20. THE SPECIFICITY OF DIAPHORASE (COENZYME FACTOR)

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THE independent discovery of diaphorase by Adler *et al.* [1937, 1] and by Dewan & Green [1937], who used the name coenzyme factor, was accompanied by disagreement with regard to the specificity of this enzyme. Dewan & Green [1938], on the basis of Thunberg experiments with the Robison ester system, using skeletal muscle as a source of the coenzyme factor, maintained that it reacted with the reduced form of codehydrogenase II (Co II) as well as that of codehydrogenase I (Co I, cozymase). Euler & co-workers [1938] and Adler *et al.* [1939, 3], employing the spectrophotometric technique and using heart muscle and yeast as starting materials, stated that partially purified preparations of diaphorase reacted only with reduced Co I. Later experiments by Adler *et al.*, however [1939, 2], indicated that the diaphorase action of crude aqueous extracts from acetone-dried tissues depended on the organ used as starting material. While, in the case of heart, the reaction with Co II was very much slower than that with Co I, in the case of adrenal gland the two reactions were of comparable velocity. Liver occupied an intermediate position. These results were explained most simply by assuming the existence of two diaphorases, the first of which acted specifically with Co I while the second was either specific for Co II or else acted with both coenzymes. These were called diaphorase I and diaphorase II respectively.

Recently Straub [1939] has obtained from heart muscle a pure flavoprotein, which Corran *et al.* [1939] showed was almost certainly identical with the heart coenzyme factor. The pure preparation was stated to be active with both coenzymes, but the reaction with Co II was not studied in detail. The relation of the coenzyme factor to the yeast flavoprotein of Haas [1938], which was also said to react with both coenzymes, is not at present clear.

The experiments presented here, which were designed to elucidate the question of specificity, clearly support the view that there are two diaphorases. One of these, which acts only with Co I, occurs in preparations from heart muscle practically unaccompanied by the other.

EXPERIMENTAL

Spectrophotometric measurements. These were kindly carried out by Mr G. Günther. The rate of reaction in the system: reduced coenzyme + diaphorase + methylene blue + O_2 was measured by decrease in the absorption at 334 mμ in the usual manner. The coenzyme solution consisted of a mixture of Co I and Co II, prepared enzymically from cozymase by the method of Adler *et al.* [1939, 1] and reduced before use by hydrosulphite.

Thunberg experiments: (*a*) the diaphorase I activity of solutions was measured from the reduction time of methylene blue in the system: CoH_2 I + diaphorase + MB. In each tube were used: 0·2 ml. CoH_2 I (100 γ), 0·25 ml. $M/2$ phosphate

*p*H 7·6, 0·25 ml. MB (1 : 5000), an appropriate quantity of the solution to be tested and water to bring the total volume to 1·5 ml. It should be noted that the non-enzymic reduction of MB by CoH_2 I is very strongly catalysed by red light [cf. Adler *et al.* 1937] and that it is therefore essential for the thermostat to be shielded from bright daylight.

(*b*) An attempt was first made to measure the diaphorase II activity of solutions by the use of the Robison ester dehydrogenase system.[1] This was abandoned when it was found that some tissue extracts, especially extracts from liver, contained a strong inhibitor of the reaction. Experiments were therefore made with the *iso*citric dehydrogenase system,[2] which Adler *et al.* [1939, 4] had found to require Co II as the specific coenzyme. In each tube were used: 0·1 ml. *iso*citrate solution, 0·25 ml. *iso*citric *apo*dehydrogenase solution (18 mg./ml. of "enzyme C" from heart muscle, practically free from diaphorase [cf. Adler *et al.* 1939, 4], 0·25 ml. Co II (12·5 μg.), 0·2 ml. of a solution containing Mn^{++} ($5\times10^{-3}M$) and Mg^{++} ($2{\cdot}5\times10^{-2}M$), 0·25 ml. veronal-acetate buffer (Michaelis) *p*H 7·66, 0·5 ml. MB (1 : 5000), an appropriate quantity of the solution to be tested and water to make the total volume up to 2·0 ml. The Co II was prepared enzymically from Co I [Adler *et al.* 1939, 1]. In this system the inhibition referred to above occurred in some cases when the concentration of the *iso*citrate solution used was $M/50$, but it was almost completely abolished by increasing this concentration to $M/10$.

