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Cobalamins and Cobalamin-Dependent Enzymes in *Candida utilis*

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Candida utilis has been shown to contain 4.7 pmol of cobalamin per g of wet cell paste. Purification of the cobalamin showed it to be a mixture of methylcobalamin and adenosylcobalamin. Two cobalamin-dependent enzyme systems have been found in the yeast: methylcobalamin-dependent methionine biosynthesis and leucine 2,3-aminomutase. The cobalamin extracted from the yeast is as effective as authentic adenosylcobalamin in stimulating leucine 2,3-aminomutase.

Early attempts to assay yeast for vitamin B_{12} activity generally found little or no activity (17). Many of these assays were based on the growth rate of chicks or rat pups. Later surveys of nutritional sources made with microbiological assays employing *Lactobacillus leichmanii*, *Euglena gracilis*, and *Ochromonas malhamensis* and the like seem to have avoided examining the yeasts on the basis of the earlier reports. Halbrook et al. (6) reported that among the organisms that were found to contribute to the B_{12} content of poultry house litter and droppings were several types of bacteria and yeasts and molds. The nature of the yeasts was not established, and evidence was alluded to that suggested that yeasts and molds play only a minor role in the production of B_{12} in such materials.

Indeed, it has long been a tenet of biology that cobalamins and related corrins are found only in bacteria and animals and that, since animals cannot synthesize corrins, bacteria are the ultimate source of all metabolically active corrins. Plants and yeasts were believed to be devoid of any B_{12} congeners. Recently these ideas have been challenged by the discovery that a variety of plants contain cobalamin-dependent enzymes (14, 15). Because these enzymes were endogenous to the plants themselves and were not the result of bacterial contamination, it must be concluded that the plants also possessed endogenous corrin compounds.

During a study of some of these plant corrins, extracts of the yeast *Candida utilis* were examined for corrin compounds. This organism was chosen because it, unlike some other yeasts, is able to grow on media unsupplemented with vitamins; any corrins and corrin-dependent enzymes it possesses must arise from de novo synthesis. This paper details the existence of such corrins and corrin-dependent reactions in the yeast, thereby demonstrating that yeasts are not unique in their metabolism, but also synthesize and utilize corrins.

MATERIALS AND METHODS

C. utilis NCYC was grown in a medium containing 8 g of sodium glutamate, 8 g of KH_2PO_4, 0.5 g of Na_2HPO_4, 3 g of K_2SO_4, 2 ml of mineral solution, and 216 mg of $CoCl_2$ per liter. The mineral solution contained (grams per liter): $MgCl_2 \cdot 6\ H_2O$, 120; $CaCl_2 \cdot 6\ H_2O$, 40; $FeCl_3 \cdot 6\ H_2O$, 1; $ZnSO_4 \cdot 7\ H_2O$, 1; $MnCl_2 \cdot 4\ H_2O$, 1; H_3BO_3, 1; KI, 0.1; $Na_2MoO_4 \cdot 2\ H_2O$, 0.1; and $CuSO_4 \cdot 5\ H_2O$, 0.1. Growth was obtained with vigorous aeration at 30°C. When the medium was supplemented with $^{57}CoCl_2$ (2 μCi), incubation was conducted in a secluded corner of the laboratory at ca. 20°C. The yield of the cells under such conditions was 4.1 g/liter, somewhat lower than the 10 g/liter obtained when the cells were grown at 30°C.

Radio-binding assay of cobalamin. The commercial kit, Phadebas B_{12} Test 50, (Pharmacia Diagnostics) was used to assay the corrin compounds. This kit depends upon the selective binding of corrin by a mucoprotein which is, in turn, attached to Sephadex beads. A constant amount of radioactive [^{57}Co]cyanocobalamin was added to all tubes followed by a constant amount of Sephadex-binder complex. Any decrease in radioactivity bound to the complex served as a measure of the non-radioactive cobalamin present. Cyanide was added to all tubes so that stability and binding variations that might result from different ligands of the corrin were avoided.

Measurement of radioactivity. Radioactivity was measured in a Beckman LS-250 liquid scintillation counter with Hydromix (Yorktown Research, Hackensack, N.J.) as the scintillant. Carbon-14 was measured directly in its appropriate energy window. Although cobalt-57 is a low-level gamma emitter, it may be counted in this scintillation counter (a beta counter) by using the energy window appropriate for tritium. Autoradiograms were made using Kodak X-Omat S film.

