STUDIES ON COENZYME III

I. L-CYSTEINESULFINIC DEHYDROGENASE AND ITS PROSTHETIC GROUP*

by

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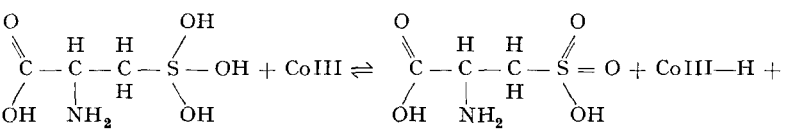
It was shown in the preceding paper that in extracts of *P. vulgaris* L-cysteinesulfinic acid is metabolized via two competing pathways. One of these commences with a transamination, while the other is initiated by a dehydrogenation step which requires an enzyme, a coenzyme, and a suitable dye for electron transport. Evidence has been presented[1,2] that the latter oxidation involves the sulfur atom and that cysteic acid is the product:



(1)

In this reaction the dehydrogenation is visualized as an attack on the hydrated sulfinic acid, in a manner analogous to the mechanism of the dehydrogenation of aldehydes. The term "CoIII" or coenzyme III is the provisional name proposed by the authors[1] for the new pyridine nucleotide which participates in the reaction.

The present paper is an account of some of the properties of L-cysteinesulfinic dehydrogenase and of its coenzyme.

MATERIALS AND METHODS

The compounds used in this work were obtained from the following sources: ATP*** (Na salt), Pabst Laboratories; FAD (Ba salt, 60% stated purity), Sigma Chemical Co.; 5-adenylic acid, Schwartz Laboratories, Inc.; *Crotalus adamanteus* venom, Ross Allen (Silver Springs, Florida). For generous gifts of various materials we are greatly indebted to Dr. F. DICKENS (pyocyanine and phenazine methosulfate), Dr. M. DIXON (Straub flavoprotein), Dr. L. J. HAYNES and Dr. A. KORNBERG (synthetic nicotinamide riboside and NMN prepared from DPN by the action of nucleotide pyrophosphatase), Dr. H. A. KREBS (samples of FAD), and Dr. F. LIPMANN (coenzyme A). The sources and preparation of the other materials used in this study, including the enzyme, have already been described[2,3].

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** Fellows of the John Simon Guggenheim Memorial Foundation, 1951–'52. Present address: Institute for Enzyme Research, University of Wisconsin, Madison, Wisc. (U.S.A.). A preliminary account of this work has appeared[1] and a summary was presented at the Second International Congress of Biochemistry, in Paris, July, 1952.

*** The following abbreviations are used: ATP, adenosine triphosphate; DPN, diphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide; NMN, nicotinamide mononucleotide; FMN, flavin mononucleotide; FAD, flavin adenine dinucleotide; TRIS, tris(hydroxymethyl)aminomethane.

References p. 299.

L-Cysteinesulfinic dehydrogenase activity was followed manometrically at the pH optimum (7.9), essentially under the conditions outlined in the legend of Fig. 1 of the preceding paper[2], except that 0.02 *M* semicarbazide was present. When purified CoIII was used as the coenzyme, 1.5 to 2.0 mg phenazine methosulfate were substituted for brilliant cresyl blue and the pH was 7.5 to 7.6 (0.033 *M* TRIS or 0.05 *M* phosphate buffer), since at higher pH values the dye is unstable. The phenazine solution was kept in the dark in the frozen state and was the last substance added to the reaction mixture. Coenzyme III content was either estimated spectrophotometrically (*cf.* text) or manometrically in the phenazine methosulfate system. In the latter assay 6 mg enzyme preparation were used and a saturation curve was constructed with a standard sample of the coenzyme from which the results were read. The latter method necessitates much smaller amounts of coenzyme than the spectrophotometric test and has the added advantage that preparations of any purity level may be used, whereas the spectrophotometric test is feasible only with materials which have been at least partially purified.

RESULTS

Coenzyme requirement. It was noted early in this investigation that in the absence of yeast extract cell-free preparations of *P. vulgaris* fail to oxidize L-cysteinesulfinic acid. When graded amounts of boiled baker's yeast extract are added, increasing amounts of O_2 uptake are obtained, until saturation is reached at about 1.5 ml yeast extract per 3.0 ml reaction volume (Fig. 1 a). Acid hydrolysis (pH 1) for 10 minutes at 100° destroys the activity of yeast, as does treatment with strongly alkaline solutions. Thus the factor in yeast extract appears to be an organic compound.

