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*A COENZYME CONTAINING PSEUDOVITAMIN B_{12}**

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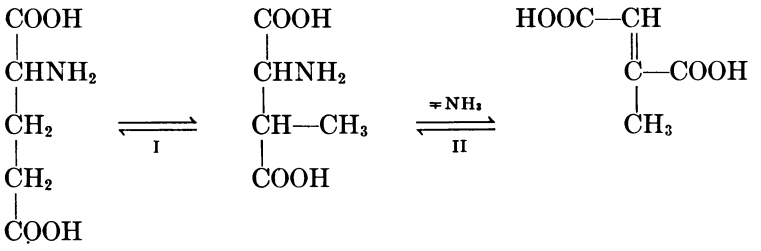
Vitamins of the B_{12} (cyanocobalamin) group apparently participate in several metabolic processes.[1] For example, Helleiner and Woods[2] reported that a cell-free extract of an *Escherichia coli* mutant, which requires either methionine or vitamin B_{12} for growth, forms methionine more rapidly in a reaction mixture containing homocysteine, serine, and adenosine triphosphate when supplemented with vitamin B_{12}. This indicates that the vitamin participates at some point in methyl group formation or transfer. More recently, Wagle *et al.*[3] found that vitamin B_{12} stimulates incorporation of amino acids into the proteins of a microsome-soluble protein system of vitamin B_{12}-deficient rats. Inhibition experiments with an unspecified anti-B_{12} compound indicated that the vitamin is involved in the activation of amino acids. Evidence for the involvement of vitamin B_{12} in the synthesis of deoxyribose by *Lactobacillus leichmanii*[4, 5] and in the reduction of dithio groups[6, 7] has also been presented.

Although these and other studies[1] have identified several metabolic processes in which the cobalamins participate directly or indirectly, the precise roles of these compounds have not been established. No specific enzymatic function has been demonstrated, and no coenzyme form of the vitamin has been described.

We wish to report the isolation of a coenzyme form of pseudovitamin B_{12} which is required for the decomposition of glutamate by particle-free extracts of *Clostridium tetanomorphum*. The coenzyme is involved in the rearrangement of the carbon skeleton of glutamate to form a branched chain amino acid, β-methylaspartate.

Previous studies in this laboratory[8] have shown that the fermentation of glutamate by *C. tetanomorphum* occurs by a sequence of reactions that is entirely different from the tricarboxylic acid cycle. A key compound in this sequence is mesaconate, a branched-chain C_5 dicarboxylic acid. The reversible conversion of glutamate to mesaconate and ammonia involves the following two reactions:



Glutamate β-Methylaspartate Mesaconate

Reaction I has been shown to require a coenzyme that can be removed from crude extracts by treatment with charcoal.[8a] Such extracts can be reactivated by the addition of boiled extract or purified coenzyme (Table 1). Estimation of the rate of reaction I and of coenzyme concentration is based upon the rate of mesaconate formation, determined spectrophotometrically at 240 mμ, in a system containing an excess of β-methylaspartase,[9] which catalyzes reaction II. For coenzyme assay, a charcoal- and protamine-treated extract, containing the apoenzyme for reaction I, is also added in excess.

