XCIX. ISOLATION AND PROPERTIES OF A FLAVOPROTEIN FROM HEART MUSCLE TISSUE

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(*Received 30 March 1939*)

SINCE Warburg & Christian [1932] isolated the first flavoprotein from yeast (flavinphosphoric acid as its prosthetic group) and Banga & Szent-Györgyi [1932] observed the presence of flavin in muscle tissue, there has been much speculation on the possible role of a flavoprotein in the mechanism of oxidations in animal tissues. As yet, however, there is no information on the state and function of flavoprotein in animal tissues.

I have purified from heart muscle tissue a flavin compound, the coenzyme of the *d*-amino-acid oxidase, which is neither free flavin, nor flavinphosphoric acid [Straub, 1938]. Warburg & Christian [1938, 1] at the same time isolated the same compound and later [Warburg & Christian, 1938, 2, 3] described it as a flavinadenine dinucleotide. I have pointed out that in muscle tissue the dinucleotide is attached to some other protein than that of *d*-amino-acid oxidase. In the present work I shall describe the isolation and properties of a flavoprotein from heart muscle tissue,[1] which has this flavinadenine dinucleotide as its prosthetic group. In a preliminary note it has been already reported that this flavoprotein is the physiological agent for the oxidation of cozymase in animal tissues; it is in fact identical with the so-called diaphorase or coenzyme factor [Straub *et al.* 1939].

Catalytic test

The concentration of the flavoprotein during the process of purification was followed with the help of the *d*-amino-acid oxidase test. The O_2 uptake was measured in Barcroft manometers containing coenzyme-free *d*-amino-acid oxidase [Straub, 1938] and *dl*-alanine, at 38° in phosphate buffer of *p*H 7·4. When flavinadenine dinucleotide is added to this mixture, it will combine with the protein to form *d*-amino-acid oxidase, thus oxidizing the alanine. The velocity of the oxidation is proportional to the amount of dinucleotide added.

If, instead of dinucleotide, a flavoprotein, which has the dinucleotide as its prosthetic group, is added to the test, no O_2 uptake is observed. If the flavoprotein solution is first heated in a boiling water bath for 3 min., the protein becomes denatured, thus liberating free dinucleotide. If this "boiled" solution is added to the test, the O_2 uptake will indicate the amount of dinucleotide, i.e. the amount of flavoprotein which was present in the solution.

The correlation between O_2 uptake and concentration of dinucleotide in the test solution has been studied by Warburg & Christian [1938, 3]. They find that

$$c = k.\frac{x}{s-x},$$

where k is a constant, x is the O_2 uptake in 10 min. in a test containing c mol. dinucleotide per litre and s is the O_2 uptake in 10 min. in a test, which contains

[1] Preliminary note [Straub, 1939].

the same amount of *d*-amino-acid oxidase as the former and an excess of dinucleotide. As this formula is valid only for one particular concentration of *d*-amino-acid oxidase, I have used it in a different form:

$$c = k \cdot \frac{s-x}{x} \cdot \frac{s}{100},$$

where c, s and x have the same meanings as before. The factor 100 is introduced only to give k a practicable value, namely, the concentration of dinucleotide in the test solution (mol./l.) which gives 50 μl. O_2 uptake in 10 min., when the same amount of enzyme with an excess of dinucleotide gives 100 μl. O_2 absorption in 10 min. The modified formula gives the same value of k for different values of s, i.e. for different amounts of *d*-amino-acid oxidase, within reasonable limits. The value of s in the present work was usually 100–150 μl. O_2 in 10 min. The value of k in this formula was found to be $2{\cdot}37 \times 10^{-7}$ under the experimental conditions mentioned above, and with air in the gas space of the manometers. Table I gives the results of one such test.

