and pyridinealdehyde-DPN.

Yeast alcohol dehydrogenase

The addition reaction between pyridinealdehyde-DPN, hydroxylamine and the yeast alcohol dehydrogenase has been described[2]. In contrast to the liver enzyme, this reaction is not shown by DPN or acetylpyridine-DPN. Mercaptans also form complexes with the yeast enzyme. For these reactions, even though DPN will still not serve as coenzyme, acetylpyridine-DPN is effective. Again the maximum is shifted 15 mμ towards shorter wavelengths when compared to the non-enzymic spectra. Substrate specificity is again apparent for the mercaptan addition reactions, in that glutathione, cysteine or 2-thioethanol are ineffective. However, all *n*-alkyl mercaptans through C_7 are capable of promoting the formation of complex.

Beef heart lactic dehydrogenase

The spectral shift in maximal absorption of bound DPNH has been reported[34]. It has been pointed out, that this shift is easier to observe with the acetylpyridine- or pyridinealdehyde analogues of DPNH[3]. The DPN-bisulfite complex has been reported to take place with this enzyme[5]. This complex occurs more favorably with the acetylpyridine- or pyridinealdehyde analogues of DPN than with the natural coenzyme. The beef heart enzyme also shows complexes with mercaptans. Substrate specificity is again significant in that mercaptoacetic acid and mercapto-succinic acid give reasonably favorable complexes, whereas ethyl mercaptan, thioethanol, glutathione or cysteine do not. It is noteworthy that there is again a shift in maximal absorption of the enzymically bound mercaptan complex toward the ultraviolet. However, the magnitude of the shift is not as clearly defined as is the case for horse liver alcohol dehydrogenase. Thus the bisulfite spectrum is shifted slightly less than 15 mμ, while the mercaptosuccinic acid spectrum is shifted slightly more.

Rabbit skeletal muscle lactic dehydrogenase

To date no spectral evidence for complex formation has been reported for this enzyme. Mercaptans, however, do form complexes with the enzyme. The same specificity prevails as found with the beef heart enzyme. A bisulfite addition complex also can be observed. It is obvious, however, that the binding of the reduced coenzyme is much less tight by this dehydrogenase than by the corresponding beef heart enzyme. Thus no spectral shift is seen in the presence of the enzyme for DPNH, while the bisulfite complex is definitely less favorable than the corresponding complex on the beef heart enzyme.

Rat liver lactic dehydrogenase

TEREYAMA AND VESTLING reported on the sulfide-DPN-enzyme complex[4]. This complex is also formed in the presence of the acetylpyridine- and pyridinealdehyde analogues of DPN. Mercapto-succinic acid, DPN and the rat liver enzyme will also form a complex. The quantity of enzyme was, unfortunately, not sufficient to perform a survey of the specificity of the mercaptan addition reaction.

In addition to the above mentioned complexes, the maximal absorption of the reduced coenzymes, when bound to the enzyme is shifted towards shorter wavelengths. The liver enzyme resembles the beef heart enzyme, in that the binding of DPNH is not strong enough to get an unambiguous shift of the 340 absorption, since at all times a significant amount of free DPNH is present. Acetylpyridine-DPNH and pyridinealdehyde-DPNH, however, show the shift clearly.

Pig heart malic dehydrogenase

Mercaptosuccinic acid will form a complex with this enzyme, when pyridinealdehyde–DPN is used as coenzyme. DPN will not promote formation of such a complex. Specificity towards substrate is again apparent, in that mercaptosuccinic acid is the only mercaptan showing an addition reaction among all mercaptans tested. Bisulfite will also give a complex with pyridine-aldehyde–DPN and enzyme. In this case acetylpyridine–DPN is also effective, but DPN fails to give a complex. Both the bisulfite and the mercaptosuccinic acid complexes show a shift in maximal absorption when bound to the pig heart catalyst. The mercaptosuccinic acid shift is very great, and even though the enzymically formed complex has a flat absorption maximum, the position of maximal absorption is shifted at least 25 mμ and probably 30 mμ towards the shorter wavelengths. The bisulfite spectrum, however, shows a shift of not more than 15 mμ.

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TABLE

SUMMARY OF THE TERNARY COMPLEXES

The complexes were ascertained in the manner as described in the legends of Figs. 1–3. When it is indicated the particular combination was not tested. The maxima reported are, in general, subject to an error of plexing agents refer to a practical concentration

Complexing agent	*Concentration (M)*	*Coenzyme*	E_{max} *non-enzymic complex* (mμ)	*liver alcohol dehydrogenase* (mμ)	*yeast alcohol dehydrogenase* (mμ)
—	—	DPNH	340	325	no shift
		APDPNH	365	350	no shift
		Py-3AlDPNH	355	340	no shift
Cyanide	$1 \cdot 10^{-3}$	DPN	325	310	no complex
		APDPN	340	330	no complex
		Py-3AlDPN	330	320	no complex
Sulfide	$1.5 \cdot 10^{-3}$	DPN	335	320	no complex
		APDPN	340	325	no complex
		Py-3AlDPN	335	325	no complex
Bisulfite	$1.0 \cdot 10^{-3}$	DPN	330	320	no complex
		APDPN	345	330	no complex
		Py-3AlDPN	345	330	no complex[b]
n-Alkyl mercaptans	$4.5 \cdot 10^{-2}$	DPN	330	315	no complex
		APDPN	355	340	340
		Py-3AlDPN	350	335	335
Hydroxylamine	$2.0 \cdot 10^{-2}$	DPN	315	300	no complex
		APDPN	340	325	no complex
		Py-3AlDPN	330	315	315
Mercaptosuccinic acid	$2.0 \cdot 10^{-2}$	DPN	330	no complex	no complex
		APDPN	350	no complex	no complex
		Py-3AlDPN	350	no complex	no complex
Mercaptoacetic acid	$2.0 \cdot 10^{-2}$	DPN	330	no complex	no complex
		APDPN	350	no complex	no complex
		Py-3AlDPN	350	no complex	no complex
2,3-Dithiopropanol	$1 \cdot 10^{-3}$	DPN	330	no complex	no complex
		APDPN	355	no complex	no complex
		Py-3AlDPN	355	no complex	no complex
2-Mercaptoethanol	$1 \cdot 10^{-1}$	DPN	330	no complex	no complex
		APDPN	340	no complex	no complex
		Py-3AlDPN	335	no complex	no complex

[a] The complex shows a finite dissociation constant, so that only indications of a spectral shift are observed. This is especially true for the beef heart lactic dehydrogenase–DPNH complex; the rat liver enzyme binds DPNH somewhat stronger.