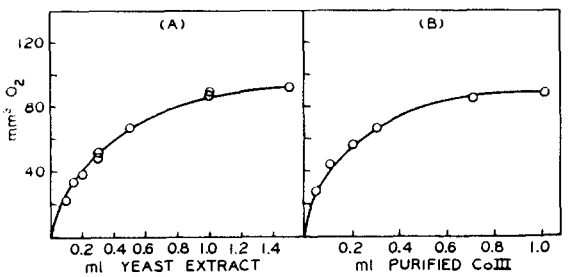


Fig. 1. Coenzyme saturation curve with phenazine methosulfate a. with yeast extract; b. with purified CoIII as the source of coenzyme. Conditions, 6 mg enzyme, 2 mg phenazine methosulfate, 0.05 *M* phosphate buffer, pH 7.6, 0.03 *M* L-cysteinesulfinate, and 0.02 *M* semicarbazide in a total volume of 3 ml. Temperature, 35°; duration, 20 min. The yeast extract and the purified coenzyme were prepared as described in the text.

A number of vitamins, vitamin derivatives, and related substances were tested in the presence and absence of ATP, alone and in combination with each other, without a discernible activating effect. The substances tested included: DPN, TPN, NMN, nicotinamide riboside, FMN, FAD, glutathione, adenylic acid (-5-phosphate as well as adenylic acids *a* and *b*), coenzyme A, pyridoxal phosphate, thiamine, thiamine phosphate, thiamine pyrophosphate, biotin, ascorbic acid, folic acid, *p*-aminobenzoic acid, and FOULKES AND PETERS' "citrate oxidation factor"[4]. It appeared, therefore, that the substance in question was a new coenzyme.

Assay of the coenzyme. For routine estimation of the coenzyme a manometric test was devised wherein phenazine methosulfate served to link the dehydrogenase system with molecular O_2. In the presence of this dye, the same maximal O_2 uptake was obtained with excess yeast extract and with saturating levels of the most purified preparation of the coenzyme (Figs. 1 a and b). One unit of coenzyme III is defined as the quantity of material which gives half-maximal activity in the manometric test, under the experimental conditions (*cf.* legend of Fig. 1). Several levels of a standard yeast preparation were usually assayed with each unknown sample in order to insure that the enzyme was fully active.

Several points may be mentioned in connection with this assay system. First, some enzyme

References p. 299.

preparations appeared to contain a very active hydrolytic enzyme which inactivated the coenzyme unless the substrate (L-cysteinesulfinate) was also present. Thus the enzyme was usually added from the side-arm of the vessel to a mixture containing substrate, coenzyme, buffer, and semicarbazide. Under these conditions no indications of coenzyme destruction were obtained in the course of 20 minute experiments, although the rate of O_2 uptake was not always linear; the slow decline with time appears to be connected with inactivation of the enzyme itself. Second, cysteinesulfinate oxidation in this system is regularly accompanied by the formation of a deep magenta-coloured dye from phenazine methosulfate and the intensity of this colour appears to parallel the O_2 consumption. No such colour was formed in the blanks (no L-cysteinesulfinate) or when glutamate oxidation was measured in the presence of TPN and phenazine methosulfate with the same enzyme preparation, nor did it form from a mixture of phenazine methosulfate, L-cysteinesulfinate, L-cysteate, and coenzyme III in the absence of the enzyme. The reaction may conceivably prove adaptable to the colorimetric measurement of L-cysteinesulfinic dehydrogenase activity.

Purification of the coenzyme. A number of baker's and brewer's yeast samples have been tested for coenzyme III content. Among yeasts available in France, Springer's baker's yeast, and among American yeasts, Fleischman's baker's yeast have been found most satisfactory.

A variety of procedures have been tested for the concentration and the purification of the coenzyme from boiled yeast extracts. The main difficulties encountered in the purification are that the heavy metal salts of the coenzyme (Hg^{++}, Ag^{+}, Pb^{++}, Ba^{++} salts) are water soluble and that adsorption-elution methods apparently can be applied with success only after removal of interfering materials present in yeast. The following procedure has been used for routine purification of coenzyme III.

I. For each kilo of fresh baker's yeast 1 liter of water is brought to about 95° and the crumbled yeast is added, under vigorous stirring, at such a rate that the temperature does not fall below 85°*. After addition of all the yeast the temperature is allowed to rise to 98–99° and the extract is then immediately cooled to room temperature. The suspension is centrifuged for 1 hour at or above 3000 *g***.