Table I

Manometer no. ...	1	2	3
Coenzyme-free *d*-amino-acid oxidase (ml.)	1·0	1·0	1·0
Purified dinucleotide solution (50 μg. lactoflavin/ml.)	—	—	1·0
Heated flavoprotein solution (0·047 mg. protein/ml.)	—	1·5	—
4·5 % *dl*-alanine solution	0·3	0·3	0·3
Phosphate buffer	2·0	0·5	1·0
μl. O_2 uptake in 10 min. at 38°	0	67·3	139
μg. lactoflavin in form of dinucleotide	—	0·39	—
% lactoflavin in flavoprotein	—	0·57	—

Spectrophotometrically found: 0·54% lactoflavin.

The fact that purified flavoprotein solutions, when added to the test, do not give rise to any O_2 absorption, means that the dinucleotide has a much greater affinity towards the protein of the flavoprotein than towards the protein of the *d*-amino-acid oxidase. If in the test a *d*-amino-acid oxidase is used which is not completely free from its coenzyme and consequently still shows some O_2 absorption without any addition of dinucleotide, this O_2 uptake will diminish on addition of a purified flavoprotein solution. Thus it appears that a small fraction (0·5–1 %) of the flavoprotein has lost its prosthetic group during the purification and this free protein is able to bind flavindinucleotide at the expense of the *d*-amino-acid oxidase.

When the flavoprotein is heated during the purification, part of it becomes denatured, and the solution will therefore contain free dinucleotide as well as flavoprotein (bound dinucleotide). For this reason a control experiment was carried out on each occasion, adding in one test the flavoprotein solution before, and in the other after, boiling. The first result gives the amount of free dinucleotide, the second the sum of free and bound forms; the difference represents the amount of undenatured flavoprotein.

From the nature of the test it appears that any protein which has the dinucleotide as its prosthetic group would be included in the determination. The purification however yields only one flavoprotein, which behaves uniformly. There is no indication that any other similar flavoprotein except that described is present in the solutions obtained.

Purification

Eleven pigs' hearts are freed from connective tissue and fat and then minced. The muscle tissue is washed 3 times with 15–20 vol. tap water for 20 min. each time with vigorous mechanical stirring. The water is pressed out through a thin cloth.

1660 g. of the washed mince are ground with 2·5 l. $M/50$ Na_2HPO_4 solution and about 500 g. sand in a mechanical mortar for 1–1½ hr.[1] The pulp is then thoroughly mixed with 2·5 l. distilled water and centrifuged.

The 4·7 l. of supernatant suspension (A) are mixed with 118 ml. M acetate buffer (pH 4·6) and centrifuged. The precipitate is suspended in 1660 ml. distilled water to which 33 g. $(NH_4)_2SO_4$ and 50 ml. ethyl alcohol are added and the mixture is heated on a water bath to 43° for 10–15 min. The insoluble muscle proteins are thus denatured and at the same time the flavoprotein becomes dissolved. When centrifuged, 1370 ml. of a somewhat opalescent strong yellow solution containing the enzyme (B) are obtained.

The solution is cooled down and then mixed with 45 ml. of an aluminium hydroxide (C_γ) gel. (1·0 g. Al_2O_3). After ½ hr. the alumina is centrifuged down. The solution is then mixed with a further 22 ml. alumina and again centrifuged. The enzyme is absorbed on the alumina and it can be eluted with alkaline phosphate. With successive lots of 50 ml. $M/5Na_2HPO_4$ solution the elution is carried on until the eluate appears to be colourless. The eluates are combined, making altogether 240 ml., and dialysed against distilled water until salt-free (C).

The precipitate, which is formed during the dialysis, is not removed but it will redissolve on addition of 2·8 g. $(NH_4)_2SO_4$ to the 280 ml. dialysate. The solution is now heated on a water bath for 5 min. to 60° and centrifuged after it has cooled down. The precipitate is discarded and 76 g. solid $(NH_4)_2SO_4$ is added to the solution (0·45 sat.). After standing in the ice chest for 1 hr., the precipitate is filtered off[2] and the solution (285 ml.) is mixed with 71 g. $(NH_4)_2SO_4$ (0·8 sat.). The precipitate is filtered off and the solution discarded. The yellow precipitate is washed from the filter paper with distilled water in about 30 ml. and dialysed overnight against 1·5 l. distilled water. The precipitate thus formed contains no enzyme, it is centrifuged off and discarded. The solution is then further dialysed against fresh distilled water. The precipitate which is now formed is usually yellow and contains some enzyme. It is not removed, but the dialysis continued until the solution is salt-free (D).