[b] There are indications that a complex exists, but the spectra are ambiguous.

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that no complex was apparent, the complex was looked for, but could not be seen. A dash indicates that about ± 3 mμ, since most spectra are relatively broad in their maximum. The concentration of the com- for the ascertainment of the ternary complexes.

E_{max} of complex bound to the dehydrogenase					
beef heart lactic dehydrogenase (mμ)	*rabbit muscle lactic dehydrogenase* (mμ)	*rat liver lactic dehydrogenase* (mμ)	*pig heart malic dehydrogenase* (mμ)	*rabbit muscle glycerophosphate dehydrogenase* (mμ)	*beef liver glumatic acid dehydrogenase* (mμ)
325[a]	no shift	325[a]	no shift	no shift	no shift
350	no shift	350	no shift	no shift	no shift
340	no shift	340	no shift	no shift	no shift
—	—	—	—	—	—
—	—	—	—	—	—
—	—	—	—	—	—
no complex	no complex	320	—	no complex	—[d]
no complex	no complex	345	—	no complex	—
no complex	no complex	335	—	no complex	—
320	320	no complex	no complex[b]	no complex	—
340	340	no complex	330	no complex	—
330	330	no complex	330	no complex	—
no complex	no complex	—	—	—	—
no complex	no complex	—	—	—	—
no complex	no complex	—	—	—	—
—	—	—	—	—	—
—	—	—	—	—	—
—	—	—	—	—	—
315	315	320	no complex	no complex	no complex
335	335	340	320	no complex	no complex
325	325	335	320	no complex	no complex
310	310	—	—	—	315
335	335	—	—	—	335
—[c]	—[c]	—	—	—	—[c]
—	—	—	—	no complex	—
—	—	—	—	no complex	—
—	—	—	—	345[e]	—
no complex	no complex	—	no complex	no complex	—
no complex	no complex	—	no complex	no complex	—
no complex	no complex	—	no complex	no complex	—

[c] The mercaptoacetic acid has a strong absorption in the region around 300 mμ. For this reason the combined non-enzymic addition and the absorption of the mercaptan itself resulted in zero transmission in the blank cuvette.

[d] The enzyme is strongly inhibited by sulfide.

[e] This complex forms with a measurable rate, rather than instantaneously.

Rabbit skeletal muscle α-glycerophosphate dehydrogenase

The only complex observed to date on this enzyme is the pyridinealdehyde–DPN 2,3-dithiopropanol-dehydrogenase complex. DPN and acetylpyridine–DPN are ineffective in replacing the pyridinealdehyde–DPN. In contrast to all other complexes reported in this paper, the complex formation is not instantaneous, but follows a definite time course. That the observed spectrum is indeed a complex, and not reduced pyridinealdehyde–DPN is indicated by the fact that the maximum of the complex is at 345 mμ, while pyridinealdehyde–DPNH absorbs maximally at 355 mμ. The maximum of the pyridinealdehyde–DPNH is not shifted in the presence of this dehydrogenase. Unfortunately, no mercaptans closer in structure to the substrate are available. It is of interest to note that the propylene glycol and glycerol dehydrogenases from *A. aerogenes* are very strongly inhibited by 2,3-dithiopropanol.

Beef liver glutamic dehydrogenase

The only mercaptan to date, which will show a ternary complex with this enzyme under the conditions examined, is mercaptoacetic acid. It is of special interest that mercaptosuccinic acid is ineffective in forming the complex. This shows a high degree of specificity of the glutamic dehydrogenase, and is in accord with the known fact that aspartic acid will not serve as a substrate for the enzyme[35].

The requirement for coenzyme and dehydrogenase in the addition reaction

The requirement for protein

The enzyme component for the formation of the ternary complex must be in the native state, since boiled enzyme fails to substitute for native enzyme in the reaction. Other methods of denaturation are equally deleterious to complex formation. This can best be illustrated by the effect of urea. Yeast alcohol dehydrogenase is completely inactivated in 5 *M* urea, and similarly, no ethylmercaptan–acetylpyridine–DPN complex is formed in 5 *M* urea in the presence of the enzyme. On the other hand, horse liver alcohol dehydrogenase retains up to 20% of its activity in 5 *M* urea. This is reflected in the retained ability to bind the DPN–ethanethiol complex.

The requirement for coenzyme

The addition of enzymes inactivating DPN to a reaction mixture containing the ternary complex completely abolishes the spectrum of the complex. This destruction can be carried out with either the *Neurospora* DPN-ase or snake venom pyrophosphatase. In the case of acetylpyridine-DPN, it is essential to use the pig brain DPN-ase since the *Neurospora* enzyme does not attack the analogues[19]. The fact that the complex once formed is destroyed by the hydrolytic enzymes, is strong evidence that the coenzymes are not acting catalytically in establishing the complex. Furthermore, the intact coenzyme is required for the formation of complex, since the various coenzyme degradation products are without activity.

A comparison between the action of DPN-ase and pyrophosphatase on the horse liver alcohol dehydrogenase–DPN–hydroxylamine complex has been published in detail[1].

The coenzyme specificity of the complex formation

Only those coenzymes, which will substitute for DPN in the catalytic activity of the enzyme, will promote complex formation on this enzyme. Thus, the acetylpyridine- and pyridinealdehyde analogues of DPN and their deaminated derivatives will promote complex formation as well as promote catalysis[21,36]. Analogues, which will fail to replace DPN in the enzymic reaction, also fail to form complexes (*e.g.* α isomer of DPN).

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The relative effectiveness, however, of a given analogue to replace DPN in catalysis and complex formation are not identical. For example, pyridinealdehyde-DPN is usually a relatively poor hydrogen acceptor[26]. On the other hand, this coenzyme analogue invariably forms the more favorable complexes. The ability of coenzymes to form ternary complexes appears to be related to their ability to form non-enzymic addition complexes[19,24] and to their oxidation–reduction potentials[19,26].

The effect of agents, promoting ternary complex formation, on reaction rates

Criteria for inhibition

Two points are of importance in establishing the inhibitory action of complexing agents. In the first place, on occasions the dehydrogenase might require sulfhydryl groups for maximal activity. In those instances mercaptans might exert a dual role, both as inhibitors, and as activators. In those instances only the extrapolated initial rate will show the actual inhibition. These rates can be experimentally difficult to ascertain if the lability of the enzyme is very great in the absence of mercaptans. This type of phenomenon has been observed with the D-lactic dehydrogenase from *L. mesenteroides*.

