# 51. THE ROLE OF COENZYMES IN DEHYDROGENASE SYSTEMS

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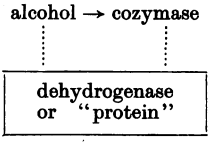
## I. INTRODUCTION

MANY dehydrogenases depend for their action on "coenzymes" (either cozymase or coenzyme II). Owing mainly to the fundamental work of Warburg the mode of action of these substances is now fairly clear. They act by becoming alternately reduced and oxidized and their function in the system is essentially that of a reversible hydrogen acceptor.

Taking the alcohol dehydrogenase system as a typical example, we may picture the process as follows. The substrate (alcohol) forms a highly dissociated compound with a very specific group in the dehydrogenase (or "protein" according to Warburg's terminology). The alcohol when combined is "activated", i.e. made more readily oxidizable. Cozymase also combines with the enzyme, likewise forming a highly dissociated compound. Two H atoms then pass from the combined alcohol to the combined cozymase, as shown by the arrow in the diagram, this being the essential reaction. The oxidized substrate and the




reduced cozymase then leave the enzyme, the former usually to be further metabolized, and the latter to combine either with a flavoprotein or with some other reversible dehydrogenase, where it is reoxidized. The enzyme then combines with further molecules of alcohol and cozymase and the process is repeated. The dehydrogenase thus essentially catalyses a bimolecular reaction between the alcohol and the cozymase:

$$\text{alcohol} + \text{Co} \rightleftharpoons \text{aldehyde} + \text{CoH}_2.$$

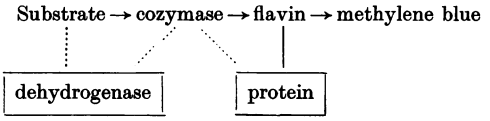
The reaction is reversible, so that presumably the group in the dehydrogenase which combines with the alcohol can also combine with aldehyde, and the group which combines with cozymase can also combine with reduced cozymase.[1]

There is a difference of opinion as to the interpretation of these facts, and two opposing schools of thought have developed during the past two or three

[1] Negelein & Wulff [1937] have measured the affinities of this dehydrogenase for the four substances involved. They find the Michaelis constants (i.e. the concentration of each substance independently at which the dehydrogenase is half saturated with it) to be as follows: cozymase, 0·0001 *M*; reduced cozymase, 0·00003 *M*; aldehyde, 0·0001 *M*; alcohol, 0·024 *M*. Thus the forces uniting the coenzyme with the dehydrogenase are not very different in magnitude from those uniting the aldehyde with the dehydrogenase. A rough calculation shows that under the usual experimental conditions practically the whole of the cozymase is in the free state: at any instant probably less than 1 part in 100 is in combination with the dehydrogenase.

years. The first view, which is held by many workers on enzymes, is that this type of reaction does not differ in any fundamental respect from other bi-molecular reactions catalysed by enzymes. The relation of the dehydrogenase protein to cozymase, no less than its relation to alcohol, is essentially that of an enzyme to its substrate. As far as this system is concerned, the cozymase is simply a very efficient H-acceptor. The second view, which is that of the Warburg school, differs fundamentally from this. According to this view the relation of dehydrogenase to cozymase is simply that of protein to prosthetic group: the complex ["protein"+cozymase] is considered to be the enzyme, it is termed a pyridine-protein (since cozymase contains a pyridine ring) and is regarded as a conjugated protein analogous to nucleoproteins, flavoproteins, haemoproteins etc. The cozymase is regarded, not as a substrate or as a coenzyme in the strict sense, but as actually a part of the enzyme itself, and in fact as its active group. The attractiveness of this view lies in the possibility it offers of arriving at a unified conception of enzymes, or at any rate of oxidizing enzymes, as conjugated proteins consisting of protein+prosthetic group. For it brings many of the dehydrogenases into line with such enzymes as peroxidase and catalase, and more particularly with diaphorase and amino-acid oxidase, which have recently been shown to be flavoproteins having a dinucleotide as prosthetic group.

It occurred to us that it might be possible to obtain evidence in favour of one or other of these opposing views by studying the behaviour of these dehydrogenases towards a wider range of H-acceptors than hitherto. Up to the present almost all the work has been done with either flavoprotein or dyes like methylene blue as H-acceptor. In any case, the dyes do not react either with the activated substrate or with the reduced cozymase but only with the reduced flavoprotein, so that their reduction occurs through flavoprotein and, as far as the dehydrogenase is concerned, using a dye is really the same as using flavoprotein itself. Flavoprotein is not reduced by the activated substrate but only by the reduced cozymase, so that the presence of cozymase is essential for the reduction of any of the above H-acceptors to take place:




On the first view—that the cozymase acts in the dehydrogenase system simply as a H-acceptor—it seemed not unlikely that other H-acceptors might be found which, like cozymase, could be directly reduced by the activated substrate; that is to say acceptors for the reduction of which cozymase would not be necessary. In fact there should be two classes of H-acceptors, namely those which like flavoprotein are only reduced in the presence of cozymase and those which are reduced even in its absence. After all, if the activated substrate can reduce cozymase why should it not reduce other substances?

On the other hand, the second view—that the cozymase is the active prosthetic group actually forming part of the enzyme itself—would lead one to expect that no matter what H-acceptors were tested, they should under no circumstances be reduced by the system in the absence of cozymase. The "protein" without cozymase should be catalytically inactive, since the essential part of the enzyme is lacking, just as for example the proteins of catalase and peroxidase are inactive without their haematin prosthetic group.

We therefore decided to take two dehydrogenases which were known to be typical members of the class reacting through cozymase and of which coenzyme-free preparations could be made, and to test their ability to reduce a number of different H-acceptors with and without cozymase. The two which were chosen were the alcohol and malic dehydrogenases of yeast. The former is the enzyme referred to above; the latter catalyses the reaction:

$$\text{malate} + \text{cozymase} = \text{oxaloacetate} + \text{reduced cozymase}.$$

One of us [Dixon, 1926] had already carried out work on a series of substances of very diverse nature which were found to act as H-acceptors for the xanthine dehydrogenase system, and the most important of these were therefore tested with the two selected dehydrogenases. Among these substances were the following: dyes, quinone, alloxan, $I_2$, $H_2O_2$, aromatic nitro compounds.

It may be said at once that the results obtained strongly supported the first of the two views outlined above. It was found that with no less than three of these H-acceptors the presence of cozymase was quite unnecessary for the reaction to take place, although with the same enzyme preparations cozymase was absolutely indispensable when methylene blue was the acceptor. In other words, the alleged prosthetic group is only necessary when certain acceptors are used; if the H-acceptor is suitably chosen the "protein" alone can catalyse the reaction without its "prosthetic group". Both the dehydrogenases tested behave in this way.

## II. Materials

The greater part of the work was carried out with enzyme preparations made as follows. Delft yeast was plasmolysed with NaCl and disintegrated by grinding for 15 min. in a high-speed ball mill, as described by Ogston & Green [1935]. The resulting viscous suspension was mixed with 3 vol. water and allowed to stand for a short time, after which the insoluble material was centrifuged off and discarded. The soluble enzymes were then purified by fractionation with ammonium sulphate, and the final precipitate was dried *in vacuo* over $H_2SO_4$ and KOH. This stable preparation contains both the malic and alcohol dehydrogenases. It also contains flavoprotein in sufficient quantities to enable the dehydrogenase systems to reduce methylene blue rapidly after the addition of cozymase. On the other hand, it is entirely coenzyme-free, as shown by the fact that no trace of reduction of methylene blue could be detected in 48 hr. in the absence of added cozymase, either by the preparation alone or after the addition of alcohol or malate. With the addition of alcohol and a small amount of cozymase complete reduction was obtained in under 1 min. The preparation was quite free from reducing material; moreover, no reduction was observed on adding cozymase without alcohol and malate.

When required for use, a portion of the dry preparation was dissolved in $M/15$ phosphate buffer, $p$H = 7·4, and the insoluble material was centrifuged off and discarded. Except where otherwise stated, 1 ml. of the solution contained the soluble material from 50 mg. of the dry preparation.

The alcohol dehydrogenase has been very highly purified by Negelein & Wulff [1937], and the most important experiments were repeated with an exceedingly pure preparation obtained by their procedure with slight modifications.

The cozymase preparation (purity about 60 %) was made by the method of Green *et al.* [1937].