0·1 % NH_3 is added drop by drop to the dialysate until the precipitate dissolves. The solution is now heated for 5 min. to 60–62° without any salt addition and the precipitate centrifuged off. 10·9 g. $(NH_4)_2SO_4$ are added to the 35 ml. solution (0·5 sat.) and the precipitate filtered off and discarded. From the 40 ml. filtrate the enzyme is precipitated by adding 4 g. $(NH_4)_2SO_4$ (0·65 sat.). The yellow precipitate is filtered off and washed from the filter with distilled water. The solution thus obtained (15 ml.) is dialysed thoroughly against distilled water. A small yellow precipitate is formed, which is centrifuged off, leaving 20 ml. of the flavoprotein solution (E). It is advisable to add a few drops of dilute alkali or phosphate, when it will remain clear.

[1] The time of grinding depends on the type of the mortar and cannot therefore be definitely stated. It is carried on until a small sample shows, after centrifuging, only a thin layer of myosin above the layers of sand and heavier muscle tissue lumps. If the grinding is continued until the whole mass becomes a uniform paste, too much myosin is extracted which cannot properly be centrifuged down, so that the supernatant suspension will be only half the volume of the added solutions, thus reducing the yield.

[2] All the filtrations have been done with Whatman no. 1 filters. No suction can be applied.

Table II. *Yield and flavin content during the purification*

	Protein dry weight g.	mg. lactoflavin present as protein-bound dinucleotide	Yield of bound dinucleotide %	Lacto-flavin %
1660 g. washed muscle	320	6·9	(100)	0·0022
(A) Phosphate extract	39	2·8	42	0·007
(B) Alcohol-$(NH_4)_2SO_4$ solution	—	0·97	14	—
(C) Alumina eluate	1·05	0·65	9·4	0·062
(D) First $(NH_4)_2SO_4$ fractionation	0·27	0·56	8·1	0·21
(E) Second $(NH_4)_2SO_4$ fractionation	0·098	0·53	7·6	0·54*

* Spectrophotometrically determined.

Properties of the flavoprotein

The colour of the flavoprotein solution is yellow, although it appears greenish because of the strong greenish fluorescence. The spectrum of the flavoprotein[1] is reproduced in Figs. 1 and 2. It shows maxima at 274, 359 and 451 mμ and minima at 250, 316 and 398 mμ. When examined under the low-dispersion microspectroscope, two distinct bands are seen in the visible region of the spectrum, one at 480–490 mμ, the other at 440–460 mμ. The existence of these bands in muscle extracts has been previously observed by Keilin & Hartree [1939].

From the absorption at 451 mμ the lactoflavin content of the purified preparations has been determined, by assuming that the value for this maximum is the same as in any other flavoprotein, i.e. $\beta_{541} = 2{\cdot}4 \times 10^7$. The flavin content thus found in the purest preparations is $0{\cdot}54 \pm 0{\cdot}02$ % lactoflavin. (Expressed as lactoflavinphosphate 0·66 %.) The molecular weight calculated from the flavin content is $\frac{100 \times 376}{0{\cdot}54} = 70{,}000$.

That the flavinadenine dinucleotide is the prosthetic group of the flavoprotein is shown by the fact that it can quantitatively replace the coenzyme of the *d*-amino-acid oxidase. No attempt has been made to isolate the prosthethic group from the purified enzyme.

The flavoprotein is reduced to the leuco compound by $Na_2S_2O_4$ and it is reoxidized when shaken with air. It is also reduced to the leuco form by dihydrocozymase.

