# A NEW PYRIDINE NUCLEOTIDE COENZYME FOR BIOLOGICAL OXIDATIONS*

by

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It was shown in the foregoing paper[1] that two pathways exist for the oxidative metabolism of L-cysteinesulfinic acid in cell-free extracts of *Proteus vulgaris*: (*A*) oxidation to cysteic acid and (*B*) transamination with an α-keto acid, followed by desulfinication of β-sulfinylpyruvic acid and oxidation of sulfite. Metabolism of the amino acid by either of these pathways requires the addition of cofactors to be found in boiled extracts of bakers' yeast. While pathway (*B*) has been duplicated by the substitution of known compounds (α-ketoglutaric acid and $Mn^{++}$) for the yeast extract, no known coenzyme or vitamin can replace the factor present in yeast extracts which functions as a coenzyme for cysteic acid formation. The following compounds, alone and in combination, were without discernible activating effect: diphosphopyridine nucleotide (DPN), triphosphopyridine nucleotide (TPN), nicotinamide mononucleotide (NMN), riboflavin phosphate, flavin-adenine dinucleotide, adenylic acid, adenosine triphosphate, glutathione, coenzyme A, pyridoxal phosphate, biotin, thiamine phosphate, thiamine pyrophosphate, ascorbic acid, *p*-aminobenzoic acid, folic acid, and a purified preparation of the coenzyme of desulfinicase[2].

In these experiments and in the actual isolation work the assay system used as a guide consisted of $O_2$ uptake measurements at pH 7.8–8.0 in tris(hydroxymethyl)aminomethane buffer at 35°, in the presence of 5 mg lyophilized enzyme preparation[1], 1.5 mg brilliant cresyl blue, and $3 \times 10^{-2}$ *M* L-cysteinesulfinic acid.

After numerous attempts at solvent fractionation, heavy metal precipitation, and chromatography on ion-exchange resins, it was found that very significant purification of the coenzyme from boiled yeast extract could be obtained in good yield by the procedure of KORNBERG AND LINDBERG[3] for the isolation of nicotinamide mononucleotide. In practice, the *iso*amyl alcohol eluate obtained by this procedure was concentrated *in vacuo* to remove the alcohol, and the resulting solution was passed through a column of Dowex 50 resin, H-cycle, to remove traces of $Mn^{++}$, and after neutralization the effluent was further concentrated *in vacuo* to a small volume. Such preparations are essentially free from flavin nucleotides and DPN and they contain only a trace of TPN.

With the purified coenzyme the oxidation can be directly measured at 340 mμ in the Beckman

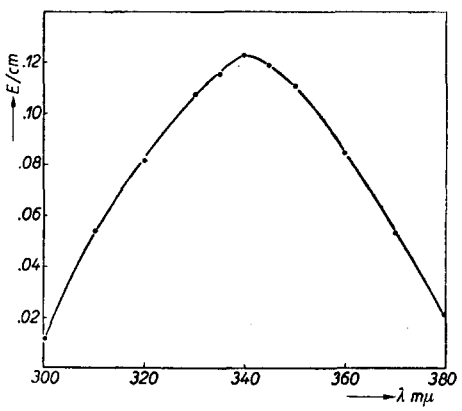


Fig. 1. Absorption spectrum of enzymatically reduced coenzyme

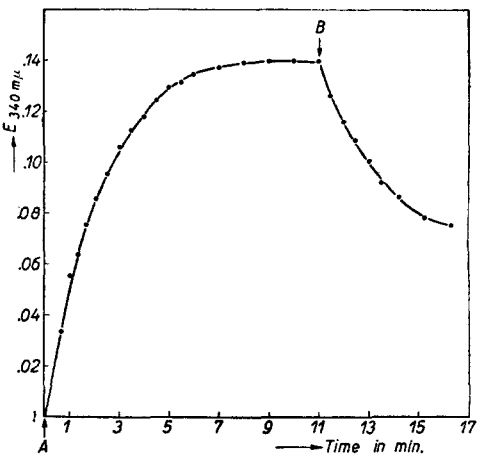


Fig. 2. Reversible oxidation of cysteinesulfinic acid by coenzyme III. Conditions: 0.4 mg protein and 2 ml purified coenzyme in a total volume of 3 ml; pH = 8.4; 60 μmols cysteinesulfinic acid added at A; 80 μmols cysteic acid added at *B*; temperature 23°