II. To each liter of the supernatant solution 42 ml of a saturated solution of Ba acetate are added and the pH is adjusted with 15% KOH to 8.3. The precipitated nucleotides are removed by brief centrifugation and the clear, yellow supernatant, containing all the coenzyme, is cooled to approximately 0° and is treated with 6 volumes of acetone (—10°). The precipitate is collected by centrifugation in the cold or by filtration through a pad of Hyflo-supercel. Acetone is removed by washing with anhydrous ether, followed by brief aeration. The brown, sticky precipitate is suspended in a small volume of 0.5 *N* H_2SO_4 and the pH is adjusted to 2.0 with 7 *N* H_2SO_4. The $BaSO_4$ is centrifuged off and the precipitate is washed twice with acidified water. The supernatant solutions are united*** and the pH is readjusted to 2.0, if necessary.

III. For each liter of yeast extract 12.5 g of charcoal are added to the solution of the coenzyme and the suspension is vigorously stirred or shaken for 20 minutes. The unadsorbed portion is filtered off through a layer of Hyflo-supercel and discarded, and after thorough washing with water the charcoal is mechanically shaken for 20 minutes with a 10% (v/v) suspension of *iso*amyl alcohol in water, using a volume of eluant which corresponds to 10% of the volume of yeast extract used. After filtration, the elution

* For small samples direct heating was applied, whereas for batches of 1 to 10 kilo of yeast a steam-coil was used for heating and the same coil served for cooling the extract after the required temperature was reached.

** With French yeasts a clear, golden yellow extract is obtained, while Fleischman's yeast gives an opalescent grey supernatant solution at this stage.

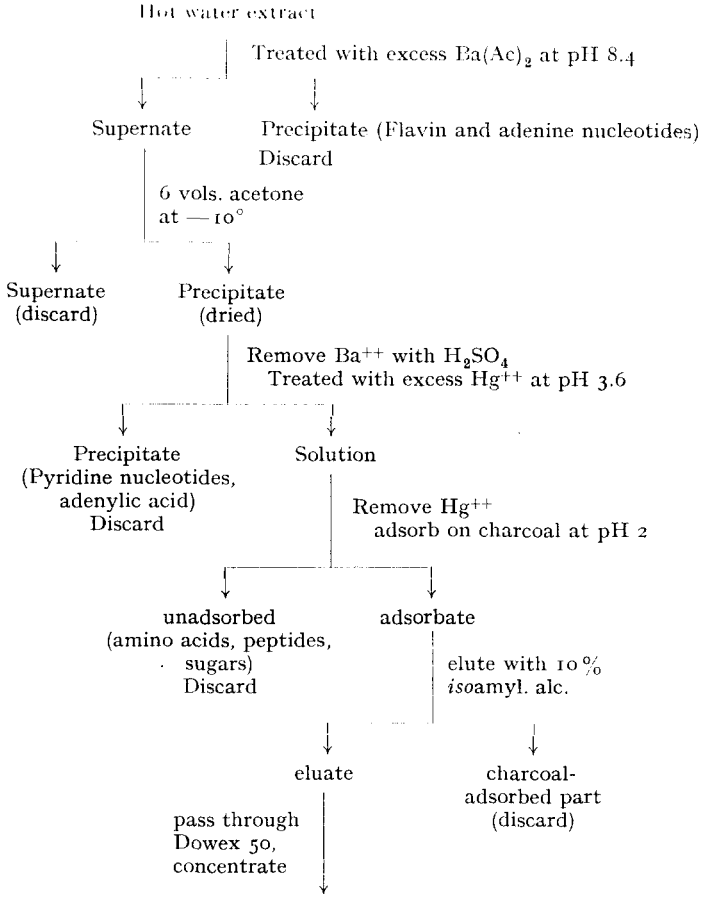
*** The Ba precipitation may be used as a concentration step; the total volume at the end of step II may be brought to about ¼th of that of the original yeast extract.

References p. 299.

with 10% *iso*amyl alcohol is repeated twice, and the united eluates are adjusted to pH 7 and concentrated *in vacuo* to a convenient small volume, using a water bath regulated at 35 to 40°.

SCHEME I

ISOLATION OF COENZYME III FROM BAKER'S YEAST



The overall yield in this procedure is 25 to 55%. Up to the end of step II the recovery is quantitative and the adsorption on charcoal is about 95% complete. Thus, essentially, the loss is due to incomplete elution. The success of the isolation depends primarily on the choice of charcoal. In France acticarbon S (acid-washed) was used; in subsequent work in America, Nuchar C 190 N (unground) was found to be satisfactory, although considerable variation was encountered in various lots of the latter.

If 5% pyridine is substituted for *iso*amyl alcohol in the elution step the yield is slightly improved, but considerable amounts of inert material are eluted along with the coenzyme. Adsorption on charcoal and elution with 10% *iso*amyl alcohol or with pyridine cannot be applied directly to the yeast extract as a concentration step, since certain materials present in yeast extract and removed in the Ba-acetone step appear to interfere with elution of the coenzyme.

