LXXV. DIAPHORASE (COENZYME FACTOR)

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DIAPHORASE (coenzyme factor), one of the enzymes capable of reoxidizing reduced cozymase, has been the subject of a number of papers coming from this Institute and from Cambridge, where it was discovered independently and approximately simultaneously [Adler *et al.* 1937; Dewan & Green, 1937]. It is found widely distributed in animal tissues [Green *et al.* 1937; Dewan, 1938; Euler & Hasse, 1938], in yeast [Euler & Günther, 1938; Green & Dewan, 1938] and in several species of bacteria [Green & Dewan, 1938]. The results reported in this paper include an extension of the knowledge concerning the distribution of the enzyme in plant tissues, a further confirmation of its specificity as an oxidizing agent for coenzyme I, and a preliminary investigation of its action in respiratory systems other than those containing cytochrome carriers.

EXPERIMENTAL

Preparation of the enzymes. As sources of the enzyme both plant and animal tissues were used. Four plant materials, pea and bean seedlings, the thin outer layer of common white potato and a mould culture predominantly *Rhizopus* (sp.) and one animal tissue, pig heart muscle, were treated in the following way:

The seedlings were grown in metal trays on moist blotting paper for about 12 days. The cotyledons and leaves were then removed and the stems and roots ground to a pulp in a mortar with washed sand. One volume of $M/2$ Na_2HPO_4 was then added and the pulp reground until a homogeneous suspension was obtained. After an equal volume of water had been added, the coarsest particles and the sand were separated by straining through cloth and the remainder of the debris removed by centrifuging at 2500 r.p.m. for 5 min. The cloudy supernatant was treated with $\frac{1}{2}$ vol. sat. ammonium sulphate and the precipitate so obtained separated by centrifuging. A volume of $M/6$ secondary phosphate equal to the volume before addition of ammonium sulphate was added to the precipitate and the treatment with ammonium sulphate repeated. The precipitate finally obtained was suspended in half the original volume of $M/6$ Na_2HPO_4 buffer (pH 8.5). This method of preparation is a slight modification of that used by Euler & Hellström [1938] for animal tissue.

By the same procedure suspensions were prepared from potato peelings and from a mould culture which had been grown for about 2 weeks on moist bread.

The method of Euler & Hellström was used for the preparation of diaphorase from pig heart muscle. After the muscle had been reduced to a pulp and the red blood cells removed with 2 % NaCl , the decolorized mass was ground with sand and extracted with $M/2$ Na_2HPO_4 . After dilution and centrifuging had been carried out for the first time, two CO_2 -acetone precipitations at 0° were made. The precipitate obtained was suspended in $M/6$ Na_2HPO_4 , treated with saturated ammonium sulphate, with CO_2 and acetone and finally with sat. ammonium sulphate again. The final precipitate was suspended in $M/6$ Na_2HPO_4 buffer (pH 8.5).

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Stability. The stability of diaphorase from peas and beans differs quite markedly from that of diaphorase from pig heart. Although the latter retained its activity over a period of 4 months, the loss of activity in plant preparations was so rapid that preparations only 2 days old were unfit for experimental use. Similar observations have been made by several investigators for other materials.

Relative concentrations of enzyme in different tissues. It is possible only roughly to determine the relative concentrations of enzyme in plant and in animal material, since the same method of preparation cannot be used advantageously for both materials. However, when the times required for active enzymes prepared as described above to decolorize the same quantity of methylene blue in a standard Thunberg test are compared with the amount of material originally used, it is found that diaphorase in peas is only 1/6 and that in beans only 1/10 as concentrated as that in pig heart muscle. The same standard methylene blue test used to assay suspensions prepared from mould and from potatoes indicated that diaphorase was absent or nearly so from these preparations. These data are summarized in Table I.

Table I. *Concentration of diaphorase in plant and in animal tissues*

A complete system contains 0.25 ml. dihydrocozymase (1.2 mg./ml.), 0.25 ml. methylene blue (1/5000), 0.25 ml. diaphorase preparation and *M*/6 Na₂HPO₄ buffer (*pH* 8.5); total volume 1.50 ml. All tubes were incubated at 30°.

The controls indicate the spontaneous breakdown of dihydrocozymase in the presence of methylene blue and the absence of H-donating systems in the enzyme preparations.

	Material	System	Decolorization time min.	Relative concentration
1	—	Without enzyme	40	—
2	Pig heart	Complete	1.5	100
3	Pig, control	Without coenzyme	>120	—
4	Peas	Complete	6	16
5	Peas, control	Without coenzyme	>120	—
6	Beans	Complete	9	10
7	Beans, control	Without coenzyme	>120	—
8	Potato	Complete	42	—
9	Potato, control	Without coenzyme	>120	—
10	Mould	Complete	>120	—
11	Mould, control	Without coenzyme	>120	—

Specificity. The Thunberg methylene blue technique and the spectrophotometric technique were employed to investigate the specificity of plant diaphorase as an oxidant of dihydrocoenzymes I and II. The data obtained by the Thunberg method are presented in Tables II*a* and II*b*; those obtained by the spectrophotometric technique [Euler & Hellström, 1938] are shown in Fig. 1.