Bioautography of cobalamin. Samples of various

preparations were examined for cobalamin content by using the procedure of Linnell (9, 10) and Nexø (11, 12), in which separations were made by thin-layer chromatography and the corrin spots were visualized by bringing the thin-layer plate into contact with agar seeded with *Escherichia coli* NCIB 9270 (ATCC 14169). This cobalamin-dependent strain grew only in the presence of cobalamins and caused the reduction of tetrazolium red included in the agar. Growth due to as little as 5 fmol of cobalamin could easily be detected.

All manipulations involving authentic or putative corrin compounds were done in dim light to avoid photo-catalyzed decomposition. The exception to this was the assay with the Phadebas kit, which converts all the corrins to their cyanide forms that are not particularly light sensitive.

Preparation of cell extracts. Cell extracts of *C. utilis* were prepared by suspending frozen cell paste in 0.02 M potassium phosphate buffer (pH 7.4) and rupturing the cells in a French pressure cell (American Instruments, Silver Spring, Md.). Whole cells and debris were removed by centrifugation at 30,000 × *g* for 30 min.

Enzyme assays. Leucine 2,3-aminomutase was measured utilizing DL-β-leucine as substrate and following the appearance of α-leucine by gas-liquid chromatography of the trimethylsilyl derivatives as described previously (14). Reaction mixtures consisted of 0.25 ml containing 100 mM triethanolamine buffer (pH 8.5), 0.5 μM flavin adenine dinucleotide, 0.5 mM coenzyme A, 0.5 mM NAD, 0.5 mM pyridoxal phosphate, 1.15 mg of protein of the crude extract of *C. utilis*, and DL-β-leucine and adenosylcobalamin as indicated. When added, adenosylcobalamin was 0.156 μM. The reaction was incubated at 37°C for 60 min and was stopped by the addition of 0.025 ml of 6 N HCl and 0.0125 ml of 10% (wt/vol) sodium tungstate. A 0.5-ml portion of the protein-free supernatant solution was derivatized with 0.1 ml of bis-trimethylsilyl-trifluoroacetamide in 0.1 ml of acetonitrile; 0.5 μmol was used as an internal standard. Separation and measure of α-leucine was obtained by using a column of OV-11 on Supelcoport (Supelco, Inc., Bellefonte, Pa.) as described by Gehrke and Leimer (5). The gas chromatograph was a Hewlett-Packard 5830A equipped with a flame ionization detector.

Methylcobalamin-dependent biosynthesis of methionine was measured by following the conversion of [$^{14}CH_3$]methylcobalamin to [$^{14}CH_3$]methionine (18, 19). The 1-ml reaction mixtures contained 100 mM potassium phosphate buffer, (pH 7.4), 1 mM S-adenosylmethionine, 50 μM flavin mononucleotide, 0.5 mM NADPH, 5 mM 2-mercaptoethanol, 0.5 mM [$^{14}CH_3$]methylcobalamin (1,066 dpm/nmol), and 1.89 mg of protein of crude cell extract of *C. utilis*. DL-Homocysteine thiolactone-hydrochloride was made up in triethanolamine buffer (pH 8.5) about 30 min before addition to the reaction mixture so as to allow the thiolactone ring to be hydrolyzed. The solution was generally clear upon addition or just beginning to show traces of opalescence, indicating that there had been some oxidation to homocystine. Reactions were carried out under argon at 37°C. After 60 min, the reaction was stopped by the addition of 0.5 ml of 0.002 M methionine in 0.2 N HCl followed by 0.5 ml of water. One milliliter of a slurry of acid-washed charcoal containing 22 mg of charcoal was added to remove the cobalamin and then centrifuged. The clear supernatant solution was exposed to strong light overnight to photolyze any residual [$^{14}CH_3$]methylcobalamin and then passed through a small column (5 by 30 mm) of Dowex-50 in the hydrogen form. The column was washed with water and then eluted with 2 N NH_4OH. An aliquot of the eluate was counted. The remaining eluate was freeze dried, in some experiments, taken up in a small amount of water, and streaked on silica gel plates. These thin-layer plates were then chromatographed in 1-butanol-acetic acid-water (5:1:2). The standard methionine and a portion of one of the samples were visualized with ninhydrin. Every 1-cm section of each of the sample tracks was counted in a scintillation vial with Hydromix.