References p. 299.

More extensive purification of the coenzyme was achieved by the inclusion of a precipitation step with mercuric acetate at pH 3.6 (which left the coenzyme in solution) prior to adsorption on charcoal and by passage of the final product at pH 7 through a column of Dowex 50, which removed residual amino acids and trace metals. The outline of this procedure is presented in Scheme 1. The product thus obtained appeared to be free from DPN, TPN, and flavin nucleotides but was contaminated with adenylic acid and NMN, as revealed by paper chromatography*.

Work on further purification of the coenzyme is in progress and will be the subject of future communications.

Properties of the coenzyme. Upon mixing a solution of the coenzyme with the dehydrogenase preparation and L-cysteinesulfinate, a rapid increase in light absorption occurs at 340 mμ (Fig. 2). The spectrum of the enzymically reduced coenzyme in the near-ultraviolet region strikingly parallels the known spectrum of reduced pyridine nucleotides and is, in fact, quantitatively identical with the latter (Fig. 3). Under the same conditions DPN, TPN, and NMN undergo no spectrophotometrically observable change, nor does addition of these nucleotides to purified coenzyme III alter the total change in light absorption at 340 mμ given by coenzyme III alone. If L-cysteic acid is added to the reduced coenzyme in the presence of the dehydrogenase, the light absorption at 340 mμ decreases, showing reoxidation of the coenzyme.

These observations indicate that coenzyme III is a pyridine nucleotide, not identical with DPN, TPN, or NMN, and that it participates in the reversible dehydrogenation of L-cysteinesulfinate in the manner shown in reaction (1).

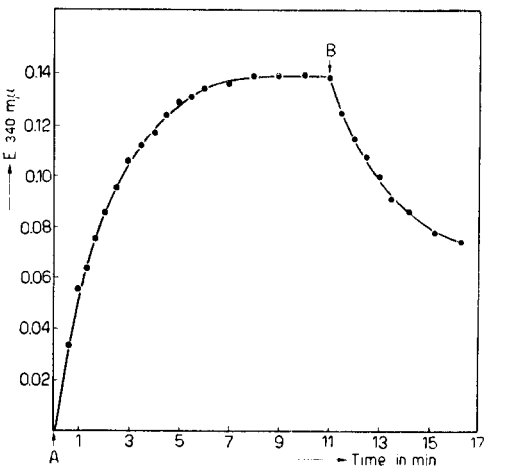


Fig. 2. Spectrophotometric measurement of L-cysteinesulfinic dehydrogenase. The cuvette contained, in a total volume of 3 ml, 70 μmoles TRIS buffer, pH 8.4, 1.0 ml of coenzyme at the last stage of purification, 60 μmoles L-cysteinesulfinate, and 0.8 mg protein. The substrate was added at point A. Readings were taken in a Beckman Quartz spectrophotometer (1 cm cuvette) at 23°. At point B 80 μmoles L-cysteate were added. The blank contained all additions, except substrate.

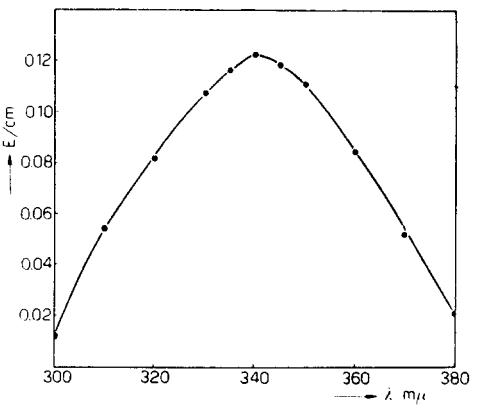


Fig. 3. Absorption spectrum of enzymatically reduced coenzyme III. Abscissa, wave-length; ordinate, light absorption in a 1 cm cell. The experiment was identical with that in Fig. 2. Readings were taken only after the light absorption at 340 mμ became constant.

In order to gain an insight into the structure of the coenzyme with the limited quantities of material available, the following experiments were performed. The acid-stability of the coenzyme was tested by heating aliquots of the coenzyme at 100° for

* This preliminary experiment was kindly performed by Dr. L. J. HAYNES in Cambridge, England.

References p. 299.