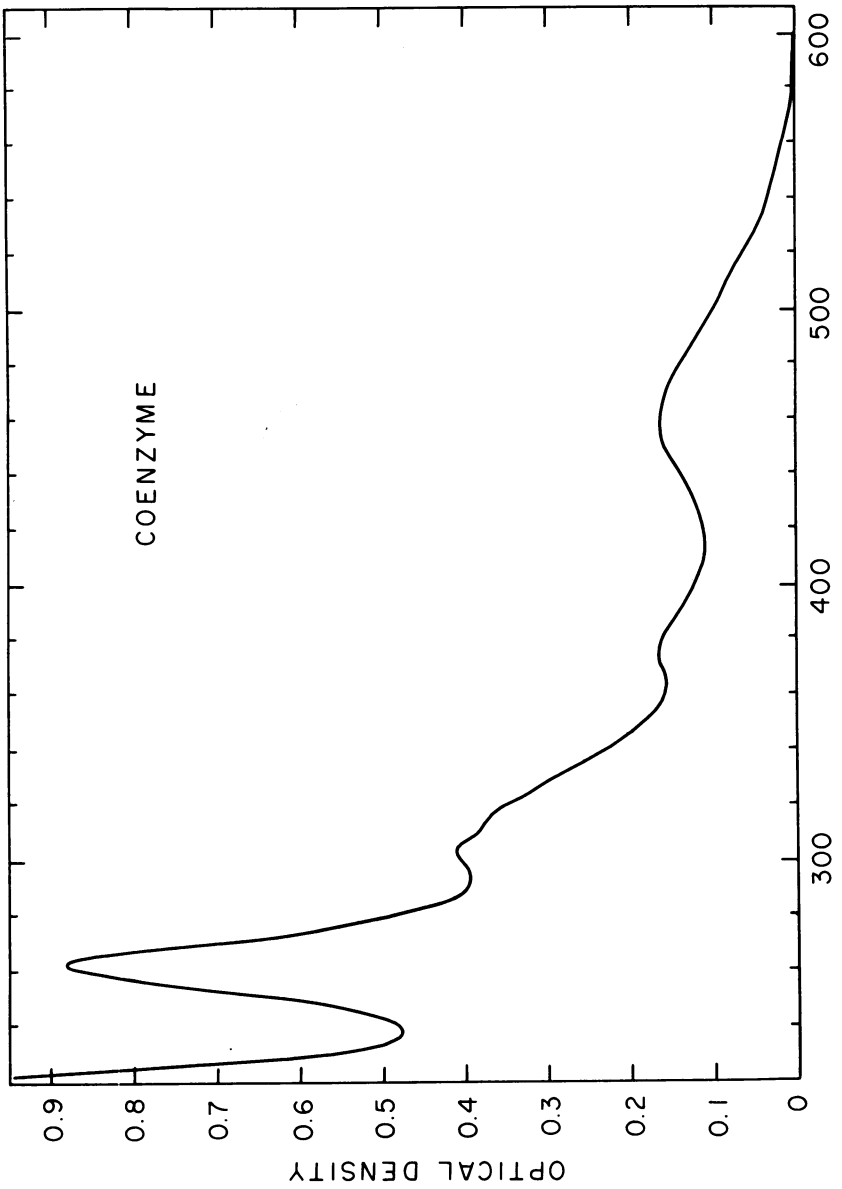


FIG. 1.—Absorption spectrum of the active coenzyme, 1.8×10^{-6} *M*, in 0.03 *M* sodium acetate pH 6.7.

We have developed a method for the purification of the coenzyme in micromolar amounts from *C. tetanomorphum* by the use of columns of alumina, Dowex-1, and Dowex-50. Fractions containing the most highly purified coenzyme are yellow-orange in color and show a distinctive absorption spectrum (Fig. 1), with maxima

TABLE 1*

COENZYME REQUIREMENT FOR CONVERSION OF GLUTAMATE TO MESACONATE

Expt.	Conditions	ΔO.D. 240 mμ/min
I	Crude extract, 0.2 ml.	0.180
	Charcoal-treated extract, 0.2 ml.	0.008
	Charcoal-treated extract, 0.2 ml. + heated extract, 0.2 ml.	0.142
II	Enzyme	0.002
	Enzyme + 0.02 ml. coenzyme solution	0.035
	Enzyme + 0.04 ml. coenzyme solution	0.076
	Enzyme + 0.08 ml. coenzyme solution	0.118

* All reaction mixtures contained Tris-chloride buffer pH 8.2 0.05 *M*, KCl 0.01 *M*, $MgCl_2$ 0.001 *M*, and potassium L-glutamate 0.01 *M* in a total volume of 1 ml. In Expt. I the crude extract contained 30 mg. protein per ml., and the charcoal-treated extract contained 28 mg. protein per ml.; the heated extract was prepared from the crude extract by heating for 10 minutes at 90° C. In Expt. II the enzyme was 0.05 ml. of a charcoal- and protamine-treated extract containing 23 mg. protein per ml.; the purified coenzyme solution was 9.1×10^{-6} *M* based on the assumption that the extinction coefficient in 0.1 *M* KCN at 367 mμ is the same as for vitamin B_{12}.