Another difficulty arises from the non-specific complexing ability of all substances tested in this study. At very high pH values enough DPN may be complexed non-enzymically to shift the equilibrium significantly. This does not represent an inhibition, even though it might appear as such when the approaching of equilibrium affects the rates. Fig. 4A illustrates this phenomenon with pig heart lactic dehydrogenase in the presence of glutathione. The initial rates are not affected by the presence of the mercaptan, but at the high pH value a significant shift in equilibrium becomes apparent. Lowering the pH by one pH unit completely abolishes the effect (Fig. 4B).

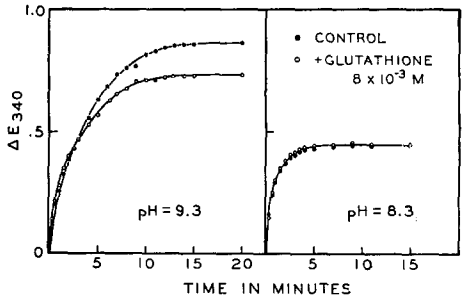

Fig. 4. The effect of glutathione on the equilibrium of pig heart lactic dehydrogenase. DPN: $6.5 \cdot 10^{-4} M$, DL-lactate: $1.7 \cdot 10^{-2} M$, in tris buffer, $0.05 M$, of the pH indicated. The reaction was started with enzyme. Final volume: 3.0 ml.

General inhibition of dehydrogenases by complexing agents

Those agents which will promote ternary complex formation will act as inhibitors for the reduction of DPN by the dehydrogenases. This inhibition is competitive with the substrate. Examples of this inhibition are given in Table II. It is of interest to note in instances where there is little inhibition with DPN or TPN, inhibition can be observed with the corresponding acetylpyridine analogues. The inhibition can be shown clearly by a classical Lineweaver-Burk plot. This latter method, however, for establishing competitive inhibition at times is difficult since the dehydrogenases in general show abnormal substrate saturation curves.

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TABLE II

THE EFFECT OF INCREASING SUBSTRATE CONCENTRATIONS ON THE INHIBITORY ACTION OF MERCAPTANS

The assay systems were as follows: For rabbit skeletal muscle lactic dehydrogenase: Tris buffer, 0.1 *M*, pH 8.0, and coenzyme, $1.5 \cdot 10^{-4} M$; For isocitric dehydrogenase: Tris buffer, 0.1 *M*, pH 8.0, TPN or acetylpyridine–TPN: $1.4 \cdot 10^{-4} M$, and $MgCl_2$, $5 \cdot 10^{-3} M$; For glucose-6-phosphate dehydrogenase: Tris buffer, 0.1 *M*, pH 8.0, TPN or acetylpyridine–TPN: $1.5 \cdot 10^{-4} M$; and for D-lactic dehydrogenase from *L. arabinosus*: Phosphate buffer, pH 8.5, 0.1 *M*, and DPN or acetylpyridine–DPN: $3.0 \cdot 10^{-4} M$. In all cases the final volume was 3.0 ml. The concentrations of the substrate, when a racemic mixture was used, is expressed as the concentration of the active isomer. The reaction was always started with the enzyme.

Enzyme	*Inhibitor*	*Inhibitor concentration (M)*	*Substrate*	*Substrate concentration (M)*	*Per cent inhibition*	
					Nicotinamide coenzymes	*Acetyl pyridine coenzymes*
Rabbit skeletal muscle lactic acid dehydrogenase	Mercaptosuccinic acid	$1.7 \cdot 10^{-2}$	Sodium DL-lactate	$1.7 \cdot 10^{-3}$	29.8	28.8
				$1.7 \cdot 10^{-2}$	0	15.0
				$1.7 \cdot 10^{-1}$	0	3.2
		$3.3 \cdot 10^{-2}$		$1.7 \cdot 10^{-3}$	33.6	63.5
				$1.7 \cdot 10^{-2}$	0	33.6
				$1.7 \cdot 10^{-1}$	0	11.7
D-lactic acid dehydrogenase	Mercaptosuccinic acid	$1.7 \cdot 10^{-2}$	Sodium D-lactate	$7.0 \cdot 10^{-4}$	73.5	70.8
				$3.5 \cdot 10^{-3}$	0	22.0
				$1.7 \cdot 10^{-2}$	0	2.0
		$3.3 \cdot 10^{-2}$		$7.0 \cdot 10^{-4}$	79.4	100
				$3.5 \cdot 10^{-3}$	25.0	78.0
				$1.7 \cdot 10^{-2}$	0	12.2
Isocitric acid dehydrogenase	Mercaptosuccinic acid	$3.3 \cdot 10^{-2}$	*dl*-isocitric acid	$1.7 \cdot 10^{-3}$	4.0	33.0
				$7.15 \cdot 10^{-3}$	2.5	0
		$1.0 \cdot 10^{-1}$		$1.7 \cdot 10^{-3}$	32.4	41.7
				$7.2 \cdot 10^{-3}$	18.7	18.2
Glucose-6-phosphate dehydrogenase	2,3-dithiopropanol	$4.5 \cdot 10^{-2}$	potassium glucose-6-phosphate	$3.3 \cdot 10^{-4}$	21.4	100
				$3.3 \cdot 10^{-3}$	2.6	87.5
				$3.3 \cdot 10^{-2}$	0	53.0
Glutamic dehydrogenase	Mercaptoacetic acid	$1.67 \cdot 10^{-2}$	L-glutamic acid	$4.4 \cdot 10^{-5}$	20.0	—
				$2.2 \cdot 10^{-4}$	—	71.4
				$4.4 \cdot 10^{-4}$	0	40.7
				$4.4 \cdot 10^{-3}$	0	34.3

It is of particular interest that the inhibition is not limited to enzymes which have been obtained in pure enough state to ascertain the formation of ternary complexes. The TPN-glucose-6-phosphate dehydrogenase is inhibited by 2,3-dithiopropanol. This inhibition is also shown by the glycerol and propylene glycol dehydrogenases from *A. aerogenes*. The effect of mercaptosuccinic acid is not limited to L-specific lactic dehydrogenases: the D-specific lactic dehydrogenase from *L. arabinosus* is equally inhibited. The specificity of the mercaptan inhibitions also extends to those enzymes which have not been extensively purified; glucose-6-phosphate dehydrogenase is not inhibited by thioethanol, whereas 2,3-dithiopropanol is an active inhibitor. The summary in Table III shows the specific action of the inhibitors on different dehydrogenases.

References p. 82/83.