The only reagent which requires special mention is alloxan. Freshly-made samples were always used, in view of the fact that the substance is somewhat

unstable and undergoes a gradual decomposition in the solid state in the course of a few months, giving a mixture of products. Alloxan was prepared from uric acid by Schlieper's [1845] method, and purified by several recrystallizations from water. Identical results were obtained with a preparation obtained from Hofmann-La Roche. Freshly made up solutions were always used.

### III. Potentiometric experiments

Methylene blue, quinone and alloxan have an important property in common, namely they all give well-defined oxidation-reduction potentials at a platinum electrode. That is to say the potential of such an electrode immersed in a solution of any one of these systems is determined (under anaerobic conditions and at a given temperature and *p*H) by the ratio of the concentrations of the reduced and oxidized forms of the substance in question. The most general and most convenient technique for following quantitatively the reduction of any of these H-acceptors is therefore the potentiometric method; any reduction of the acceptor by the enzyme system is clearly shown by a change of the potential in a negative direction. This method was in fact used by Dixon [1926] in studying the reduction of quinone and alloxan by xanthine dehydrogenase.

For this work a special cell had to be devised, since none of those hitherto used was suitable. The main special requirements were: (*a*) a convenient arrangement permitting the successive addition of several reagents (e.g. substrate, H-acceptor, coenzyme etc.) to the solution without introducing any $O_2$ into the apparatus, (*b*) the complete elimination of every possibility of contamination (especially with traces of cozymase) from a previous experiment, e.g. by diffusion from a salt bridge forming part of the apparatus. The latter point was important, because the possibility of the presence of traces of cozymase would have destroyed the evidential value of our experiments. The apparatus which was devised for this work has been described in the preceding paper [Zerfas & Dixon, 1940]. The details of the method are described there, and it is only necessary here to give the general procedure, which was as follows. The enzyme solution, suitably buffered to *p*H 7·4, was placed in the electrode vessel and the reagents to be added were placed in the small suspended cups. After evacuating and washing out the apparatus several times with pure $N_2$, it was left evacuated and placed in the water bath at room temperature. After allowing a short time for the pyrogallol in the side-bulb to absorb any residual traces of $O_2$ the H-acceptor (methylene blue, quinone or alloxan) was mixed with the enzyme solution. The steady potential of the acceptor was rapidly set up, and after waiting for a sufficient period to make sure that no spontaneous reduction of the acceptor was occurring, the reagents could be added at suitable time-intervals, and the course of the reaction followed by the potential measurements. The solution was kept mixed by gentle oscillation during the experiment.

The amount of the acceptor reduced at any instant could theoretically be approximately calculated from the potential reading, making use of the values given in the literature for the normal potentials of the systems. The exact calculation however presents certain difficulties in practice. Fortunately it is not necessary for our present purpose, since the absolute amount of the reduction is unimportant and we only require to know the general course of the reaction. We have therefore preferred simply to take the potential as an index of the extent of the reduction on an arbitrary scale and to plot the observed potentials directly. In the figures therefore the ordinates represent the observed potentials with reference to the standard quinhydrone half-cell (*p*H = 2·0), expressed in mV.,

and the abscissae represent time in min. after adding the acceptor. It will be noticed that a rise in the curve corresponds with a more negative potential and therefore with a reduction of the acceptor.

### (*a*) *The alcohol dehydrogenase*

Fig. 1 shows the reduction of methylene blue by the alcohol dehydrogenase system. The enzyme and methylene blue were present initially and the potential is that of the methylene blue. On adding the alcohol no change whatever was produced, showing that the system was quite free from cozymase. On the subsequent addition of cozymase the methylene blue was rapidly reduced, as shown by the abrupt rise in the curve. As stated above, this enzyme preparation contained some flavoprotein. The addition of flavoprotein was therefore unnecessary and the methylene blue was reduced immediately upon adding the cozymase.

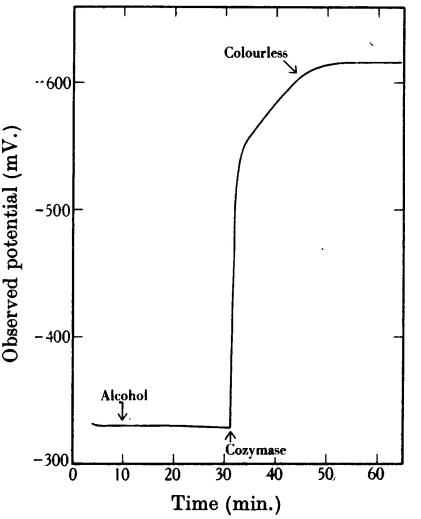



Fig. 1. Reduction of methylene blue by alcohol dehydrogenase. Initially present: 1 ml. enzyme solution + 3 ml. *M*/15 phosphate *p*H 7·3 + (added at zero time) 1 ml. *M*/2000 methylene blue. Added later as shown: 1 ml. *M* ethyl alcohol; 0·2 ml. (=0·5 mg.) cozymase. Temperature 18°.

Fig. 2 shows a similar experiment carried out with the highly purified enzyme preparation mentioned later, which was free from flavoprotein. In contrast to Fig. 1, no reduction occurred on the addition of cozymase and the presence of flavoprotein as well was necessary. Thus methylene blue is only reduced by the alcohol dehydrogenase if alcohol, cozymase and flavoprotein are all present. This fact is of course very well known already, but it is shown very clearly by this method.

The case of quinone as H-acceptor is slightly different. Fig. 3 shows the reduction of quinone by the alcohol dehydrogenase system. No methylene blue was present. Enzyme and quinhydrone were present initially; the potential is that of the latter and any change shows a reduction of the quinone. As before, no reduction occurred without cozymase. The presence of flavoprotein however was not found to be necessary, so that it appears that the reduced coenzyme can react directly with quinone. The same thing was recently found by Kubowitz [1937] for *o*-quinone, the oxidation product of catechol. Another acceptor of this type is ferricyanide, the reduction of which by dehydrogenases of this kind requires coenzyme but not flavoprotein, as shown by Quastel & Wheatley [1938].

Dickens & McIlwain [1938] also showed that phenazines, unlike other dyes, react directly with reduced coenzyme II and do not require flavoprotein for their reduction.

It should be mentioned that we found quinone to be somewhat toxic to the enzyme if added as such, owing apparently to a direct oxidizing action on the enzyme itself, an effect which has been observed with other enzymes. We found however that if added in the form of quinhydrone it was very much less toxic and this was done in the experiment of Fig. 3.

We may mention here in parenthesis that we found *m*-dinitrobenzene to be reduced by the alcohol dehydrogenase system with the addition of cozymase.

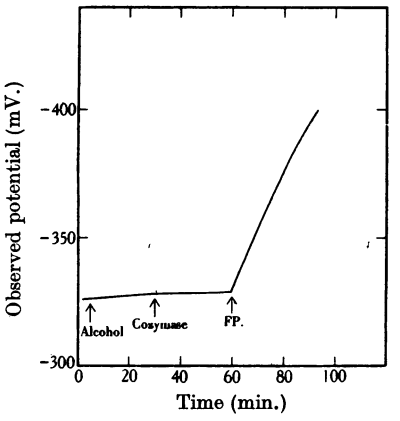



Fig. 2.

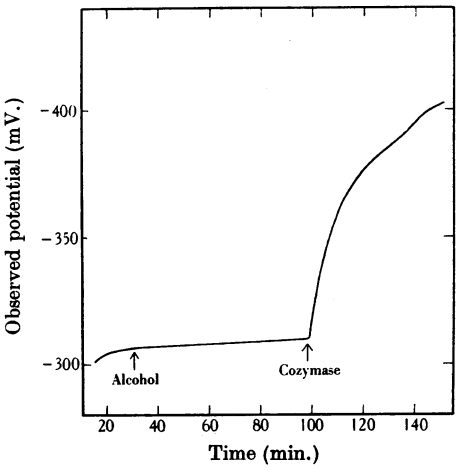



Fig. 3.

Fig. 2. Reduction of methylene blue by highly purified alcohol dehydrogenase. Initially present: 1 ml. purified enzyme solution + 2 ml. *M*/15 phosphate *p*H 7·4 + (added at zero time) 0·5 ml. *M*/2000 methylene blue. Added later as shown: 1 ml. *M* alcohol; 0·2 ml. (=0·6 mg.) cozymase; 0·1 ml. heart flavoprotein (=0·2 μg. flavin). Temperature 17°.