All diaphorase activities measured by Thunberg experiments were controlled by comparison with the activity of a standard "old" flavin enzyme solution.

Preparation of extracts from acetone-dried tissues

Extracts were prepared from heart, liver and adrenal gland as follows. The fresh material was freed from fat and connective tissue, minced, and then dried by shaking twice with four times its volume of acetone and subsequent exposure to air in a thin layer. 5 g. of the dry powder were then ground with 2·5 g. sand and 25 ml. water for $\frac{1}{2}$ hr. and the resulting mixture pressed through muslin and centrifuged. The solution was dialysed for 20 hr. against running tap-water and the precipitate which formed centrifuged off. In this manner clear reddish solutions were obtained from heart and liver (12 and 17 ml. respectively). The solution from adrenal gland (12 ml.) could not be centrifuged clear.

Partial purification of heart and adrenal gland diaphorase by the method of Straub [1939]

In the case of heart this purification was carried as far as the elution from alumina Cγ. The eluate was dialysed against running tap-water for 40 hr., brought to *p*H 7·5 with $N/10$ NaOH and centrifuged. From one pig heart there were obtained 30 ml. of a clear yellowish solution.

The purification of adrenal gland diaphorase was carried out as follows: 700 g. adrenal gland were freed from fat and minced. The mince was washed 10 times with 500 ml. of water, the liquid being pressed out gently through muslin. The residue (430 g.) was ground for 1 hr. with sand and 600 ml. of $M/50$ Na_2HPO_4 (added in small portions); 200 ml. water were then added and the mixture centrifuged. To the resulting solution were added 25 ml. of $M/1$ acetate buffer *p*H 4·5. The precipitate was centrifuged off, suspended in 400 ml. water + 8 g.

[1] In this connexion it may be noted that extracts from adrenal gland contain a strong, Co II-specific, Robison ester dehydrogenase.

[2] We are grateful to Dr C. Martius, of Tübingen, for a gift of *iso*citric acid.

$(NH_4)_2SO_4$ + 12 ml. ethyl alcohol and the suspension heated 15 min. at 43°. After cooling and centrifuging the solution was dialysed for 18 hr. Adsorption was then effected by the addition of two 15 ml. portions and then one 10 ml. portion of alumina Cγ (1·7 %). The remaining solution had a weak brownish-yellow colour. Elution from the alumina was carried out with 12 ml. portions of $M/5$ Na_2HPO_4 and the eluate dialysed for 40 hr. against running tap-water. The solution was brought to *p*H 7·5 with $M/10$ NaOH and the small white precipitate centrifuged off. This procedure gave 140 ml. of a clear yellowish solution.

Results

Spectrophotometric investigations with diaphorase solutions from heart and adrenal gland

When it had been established that in Thunberg experiments certain tissue extracts inhibited the reaction designed to measure the activity of diaphorase II the possibility was considered that such an inhibition was responsible for the Co I specificity, previously reported from this Institute, of diaphorase solutions prepared from heart and yeast. Fig. 1 shows clearly that this is not the case.

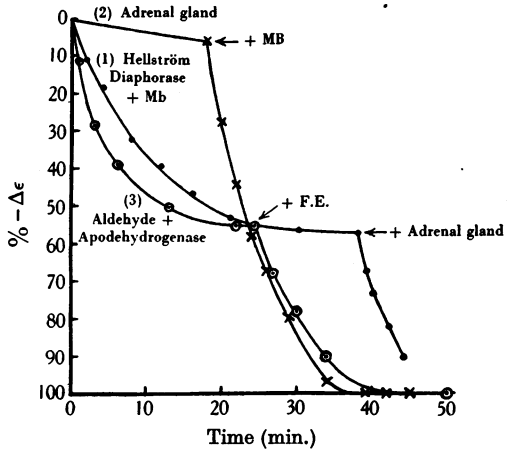

Fig. 1. Spectrophotometric experiments. Oxidation of a mixture of CoH_2I and CoH_2II. Curve (1) corrected for non-enzymic reaction between CoH_2I and Mb [cf. Adler *et. al.* 1939, 3].