10 minutes at various pH values. The results showed that the coenzyme was more labile to acids than could be attributed to the nicotinamide-ribose linkage, thus suggesting the presence of a structure with the lability of a pyrophosphate bond. Thus, in 0.1 *N* HCl in 10 minutes at 100°, 70% of the substance was hydrolyzed, as judged by spectrophotometric estimation of the residual activity at 340 mμ. Confirmatory evidence for the probable presence of a pyrophosphate linkage in the molecule was obtained by treatment of the purified coenzyme (1 ml solution at the end of step III) with 9.4 units[5] of highly purified nucleotide pyrophosphatase (554 units per mg protein) in TRIS buffer, pH 8.5, for 30 minutes at 35°. This treatment destroyed the activity of the coenzyme sample completely. Incubation, under identical conditions, with 0.04 mg of the venom of *Crotalus adamanteus* (a rich source of 5-nucleotidase[6] and of phosphodiesterase), with added $MgCl_2$ to insure maximal activity of the 5-nucleotidase, failed to affect the coenzyme.

As previously mentioned, the bacterial extracts used in this study contained an enzyme which hydrolyzes the coenzyme, unless cysteinesulfinate is also present during the incubation. This fact could be readily ascertained by measurement of the total reduction at 340 mμ before and after incubation of a standard coenzyme preparation with the enzyme at 35°, in the absence of substrate. The hydrolytic enzyme seems to act on a phosphate linkage rather than on the nicotinamide-ribose bond, since 0.01 *M* nicotinamide failed to protect against its action, while 0.005 *M* 5-adenylic acid protected the coenzyme to a significant extent. It may be added that the action of this hydrolytic enzyme, upon cell disruption, may explain why L-cysteinesulfinic dehydrogenase occurs in the apoenzyme stage in the bacterial extracts.

Properties of the dehydrogenase. In the brilliant cresyl blue assay (with boiled yeast extract as a source of the coenzyme) L-cysteinesulfinic dehydrogenase acts optimally at pH 7.8 (Fig. 4). The activity is the same in phosphate and in TRIS buffers at the same pH. Half saturation of the enzyme is reached at $4.1 \cdot 10^{-3}$ *M* L-cysteinesulfinate, as determined by the method of LINEWEAVER AND BURK (Fig. 5).

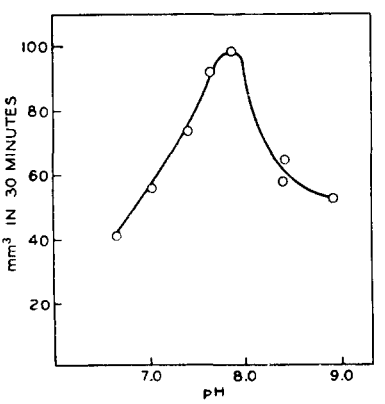


Fig. 4. Relation of pH to L-cysteinsulfinic dehydrogenase activity. Conditions as in MATERIALS AND METHODS under brilliant cresyl blue assay, except that 120 μmoles substrate were present. The pH values are those measured in the reaction mixture at room temperature. Buffers: phosphate from pH 6.65 to 7.02; TRIS from pH 7.38 to 8.40; NH_3 from pH 8.37 to 8.91.

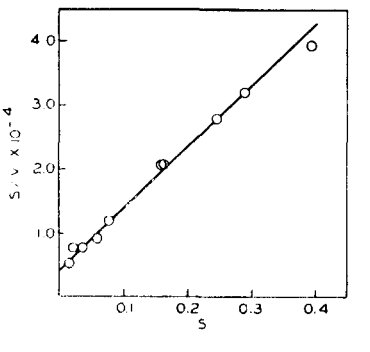


Fig. 5. Effect of substrate concentration on L-cysteinesulfinic dehydrogenase activity. Abscissa, average molar concentration of L-cysteinesulfinic acid in the course of the experiment × 10; ordinate, substrate concentration/velocity (in mm^3 O_2 uptake per 30 min). Brilliant cresyl blue assay; 1.5 ml yeast extract as the source of coenzyme III, and 70 μmoles TRIS buffer, pH 8, per vessel, in a total volume of 3.5 ml.

References p. 299.

The enzyme appears to be rather labile, particularly if the extracts are prepared by grinding with alumina. It may be stored, however, for several weeks at —20° in the lyophilized state, and solutions may be kept for a day at 0° without loss of activity*. Fractionation with acetone or alcohol at low temperatures invariably resulted in marked inactivation.