Fig. 3. Reduction of quinone by alcohol dehydrogenase. Initially present: 1 ml. enzyme solution + 2 ml. phosphate *p*H 7·3 + (added at zero time) 1 ml. *M*/2000 quinhydrone. Added later as shown: 1 ml. *M* alcohol; 0·2 ml. (=0·5 mg.) cozymase. Temperature 18°.

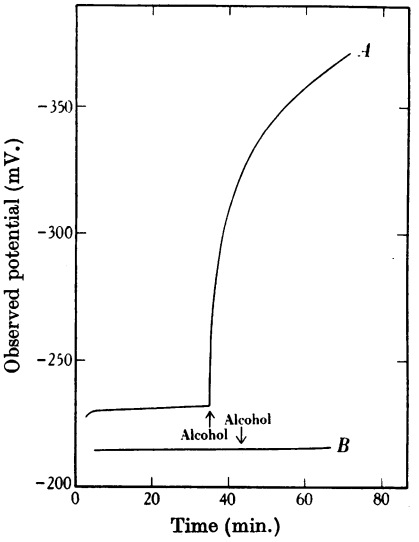



Fig. 4.

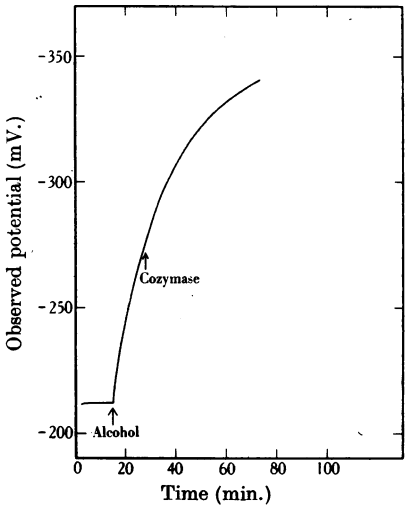



Fig. 5.

Fig. 4. Reduction of alloxan by alcohol dehydrogenase. Curve *A*: initially present: 1 ml. enzyme solution + 3 ml. *M*/15 phosphate *p*H 7·4 + (added at zero time) 0·5 ml. *M*/2000 alloxan. Added later as shown: 1 ml. *M* alcohol. Curve *B*: initially present: 1 ml. enzyme solution previously boiled 3 min. + 2 ml. phosphate *p*H 7·3 + (added at zero time) 1 ml. *M*/2000 alloxan. Added later as shown: 1 ml. *M* alcohol. Temperature 17°.

Fig. 5. Reduction of alloxan by alcohol dehydrogenase. Initially present: 1 ml. enzyme solution + 2 ml. phosphate *p*H 7·3 + (added at zero time) 1 ml. *M*/2000 alloxan. Added later as shown: 1 ml. *M* alcohol; 0·2 ml. (=0·5 mg.) cozymase.

Lipschitz [1921] has shown that this substance acts as a H-acceptor with dehydrogenases generally in a very similar way to methylene blue. We found that on incubating the dehydrogenase with alcohol, cozymase and a suspension of dinitrobenzene, a strong red colour, due to the reduction product, was produced by the addition of alkali. In a duplicate control without cozymase no trace of colour was produced. The reduction therefore, like that of methylene blue, takes place through cozymase. It was not definitely determined whether it goes through flavoprotein also, but the close similarity between dinitrobenzene and methylene blue in their behaviour with dehydrogenase systems suggests that this is the case.

All the hydrogen acceptors mentioned hitherto, whether they require flavoprotein or not, depend absolutely on the presence of cozymase for their reduction by the alcohol dehydrogenase system. The case of alloxan is very different. Fig. 4, curve *A*, shows a typical experiment. No methylene blue or quinone was present. The enzyme and alloxan were mixed initially and the steady potential due to the alloxan was quickly set up. In contrast to the other cases, as soon as the alcohol was added a rapid reduction of the alloxan occurred, in spite of the fact that cozymase was completely absent, as shown by the previous results. Thus with this acceptor the "protein" part of the system alone can catalyse the reaction and cozymase is not necessary. This striking result has been obtained with more than a dozen different preparations of the enzyme and with several different samples of alloxan. Moreover, the reaction is not accelerated if cozymase is added while it is proceeding, as Fig. 5 shows.

The reaction is of course due to the action of the enzyme and no effect is obtained if the enzyme solution is first boiled, as shown in Fig. 4, curve *B*. In different preparations the rate of change of potential runs roughly parallel with the alcohol dehydrogenase activity as tested in the usual way by the Thunberg technique. It may also be mentioned that the enzyme solutions alone, in the absence of alloxan or any other added H-acceptor, gave no potential change on the addition of alcohol. On adding both alcohol and cozymase a slight drift of potential in a negative direction was observed, which was probably due to the small amounts of flavoprotein present in the enzyme solutions. This effect was negligible in comparison with those obtained with the added aceptors.

Like quinone, alloxan was found to have a toxic action on the enzyme, an effect which has been previously found with other dehydrogenases. This does not seem to be marked with the alloxan concentrations used in these experiments, but much higher concentrations should not be used if the toxic effect is to be avoided.[1]

In order to establish our contention, we have to show not only that alloxan is reduced by the system, but that cozymase is completely absent. For this purpose direct chemical tests are of course useless, owing to their comparative insensitivity, and the most stringent test of its absence is the non-occurrence of some reaction which is rapidly catalysed by it. The most suitable reaction for this purpose is the reduction of methylene blue by dehydrogenases in presence of flavoprotein. If no reduction of the dye occurs in the absence of added cozymase, whereas the addition of a little cozymase causes a rapid reduction, it can be taken as proved that no cozymase was present initially. Such evidence

[1] The inactivation by quinone and alloxan is presumably due to the oxidation of some group in the enzyme. We have obtained some indications that after partial inactivation of the enzyme by moderately high concentrations of alloxan it may be reactivated by incubation with alcohol and cozymase. It is possible that this is due to a reduction of the group in question by reduced cozymase.

has already been presented in Fig. 1, which shows that there is not the slightest trace of reduction of methylene blue by the enzyme solution containing flavoprotein and alcohol, but a very rapid reduction on adding cozymase. This can be shown even more convincingly by the Thunberg method. In view of the results given in Table 1 there can be no doubt that our preparations were completely free from traces of cozymase. The catalytic action of small amounts of cozymase is not enhanced by the addition of alloxan.

Table 1

Quantities as follows: enzyme 1 ml. (=50 mg. original prep.); methylene blue 0·5 ml. $M/2000$; alcohol 1 ml. $M$; malate 1 ml. $M/10$; cozymase 0·5 ml. (=0·5 mg.); total volume 5 ml., made up with $M/15$ phosphate buffer $p$H 7·3. Temperature 37°. Thunberg tubes with hollow stoppers were used and the methylene blue and cozymase were placed in the stoppers. The tubes were alternately evacuated and filled with pure $N_2$ twice and finally evacuated, so as to remove all traces of $O_2$. The contents of the tubes were mixed after they had attained the temperature of the thermostat.

| | |
|---|---|
| Tube 1. Enzyme + methylene blue | No trace of reduction in 48 hr. |
| Tube 2. Enzyme + methylene blue + alcohol | No trace of reduction in 48 hr. |
| Tube 3. Enzyme + methylene blue + malate | No trace of reduction in 48 hr. |
| Tube 4. Enzyme + methylene blue + alcohol + cozymase | Complete reduction in 45 sec. |
| Tube 5. Enzyme + methylene blue + malate + cozymase | Complete reduction in 6 min. |

Although this evidence would seem to be adequate, we have carried it still further by making a very highly purified preparation of alcohol dehydrogenase. Negelein & Wulff [1937], by a complicated series of fractionations and precipitations, have obtained the enzyme from autolysed brewer's bottom yeast in a pure crystalline form, and we have made use of this method. In spite of every effort to follow their procedure exactly, we did not succeed in obtaining crystalline preparations or in attaining the very high activities reported by them, probably owing to the use of a different yeast. The yeast available to us was a bottom yeast obtained from Belgium, and the enzyme was found at almost every stage to precipitate under slightly different conditions from those given by Negelein & Wulff, so that many slight modifications were necessary. Nevertheless, by carrying through the whole procedure with these modifications we obtained a highly purified and very active preparation of alcohol dehydrogenase, completely free, as far as we could determine, from every other enzyme and coenzyme and from flavoprotein.