TABLE III

SUMMARY OF TERNARY COMPLEX FORMATION ON DEHYDROGENASES

Compounds complexing with all DPN molecules on the dehydrogenase		*Compounds complexing with a limited number of DPN molecules on the dehydrogenase*	
Compound	*Inhibitor for*	*Compound*	*Inhibitor for*
Bisulfite	most substrates	Hydroxylamine	ethanol
Sulfide	tightly bound substrates	*n*-Alkyl mercaptans	ethanol
Cyanide	very tightly bound substrates	Mercapto-acetic acid	lactic acid, glutamic acid
		Mercapto-succinic acid	lactic acid, malic acid, isocitric acid
		2,3-Dithiopropanol	glucose-6-phosphate, glycerol-1-phosphate, glycerol, propylene glycol.

In certain instances a given agent would not give a complex with DPN, yet it did inhibit the reaction with DPN as hydrogen acceptor. This has been previously shown for the action of hydroxylamine on yeast alcohol dehydrogenase[37]. This is again true for ethylmercaptan. In contrast to the action of hydroxylamine on liver alcohol dehydrogenase[37,38] no lag in the inhibitory action of ethanethiol on the yeast or liver enzyme is apparent.

The correlation between inhibitory activity and complex formation

Even though a given complexing agent might inhibit the reaction with DPN in the absence of a corresponding complex formation, the inhibition is generally greater with acetylpyridine-DPN or with pyridinealdehyde-DPN. The magnitude of inhibition appears to be related to the extent of complex formation (Table IV). This is true also for those dehydrogenases in which ternary complexes with DPN as well as with the analogues are observable (*e.g.*, rat liver lactic dehydrogenase).

The effect of complexing agents on the oxidation of DPNH

The complexing agents do not react with the reduced form of the coenzyme. However, as the reaction proceeds from DPNH to DPN, the accumulated oxidized coenzyme shows a tendency to form complexes, thus inhibiting further reaction by competition with DPNH. This is especially marked, when the complex is very favorable, as is the case for the bisulfite–DPN–beef heart lactic dehydrogenase complex. In this reaction as little as 3 γ of bisulfite/3 ml will inhibit the reaction 50%[5]. To some degree this inhibition holds true for the mercaptans. But since the complex is far more favorable with acetylpyridine-DPN, the oxidation of acetylpyridine-DPNH is far more strongly inhibited than the oxidation of DPNH. For example, with beef heart lactic dehydrogenase $3.3 \cdot 10^{-2} M$ mercaptosuccinic acid does not inhibit DPNH oxidation at all, whereas acetylpyridine-DPNH oxidation is inhibited 50%*.

* The experiment was performed in phosphate buffer, pH 8.5, 0.1 M, pyruvate $1 \cdot 10^{-2} M$, and a coenzyme concentration of $1 \cdot 10^{-4} M$.

References p. 82/83.

TABLE IV

THE EFFECTIVENESS OF THE COMPLEXING AGENTS AS INHIBITORS OF DEHYDROGENASES USING DIFFERENT COENZYMES

The following reaction mixtures were used, all with a final volume of 3.0 ml: Glycerolphosphate dehydrogenase: Tris buffer, pH 9.3, 0.1 *M*, DPN, $3.0 \cdot 10^{-4}$ *M*, and α-glycerolphosphate: $1.7 \cdot 10^{-2}$ *M*; Malic dehydrogenase: with mercaptosuccinic acid as inhibitor: DPN: $1.5 \cdot 10^{-4}$ *M*, L-malate: $1.0 \cdot 10^{-2}$ *M*, in tris buffer, pH 9.3, 0.1 *M*, and with bisulfite as inhibitor: pyrophosphate buffer, pH 9.3, 0.1 *M*, with identical concentrations of substrate and coenzyme; for rat liver lactic dehydrogenase: Tris buffer, 0.1 *M*, pH 9.3, DPN: $1.5 \cdot 10^{-4}$ *M*, and sodium DL-lactate $2.7 \cdot 10^{-2}$ *M*. In all cases the reaction was initiated with the enzyme. Preincubation of enzyme, coenzyme and substrate did not alter the results obtained. Rates are expressed as change in optical density per minute.

Enzyme	*Inhibitor*	*Concentration (M)*	*Rate of coenzyme reduction*					
			DPN		*Acetylpyridine-DPN*		*Pyridine aldehyde-DPN*	
			E_{340}	*% Inhibition*	E_{365}	*% Inhibition*	E_{355}	*% Inhibition*
Rabbit skeletal muscle glycerol phosphate dehydrogenase	2,3-dithiopropanol	—	0.087	—	—	—	0.012	—
		$4.0 \cdot 10^{-2}$	0.081	6.9	—	—	0.005	58.3
Pig heart malic dehydrogenase	mercaptosuccinic acid	—	0.084	—	—	—	0.018	—
		$1.0 \cdot 10^{-2}$	0.075	10.7	—	—	0.003	83.3
	bisulfite	—	0.198	—	0.297*	—	0.036	—
		$0.9 \cdot 10^{-3}$	0.078	60.6	0.047	84.2	0.004	88.9
Rat liver lactic dehydrogenase	mercaptosuccinic acid	—	0.154	—	0.030	—	0.011	—
		$1.7 \cdot 10^{-2}$	0.162	0	0.022	26.7	0.000	100
		$5.0 \cdot 10^{-2}$	0.147	4.5	0.013	56.7	0.000	100
		$9.9 \cdot 10^{-2}$	0.119	22.7	0.000	100	0.000	100

* The activity of acetylpyridine-DPN compared to DPN is far greater in pyrophosphate than in tris or phosphate buffer.

The correlation between substrate and mercaptan specificity

Structural specificity

As mentioned before, one of the striking features of the mercaptan addition reaction is the specificity of the enzyme for the mercaptan structure, both for the addition reaction and the inhibition. Yet, both yeast and liver alcohol dehydrogenase will form complexes with all *n*-alkyl mercaptans. This is in line with the substrate specificity of these enzymes; yeast alcohol dehydrogenase in particular will attack a variety of primary and secondary alcohols[39]. The effectiveness of the mercaptan addition and the effectiveness of the corresponding alcohol to serve as a substrate compares reasonably well on the two dehydrogenases (Table V). While butanol[1] reacts only about 60% of the rate of ethanol on the yeast enzyme, the liver enzyme oxidizes butanol 50% *faster* than ethanol. This is correlated with the dissociation constants of the corresponding mercaptan complexes. The butyl mercaptan complex has, on the yeast enzyme, nearly twice the dissociation constant of the ethyl mercaptan. On the liver enzyme, however, the dissociation constants are nearly identical.