Curve (3) shows the relative proportions of CoH_2 I and CoH_2 II in the coenzyme solution used, aldehyde in the presence of alcohol *apo*dehydrogenase oxidizing only the CoH_2 I and the remaining CoH_2 II being subsequently oxidized by the addition of "old" flavin enzyme (F.E.). Curve (2) shows the effect of using an extract of acetone-dried adrenal gland as a source of diaphorase. It is evident, (*a*) that there is very little aerobic reaction in the absence of MB, and (*b*) that the diaphorase transfers hydrogen from both reduced coenzymes to MB. Curve (1) shows that diaphorase prepared from heart by the method of Euler & Hellström [1938] transfers hydrogen only from reduced Co I, but that on addition of adrenal gland extract CoH_2 II also reacts. The difference in the diaphorase actions of the solutions obtained from heart and adrenal gland is therefore not due to the presence of an inhibitor in the former solution.

Comparison of the diaphorase I and diaphorase II activities of acetone-dried tissue extracts by the Thunberg technique

In all the following Figs. the diaphorase activity is expressed in the form $(I/T - I/T_0) \times 100$. T is the time, in minutes, of MB decoloration in the presence of the given diaphorase solution and T_0 is the corresponding time in the absence of diaphorase. The activity is plotted against the volume, in ml., of the solution used. In each case the "old" flavin enzyme solution used as a control is of the same concentration.

In Table 1 are given figures relating to the diaphorase I activities of extracts from liver, adrenal gland and heart. F.E. denotes the standard "old" flavin enzyme solution.

Table 1

Diaphorase I

Enzyme	T (min.)	$(I/T - I/T_0) \times 100$
0·2 ml. F.E.	2·75	34
0·1 ml. F.E.	5·0	18
0·05 ml. F.E.	9·0	9
0·025 ml. F.E.	16·0	4
0·1 ml. liver	4·0	23
0·05 ml. liver	7·75	12
0·025 ml. liver	14·0	5
0·1 ml. ad. gland	2·75	34
0·05 ml. ad. gland	5·0	18
0·025 ml. ad. gland	9·0	9
0·1 ml. heart	1·66	58
0·05 ml. heart	3·25	29
0·025 ml. heart	6·0	15
—	60 ($=T_0$)	0

Fig. 2 shows the relative diaphorase II activities of the same solutions, measured in the *iso*citric acid dehydrogenase system. In this system the decoloration time without diaphorase (T_0) was 80 min. The increase in activity on adding flavin enzyme to experiments already containing solutions from heart and adrenal gland indicates that there is no inhibition of the reaction, but in the case of liver a small inhibition appears to occur. From a consideration of Table 1 and Fig. 2 an approximate estimate can be made of the ratio, diaphorase I activity/diaphorase II activity, in the three solutions. These are as follows:

Adrenal gland	4·5 : 1
Liver	20 : 1
Heart	130 : 1

The value for liver is a maximum as a certain amount of inhibition of the *iso*citric dehydrogenase appears to occur in this case.

The values in Fig. 2 were obtained by the use of $M/10$ *iso*citrate (0·1 ml.) in the test system. Fig. 3 shows the effect of employing $M/50$ *iso*citrate. It is apparent that, while there is still no inhibition with adrenal gland, in the case of liver this has become very marked. With the solutions obtained from heart the inhibition was so strong that all values of $(I/T - I/T_0)$ were negative: for this reason they are not shown.

In the main the results described in this section confirm those previously obtained by Adler *et al.* [1939, 2], with extracts from different acetone-dried tissues, using the spectrophotometric technique.