Spectrophotometric experiments. Exp. 1. (See below, (*a*) and (*b*).) The cell contained originally 1.0 ml. phosphate buffer (*pH* 7.2), 0.05 ml. diaphorase from peas, 3.7 ml. water and 0.15 ml. dihydrocodehydrase I (0.6 mg./ml.). After 10 min. 0.1 ml. methylene blue (1/5000) was added. Exp. 2. (See below, (*c*), (*d*) and (*e*).) The cell contained originally 1.0 ml. phosphate buffer (*pH* 7.2), 0.05 ml. diaphorase from peas, 0.85 ml. water and 3.00 ml. dihydrocodehydrase II (prepared enzymically). After 10 min. 0.1 ml. methylene blue (1/5000) and after 30 min. 0.1 ml. flavin enzyme were added. In both experiments the decrease in extinction at 334 mμ was measured.

(*a*) Dihydrocodehydrase I, diaphorase and buffer. No reaction in 10 min. No autoxidizable flavoprotein or cytochrome present.

Fig. 1. Specificity of diaphorase for dihydrocodehydrase I.

Table IIa. *Specificity test. Dihydrocodehydrase I (cozymase) and diaphorase*

A complete system contains 0.25 ml. dihydrocodehydrase I (1.2 mg./ml.); 0.25 ml. diaphorase from peas; 0.25 ml. methylene blue (1/5000) and phosphate buffer (pH 8.5) to make 1.50 ml. total volume.

	System	Decolorization time, min.
1	Complete	8
2	Without coenzyme	> 80
3	Without diaphorase	37.5

Table IIb. *Specificity test. Dihydrocodehydrase II¹ and diaphorase*

A complete system contains (a) 0.25 ml. Robison ester; (b) 0.25 ml. Robison ester dehydrogenase; (c) 0.25 ml. codehydrase II; (d) 0.25 ml. diaphorase from peas; (e) 0.50 ml. methylene blue (1/5000); (f) phosphate buffer (pH 8.5) to make a total volume of 1.75 ml.

In tube 6 diaphorase has been replaced by 0.25 ml. flavin enzyme.

	System	Decolorization time, min.
1	Complete	28.5
2	Without (a)	> 120
3	Without (b)	> 120
4	Without (c)	> 120
5	Without (d)	28.5
6	Flavin enzyme	9.5
7	Without (a) and (b)	> 120
8	Without (a), (b) and (c)	> 120

(b) As (a) but with methylene blue added. Decrease in extinction = 0.24 unit in 35 min.

(c) Dihydrocodehydrase II, diaphorase and buffer. No reaction in 10 min. No autoxidizable flavoprotein or cytochrome present.

(d) As (c) but with methylene blue. No reaction in 20 min. No reaction between coenzyme and diaphorase.

(e) As (d) with flavin enzyme. Decrease of 0.1 unit in 3 min. Oxidizable coenzyme present.

The results of these experiments confirm the fact already reported by this Institute that diaphorase is a specific oxidant for dihydrocodehydrase I (cozymase).

¹ The dihydrocodehydrase II was formed *in situ* from codehydrase II and H made available from Robison ester by the action of Robison ester dehydrogenase.

The role of diaphorase in non-cytochrome systems

No statement has been made concerning the possible role of diaphorase in respiratory systems other than those involving cytochrome carriers. One system found in some plants both of higher and lower orders is the system in which quinones play a dominating part as H acceptors. It was felt that the best method of approach would be to study reactions involving dihydrocozymase and simple quinones, both in the presence and in the absence of diaphorase.

Procedure. Methods which employed the Thunberg technique and the Zeiss photometer and depended upon the disappearance of colour from solutions of quinones after the addition of dihydrocozymase were found to be inapplicable to this work. The method finally adopted depends upon the destruction of cozymase formed during the reaction between dihydrocozymase and quinone by heat in a strongly alkaline solution and subsequent measurement of residual dihydrocozymase by a fermentation technique [Myrbäck, 1933].