The data in Table V for the yeast enzyme are for acetylpyridine-DPN whereas the dissociation constants for the liver enzyme are with DPN. Since the dissociation

References p. 82/83.

TABLE V

THE ACTIVITY OF MERCAPTANS OF VARYING CHAINLENGTHS IN COMPLEX FORMATION ON ALCOHOL DEHYDROGENASES

The rates were determined at alcohol concentrations of $2.5 \cdot 10^{-3} M$, with a DPN concentration of $1.5 \cdot 10^{-4} M$. For yeast alcohol dehydrogenase the buffer employed was Tris, pH 9.3, $0.05 M$. For the horse liver enzyme the buffer employed was phosphate, pH 7.5, $0.1 M$. The dissociation constants for the mercaptan addition complexes were calculated from the formula:

$$(C_b)/(E_t) = -K_{app.}\,[(C_b)/(E_t)]/(C_f) + m$$

where $K_{app.}$ is defined by the equation:

$$K_{app.} = (H^+)/(RSH) \cdot K_{diss.}$$

(RSH) represents the concentration of the mercaptan. The complexes were determined in phosphate buffer, $0.1 M$, pH 7.5. For yeast alcohol dehydrogenase acetylpyridine–DPN was used, for the liver enzyme DPN. The non-enzymic dissociation constant represents the value obtained with pyridinealdehyde–DPN, and is calculated from previously published data[24].

n-alkyl mercaptan or n-*alkyl carbinol used*	K_{diss} *non-enzymic complex*	*Yeast alcohol dehydrogenase*		*Liver alcohol dehydrogenase*	
		K_{diss} *complex*	*Relative rate*[a]	K_{diss} *complex*	*Relative rate*[a]
C_2	$1.0 \cdot 10^{9}$	$4.4 \cdot 10^{-3}$	100	$3.7 \cdot 10^{-4}$	100
C_3	—[b]	—[b]	66	—[b]	150
C_4	$1.4 \cdot 10^{9}$	$7.4 \cdot 10^{-3}$	61	$3.7 \cdot 10^{-4}$	162
C_5	$1.7 \cdot 10^{9}$	$13.0 \cdot 10^{-3}$	—[c]	$4.0 \cdot 10^{-4}$	—[c]
C_6	$3.0 \cdot 10^{9}$	$46.0 \cdot 10^{-3}$	41	$4.8 \cdot 10^{-4}$	156

[a] The relative rate of ethanol is taken arbitrarily as 100.

[b] The purity of our propanethiol preparation was in question. Since no facilities were available for distillation of mercaptans, the value has been deleted.

[c] *n*-Amyl alcohol contains an inhibitor, which can be partially, but never completely removed by fractional redistillation. The inhibitor appears to act stronger on the yeast enzyme than on the liver enzyme.

constants are usually about ten times higher for DPN as compared to acetylpyridine-DPN, the liver enzyme appears to bind the mercaptans (and by analogy the substrate) about 100 times stronger than the yeast enzyme.

Stoichiometry of the addition reaction

Types of stoichiometry observed

When the stoichiometry of the complex reaction was determined for those dehydrogenases for which the molecular weight was known, it became apparent that the complexing agents fell into two groups. One group gave the number of complexing agents bound per mole of enzyme identical with the number of DPN or DPNH molecules bound. The second group, however, showed a discrepancy between these two entities. To the first group belong ions, like sulfide, cyanide and bisulfite. The second group consists of substances closely related in structure to the substrate (*e.g.*, hydroxylamine and mercaptans). We have considered binding of DPNH as a member of the first class, since it represents a complex between enzyme, DPN and a hydride ion.

The stoichiometry of complex formation is summarized in Table VI; the individual enzymes are discussed below.

Horse liver alcohol dehydrogenase

Since the classical work by THEORELL AND BONNICHSEN[27], it has been known that the enzyme binds two moles of DPNH per mole of enzyme. Similarly, two moles of the DPN-cyanide complex

TABLE VI

THE STOICHIOMETRY FOR COENZYME AND SUBSTRATE BINDING BY DEHYDROGENASES

All determinations were performed in phosphate buffer, pH 7.5, usually 0.1 *M*; in some instances, where the enzyme could not stand exhaustive dialysis, higher concentrations of phosphate buffer had to be employed, to overcome the pH lowering effect of the ammonium sulphate. The sulphate ion *per se* has no effect on the addition reaction, either qualitatively or quantitatively, but the pH is critical. The experimental points were obtained in the manner, described in the legend of Figs. 1–3; the concentrations of the complexing agents were those reported in Table I. All figures are the result of extrapolation to infinite coenzyme concentrations, as described in the text. The figures in parentheses refer to the literature references, in those instances where the particular determination was taken from the literature. The theoretical ratio was obtained by making the assumption that all figures are integers.

Enzyme	*Coenzyme*	*Moles coenzyme bound/mole of enzyme*			*Moles of substrate bound/mole of enzyme*			*Ratio total moles coenzyme bound/ moles of substrate complex*	*Theoretical ratio*
		Complexing agent used	*Found*	*Average*	*Complexing agent used*	*Found*	*Average*		
Yeast alcohol	DPN	—[a]	3.6 (*ref.* [28])						
dehydrogenase	DPNH	—[a]	3.6 (*ref.* [28])						
	APDPN[b]				ethanethiol	0.60			
	APDPN				propanethiol	0.80			
	APDPN				butanethiol	1.40			
	APDPN				pentanethiol	1.00			
	APDPN				hexanethiol	1.33			
	Py3AlDPN[b]				hydroxylamine	0.80 (*ref.* [2])			
	Py3AlDPNH	—[a]	3.9 (*ref.* [2])	3.7			0.97	3.8	4
Liver alcohol	DPN				hydroxylamine[c]	1.01			
dehydrogenase	DPN				ethanethiol	1.00			
	DPN				propanethiol	0.95			
	DPN				butanethiol	1.00			

	DPN				pentanethiol	0.99			
	DPN				hexanethiol	0.97			
	DPN				heptanethiol	0.95			
	DPN				octanethiol	0.90			
	DPNH	—[d]	2.0 (*ref.* [27])	2.0			0.97	2.1	2
Beef heart	DPN	—[a]	3.6 (*ref.* [40])		Mercapto-	2.10			
lactic dehydrogenase	DPN	bisulfite	4.0 (*ref.* [5])		succinic acid				
	APDPNH	—[d]	4.25	3.95			2.10	1.9	2
Rat liver	DPN	sulfide	2.0 (*ref.* [4])		Mercapto-	1.15			
lactic dehydrogenase	APDPNH	—[d]	1.97	1.98	succinic acid		1.15	1.7	2
Rabbit muscle	Py3AlDPN	bisulfite	3.94		Mercapto-	1.90		2.1	2
lactic dehydrogenase[e]					succinic acid				
Pig heart	APDPN	bisulfite	2.10		Mercapto-	0.80			
malic dehydrogenase[e,f]	Py3AlDPN			2.10	succinic acid	1.12	0.96	2.2	2

[a] Determined by the ultracentrifugational separation technique.