It is well known that cozymase undergoes rapid destruction when yeast is ground up or autolysed, and even in the early stages of purification the preparations were found to be free from any trace of cozymase by the methylene blue test. Even if minute traces had been present, we consider that it is inconceivable that any could have survived the subsequent thorough-going purification processes.

With this preparation, diluted to give a reasonable activity, the experiment of Fig. 6 was carried out. It will be seen that the purified enzyme "protein", without any additions, is able to catalyse the reaction between alcohol and alloxan just as the ordinary preparations do.

The reduction of alloxan occurring in the first few minutes of the experiment, before any alcohol had been added, was only obtained with this preparation. It is most probably due to traces of alcohol already present in the enzyme preparation. The last step in the purification of the enzyme is a precipitation by

alcohol, and it is possible that the preparation still contained a certain amount. This would cause a slight initial reduction of alloxan, such as that shown by the curve, which however soon comes to a standstill and does not interfere in any way with the main part of the experiment.

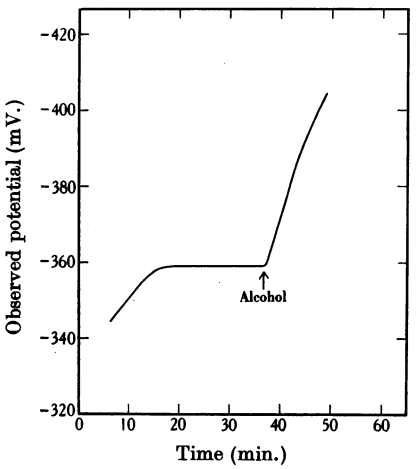



Fig. 6.

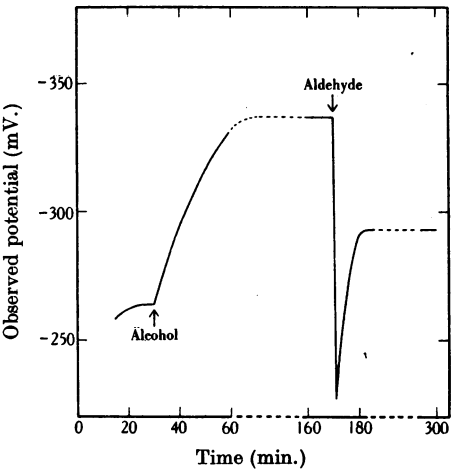



Fig. 7.

Fig. 6. Reduction of alloxan by highly purified alcohol dehydrogenase. Initially present: 1 ml. purified enzyme solution + 2 ml. *M*/15 phosphate *p*H 7·4 + (added at zero time) 0·1 ml. *M*/50 alloxan. Added later as shown: 0·5 ml. *M* alcohol. Temperature 19°.

Fig. 7. Reversibility of reaction between alcohol dehydrogenase and alloxan. Initially present: 1 ml. enzyme solution + 2 ml. phosphate *p*H 7·3 + (added at zero time) 1 ml. *M*/2000 alloxan. Added later as shown: 1 ml. *M*/10 alcohol; 1 ml. *M*/5 acetaldehyde.

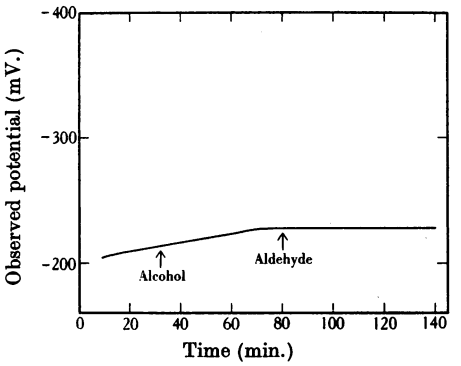



Fig. 8. Repetition with boiled enzyme. Details exactly as for Fig. 7, except that the enzyme solution was previously boiled 3 min.

Before proceeding to the malic dehydrogenase a further observation on the alcohol dehydrogenase may be of interest. The reaction between alcohol and cozymase catalysed by the enzyme is reversible and it was thought that this might also be the case for the reaction between alcohol and alloxan:

alcohol + alloxan = aldehyde + dialuric acid.

The experiment shown in Fig. 7 seems to show that this is so, although we do not wish to lay too much stress on the point, as this was the only such experiment carried out. After allowing the alcohol (one-tenth usual amount) and alloxan to react, neutral acetaldehyde was tipped in and, after a transient "kick", produced a definite change of potential in the expected direction. This effect was definitely enzymic, as Fig. 8 shows that it is not observed with boiled enzyme solution.

*(b) The malic dehydrogenase*

The behaviour of the malic dehydrogenase is similar in all essential respects to that of the alcohol system. Figs. 9–11 show the reductions of methylene blue,

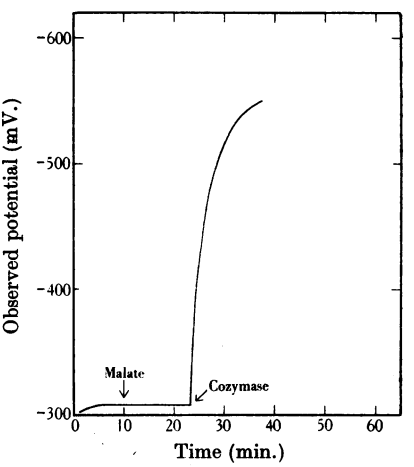



Fig. 9.

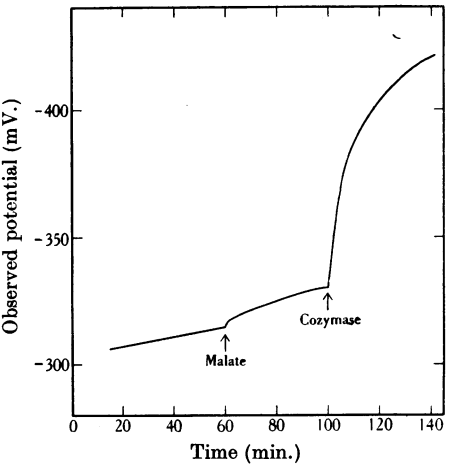



Fig. 10.

Fig. 9. Reduction of methylene blue by malic dehydrogenase. Initially present: 1 ml. enzyme solution + 2 ml. phosphate *p*H 7·3 + (added at zero time) 1 ml. *M*/2000 methylene blue. Added later as shown: 1 ml. *M*/10 malate; 0·2 ml. (=0·5 mg.) cozymase.

Fig. 10. Reduction of quinone by malic dehydrogenase. Initially present: 1 ml. enzyme solution + 3 ml. *M*/15 phosphate *p*H 7·4 + (added at zero time) 1 ml. *M*/2000 quinhydrone. Added later as shown: 0·1 ml. *M* malate; 0·2 ml. (=0·5 mg.) cozymase.

quinone and alloxan respectively, and may be compared with Figs. 1, 3 and 4. No purification of the malic enzyme comparable with that of the alcohol enzyme has yet been accomplished, so that we have no experiment corresponding with Fig. 2.

It will be seen that, as in the case of the alcohol dehydrogenase, cozymase is essential when methylene blue or quinone are used as H-acceptors; but when alloxan is used as acceptor the oxidation of malate is catalysed by the dehydrogenase in the complete absence of cozymase. The absence of cozymase from this enzyme preparation has already been shown by the results of Table 1. The reduction of alloxan is a little slower than it is with the alcohol dehydrogenase, as the malic enzyme is less active (cf. Table 1). It is easy to show that the oxidation of malate by alloxan, like that of alcohol, is enzymic, for no effect is obtained with a boiled enzyme solution, as shown in Fig. 11, curve *B*. There is no doubt that the catalysis of the reaction between malate and alloxan is due to the malic dehydrogenase; a preparation of alcohol dehydrogenase which has been freed from malic dehydrogenase gives no reduction of alloxan by malate, but a rapid reduction by alcohol.

Thus we have shown that two typical members of the group of cozymase-dependent dehydrogenases (or "pyridine-proteins") are active without cozymase, provided that the H-acceptor is suitably chosen.