Electron transfer from coenzyme III to O_2. As noted in Fig. 1, in the presence of phenazine methosulfate, the same maximal activity is obtained at saturating levels of yeast extract and of the most purified fraction of coenzyme III. In the brilliant cresyl blue assay, with unfractionated yeast extract as a source of the coenzyme, the same activity is obtained as in the phenazine methosulfate assay system. With the purified coenzyme only a fraction of the maximal O_2 uptake is obtained. When methylene blue or pyocyanine are substituted for brilliant cresyl blue as an electron carrier from coenzyme III to O_2, the rate of O_2 uptake is again only about 30% of that obtained in the phenazine methosulfate assay. These observations are readily interpreted in the light of the facts that dyes like methylene blue, brilliant cresyl blue, and pyocyanine do not react readily with dihydropyridine nucleotides, whereas there is good reason to believe that phenazine methosulfate does[7]. It is well known that flavoproteins are required for the catalysis of the reaction between methylene blue or brilliant cresyl blue and reduced DPN and TPN. Very likely, the same requirement may exist for the reoxidation of reduced coenzyme III. Thus it appears that a diaphorase (or cytochrome reductase) concerned with the reoxidation of reduced CoIII is present in the bacterial extract and, like cysteinesulfinic dehydrogenase, it is obtained largely in the apoenzyme state. Crude yeast extract, then, supplies two cofactors for the brilliant cresyl blue assay system (coenzyme III and a prosthetic group for the diaphorase, which will be referred to as "codiaphorase") but only one substance (coenzyme III) for the phenazine methosulfate assay. The codiaphorase is largely removed in the course of the purification of coenzyme III; a substantial part of it is lost in the first Ba precipitation.

The diaphorase concerned with the reoxidation of coenzyme III is at least partly autooxidizable. Thus, in the presence of excess yeast extract, about half of the maximal O_2 uptake measurable in the presence of brilliant cresyl blue is manifested in the absence of the dye.

A satisfactory measure of the codiaphorase may be achieved with an excess of purified coenzyme III in the manometric brilliant cresyl blue method, whereby addition of unfractionated yeast extract boosts the small level of O_2 uptake obtained with the coenzyme alone to that measured with phenazine methosulfate as carrier. By means of this procedure it was established that neither riboflavin, FMN, FAD, nor a combination of other known cofactors could substitute for the codiaphorase at any concentration tested. A small but significant codiaphorase activity was observed upon incubation of FMN + ATP (but not of riboflavin + ATP) with the enzyme preparation, indicating possible synthesis of the codiaphorase.

These experiments suggest that there is in yeast extract a second new coenzyme. It is interesting to speculate that it may be a flavin nucleotide analogous to the proposed structure of coenzyme III[1]. Experiments designed to test this hypothesis are now in progress.

Certain other observations relating to the general question of the oxidation of di-

* One preparation, grown in liquid culture with forced aeration, gave a high yield of enzyme, but the lyophilized extract deteriorated very rapidly, even at —10°, and this technique of growth was thereafter abandoned.

References p. 299.

hydrocoenzyme III may be briefly mentioned. Among dyes thought to oxidize dihydropyridine nucleotides without the mediation of flavins, besides phenazine methosulfate, ferricyanide[8] and 2,6-dichlorophenol-indophenol[9] have been tested. Both of these oxidants reacted rapidly with the reduced coenzyme in spectrophotometric tests*. The spectrophotometric measurement of the reduction of 2,6-dichlorophenol-indophenol is a convenient measure of the activity of L-cysteinesulfinic dehydrogenase, but ferricyanide cannot be used for this purpose, since it appears to be very inhibitory to the enzyme.

Heart flavoprotein, purified by the method of STRAUB[10], failed to catalyze the reoxidation of dihydrocoenzyme III by methylene blue.

DISCUSSION

The facts presented in the present and the preceding[2] paper lead to the inevitable conclusion that the L-cysteinesulfinic dehydrogenase of *P. vulgaris* requires a cofactor for activity and that this substance is present in yeast but is not identical with a large number of substances tested. If one accepts the conclusion, based mainly on the spectrophotometric observations reported above, of the pyridine nucleotide nature of this cofactor, the inactivity of a variety of highly purified DPN, TPN, and NMN samples in both the manometric and spectrophotometric tests, points clearly to a new coenzyme in the pyridine series. The argument is strengthened by the recent observations of the authors that soluble L-cysteinesulfinic dehydrogenases prepared from numerous tissues of several species of higher animals also require added coenzyme III for activity, and that DPN and TPN substitute very poorly, or not all, for coenzyme III in the animal dehydrogenases[11].

One may wonder why this substance has escaped detection through the numerous years of investigations of pyridine nucleotide enzymes. In this regard, it may be well to recall that it has not been critically shown that DPN and TPN account for the total pyridine nucleotide content of biological materials. As a matter of fact, it is mentioned in the present paper that the purified coenzyme preparation, isolated from baker's yeast, contains a substance which is paper-chromatographically indistinguishable from NMN, although the presence of the latter compound in yeast, while predictable[12], has never been reported. Also, the concentration of coenzyme III in yeast appears to be appreciably lower than that of DPN, and it may be low enough to escape detection in an analysis of total pyridine nucleotides.