[b] APDPN and Py3AlDPN stand for the acetylpyridine and pyridinealdehyde analogues of DPN. APDPNH and Py3AlDPNH stand for the reduced form of APDPN and Py3AlDPN resp.

[c] This experiment was performed with a new preparation of enzyme, compared to the one reported in Fig. 5.

[d] Determined by the shift in maximum of the spectrum of the reduced coenzyme, when bound to the dehydrogenase.

[e] The stoichiometry data were obtained under the assumption that the enzyme preparation had an extinction coefficient of $2.0 \cdot 10^5$/mole/liter.

[f] Even though the molecular weight and extinction coefficient have been reported for this enzyme[32], the preparation employed by us was not of sufficient purity to employ these quantities for calculation.

are bound. However, the DPN-hydroxylamine complex shows a stoichiometry of only one mole per mole of enzyme. This held true, whether DPN, acetylpyridine-DPN or pyridinealdehyde-DPN was used as a coenzyme. This stoichiometry is in contrast with that previously reported of two moles per mole of enzyme[1]. This conclusion was drawn under the assumption that the equilibrium constant of the complex formation was very much in favor of the binding. Fig. 5 shows the data reported by KAPLAN AND CIOTTI[1], by calculating the actual amount of complex formed in the reaction mixture. The same stoichiometry of one mole per mole of enzyme is shown by all mercaptans through C_8.

Yeast alcohol dehydrogenase

It has been shown previously that this enzyme binds four moles of DPN, DPNH[28] or pyridinealdehyde–DPNH[2]*. On the other hand, as reported previously[2], only 1 mole of the pyridinealdehyde–DPN-hydroxylamine complex is bound per mole of enzyme. This also holds true for the *n*-alkylmercaptan complexes, whether acetylpyridine–DPN or pyridinealdehyde–DPN is used as a coenzyme. A saturation curve (as used for horse liver alcohol dehydrogenase in Fig. 5) could conceivably indicate that one DPN complex is bound rapidly, while the other complex is bound very unfavorably. This objection would be even more pertinent with the yeast enzyme, where the discrepancy between the number of complexes formed and the number of molecules of DPN bound is very large. For this reason the stoichiometry has been calculated by extrapolation to infinite coenzyme concentration in the presence of a very large excess of complexing agent. This can be done by either of the following two equations**:

$$(C_b)/(E_t) = -K\,[(C_b)/(E_t)]/(C_f) + m \qquad (1)$$

where (C_b), (C_f), and (E_t) stand for the concentration of bound complex, free coenzyme, and total enzyme, respectively; m equals the number of complexes maximally bound to the enzyme.

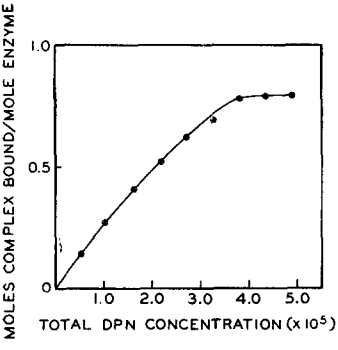

Fig. 5. The saturation curve for the hydroxylamine–DPN–liver alcohol dehydrogenase complex. 0.001 M neutralized hydroxylamine (pH 7.5) using 0.1 M phosphate buffer. Enzyme concentration $3.09 \cdot 10^{-5} M$. The molarity of the complex was estimated from the increase in absorption at 300 mμ.

* It has recently been reported that yeast alcohol dehydrogenase binds 5 moles of DPN/mole of enzyme[39a] rather than four; 5 moles of zinc rather than four were also found bound to the enzyme[39b]. Under special conditions, by binding large amounts of zinc to the enzyme, as high as 28 moles of DPN can be bound[39b]. The interpretation of these observations is not clear. Our data indicate that the binding of the reduced coenzyme shows a binding of only 4 moles DPNH/mole of enzyme.

** The equations can be derived as follows: between the coenzyme (C), the nucleophilic compound (SH), and that fraction of enzyme which binds one mole of nucleophilic compound/mole (E), we have the free and independent series of equilibria:

$$C + SH \overset{K_0}{\rightleftharpoons} C\cdot S + H^+ \quad \text{through } E\text{–}C_n + SH \overset{K'}{\rightleftharpoons} E\text{–}C_{(n-1)}\text{–}C\cdot S + H^+$$

$$E\text{–}C\cdot S + C \overset{K''}{\rightleftharpoons} E\text{–}C\text{–}C\cdot S \text{ through } E\text{–}C_{(n-2)}\text{–}C\cdot S + C \overset{K''}{\rightleftharpoons} E\text{–}C_{(n-1)}\text{–}C\cdot S$$

$$E + C\cdot S \overset{K'''}{\rightleftharpoons} E\text{–}C\cdot S \quad \text{through } E\text{–}C_{(n-1)} + C\cdot S \overset{K'''}{\rightleftharpoons} E\text{–}C_{(n-1)}\text{–}C\cdot S$$

$$E + C \overset{K''}{\rightleftharpoons} E\text{–}C \quad \text{through } E\text{–}C_{(n-1)} + C \overset{K''}{\rightleftharpoons} E\text{–}C_n$$

We define C_b (*i.e.* total amount of enzymically bound complex) by:

$$(C_b) = (E\text{–}C\cdot S) + (E\text{–}C\text{–}C\cdot S) + \text{----------} + (E\text{–}C_{(n-1)}\text{–}C\cdot S)$$

From the equilibria, defined above, we can calculate (C_b) by expressing all terms in $(E\text{–}C_{(n-1)}\text{–}C\cdot S)$, as:

$$(C_b) = (E\text{–}C_{(n-1)}\text{–}C\cdot S) \sum_{(n=0)}^{(n-1)} {}_1/(K''\,(C))^n \qquad (3)$$

Since $K''\,(C) \ll {}_1$, and hence ${}_1/K''\,(C) \gg {}_1$, we can solve (3) to give:

$$(E\text{–}C_{(n-1)}\text{–}C\cdot S) = (C_b)\,(K''\,(C))^{(n-1)} \qquad (4)$$

References p. 82/83. (*Continued on p. 79*)

Alternately one may use equation (2):

$$1/(C_b) = K'/m(E_t) \times 1/(C_f) + 1/m(E_t) \quad (2)$$

Calculation of the stoichiometry by the use of these equations is illustrated in Fig. 6 for the case of pentanethiol, acetylpyridine–DPN and yeast alcohol dehydrogenase. All data in Table VI are calculated by extrapolation to infinite coenzyme concentration.