Extraction of corrin material from yeast. Frozen cell paste (100 g) was thawed in 400 ml of absolute ethanol and heated at 70°C for 60 min. The cells were filtered, and the clear yellow filtrate was concentrated under vacuum on a rotary evaporator. A gummy brown precipitate formed as the alcohol was removed. The precipitate was extracted with about 10 ml of water, and the resulting solution was passed through a small column (5 by 30 mm) of Amberlite XAD-2 (60/100 dry mesh) prepared according to the method of Fenton and Rosenberg (4). The column was washed with about 10 ml of acetic acid-water (1:100), and the effluent and wash were discarded. The column was then eluted with methanol-water-acetic acid (50:50:1).

RESULTS

Direct measurement of corrin. When a crude cell extract of *C. utilis* was examined with the Phadebas test kit, it was treated in the same fashion as though it were blood serum (for which the kit was designed) in that it was heated in glutamate buffer (pH 3.3) containing 0.002% potassium cyanide for about 15 min to convert any bound corrin to a freely soluble cyanide derivative. When measured directly, the crude extract contained 1,720 pmol of cobalamin per liter of extract, which corresponded to 5.6 pmol/g of packed cell paste. When more precise assays were conducted by the method of additions (Fig. 1), 1,450 pmol/liter or 4.7 pmol/g of wet cell paste were found.

Bioautography. When the isolated corrin was chromatographed on silica gel plates and exposed to the agar seeded with the *E. coli* mutant, strong growth was seen in areas characteristic of both methyl- and adenosylcobalamin. The R_f of the upper spot of the *C. utilis* extract was 0.77, which compares well with that of 0.79 for methylcob(III)alamin. The R_f for the lower spot of the *C. utilis* extract was 0.61, which compares with 0.66 for adenosylcob(III)alamin.

When cells grown in the presence of $^{57}CoCl_2$ were extracted, the corrin was found to be radioactive. It bound to the Sephadex-binder complex

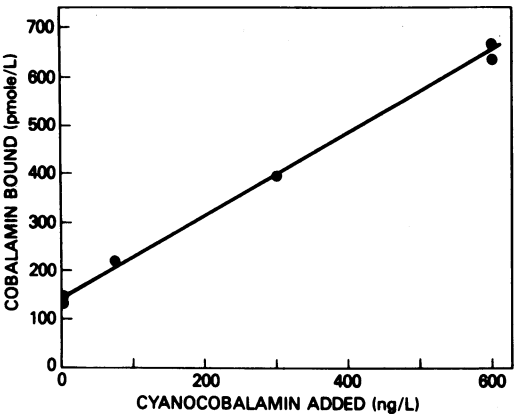

FIG. 1. *Effect of added cyanocobalamin on the binding assay of cobalamin from C. utilis. Crude cell extract of C. utilis was treated as described in the text, and 0.1-ml portion was added to the Phadebas assay. Standard solutions of cyanocobalamin from the Phadebas kit were added to give the concentrations indicated, and the tubes were made up to 1.0 ml with appropriate volumes of glutamate buffer (Phadebas), pH 4.1, containing 0.002% KCN. The resulting solutions were assayed according to the manufacturer's directions and are plotted here exactly as reported in the assay, i.e., as picomoles per liter of fluid examined. Because of dilution, the actual value at the intercept (no cyanocobalamin added) is 1,450 pmol/liter.*

of the Phadebas test kit in the same fashion as the non-radiolabeled corrin and exhibited the same isotope dilution as that seen with authentic cobalamin. Autoradiograms of the radioactive corrin were unsuccessful because of the low yield of the corrin in the extract coupled with the low specific radioactivity. Cochromatography with authentic cobalamin and with unlabeled yeast corrin showed normal chromatography on bioautograms.

Leucine 2,3-aminomutase. Table 1 shows the results of the assay of cell extracts of *C. utilis* for leucine 2,3-aminomutase activity. In experiment 1, there was some endogenous activity which clearly responded to the addition of the substrate β-leucine. The activity, however, was greatly stimulated by the addition of exogenous B_{12} coenzyme. Moreover, as is shown in experiment 2, the addition of corrin extracted from yeast stimulates the leucine 2,3-aminomutase of yeast in the same pattern as authentic adenosylcobalamin.