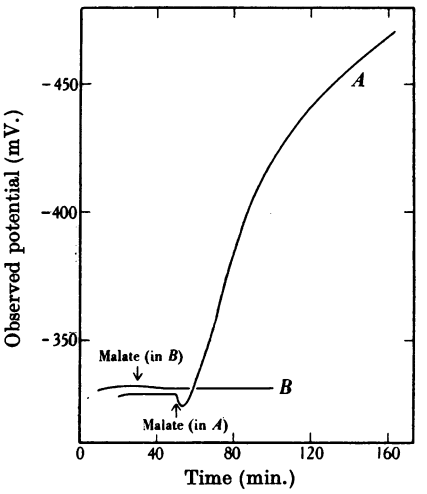



Fig. 11. Reduction of alloxan by malic dehydrogenase. Initially present: 1 ml. enzyme solution + 2 ml. phosphate *p*H 7·3 + (added at zero time) 1 ml. *M*/2000 alloxan. Added later as shown: 1 ml. *M*/10 malate. Curve *A* with fresh enzyme; curve *B* with boiled enzyme. Temperature 18°.

### (*c*) *Other dehydrogenases*

Similar results were obtained with a number of other dehydrogenases. The xanthine [Dixon, 1926], succinic and yeast lactic dehydrogenases were all found

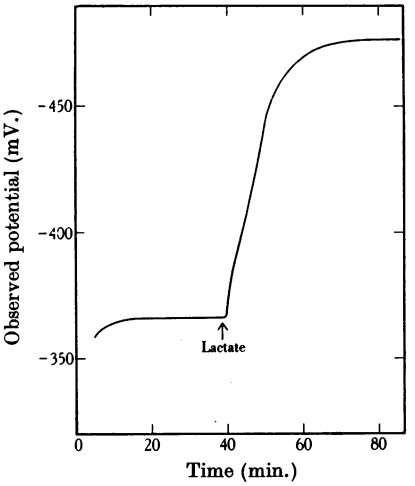



Fig. 12.

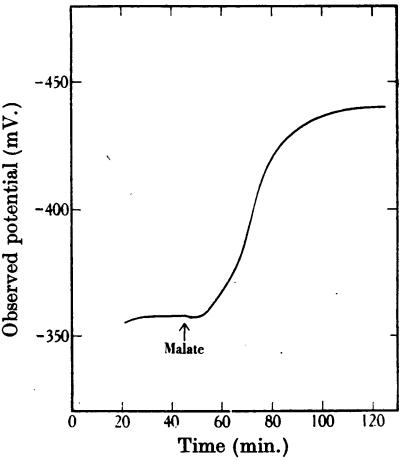



Fig. 13.

Fig. 12. Reduction of alloxan by lactic dehydrogenase of heart. Initially present: 1 ml. pig heart enzyme + 2 ml. *M*/15 phosphate *p*H 7·4 + (added at zero time) 0·3 ml. *M*/142 alloxan. Added later as shown: 0·5 ml. *M* lactate. Temperature 20°.

Fig. 13. Reduction of alloxan by malic dehydrogenase of heart. Details as for Fig. 12, but with 0·5 ml. *M* malate instead of the lactate.

to reduce alloxan in the absence of cozymase, but this is not surprising, as they do not depend upon cozymase even for their reduction of methylene blue. The lactic and malic dehydrogenases of heart muscle, however, are known to reduce dyes only in presence of cozymase. With alloxan, nevertheless, cozymase is unnecessary, just as with the two dehydrogenases first dealt with, as shown in Figs. 12 and 13 respectively. These experiments were carried out on a preparation of the enzyme obtained from pig heart muscle which was kindly given to us by Dr Dewan. These experiments have not the same evidential value as those given above, as it was not possible to be equally certain that all traces of cozymase were absent. Nevertheless, the results obtained by the Thunberg method with this preparation, given in Table 2, show that such traces, if present, are very small, and it is highly probable that these dehydrogenases also can dispense with cozymase under such conditions. The slight residual reduction is probably not due to cozymase but to traces of spontaneously reducing substances.

Table 2

Quantities as follows: enzyme 1 ml.; methylene blue 0·5 ml. $M/2000$; Na malate 0·5 ml. $M$; Na lactate 0·5 ml. $M$; cozymase 0·2 ml. (=0·6 mg.); total volume 5 ml., made up with $M/15$ phosphate buffer $p$H 7·4. Temperature 37°. Procedure as in Table 1.

| | | Reduction time |
|---|---|---|
| Tube 1. | Enzyme + methylene blue + malate + cozymase | 70 sec. |
| Tube 2. | Enzyme + methylene blue + lactate + cozymase | 100 sec. |
| Tube 3. | Enzyme + methylene blue + malate | 3 hr. |
| Tube 4. | Enzyme + methylene blue + cozymase | 4 hr. |

## IV. Estimations of the oxidation product

Not wishing to rely entirely on potentiometric measurements, we have confirmed the above results and extended them to some other H-acceptors by an entirely independent method. In the case of the malic dehydrogenase, the reaction

$$\text{malate} + \text{alloxan} = \text{oxaloacetate} + \text{dialuric acid}$$

can obviously be followed either by measuring the degree of reduction of the alloxan, as above, or by estimating the amount of oxaloacetate formed. Small amounts of oxaloacetate are readily estimated manometrically by the aniline citrate method worked out by Ostern [1933] and Edson [1935], and we have made use of this method in order to study the behaviour of the malic dehydrogenase towards not only the acceptors already dealt with but also others for which the potentiometric method is not suitable.

It was found that by making several slight modifications in the method its reliability and sensitivity could be considerably increased. One of the chief causes of inaccuracies is the pressure change, not completely explained, which develops for some minutes after the aniline-citrate mixture is diluted by mixing with the main solution, even in the absence of oxaloacetate. This is usually regarded as a "blank" to be subtracted from the reading with oxaloacetate. The compensation is not always perfect, however, as it is difficult to duplicate the conditions exactly in the control manometer. We have carried out most of our estimations as follows. Barcroft differential manometers were used, and the aniline citrate was added to both flasks of each manometer. 2 ml. water + 0·3 ml. glacial acetic acid were placed in the main body of the flasks, the aniline citrate (0·3 ml. of a mixture of equal parts of aniline and 50 % citric acid) was mixed with this at the beginning and the manometers shaken in the thermostat at 37°

for 10 min., with the taps open, by which time equilibrium had been reached. The taps were then closed, and the initial readings taken. Only then was the solution to be tested, acidified with glacial acetic acid, tipped into the solution in the right-hand flask, an equal amount of water containing acetic acid being added to the left-hand flask. The reaction was found to be complete after a further 20 min. shaking and the final readings were then taken. The manometers were read to the nearest 0·1 mm.

The use of a temperature of 37° reduced the time required for the estimations, but it is known that oxaloacetic acid decomposes spontaneously to some extent at this temperature, and experiments were therefore carried out with a solution containing a known amount of oxaloacetate in order to see whether this source of error was serious. As can be seen from Table 3, the magnitude of the error was unimportant for our purpose.[1] The results are expressed in $\mu$l. of $CO_2$, 1 $\mu$mol. oxaloacetate corresponding to 22·4 $\mu$l. $CO_2$. The table also shows that very small amounts of oxaloacetate added to the enzyme preparation can be determined satisfactorily. The blank for the enzyme was found to be zero. Some samples of malate however were found to give a small consistent blank of a few $\mu$l., even in absence of the enzyme, and the figures given below have been corrected for this where necessary.

Table 3

| | Oxaloacetate added $\mu$l. | Oxaloacetate found $\mu$l. |
|---|---|---|
| Oxaloacetate | 170 | 160 |
| Enzyme + oxaloacetate | 17 | 16·5 |
| Enzyme + oxaloacetate | 17 | 14 |
| Enzyme | 0 | 0 |

In carrying out the main experiments the dehydrogenase, malate and H-acceptor were incubated together at 30° under anaerobic conditions to allow the reaction to take place. The enzyme was then destroyed by adding glacial acetic acid and the oxaloacetic acid formed was estimated in samples of the liquid by the method just described. The malic dehydrogenase preparation used in these experiments was the same as that used for the potentiometric work.

The results obtained with alloxan as acceptor are given in Table 4. No cozymase was present in any of these experiments. Where two figures are given they refer to separate experiments. A + sign denotes that the substance in question was present in the concentration specified, a − sign indicates that it was absent.