Regarding the structure of coenzyme III, the following facts are pertinent. The spectrum of the enzymically reduced coenzyme and the specificity of the electron acceptors which react with the reduced form (phenazine methosulfate, ferricyanide, 2,6-dichlorophenol-indophenol, but not methylene blue, brilliant cresyl blue, or pyocyanine) strongly suggest a pyridinium compound. Other evidence relating to the structure is necessarily indirect, in view of the fact that pure samples of the compound have not yet been prepared. The solubility of all the heavy metal salts of the coenzyme contraindicates any dinucleotide structure containing an adenylic acid moiety. The action of nucleotide pyrophosphatase suggests the presence of a pyrophosphate linkage. While the nucleotide pyrophosphatase used in this work may have contained traces of phosphatases, the marked lability of the compound to acids reinforces the tentative

* Ferricyanide reduction was followed at 420 mμ and 2,6-dichlorophenol-indophenol reduction at 610 mμ.

References p. 299.

conclusions regarding the presence of a pyrophosphate linkage. Finally, it should be pointed out that the procedure developed for the isolation of the coenzyme is an adaptation of the method of KORNBERG AND LINDBERG[13] for the purification of NMN. In fact, of a variety of initial concentration steps tested, this has been the only one suitable for the purpose. The striking similarity of the behaviour of coenzyme III to NMN in numerous fractionation procedures suggests that the two compounds may be closely related. The structure nicotinamide-ribose-(5)-pyrophosphate would be in accord with the above observations, and would explain also the inability of 5-nucleotidase to hydrolyze the compound. The above structure remains, obviously, only a working hypothesis until current efforts at establishing nicotinamide: ribose: PO_4 ratios in highly purified samples are successfully completed.

Coenzyme III may have numerous metabolic functions besides its rôle as the prosthetic group of L-cysteinesulfinic dehydrogenase. Its importance in the oxidative metabolism of animal tissues is shown not only by its specific function as the prosthetic group of dehydrogenases of animal origin but also by the presence of coenzyme III in every animal tissue so far examined[11].

Experiments on the nature of codiaphorase and on the structure and metabolic functions of coenzyme III are being continued.

This paper is dedicated to Prof. C. FROMAGEOT, in grateful acknowledgment of his constant and enthusiastic support of this work.

SUMMARY

1. L-Cysteinesulfinic dehydrogenase, the enzyme which catalyzes the dehydrogenation of L-cysteinesulfinic acid, requires a coenzyme for activity. The coenzyme requirement is not fulfilled by known vitamins, coenzymes, or related substances, but boiled yeast extract is a good source of the cofactor.

2. The new coenzyme, provisionally named coenzyme III, has been extensively purified from baker's yeast. When the dehydrogenase oxidizes cysteinesulfinic acid in the presence of the purified coenzyme, the latter is reduced to a substance with an absorption spectrum characteristic of dihydropyridine nucleotides. Upon addition of L-cysteic acid, the reduced spectrum disappears, indicating reversal of the dehydrogenation.

3. The purified coenzyme is rapidly destroyed by 0.1 *N* acids at 100° and by nucleotide pyrophosphatase but is not acted upon by 5-nucleotidase or phosphodiesterase. The heavy metal salts of coenzyme III are water soluble. In charcoal chromatography it behaves very much like nicotinamide mononucleotide.

4. On the basis of its behaviour to chemical reagents and to hydrolytic enzymes, the structure of coenzyme III is tentatively suggested to be nicotinamide-ribose-(5)-pyrophosphate.

5. Some of the salient properties of L-cysteinesulfinic dehydrogenase have been investigated.

6. On the basis of a detailed study of the electron transport from coenzyme III to O_2, it is concluded that a diaphorase (or cytochrome reductase), concerned with the reoxidation of coenzyme III, is present in the extracts in the apoenzyme form. This protein requires a second new coenzyme, which is not identical with FMN or FAD, but is likely to be a flavin. The latter cofactor is present in yeast extract and is readily separated from coenzyme III.

7. It is pointed out that coenzyme III also functions as the prosthetic group of dehydrogenases of animal origin and that it appears to be of wide distribution.

RÉSUMÉ

1. La L-cystéinesulfinique déhydrogénase, enzyme qui catalyse la déshydrogénation de l'acide L-cystéinesulfinique, exige la présence d'un coenzyme. Les vitamines et les coenzymes connus, ou des substances voisines, ne peuvent satisfaire cette exigence, mais un extrait de levure bouilli est une bonne source de cofacteur.