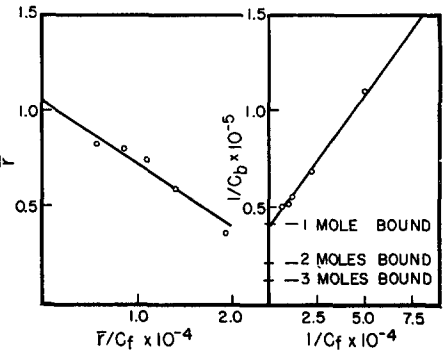

Fig. 6. Calculation of the stoichiometry, employing extrapolation to infinite coenzyme concentrations. The enzyme used was yeast alcohol dehydrogenase. Enzyme concentration $2.42 \cdot 10^{-5} M$, in phosphate buffer, pH 7.5, 0.1 M. Mercaptan concentration 0.083 M. The curve on the left is a plot of:

$$(C_b)/(E_t) = -K[(C_b)/(E_t)]/(C_f) + m$$

The average number of complexes bound/mole of enzyme $(C_b)/(E_t)$ is expressed on the graph as $\bar{r}$. The intercept, m, represents the number of complexes at infinite coenzyme concentration. The graph on the right is a plot of:

$$1/(C_b) = K/m \cdot (E_t) \times 1/(C_f) + 1/m \cdot (E_t)$$

On the figure are indicated the position of intercept (*i.e.* the reciprocal of enzyme concentration times the number of complexes bound) if 1, 2 or 3 complexes were bound/mole of enzyme.

Beef heart lactic dehydrogenase

TAKENAKA AND SCHWERT[40] have shown by ultracentrifugational studies, that 4 moles of DPN or DPNH are bound/mole of enzyme. This is in agreement with the report of PFLEIDERER *et al.*[5] that four bisulfite complexes are bound/mole of enzyme. This figure can be verified by employing the shift in maximal absorption when acetylpyridine-DPNH is bound to the enzyme. However, when the mercaptosuccinic acid complex was measured, only 2 moles of complex were formed maximally/mole of enzyme.

Defining $E_f = E - C_b$ we can derive for E_f from the equilibria along the same lines equation (5):

$$(E\text{–}C_n) = (E_f)\,(K''\,(C))^n \quad (5)$$

Expressing now $(E\text{–}C_{(n-1)}\text{–}C \cdot S)$ in $(E\text{–}C_n)$ from the independent equilibria, introducing the solution in equation (4) and then equating (4) and (5), we get, remembering that $(E_f) = (E) - (C_b)$:

$$(C_b) = \frac{(E)}{1 + \dfrac{(H^+)}{K'K''\,(C)\,(SH)}} \quad (6)$$

If E_t represents the total amount of enzyme, and m the moles of complexes bound/mole of enzyme, we can rewrite equation (6) as:

$$(C_b) = \frac{m(E_t)}{1 + \dfrac{(H^+)}{K'K''\,(C)\,(SH)}} \quad (7)$$

A simple arrangement yields equation (1) and (2), described in the text. It is clear that the equilibrium constant in these equations has the value:

$$K = (H^+)/(SH) \cdot K'K'' \quad (8)$$

In this development the approximation is introduced that the free coenzyme (C) equals total coenzyme minus complex concentration. If the number of complexes coincides with the number of DPN maximally bound (in the derivation $m \cdot n = m$), this is true. If the binding of the complex is weak, so that the free coenzyme exceeds greatly the concentration of complex, the approximation is valid. If, however, the concentration of free coenzyme is small compared to the complex bound, and if $m \cdot n > m$, the approximation is invalid, and a correction is required. This is apparently necessary for horse liver alcohol dehydrogenase. In this case, the binding of uncomplexed coenzyme can be approximated by the equation:

$$(C) = (C_t) - n(C_b) \quad (9)$$

For the horse liver enzyme n (the ratio of total DPN bound to complex bound) equals 2.

References p. 82/83.

Rat liver lactic dehydrogenase

TEREYAMA AND VESTLING have reported that 2 moles of DPN-sulfide complex are bound/mole of enzyme[4]. The binding of 2 moles of coenzyme/mole of enzyme could again be verified by the binding of acetylpyridine-DPNH. The mercaptosuccinic acid complex, however, showed only close to 1 mole of complex/mole of enzyme.

Other dehydrogenases

When the molecular weight of the dehydrogenases was not known, the number of complexes bound per unit enzyme could still be ascertained, and the two groups of complexes could be compared. Table VI shows that for both rabbit muscle lactic dehydrogenase and pig heart malic dehydrogenase, an approximate ratio of 2 is obtained for the stoichiometry as determined by the complexes formed with ions, and those with mercaptosuccinic acid.

In fact, the stoichiometry of the substrate binding can be employed to estimate a minimal molecular weight from the specific extinction of the enzyme. The specific extinction of rabbit muscle lactic dehydrogenase was found to be $1.19 \cdot 10^5$/100,000 g protein. Therefore, from the data in Table V it is clear that the minimal molecular weight of the enzyme is in the order of 85,000.

The effect of pH on the reaction and reaction stoichiometry

THEORELL AND BONNICHSEN reported that the binding of liver alcohol dehydrogenase for DPNH shows a decline from 2 moles/mole to 1 mole/mole if the pH was increased to 10.0[27]. Unfortunately, the non-enzymic complexes must be taken into consideration in the ternary complex formation when the pH is increased, since all complexes are favored by a low hydrogen ion concentration. For the addition reaction of hydroxylamine, however, the stoichiometry remained the same over the pH range of 6 to 8. The reaction often does not proceed at all at low pH values: for example, beef heart lactic dehydrogenase does not show a mercaptosuccinic acid complex below pH 7.0.

DISCUSSION

The theory of action of the dehydrogenases employed in this study has been proposed in previous reports[41,42]. This proposal, mainly based on the ternary complexes as intermediates in the reaction of alcohol dehydrogenases[25,43], suggested that each dehydrogenase employed 2 molecules of DPN for oxidation of 1 molecule of substrate. The first DPN acts as the acceptor of the substrate by binding through the *para*-position. The second DPN acts as hydrogen acceptor of the bound substrate. The evidence, reported in this paper, which supports the two DPN–one substrate theory can be summarized as follows:

1. Compounds, which are related in structure to the substrate and which form non-enzymic complexes with DPN, will have the complex made more favorable in the presence of the dehydrogenase.