One set of solutions was prepared as follows: 2.0 ml. quinone (equivalent to 350 μ g. coenzyme), 0.17 ml. diaphorase and 0.13 ml. dihydrocozymase (2.6 mg./ml.). A second set of solutions contained no diaphorase but an equal volume of *M*/6 Na_2HPO_4 buffer (*pH* 8.5). Controls containing neither dye nor diaphorase were also prepared. One tube of the first set and a corresponding tube of the second set were allowed to react for a definite period, after which the reaction was stopped by adding 0.50 ml. *N*/10 NaOH, to give a *pH* > 10. The tubes were sealed and heated in boiling water for 10 min., then the solution was brought back to *pH* 8 by adding 0.50 ml. *N*/10 acetic acid. Pairs of tubes were treated in the same manner after the reactions had continued for varying times. Two controls were carried through the same procedure, the one heated after 15 min. and the other not heated at all. By this procedure any cozymase formed during the reaction was destroyed.

A fermentation mixture corresponding to each solution to be tested for residual dihydrocozymase contained the following components: 400 mg. apozymase, 100 mg. glucose, 0.45 ml. phosphate buffer (*pH* 6.3), 0.15 ml. hexosediphosphate, 1.05 ml. water and 0.35 ml. test solution. For best results an untreated test solution should contain between 50 and 100 μ g. coenzyme per ml. Fermentations were carried out at 30° and the fermentation rate for each test solution determined from the volume of CO_2 evolved during the hour of most rapid fermentation. The fermentation rate is a measure of the amount of dihydrocozymase remaining in the original solution. The results of the reaction between dihydrocozymase and three quinones, *o*- and *p*-benzoquinone and β -naphthoquinone, both in the presence and in the absence of diaphorase, are shown in Fig. 2.

o-Benzoquinone was prepared according to a modification of methods reported by Kvalnes [1934] and by Willstätter & Pfannenstiel [1904] and

Fig. 2. Reduction of quinones by dihydrocozymase. Effect of diaphorase. Curve A, $\times \times \times$ *o*-benzoquinone without diaphorase. Curve B, $\odot \odot \odot$ *o*-benzoquinone with diaphorase. Curve C, $\times \times \times$ *p*-benzoquinone without diaphorase. Curve D, $\odot \odot \odot$ *p*-benzoquinone with diaphorase. Curve E, $\times \times \times$ β -naphthoquinone without diaphorase. Curve F, $\odot \odot \odot$ β -naphthoquinone with diaphorase.

Willstätter & Müller [1908] starting with pyrocatechol, Ag_2O and fused Na_2SO_4 in absolute ether and light petroleum. β -Naphthoquinone was purified by recrystallization from alcohol. *p*-Benzoquinone was purified by recrystallization from alcohol and sublimation. The diaphorase used throughout the work was prepared from pig heart muscle. There was no loss of activity during the period of investigation.

The results indicate, in the cases of *o*- and *p*-benzoquinones, that diaphorase apparently plays no part in the transfer of H from dihydrocozymase to these simple quinones, but, in the case of β -naphthoquinone, that diaphorase accelerates H-transfer and that the reaction is extremely rapid during the first minutes. From these results no definite conclusion can be drawn concerning the part, if any, played by diaphorase in a quinone respiratory system. If, however, the result obtained with β -naphthoquinone can be discounted, the fact that two plant tissues (potato and mould), containing quinone respiratory systems, have little or no diaphorase parallels the observation that diaphorase did not accelerate the oxidizing action of the other quinones. A tentative conclusion, then, is that diaphorase is not necessary in respiratory systems involving quinones. This will be confirmed or denied by future investigation.

SUMMARY

1. Diaphorase is present in the tissues of some higher plants and absent or nearly so in those of other higher plants and fungi.
2. Plant diaphorase is extremely unstable.
3. Plant diaphorase is a specific oxidant for dihydrocodehydrase I (cozymase).
4. The role of diaphorase as H transporter in respiratory systems other than those containing cytochrome carriers is still undetermined, although tentatively it is concluded that the enzyme is not necessary in quinone systems.

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REFERENCES

- Adler, Euler & Hellström (1937). *Sv. Vet. Akad. Ark. Kemi*, **12**, No. 38.
Dewan (1938). *Biochem. J.* **32**, 1381.
— & Green (1937). *Nature, Lond.*, **140**, 1097.
Euler & Günther (1938). *Naturwissenschaften*, **26**, 676.
— & Hasse (1938). *Naturwissenschaften*, **26**, 187.
— & Hellström (1938). *Hoppe-Seyl. Z.* **252**, 31.
Green & Dewan (1938). *Biochem. J.* **32**, 1200.
— Needham & Dewan (1937). *Biochem. J.* **31**, 2327.
Kvalnes (1934). *J. Amer. chem. Soc.* **56**, 2487.
Myrbäck (1933). *Ergebn. Enzymforsch.* **2**, 167.
Willstätter & Pfannenstiel (1904). *Ber. dtsh. chem. Ges.* **37**, 4744.
— & Müller (1908). *Ber. dtsh. chem. Ges.* **41**, 2580.