Methionine biosynthesis. When radiolabeled methylcobalamin and homocysteine were incubated with cell extracts of *C. utilis*, label was recovered in the amino acid fraction. This incorporation was proportional to homocysteine concentration (Fig. 2). Because the radiolabel was in the methyl group of the methylcobalamin, it is reasonable to conclude that the product of the incorporation was the methylated homocysteine, i.e., methionine. Evidence consistent with such an interpretation was provided by thin-layer chromatography of the amino acid fraction. The radiolabel in each of the samples migrated with the same R_f (0.43) as that of authentic methionine.

DISCUSSION

The chromatographic evidence indicates that the corrin in *C. utilis* is cobalamin in its methyl and adenosyl forms. This is clearly consistent with the binding seen in the isotope dilution experiments with the Phadebas test kit, whether

TABLE 1. *Leucine 2,3-aminomutase activity in C. utilis*[a]

Exp	DL-β-Leucine added (μmol/ml)	α-Leucine formed (nmol/ml)			
		No addition	Adenosylcobalamin added	Yeast cobalamin added	Adenosylcobalamin + yeast cobalamin added
1	0	0	286		
	4	155	428		
	20	174	726		
2	8	80	161	197	153

[a] Reaction conditions are those described in the text. When added, adenosylcobalamin was 0.156 μM; yeast cobalamin was approximately the same concentration based on the relative intensity of the adenosylcobalamin spot on the bioautogram.

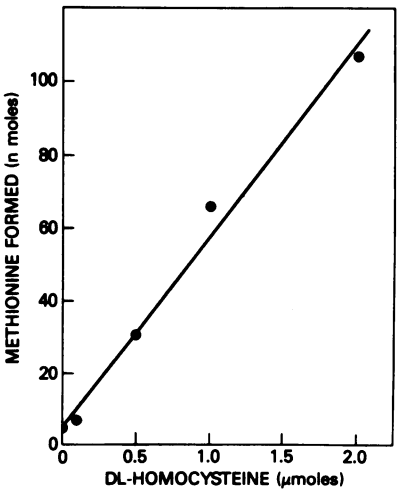

FIG. 2. *Dependence upon DL-homocysteine of methylcobalamin-dependent synthesis of methionine by C. utilis.*

the immobilized binder is intrinsic factor, as indicated by the manufacturer, or a binder of broader corrinoid specificity, as suggested by Kolhouse et al. (8). Intrinsic factor is the cobalamin-specific mucoprotein produced in mammalian stomachs with which cobalamin must bind before it can be absorbed in the lower small intestine. The competition for binding to the Sephadex-intrinsic factor complex between authentic cobalamin and the corrin from the yeast is exactly the sort one would predict for cobalamin.

The detection of methylcobalamin on the bioautograph suggested that the methylcobalamin-dependent pathway of methionine synthesis might be a normal pathway for *C. utilis*. Accordingly, since [$^{14}CH_3$]methylcobalamin was shown to give rise to a labeled amino acid when incubated with extracts and homocysteine, it is reasonable to conclude that this pathway of methionine biosynthesis is operative in *C. utilis*.

The other cobalamin-dependent enzyme found, leucine 2,3-aminomutase (EC 5.4.3.--), seems to be an enzymic activity that is very widespread in nature. It has been found in bacteria (13), mammals (13), plants (14, 15), and now yeast. In bacteria this enzyme seems to have a role in the catabolism of α-leucine. Whether this is so in animals and plants is not certain. Whether catabolic, anabolic, or amphibolic, however, its role must be important for the activity to be so widely dispersed in nature.

The value of 4.7 pmol of corrin per g of packed cell paste is not a trivial amount. Liver is recognized as probably the best source of dietary cobalamin, and such values as 33 pmol/g of moist rat liver (2), 64 pmol/g of moist chicken liver (2), and 222 pmol/g of moist bovine liver (16) have been reported. It is estimated that the minimum daily requirement for cobalamin by humans is about 1 μg (0.739 nmol) per day (3, 6) (not recommended daily allowance, but, rather, the amount of cobalamin necessary to alleviate the symptoms of pernicious anemia). A single-cell organism which can provide not only protein and several vitamins, but also about 1% of the daily human requirement of cobalamin per g of packed cell paste warrants serious consideration as a dietary supplement.

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