Table 4

Final volume 25 ml. in each case, made up with $M/15$ phosphate buffer $p$H 7·4, and containing 500 mg. enzyme preparation and the following concentrations of the other reagents, where present: alloxan, 0·001 $M$; Na malate, 0·02 $M$ except in the controls without alloxan, where it was increased to 0·2 $M$.

| Enzyme | Malate | Alloxan | Time of incubation min. | Oxaloacetate found $\mu$l. |
|---|---|---|---|---|
| + | + | + | 0 | 0; 5 |
| + | + | + | 10 | 40 |
| + | + | + | 20 | 92 |
| + | + | + | 60 | 93; 94 |
| + | − | + | 60 | 0 |
| + | + | − | 60 | 0; 0 |
| Boiled | + | + | 60 | 0 |

[1] The results agree with those of Breusch [1939] just published.

These results give a complete confirmation of those obtained by the potentiometric method. No oxaloacetate is formed during the incubation when either the malate or the alloxan is missing, or when both are incubated with boiled enzyme; none is found when all the components are present but the mixture is given no time to react. But when the complete system is incubated oxaloacetate is fairly rapidly produced. Thus there is no doubt that the malic dehydrogenase can catalyse the oxidation of malate to oxaloacetate by alloxan in the absence of cozymase.

The results with alloxan can be contrasted with those obtained with methylene blue as acceptor, shown in the first part of Table 5. The fact that oxaloacetate

Table 5

Conditions as in Table 4, except that the concentration of malate in the mixture was 0·04 *M*. The same enzyme preparation was used. (diBrPhI = 2:6-dibromophenolindophenol.)

| Enzyme | Malate | Cozymase | Acceptor | Conc. of acceptor | Incubation time min. | Oxaloacetate found $\mu$l. |
|---|---|---|---|---|---|---|
| + | + | – | Meth. blue | 0·0005 *M* | 60 | 0 |
| + | + | 3 mg. | Meth. blue | 0·0005 *M* | 20 | 147 |
| + | + | 4·7 mg. | Meth. blue | 0·0005 *M* | 20 | 169 |
| + | + | – | Quinhydrone | 0·0005 *M* | 20 | 0; 5 |
| + | – | – | Quinhydrone | 0·0005 *M* | 20 | 0 |
| + | + | – | Iodine | 0·0005 *M* | 20 | 114; 94 |
| + | + | – | Iodine | 0·00065 *M* | 20 | 138 |
| – | + | – | Iodine | 0·00065 *M* | 20 | –4 |
| + | + | – | – | 0 | 20 | 0 |
| + | + | – | $H_2O_2$ | 0·002 *M* | 20 | 97 |
| + | + | – | – | 0 | 20 | 5 |
| + | + | – | $K_3Fe(CN)_6$ | 0·0005 *M* | 20 | 0 |
| + | + | – | $KMnO_4$ | 0·0005 *M* | 20 | 0 |
| + | + | – | diBrPhI | 0·001 *M* | 20 | 69 |
| + | – | – | diBrPhI | 0·001 *M* | 20 | 0 |

was rapidly formed in presence of cozymase and methylene blue, but that none at all is formed with methylene blue alone, even with an incubation three times as long, gives further evidence of the freedom of the enzyme preparation from cozymase. Some figures are also given for quinhydrone as acceptor, but it is possible that these are not so reliable as the others, as quinone seems to interfere to some extent with the aniline citrate reaction.

Certain other substances which had previously been found to act as H-acceptors with xanthine dehydrogenase are also included in the table. These substances are all reduced rather rapidly even in the absence of malate by substances present in the enzyme preparation. This made it impossible to study their reaction with the malate system by the usual method, i.e. by measuring their rate of reduction. They could however be studied by estimating the oxaloacetate formed, which would show whether they reacted with the malic system even if they were mainly used up in oxidizing other substances, or in the case of $H_2O_2$ by catalase, which was present in small amounts. In actual fact no less than three of these substances (iodine, $H_2O_2$ and dibromophenolindophenol) behave like alloxan, in that the malic dehydrogenase does not need cozymase in order to react with them. Iodine acts particularly well, although it has no action in the absence of the enzyme. The effective concentration was certainly much less than that given, as it was reduced fairly rapidly by other substances present in the enzyme preparation. In higher concentrations it was found to be toxic to the enzyme. $H_2O_2$ also acts as a direct H-acceptor, as with

xanthine dehydrogenase; it was not found to be toxic, possibly owing to the presence of some catalase. It is probably more effective than it appears, since the catalase no doubt reduced its concentration to a much lower figure. Ferricyanide had already been shown by Quastel & Wheatley to require cozymase in order to react with cozymase-dependent dehydrogenases.

The case of the indophenol dye is interesting, as all the other dyes hitherto tested have been found to react with such systems only through cozymase. This dye has an unusually high oxidation potential, but it is not clear whether its behaviour is due to this or whether other members of the indophenol series would behave similarly. Owing to its high potential it is reduced fairly rapidly by the enzyme solution itself and the presence or absence of malate has little effect on its reduction time as determined by the Thunberg method. Nevertheless, at the conclusion of the Thunberg experiments also the presence of oxaloacetate was definitely confirmed in the tubes containing the enzyme, malate and indophenol, though none could be detected when one of the components was missing. Thus this dye can be reduced by the activated malate directly. But it can also be reduced, like other dyes, through cozymase and flavoprotein, for the addition of cozymase did accelerate its reduction by the malic system.

It should be possible to study the reaction of alloxan with the alcohol dehydrogenase, like the malic dehydrogenase, by estimations of the oxidation product. Owing to certain technical difficulties, however, we have not obtained satisfactory quantitative results with this system. The difficulty is in estimating very small amounts of aldehyde in solutions of the enzyme preparation containing comparatively large amounts of alcohol. Only small amounts of aldehyde can be expected, for the alloxan concentration is small and cannot be increased because of its toxicity to the enzyme. The alcohol concentration, on the other hand, must be kept up owing to the rather small affinity of the enzyme for alcohol. We have not succeeded in finding a satisfactory method for use under these conditions. Though we were able, by the isolation of the dinitrophenylhydrazone from distillates, to obtain qualitative evidence that aldehyde was formed in the reaction with alloxan in the absence of cozymase, we have no quantitative results on this system to offer comparable with Tables 4 and 5. In view of the definiteness of the potentiometric results, however, and the close similarity in the behaviours of the two systems, we do not feel that this is a serious omission.

*Further comments on the above experiments*

It has been shown that several of the substances tested can replace cozymase as H-acceptors in the dehydrogenase systems. In order to avoid misunderstandings it should be made clear that we do not claim that these substances can act as coenzymes for these systems. Cozymase acts as a coenzyme because it is not only reduced by the dehydrogenases but is then reoxidized by other systems (flavoproteins, etc.). Alloxan and the other substances do not seem to act as coenzymes because, although they are reduced like cozymase by the dehydrogenases, they are not re-oxidized by the other systems. In the case of alloxan it might be thought that it should act as a coenzyme, because its reduced form, dialuric acid, can reduce methylene blue and is also oxidized by $O_2$. No coenzyme action, however, could be observed. But dialuric acid itself, when added to the enzyme solution, failed to reduce methylene blue or to take up $O_2$, although it did so readily in the absence of the enzyme. The fact is that the oxidation of dialuric acid is due to a rather complicated heavy-metal catalysis, and it is well known that many proteins and tissue preparations are apt to

inhibit such reactions, presumably by forming inactive complexes with the catalytic metals. There is no doubt that this, and not a failure to become reduced by the dehydrogenase, is the reason why alloxan did not act as a co-enzyme in our experiments. The mechanism of the oxidation of dialuric acid is a quite separate problem, with which we are not at the moment concerned.

By a curious coincidence, while this work was in progress Bernheim [1938] published a paper showing that the aerobic oxidation of alcohol by liver suspensions is strongly catalysed by alloxan. In some cases the rate of oxidation was increased by 1000% by alloxan in a concentration of $M/10{,}000$. This effect was not explained, but it seems probable that it is connected with the effects reported here, though one cannot be dogmatic on the point, for Bernheim obtained negative results with the oxidation of lactate, whereas in our experiments (Fig. 12) the lactic dehydrogenase behaved towards alloxan in the same way as the alcohol dehydrogenase.