The fluorescence of the flavoprotein solutions is just as strong as that of the free flavin. It is not due to the presence of free flavin and it is completely extinguished on the addition of reduced cozymase. The old yellow enzyme of Warburg & Christian does not show any fluorescence. The difference might be explained if the alloxazin ring is not bound to the protein in the case of the heart flavoprotein as it is in the case of the old yellow enzyme [Kuhn & Boulanger, 1936]. This supposition is supported by the fact that the absorption band of the flavoprotein coincides with the bands of the free flavin.

The flavoprotein is in an insoluble state in the tissue, but this is not an intrinsic property of the enzyme. During the purification, when heated with 3 % alcohol, the enzyme is detached from the insoluble muscle proteins and becomes soluble in salt solution. After removing impurities it is soluble even in salt-free solutions.

The N content of the purified flavoprotein was found to be 15·7 %.

[1] The spectrum was taken with a Hilger "Spekker" apparatus. I am indebted to Mr H. S. Corran for carrying out the measurements.

The heat stability of the flavoprotein is remarkable, and it increases during the purification. When it is already in a soluble form, heating for 5 min. to 60° does not cause any destruction, while heating for 5 min. to 70° only destroys

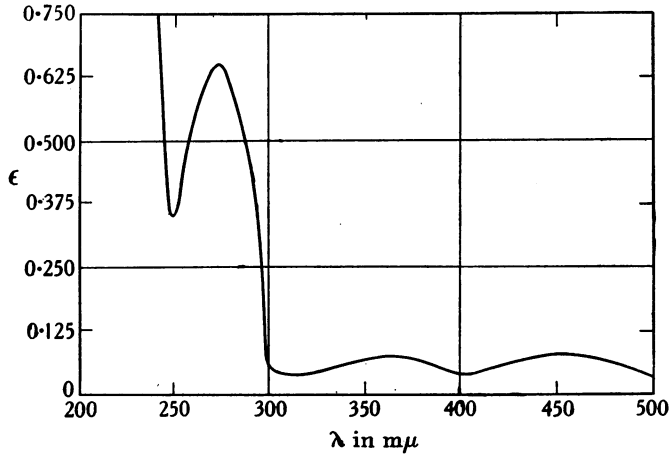

Fig. 1. Absorption spectrum of a 0·0265 % solution of the flavoprotein ($d = 2$ cm.).

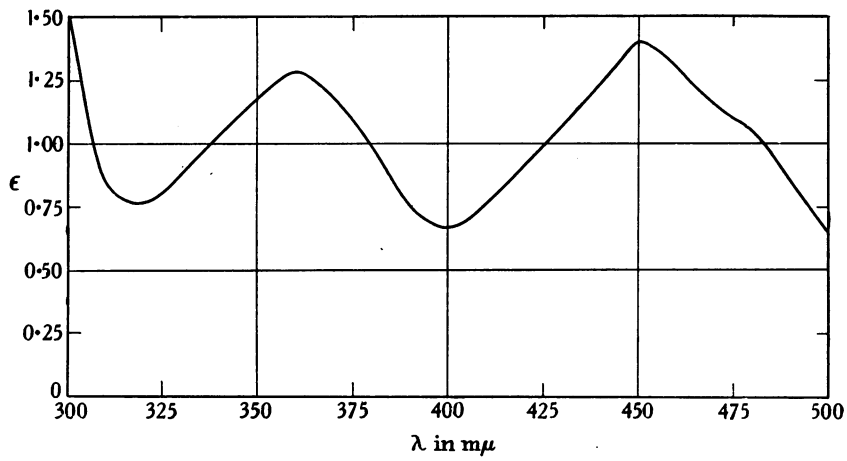

Fig. 2. Absorption spectrum of a 0·47 % solution of the flavoprotein ($d = 2$ cm.).

about 10 %. The heat stability of the purified preparation will be discussed in a subsequent paper.

I wish to express my deep gratitude to Prof. D. Keilin for his constant help and interest in this work.

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