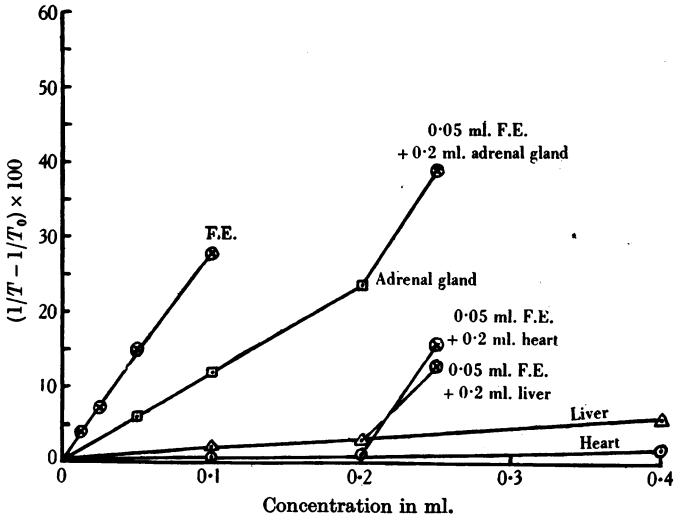

Fig. 2. Thunberg experiments. Extracts from acetone-dried organs. Diaphorase II activity ($M/10$ *iso*citrate).

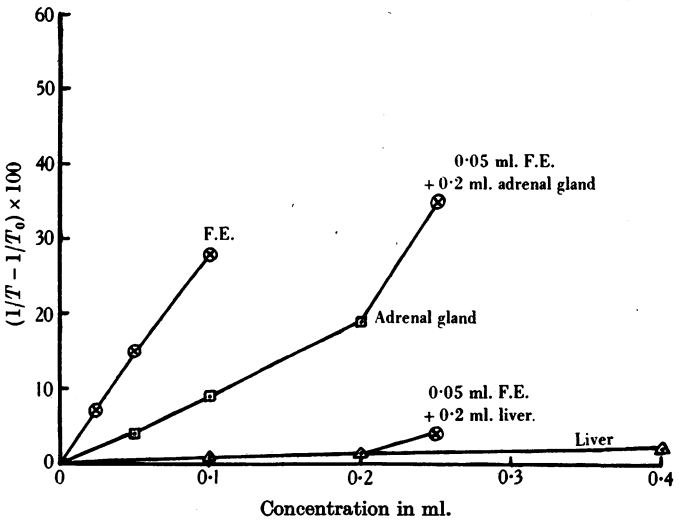

Fig. 3. Thunberg experiments. Extracts from acetone-dried organs. Diaphorase II activity ($M/50$ *iso*citrate).

Comparison of the diaphorase I and diaphorase II activities of heart and adrenal gland extracts partially purified by the method of Straub [1939]

In view of the fact that the pure flavoprotein of Straub was said to be active with both coenzymes it was considered of interest to compare the diaphorase I and diaphorase II activities of extracts from heart and adrenal gland which had been partially purified by the same procedure. The purification has already been described.

The relative diaphorase I and diaphorase II activities are shown in Figs. 4 and 5. In both cases there was no inhibition in the *iso*citric dehydrogenase system when *M*/50 *iso*citrate was used. It is evident that the diaphorase II

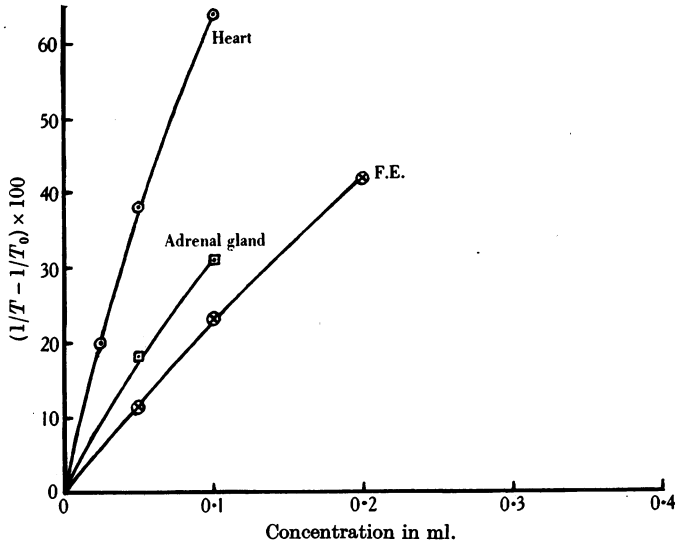

Fig. 4. Thunberg experiments. Heart and adrenal gland extracts partially purified by the method of Straub. Diaphorase I activity.