Bernheim tested alloxan because of its relation to the flavins; it is used in their synthesis and can be regarded as forming part of the flavin molecule. It has been suggested to us that our results may be connected with that relationship. We do not think that this is the case, for the following reasons: (1) The relationship is not really very close from an oxidation-reduction point of view: the part of the alloxan molecule which is active in these reactions, namely the group which is reduced to form dialuric acid, has disappeared in the flavin molecule. Also the flavin group itself, without the carbohydrate side chain, phosphoric acid and protein, is inactive in any case. (2) The suggested relationship is not to cozymase but to flavin, which does not react with the dehydrogenases and has quite a different function. (3) We have shown that other quite unrelated substances can act in the same way as alloxan.

It should be made clear that the substances which we have shown to act like cozymase as direct H-acceptors do not equal cozymase in efficiency. Data for an exact comparison are lacking, but in the case of the malic system a rough calculation of relative velocities can be made from the data of Tables 1 and 4, making due allowance for the difference in conditions. This indicates that cozymase is reduced from three to six times as rapidly as alloxan by the malic enzyme under similar conditions. The difference is probably not so great in the case of $I_2$, but the effective concentration of $I_2$ cannot be determined. It is of course to be expected that the enzyme would react most readily with its natural H-acceptor, cozymase.

Finally, it may be objected that alloxan is unstable in aqueous solution. Richardson & Cannan [1929] have stated, purely on the basis of a drift in their observed potentials and without any chemical evidence, that in alkaline and even in neutral solutions alloxan rapidly changes into alloxanic acid, the half-life period of alloxan at *p*H 7 being only 1 min. at 30°. This is definitely not the case. Hill & Michaelis [1933] have shown that the potential drift in question is abolished by the addition of small amounts of iron salts, which catalyse the reaction of the system with the electrode. It is therefore due to some other cause. Alloxan is in fact reasonably stable in neutral solution. The change to alloxanic acid, which involves the opening of the 6-membered ring and reclosure to give a 5-membered ring, requires heating of the alloxan with strong alkali. Behrend & Zieger [1915] have shown that if KOH is added to a solution of alloxan (*p*H about 8) the product which crystallizes after some time (in 70% yield) is the pure potassium salt of alloxan and not that of alloxanic acid. The potassium salt of alloxanic acid is produced only on allowing the mixture to stand "for some hours" or on warming. Liebig & Wöhler [1841] showed that

on boiling aqueous solutions of alloxan it is decomposed into alloxantin, parabanic acid and $CO_2$, but the process is a slow one, even at the boiling point, and would certainly be negligible at room temperature. It is true that alloxan solutions undergo a change in conductivity on standing for many hours [Wood, 1906]. The change is most likely a simple one, such as the enolization HN—CO $\rightleftharpoons$ N=C(OH) which occurs in the analogous purine compounds.

The question does not really affect our work in any vital respect, for it is immaterial to our argument whether the substance which acts as a direct H-acceptor with the dehydrogenases is alloxan itself or some other substance derived from it. In particular, the most likely derivative, alloxantin, could have been used in our experiments instead of alloxan, as it bears the same relation to alloxan as quinhydrone does to quinone. We have indeed repeated the most important potentiometric experiments using alloxantin and obtained similar results. We believe, nevertheless, that no appreciable decomposition can have taken place under the conditions of our experiments, which were of short duration, at almost neutral *p*H and at reasonably low temperatures. Moreover, our results do not depend only on experiments with alloxan.

## V. Discussion

The view that the two coenzymes form the prosthetic groups of dehydrogenases is undoubtedly very attractive. The close analogy with flavoprotein enzymes like the *d*-amino-acid oxidase, in which the prosthetic flavin group is chemically related to cozymase and is reduced by the substrate in much the same way, is admittedly a strong argument in its favour. It is however the only argument advanced in its favour, and there are several strong arguments against it. Even this argument from analogy can be used in two ways, for nucleotides not only form prosthetic groups but also act as carrier-substrates. For instance, adenylic acid, which also is chemically related to cozymase, plays very much the same role in phosphate transport as cozymase does in hydrogen transport: it comes into relation with many enzymes, but it is not assigned as prosthetic group to any of them. Cozymase likewise does not remain permanently attached to any one enzyme; it comes into relation with many enzymes, but like adenylic acid is for the most part free in solution.

The following are some of the arguments which may be advanced against the view that cozymase is to be regarded as the prosthetic group of the dehydrogenases with which it acts:

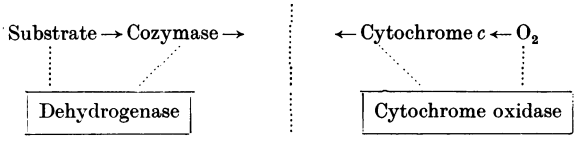
(1) The type of union between coenzyme and dehydrogenase is that which we have come to associate with the union of enzyme and substrate, rather than the type which is met with in conjugated proteins. In conjugated proteins the prosthetic group is attached to the protein by a definite chemical combination, and it can only be separated from the protein by splitting with acid or some equally drastic process. The combination of coenzyme with dehydrogenase, like that of substrate with enzyme, is highly dissociated, almost instantaneously reversible when the concentration of free coenzyme is altered and comes to a definite equilibrium when only a small part of the total coenzyme is combined. The value of the affinity of the enzyme for cozymase is quite a common one for enzyme-substrate combinations. Thus the analogy with conjugated proteins breaks down in this respect.

(2) The dehydrogenase catalyses the reaction between two substances, the specific substrate of the enzyme and cozymase (see the introduction), and it is difficult to see why one should be chosen as the prosthetic group of the enzyme

rather than the other. There seems in fact to be no criterion for such a distinction. They both combine dissociably with the enzyme in much the same way; as we have seen the affinity of the enzyme for aldehyde is the same as its affinity for cozymase, so that the strength of the forces involved is the same in each case; the combination is specific in both cases—if anything it is less specific for cozymase, which combines with many different enzymes. If we are to speak of "pyridine-proteins" it seems that we should be equally justified in speaking of "aldehyde-proteins", a term which would include the Schardinger enzyme, the aldehyde oxidases of liver and potato, alcohol dehydrogenase and aldehyde mutase. The fact that cozymase has a carrier function in the cell, which most of the other substrates have not, is not a valid reason for making the distinction; for the fact that the cozymase goes on to react with other systems after leaving the dehydrogenase is irrelevant so far as the dehydrogenase itself is concerned.

(3) There is evidence [Haas, 1937] that cozymase combines with flavoprotein when it reduces the flavin group, just as it combines with the dehydrogenase when it oxidizes the substrate. If it is to be regarded as a prosthetic group when combined with the dehydrogenase it must logically be so regarded when combined with flavoprotein also. Thus the flavoprotein must then be considered a pyridine-protein, and would have two prosthetic groups.

(4) The two terminal systems of the main catalytic chain in the respiratory mechanism can almost be regarded as functionally mirror images of one another:




The relationship of cytochrome *c* to cytochrome oxidase shows a close analogy (when we substitute oxidation for reduction) with that of cozymase to the dehydrogenase. Logically therefore we should have to consider cytochrome *c* as the prosthetic group of cytochrome oxidase.

(5) Cozymase is hydrolysed by a phosphatase, which of course combines with it in order to bring about the reaction. There seems to be no logical basis for making a distinction between the combination of cozymase with phosphatase and its combination with dehydrogenase, so that the former would also have to be considered a pyridine-protein, though only when it catalyses this particular reaction. Thus it would sometimes be a pyridine-protein and sometimes not, just as the dehydrogenase would have to be considered as sometimes a pyridine-protein and sometimes not (e.g. when it catalyses the reaction with alloxan, etc.).

The three last points are given as examples of the confusion which would follow from the logical application of the idea.

(6) In addition to its normal reaction, succinic dehydrogenase catalyses the reverse reaction:

$$\text{fumarate} + \text{CoH}_2 = \text{succinate} + \text{Co},$$

where Co represents cozymase [Dewan & Green, 1937]. This reaction is exactly analogous to the reverse reaction of the alcohol dehydrogenase:

$$\text{aldehyde} + \text{CoH}_2 = \text{alcohol} + \text{Co}$$

and the corresponding reactions of other cozymase-dependent dehydrogenases. The closeness of the analogy is such that if the cozymase is to be regarded as the prosthetic group in these cases it must also be so regarded in the succinic system.