at 263, 303, 374, and 457 mμ. When a solution of the coenzyme is exposed to light from a tungsten lamp, the color changes gradually from yellow-orange to red, and the absorption spectrum is modified to give maxima at 261, 351, 407, 495, and 525 mμ (Fig. 2). The latter spectrum is similar to that of vitamin B_{12} and related compounds.[10] The change in the spectrum accompanies inactivation of the co-

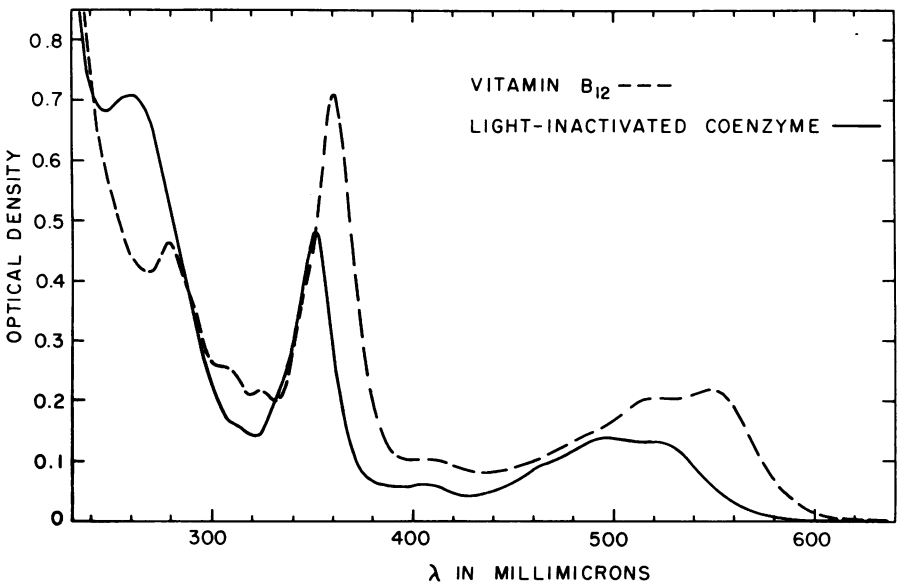


FIG. 2.—Absorption spectra of 2.6×10^{-5} *M* vitamin B_{12} and 1.8×10^{-5} *M* coenzyme inactivated by exposure to a 100-watt tungsten lamp for 105 minutes.

enzyme (see below). When 0.1 *M* KCN is added to the coenzyme, the color changes to reddish-purple, and the spectrum is characteristic of the dicyanide form[10] of the vitamin B_{12} group (Fig. 3). Further evidence for a structural similarity with this group is provided by the demonstration that the coenzyme contains approximately equimolar amounts of cobalt[11] and cyanide. Adenine, ribose, ammonia, and

phosphate are released from the coenzyme by acid hydrolysis, indicating that it is a derivative of pseudovitamin B_{12}.[12] A conspicuous difference between the spectra of the vitamin B_{12} group and the coenzyme is the relatively higher absorption of the latter in the 263–276 mμ region. This indicates that the coenzyme contains one or more additional light-absorbing groups. In confirmation of this conclusion we have