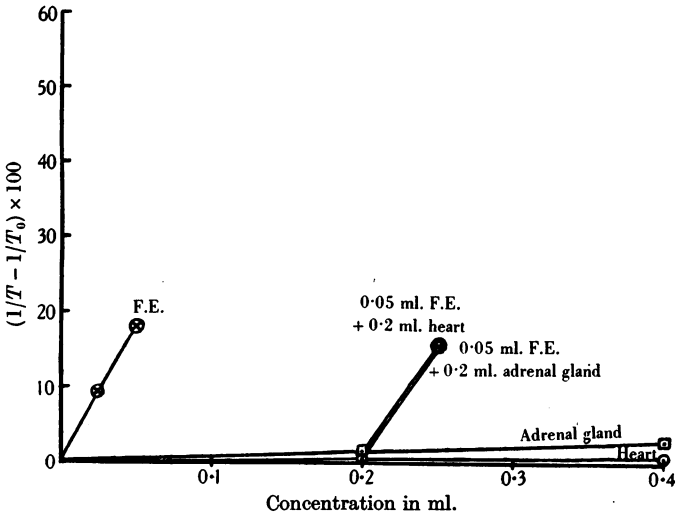

Fig. 5. Thunberg experiments. Heart and adrenal gland extracts partially purified by the method of Straub. Diaphorase II activity (*M*/50 *iso*citrate).

activity of the solution from heart is negligible, while in the case of adrenal gland it is very much smaller, relatively, than that of the extract from acetone-dried adrenal gland powder. In the present case the ratio, diaphorase I activity/ diaphorase II activity, was approximately 180 : 1. It thus appeared that in

some step of the purification the diaphorase II had been almost entirely removed. This was not due to the removal of a heat-stable substance: addition of acetone-dried adrenal gland extract, which had been heated to 80° for 5 min., had no effect on the activity.

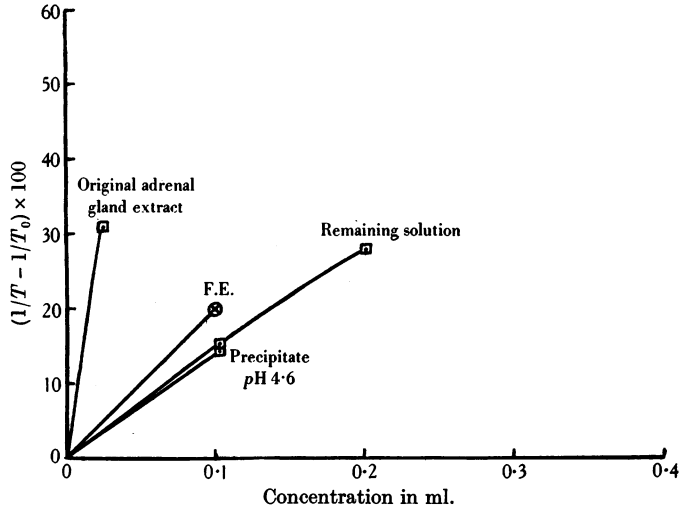

Fig. 6. Thunberg experiments. Acetone-dried adrenal gland extract; precipitate by acetate buffer *p*H 4·5, and remaining solution. Diaphorase I activity.

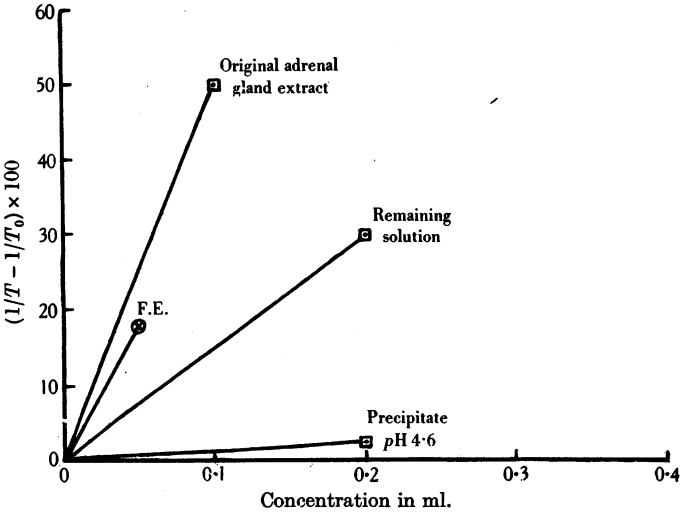

Fig. 7. Thunberg experiments. Acetone-dried adrenal gland extract; precipitate by acetate buffer *p*H 4·5, and remaining solution. Diaphorase II activity (*M*/50 *iso*citrate).