But the succinic dehydrogenase is one of the coenzyme-independent enzymes; it is not a pyridine-protein, for its normal forward reaction succinate + methylene blue (etc.) = fumarate + $MBH_2$ (etc.) is quite independent of the presence of either of the coenzymes.

(7) The most fundamental distinction between a substrate and a prosthetic group of an enzyme is as follows.[1] If to a solution containing the substance in question (prosthetic group or substrate), together with excess of the specific substrate of the enzyme, a small amount of the enzyme protein is added and allowed time to act, an unlimited amount of the substance will be reduced if it is a substrate, whereas if it forms the prosthetic group the amount reduced cannot be more than stoichiometrically equivalent to the enzyme protein added. That is to say if we add 100 molecules of the enzyme protein, each molecule of which combines with one molecule of the substance as its prosthetic group, not more than 100 molecules of the substance can become reduced. On the other hand if the substance is a substrate of the enzyme, 100 enzyme molecules, given time, could reduce an unlimited amount of it. Let us apply this test, for purposes of comparison, to the amino-acid oxidase and the alcohol dehydrogenase. Warburg & Christian [1938] have shown that if a small amount of the protein of the oxidase is added to a mixture of flavin-adenine-dinucleotide and alanine no visible reduction of the flavin occurs. Only that small fraction which is combined as part of the enzyme is reduced by the alanine: the free flavin-dinucleotide does not form a substrate for the enzyme, and is not reduced. In order to reduce all of the dinucleotide an amount of the oxidase protein equivalent to it must be added, when it all becomes combined as part of the enzyme. If on the other hand a small amount of the alcohol dehydrogenase protein is added to a mixture of cozymase and alcohol the whole of the cozymase is rapidly reduced. By this criterion therefore the flavin nucleotide forms the prosthetic group of the amino-acid oxidase, whereas cozymase is definitely a substrate of the dehydrogenase.

(8) The complex [dehydrogenase + cozymase], *as such*, cannot be regarded as a catalyst in the strict sense. The dehydrogenase is a true catalyst, catalysing the reaction between cozymase and, say, alcohol. The cozymase is also a true catalyst, catalysing the reaction between the dehydrogenase-alcohol complex and flavoprotein. The dehydrogenase-cozymase complex, however, cannot as such be regarded as catalysing any reaction. One part of the complex is reduced by alcohol; the complex then breaks down, the cozymase part migrates to form new complexes with other systems, while the dehydrogenase part combines with another cozymase molecule and the process is repeated. If the complex is not strictly a catalyst it seems undesirable to apply the term enzyme to it.

(9) The final argument is provided by the observations described in this paper. We have shown that the dehydrogenase proteins, under proper conditions, can bring about their respective oxidations in the complete absence of the alleged prosthetic group. They are therefore complete enzymes in themselves. A prosthetic group which does not form an essential part of the enzyme seems to be a contradiction in terms, and since the enzymes can act without cozymase we do not think it is any longer possible to regard cozymase as their prosthetic group. The term pyridine-protein consequently becomes misleading and its use should be discontinued.

We have not dealt with coenzyme II in our experiments, but its general behaviour and mode of action are so similar to those of cozymase that there is little doubt that the same considerations apply to it.

[1] This criterion was suggested to us by Prof. Keilin.

By no means all anaerobic dehydrogenases depend for their power of reducing methylene blue etc. on one or other of these two coenzymes. Quite a number are altogether independent of these coenzymes and their properties strongly suggest that they do not depend on a coenzyme at all. In this group may be included the following dehydrogenases: succinic, lactic of yeast, α-hydroxyglutaric, α-glycerophosphoric (insoluble), choline dehydrogenases and some others. These can all reduce methylene blue in the absence of cozymase and coenzyme II. The view expressed in this paper brings the coenzyme-dependent dehydrogenases more into line with these coenzyme-independent dehydrogenases, and makes it possible to consider the anaerobic dehydrogenases as one group of enzymes. The difference becomes simply one of enzyme specificity towards the H-acceptor: one group of enzymes can react directly with many acceptors, including methylene blue, quinone, alloxan and other substances; the other is more specific and cannot react directly with methylene blue or quinone, but can react with a number of other substances, including alloxan and cozymase.

On the view that the coenzymes form prosthetic groups it is of course still possible to bring the two classes of enzymes into line by assuming that the coenzyme-independent dehydrogenases contain other prosthetic groups of unknown nature. There is, however, no indication that this is the case. Attempts to decide the point are at present being made.

In conclusion we would suggest that the growing tendency to refer to dehydrogenases[1] and certain other enzymes simply as proteins is not one to be encouraged, as it tends to obscure their essentially enzymic character. No doubt enzymes are proteins, but not all proteins are enzymes, and the great bulk of the protein of the body is catalytically inactive. It is important to retain some term by which these highly specialized catalytic proteins may be distinguished from ordinary proteins; just as for example it is convenient to retain the term virus, although the viruses are also proteins.

Even in the enzymes which have prosthetic groups, such as the flavoproteins, the protein parts are by no means to be regarded as ordinary proteins. It is the protein part which determines the specificity and which contains the "active centre", or group which specifically combines with the substrate and thereby produces that profound change in its properties which we speak of as activation.

[1] The term dehydrogenase (or dehydrase) has been in regular use for a great many years in the sense of its original definition, namely to denote an enzyme which, with or without the addition of a coenzyme, brings about the dehydrogenation of its substrate by hydrogen acceptors. The term "oxidase system" has been kept for the complete system (usually containing more than one enzyme) which enables the substrate to react with $O_2$. For example the succinoxidase system consists of the succinic dehydrogenase, which enables succinate to reduce methylene blue or cytochrome, together with cytochrome *c* and cytochrome oxidase. Very recently however some workers have begun to use the term dehydrogenase in a quite unorthodox sense, namely to denote complete oxidase systems. For example it is applied to a system consisting of dehydrogenase + coenzyme + diaphorase, or one artificially built up from alcohol dehydrogenase + cozymase + catechol + catechol oxidase. This is calculated to cause the greatest confusion, and we suggest that the term should be retained with its original significance.

The argument that the "proteins" are not dehydrogenases because they cannot by themselves dehydrogenate substances cannot be accepted. The succinic dehydrogenase cannot dehydrogenate succinate without the addition of methylene blue or its equivalent, but it is none the less a dehydrogenase. In fact no enzyme can act in the absence of its substrate. Although the "proteins" may require the addition of coenzyme before any action occurs, they are dehydrogenases in the strictest sense, since they catalyse the dehydrogenation of their substrates by coenzyme much as the succinic dehydrogenase catalyses the dehydrogenation of succinate by methylene blue.

The prosthetic flavin group, which is common to many enzymes, merely acts as an acceptor of hydrogen from the substrate so activated. Thus the protein parts of these enzymes, and the anaerobic dehydrogenases themselves, may be regarded as analogous to the hydrolysing enzymes, which are proteins containing active centres but in all probability no prosthetic groups, as pointed out by Northrop [1935].

SUMMARY

1. Two typical cozymase-dependent dehydrogenases, the alcohol and malic dehydrogenases of yeast, have been studied with a wider range of hydrogen acceptors than hitherto. The substances used were those previously shown to function as acceptors with the xanthine dehydrogenase.

2. The H-acceptors fall into three groups:

(*a*) those which require the presence of cozymase and flavoprotein: dyes (with certain exceptions), *m*-dinitrobenzene (probably), cytochrome etc.;

(*b*) those which require the presence of cozymase but not flavoprotein: benzoquinone, *o*-quinone, phenazine dyes, $K_3Fe(CN)_6$;

(*c*) those which do not require the presence of either cozymase or flavoprotein: alloxan, $I_2$, $H_2O_2$, dibromophenolindophenol.

Thus cozymase is not necessary for all acceptors. Cozymase is itself only one of several acceptors which react directly with the activated substrate.

3. It has been shown by two independent methods that the dehydrogenases alone (i.e. the so-called "proteins") can bring about the oxidation of their respective substrates in the complete absence of cozymase when acceptors of group (*c*) are used. Thus the "proteins" are in fact complete enzymes.

4. The relationship of dehydrogenase to coenzyme is that of enzyme to substrate rather than that of protein to prosthetic group. A number of arguments is given against the view that the coenzymes are to be regarded as the prosthetic groups of the dehydrogenases, and it is suggested that the conception of "pyridine-proteins" is misleading and should be abandoned.

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