2. The stoichiometry of this class of complexes appears to be maximally one complex per two DPN molecules. This has been found to be the case with all dehydrogenases studied with the exception of yeast alcohol dehydrogenase, for which an experimental ratio of one complex per four DPN molecules was obtained.

3. All DPN molecules are equivalent in binding small nucleophilic compounds to the dehydrogenase.

4. The ability of the dehydrogenase to form ternary complexes with mercaptans appears to correspond to the relative effectiveness of the equivalent alcohol in serving as a substrate for the dehydrogenase. The kinetic evidence for the above theory will be given in a subsequent publication.

A few points merit special discussion. Even though the position of maximal absorption is invariably shifted when the complex is bound to the dehydrogenase, the

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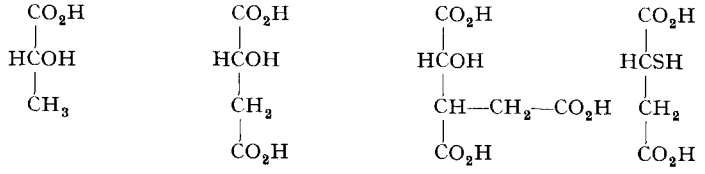
magnitude of the shift varies from as little as 10 mμ to as much as 30 mμ. It is clear that the magnitude of the shift is dependent on the given ternary complex, and not on the specific dehydrogenase. In fact, some dehydrogenases show a large variation in shift depending on the complex studied (*e.g.*, malic dehydrogenase). Also, the shift may differ, using acetylpyridine-DPN instead of DPN, with the same dehydrogenase and the same nucleophilic agent (*e.g.*, beef heart lactic dehydrogenase). It is clear therefore, that no theory of the mechanism of induction of decreased resonance energy can be deduced from the magnitude of the shift.

It is interesting that the reaction from hemi-acetals to lactones fall in the group of "alcohol" dehydrogenases. This is in agreement with the finding that the oxidation of glucose-6-phosphate or glucose results in a lactone[44,45,46].

The dehydrogenases, discussed in this paper, are in general quite unspecific enzymes, both with respect to coenzyme[21,26] and with respect to substrate. For instance, beef heart lactic dehydrogenase will attack a variety of α-hydroxy acids[47]. Even though the mercaptan addition reaction is structurally specific, a great amount of overlap occurs between the compounds, which can act as structural analogues of the substrate. However, if one compares the structures of the substrates of the enzymes, sensitive to mercaptosuccinic acid, a strong similarity is observable:



Similarly, 2,3-dithiopropanol is restricted in action to enzymes, which utilize polyhydroxy compounds as substrates.

It is probable that the enzymic addition reactions occur at the same position of the DPN molecule as do the non-enzymic addition reactions. For at least two known addition reactions, *i.e.* the cyanide[48] and hydrosulfite[49] complexes, it has been shown that the position of reaction is the *para*-position of the nicotinamide ring of the DPN molecule. This is the same position where the oxidation–reduction transformation is localized[50].

The report by BOYER AND THEORELL[51] that not only the position of maximal absorption shifts when DPNH is bound to horse liver alcohol dehydrogenase, but also the maximum of the emitted fluorescent spectrum, is of special interest, since it supplies a method of greatly enhanced sensitivity in the detection of complex formation. The present investigation did not pursue this aspect of the problem, but it might prove fruitful in the future, since complexes, formed non-enzymically have fluorescent spectra, very similar to the one exhibited by DPNH.

SUMMARY

1. The reaction between a number of dehydrogenases, DPN or DPN analogues, and nucleophilic substances, known to form addition complexes with DPN has been described. The dehydrogenases include lactic acid-, malic acid-, propylene glycol-, glycerol-, glycerol phosphate-, glucose-6-phosphate-, isocitric acid-, glutamic acid-, and alcohol dehydrogenases.

2. The nucleophilic substances in many instances have their reaction with the coenzyme

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favored by the presence of the dehydrogenases. The compounds fall into two classes: those, which are bound to the DPN on the dehydrogenase in equal molar amount to the moles of DPN bound; and those which show a discrepancy in this correlation, in that maximally 1 mole of complexing agent is bound for each 2 moles of DPN bound. This has been found true for all dehydrogenases studied except yeast alcohol dehydrogenase, for which a ratio of 4 was obtained.

3. The nucleophilic substances, which show the above discrepancy, are all compounds structurally related to the substrate. Mercaptans and hydroxylamine fall into this class. The dehydrogenases show specificity towards the structure of these compounds; a close resemblance between the structure of the agent and the substrate must exist before the reaction is apparent.

4. The complexing agents are, in general, competitive inhibitors for the dehydrogenases.

5. The affinity of the alcohol dehydrogenases in forming complexes with mercaptans corresponds to the effectiveness of the analogous alcohol to serve as substrate in the enzymic reaction.

6. The data are discussed with respect to the postulation that one substrate is acted upon by the intervention of 2 enzyme bound DPN molecules. This mechanism appears to hold for the group of dehydrogenases which oxidize alcohols to aldehydes and ketones, primary amines to ketones plus ammonia, and hemiacetals to lactones.

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STUDIES ON THE MECHANISM OF OXIDATIVE PHOSPHORYLATION

III. PHOSPHORYLATING PARTICLE TYPES FROM BEEF HEART

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Previous reports from this laboratory have indicated the presence of several particle types in crude mitochondrial suspensions, prepared from beef heart, which are capable of catalysing oxidative phosphorylation[1]. These crude suspensions of mitochondria, readily prepared in large amounts from slaughterhouse material[2], can be further fractionated to yield preparations which are very active both with respect to oxidation and to phosphorylation. The early results along these lines have been briefly mentioned[1].

It is the purpose of this communication to describe the method of isolation and the enzymic properties of these particles. A preliminary report on this work has been presented earlier[3].

METHODS

Assay

The assay employed for measuring oxidative phosphorylation has been partially modified from that previously reported[1]. Oxygen uptake was measured by the standard Warburg technique at 30°C with an equilibration period of six minutes, during which time the oxygen uptake was assumed to be linear and equivalent to that in the subsequent six minute period. In a final volume of 3 ml the following components were added: three to eight mg particle protein, 20 to 80 μmoles phosphate

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