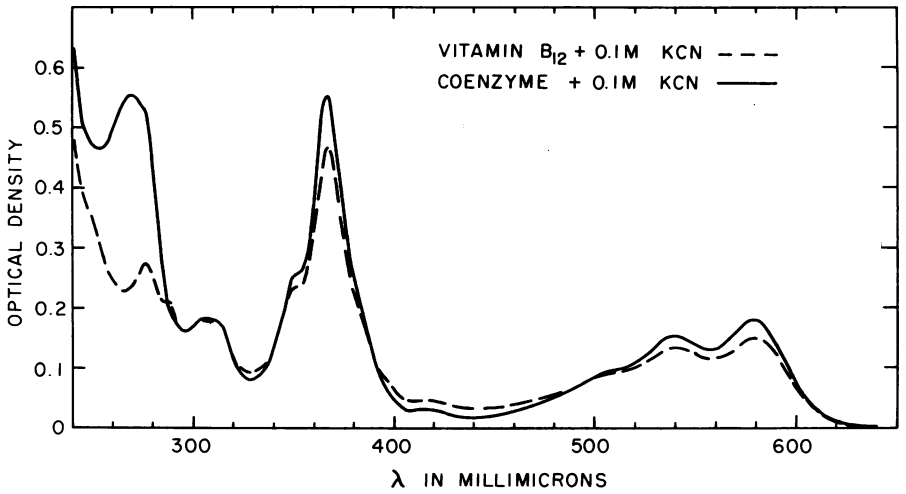


FIG. 3.—Absorption spectra of 1.5×10^{-5} *M* vitamin B_{12} and 1.8×10^{-5} *M* coenzyme in 0.1 *M* KCN.

observed that exposure of the coenzyme to light or to mild acid hydrolysis splits off a colorless compound that contains adenine or a closely related substance.

The connection between the yellow-orange compound (Fig. 1) and coenzyme activity was established in several ways. Throughout purification of the coenzyme, the activity was observed always to accompany the yellow-orange color. Quantitative enzymatic assays on highly purified column peak fractions showed a close proportionality between activity and optical density at 263, 303, and 457 mμ. Further evidence was provided by an experiment in which the change in absorption spectrum, on exposure of the coenzyme to light for various intervals, was correlated with enzymatic ac-

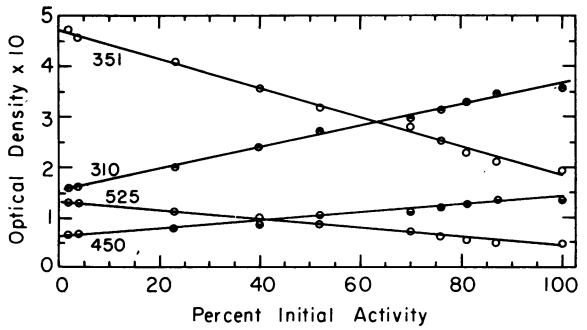


FIG. 4.—Proportionality between enzymatic activity and spectral changes during inactivation of the coenzyme by visible light. A 1.8×10^{-5} *M* solution of purified coenzyme in 0.03 *M* sodium acetate pH 6.8 was exposed at 0° C. to a 100-watt tungsten lamp at a distance of 6–30 cm. At intervals during 105 minutes, samples were removed for determining coenzyme activity, and the spectrum by means of a Cary spectrophotometer. The indicated wave lengths are expressed in millimicrons.

tivity. The decline in activity was found to be directly proportional to the change in optical density at several different wave lengths (Fig. 4). These observations indicate that coenzyme activity and the spectrum belong to the same compound. Neither vitamin B_{12} nor pseudovitamin B_{12}[12] is active in the enzymatic reactions.

We conclude that the coenzyme is a derivative of pseudovitamin B_{12} containing one or more additional purine moieties. The light-sensitivity of the coenzyme and the large shift of the peak in the visible region to longer wave lengths on exposure to light suggest that a functionally important substituent group is attached both to cobalt and to some part of the conjugated double-bond system of the porphyrin-like structure.

The precise role of the coenzyme in the interconversion of glutamate and β-methylaspartate is not yet known.

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[11] We are indebted to Dr. C. C. Delwiche, Kearny Foundation, University of California, for identifying and estimating cobalt by means of an X-ray fluorescence spectrometer.

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*ON THE REGULATION OF FATTY ACID BIOSYNTHESIS BY LIPOGENIN**

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We have shown[1] that a substance could be extracted from liver or yeast which, in catalytic amounts, markedly increased the rate of fatty acid synthesis in cell-free preparations from fasted rats. We now propose the name of "lipogenin" for this substance. The lipogenin used in these experiments was prepared from yeast