*Effect of precipitation of acetone-dried adrenal gland extract by acetate buffer p*H *4·5*

With the above results in mind it was decided to repeat the purification procedure with the extract from acetone-dried adrenal gland known to be active with respect to diaphorase II. To 8·5 ml. of such a solution was added 0·2 ml.

of $M/1$ acetate buffer pH 4·5. The precipitate was centrifuged off, suspended in water and brought to pH 7·5 by $N/10$ NaOH (vol. 10 ml.). The remaining solution was brought to pH 7·5 by $N/10$ NaOH and the volume made up to 10 ml. These two solutions were tested in the usual manner and compared with the original solution before precipitation. Figs. 6 and 7 show that, while the diaphorase I activity was approximately evenly divided between the precipitate and the remaining solution, the diaphorase II activity was associated almost entirely with the solution. This indicates the explanation of the results reported in the previous section. In the present case the ratios, diaphorase I activity/diaphorase II activity, are approximately as follows:

Original dialysed extract	4·5 : 1
Solution remaining after precipitation with acetate buffer	1·8 : 1
Precipitate resuspended	180 : 1

Although a considerable loss of both diaphorase I and diaphorase II activity occurred during the precipitation a solution was obtained by this procedure in which the ratio of the diaphorase II to diaphorase I activity was more than doubled.

On precipitating the last solution with $(NH_4)_2SO_4$ at 0·45 and 0·8 saturation (pH 6·6) most of the diaphorase I and diaphorase II appeared in the second fraction and the activity ratios were not significantly altered.

Conclusions

Both spectrophotometric and Thunberg experiments show that the ratios of diaphorase activity, measured in systems involving Co I and Co II, differ widely with solutions prepared from different organs. These differences are real and not explicable in terms of inhibition, by certain components of the solutions, of the test system using Co II. Moreover, in the case of an extract from acetone-dried adrenal gland the above ratio can be changed considerably by precipitation with acetate buffer pH 4·5.

The only simple explanation of these facts is that there are two diaphorases. The preparation of solutions which act only with Co I shows that one of these (diaphorase I) is specific for this coenzyme. As no solution has been obtained which acts only with Co II it is not decided whether the second (diaphorase II) acts specifically with Co II or with both coenzymes. For this reason the activity ratios in the Co I and Co II systems cannot be used to make a quantitative comparison of the amounts of the two diaphorases present in the various solutions: part of the diaphorase I activity may be attributable to diaphorase II.

In agreement with previous experience in this Institute with diaphorase solutions prepared from heart muscle, the heart diaphorase partially purified by the method of Straub showed practically no activity with respect to Co II. In fact the relative diaphorase II activity appeared to be even smaller, in this case, than that of crude extracts from acetone-dried heart muscle. A preparation from adrenal gland, which had been partially purified by the same method, also showed very little diaphorase II activity, in sharp contrast to the crude extract from acetone-dried material. Further experiments with the latter suggested that these results were due to the effect of precipitation with acetate buffer pH 4·5, only diaphorase I being carried down by the precipitate.

SUMMARY

1. An investigation was made of the diaphorase activities of various solutions with respect to Co I (diaphorase I activity) and to Co II (diaphorase II activity). With the Thunberg technique diaphorase II activity was measured by the use of the *iso*citric dehydrogenase system.

2. Spectrophotometric experiments confirmed the finding that diaphorase prepared from heart according to Euler & Hellström had only diaphorase I activity, but that extracts from acetone-dried adrenal gland possessed also diaphorase II activity. It was shown that negative results with respect to diaphorase II were not due to inhibition of the test system.

3. Thunberg experiments also showed that the ratio, diaphorase II activity/diaphorase I activity, varied widely with different extracts (adrenal gland > liver > heart). In the case of adrenal gland the ratio could be changed considerably by precipitation with acetate buffer *p*H 4·5.

4. Very little diaphorase II activity could be detected in preparations either from heart or adrenal gland which had been partially purified by the method of Straub.

5. These results are considered to show clearly the existence of two diaphorases.

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