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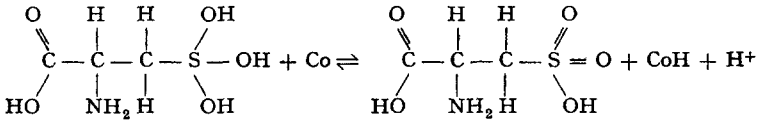
spectrophotometer in the simple system consisting of enzyme, substrate, and purified coenzyme, since the reduced form of the latter exhibits the typical spectrum of pyridine nucleotides (Fig. 1). In the absence of cysteinesulfinic acid no reduction occurs; upon addition of the substrate the band of the reduced coenzyme appears immediately and the reduction continues until equilibrium is reached (Fig. 2). If at any time after equilibrium is attained cysteic acid is added, the process is reversed and oxidation of the reduced coenzyme takes place (point *B* in Fig. 2), since at pH 7.8 the equilibrium is probably in favor of the reduction of cysteic acid by the dihydrocoenzyme. The reaction may then be represented as



wherein the term Co denotes the coenzyme and cysteinesulfinate is represented in the hydrated form, by analogy with the known mechanism of dehydrogenation of aldehydes. It should be added that DPN, TPN, and NMN are completely inactive in spectrophotometric assays, just as in manometric experiments.

The evidence quoted strongly suggests that the new coenzyme is a pyridine nucleotide derivative. As other pyridine nucleotides[4], it can be coupled with 2,6-dichlorophenol-indophenol and the reduction of the dye, estimated spectrophotometrically at 610 mμ, is then a measure of the activity of the dehydrogenase system.

Acid hydrolysis of the oxidized coenzyme at 100° indicates that it has a much more pronounced lability than could be accounted for by the nicotinamide-ribose linkage, suggesting the presence of a more acid-labile bond, possibly of pyrophosphate nature. The presence of a pyrophosphate bond in the molecule was established with the aid of highly purified nucleotide pyrophosphatase[5] (kindly supplied by Dr A. KORNBERG), which rapidly and completely destroyed the coenzyme. These observations, as well as other data on the solubility of heavy metal salts of the coenzyme, and its very similar behavior to NMN in charcoal chromatography suggest that the compound is a mononucleotide, closely related to NMN, but containing a pyrophosphate bond. The data are compatible with the structure: nicotinamide-ribose-(5)-pyrophosphate. Until the structure is definitely established by isolation in the pure state or by synthesis, as a provisional name "Coenzyme III" is suggested for the compound.

It should be added that our enzyme extracts appear to contain an enzyme which slowly hydrolyzes the coenzyme. The presence of this enzyme and the apparently high dissociation constant of cysteinesulfinic dehydrogenase may explain why the latter occurs almost entirely in the split-protein or apoenzyme state in our preparations. L-cysteinesulfinic acid and efficient metal-complexing agents, such as Complexone III, protect the coenzyme III from this enzymatic destruction, but nicotinamide has no such effect.

While crude yeast extract and partially purified preparations of Coenzyme III are highly active in the manometric test outlined, with our most purified preparations of the coenzyme only a fraction of the total dehydrogenase activity can be measured as $O_2$ uptake, although these show excellent activity in the spectrophotometric assay and cysteic acid is the product in all cases. Since brilliant cresyl blue does not react directly with reduced pyridine nucleotides, it appears that a diaphorase is present in our enzyme preparation, which requires the addition of an external source of prosthetic group for full activity and that this factor is removed in the course of the purification of Coenzyme III. Riboflavin, flavin mononucleotide, and flavin-adenine dinucleotide failed to reactivate the diaphorase. The mechanism of hydrogen transport from Coenzyme III to $O_2$ will be the subject of another communication.

The presence of coenzyme III in relatively high concentration in yeast suggests that it may also play a rôle in the metabolism of yeast cells. We are planning to ascertain whether the coenzyme is involved in the sulfur metabolism of animal tissues and in other bacterial dehydrogenases.

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## REFERENCES

[1] E. B. KEARNEY AND T. P. SINGER, *Biochim. Biophys. Acta*, 8 (1952) 698.
[2] B. BERGERET, F. CHATAGNER, AND C. FROMAGEOT, *Biochim. Biophys. Acta*, 4 (1950) 244.
[3] A. KORNBERG AND O. LINDBERG, *J. Biol. Chem.*, 176 (1948) 665.
[4] E. HAAS, *J. Biol. Chem.*, 155 (1944) 333.
[5] A. KORNBERG AND W. E. PRICER, JR., *J. Biol. Chem.*, 182 (1950) 763.

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