2. Le nouveau coenzyme, appelé provisoirement Coenzyme-III, a été extrait et fortement purifié à partir de levure de boulangerie. Au cours de l'oxydation de l'acide cystéinesulfinique par la déhydrogénase en présence de coenzyme purifié, ce dernier est réduit en une substance possédant

References p. 299.

le spectre d'absorption caractéristique des dihydropyridines nucléotides. Après addition d'acide L-cystéique, la disparition du spectre réduit indique le retour à la forme oxydée.

3. Les acides 0.1 *N* à 100° et la nucléotide pyrophosphatase détruisent rapidement le coenzyme purifié, mais la 5-nucléotidase ou la phosphodiestérase sont sans action. Les sels de métaux lourols coenzyme III sont solubles dans l'eau. Le comportement au cours de la chromatographie sur charbon est très semblable à celui du nicotinamide mononucléotide.

4. Le comportement vis à vis des réactifs chimiques et des enzymes hydrolytiques permet de proposer, pour le coenzyme III, la structure hypothétique: nicotinamide-ribose-(5)-pyrophosphate.

5. Quelques-unes des propriétés caractéristiques de la L-cystéinesulfinique-déhydrogénase ont été étudiées.

6. Une étude détaillée du transport d'électrons du coenzyme III à l'oxygène montre qu'une diaphorase (ou cytochrome réductase), qui joue un rôle dans la réoxydation du coenzyme III, se trouve dans les extraits sous forme apoenzyme. Cette protéine exige un second nouveau coenzyme, différent de FMN ou FAD, mais semblable à une flavine. Ce dernier cofacteur existe dans l'extrait de levure, et est facilement séparé du coenzyme III.

7. Le coenzyme III fonctionne également comme groupement prosthétique de déhydrogénases d'origine animale, et semble être largement répandu.

ZUSAMMENFASSUNG

1. L-Cysteinsulfindehydrogenase, das Enzym das die Dehydrogenierung der L-Cysteinsulfinsäure katalysiert, erfordert ein Coenzym um Aktivität zu erlangen. Die Anforderungen, die an das Coenzym gestellt werden müssen, werden nicht von den bekannten Vitaminen, Coenzymen oder verwandten Substanzen erfüllt; gekochter Hefeextrakt bildet dagegen eine gute Quelle für den Cofaktor.

2. Das neue Coenzym, das vorläufig Coenzym III genannt wird, wurde ausgehend von Bäckerhefe weitgehend gereinigt. Bei der Oxydation der Cysteinsulfinsäure mit Dehydrogenase in Gegenwart des gereinigten Coenzyms wird das letztere zu einer Substanz reduziert, die ein für Dihydropyridinnucleotide charakteristisches Absorptionsspektrum besitzt. Setzt man L-Cysteinsäure zu, so verschwindet das Spektrum der reduzierten Substanz, was eine Umkehr der Dehydrogenierung anzeigt.

3. Das gereinigte Coenzym wird rasch von 0.1 *N* Säuren bei 100° und von Nucleotidpyrophosphatase zerstört, dagegen wirken 5-Nucleotidase und Phosphodiesterase nicht ein. Die Schwermetallsalze des Coenzyms III sind wasserlöslich. Bei der Chromatographie mit Kohle verhält es sich weitgehend wie Nicotinamidmononucleotid.

4. Auf Grund seines Verhaltens gegenüber chemischen Reagenzien und hydrolysierenden Enzymen wird vorgeschlagen das Coenzym III versuchsweise als Nicotinamid-Ribose-(5)-pyrophosphat zu betrachten.

5. Einige der hervorstehendsten Eigenschaften der L-Cysteinsulfinsäuredehydrogenase wurden untersucht.

6. Auf Grund einer eingehenden Untersuchung der Elektronenübertragung von Coenzym III auf Sauerstoff, wurde geschlossen, dass eine Diaphorase (oder Cytochromreduktase), die an der Reoxydation des Coenzyms III beteiligt ist, im Extrakt in der Apoenzymform vorliegt. Dieses Protein erfordert ein zweites neues Coenzym, das nicht mit FMN oder FAD identisch ist, aber sehr wahrscheinlich ein Flavin ist. Der letztere Faktor kommt im Hefeextrakt vor und kann leicht vom Coenzym III getrennt werden.

7. Es wird darauf hingewiesen, dass das Coenzym III auch als prostethische Gruppe für Dehydrogenasen tierischen Ursprungs fungiert und dass es sehr weit verbreitet zu sein scheint.

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