

was determined from *Z* by the use of normal tables. The relatively low *p* value obtained for the isomerase-cyclohydrolase interval probably reflects the unreliable nature of the cyclohydrolase assay in these experiments.

¹³ Goldstein, A., D. B. Goldstein, and L. T. Lowney, *J. Mol. Biol.*, **9**, 213 (1964).

¹⁴ Kepes, A., and S. Beguin, *Biochem. Biophys. Research Commun.*, **18**, 377 (1965).

¹⁵ Alpers, D. H., and G. M. Tomkins, these PROCEEDINGS, **53**, 797 (1965).

¹⁶ Martin, R. G., *J. Biol. Chem.*, **238**, 257 (1963).

¹⁷ Whitfield, H. J., Jr., D. W. E. Smith, and R. G. Martin, *J. Biol. Chem.*, **239**, 3288 (1964).

¹⁸ The possibility of a polycistronic message for the histidine operon has been discussed by Martin, R. G., in *Cold Spring Harbor Symposia on Quantitative Biology*, vol. 28 (1963), p. 357.

REQUIREMENT OF RIBONUCLEOTIDE REDUCTASE FOR COBAMIDE COENZYME, A PRODUCT OF RIBOSOMAL ACTIVITY*

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Communicated by Herman M. Kalckar, May 21, 1965

Until recently, vitamin B₁₂ was implicated in the enzymatic conversion of ribonucleotides to deoxyribonucleotides only by the following indirect evidence:¹ (1) the ability of deoxyribonucleosides to eliminate the nutritional requirement for vitamin B₁₂ of certain lactobacilli;² (2) isotopic data showing poor conversion of ribosyl groups to DNA deoxyribose in the absence of vitamin B₁₂;³⁻⁷ (3) decreased levels of intracellular acid-soluble deoxyribosyl compounds in vitamin B₁₂-deficient *Lactobacillus leichmannii* with abrupt increases following repletion;⁸ and (4) typical unbalanced growth when *L. leichmannii* and other vitamin B₁₂-dependent organisms are deprived of vitamin B₁₂.^{9, 10}

The attempts of recent years by several laboratories to obtain direct enzymological evidence of a relationship between vitamin B₁₂ and deoxyribosyl synthesis culminated in the recent demonstration by Blakley and Barker that deoxyribosyl formation from CMP-C¹⁴ by crude Dowex-1-(Cl)-treated extracts of *L. leichmannii* (ATCC 4797) diminished to about 5 per cent of the optimal rate in the absence of DBC coenzyme.¹¹ Extracts also required 2-mercaptoethanol, glucose 6-phosphate, ATP, and Mg⁺⁺, and under optimal conditions formed approximately 1.6 μmoles of deoxyribosyl per hour per mg of protein. It is of interest that a cobamide requirement has not yet been reported for purified ribonucleotide reductases of Novikoff rat hepatoma¹² and the vitamin B₁₂ prototroph, *E. coli* B,¹³ or for the reductase of *E. coli*_{15T}- derepressed by thymine withdrawal,¹⁴ although the last is slightly stimulated by CN-B₁₂ and DBC coenzyme.¹⁵

Because our extracts of *L. leichmannii* had displayed feeble reductase activity, we tested the possibility that this enzyme, like that of *E. coli*_{15T}-,^{14, 15} is under repressor control, in which case activities could be enhanced by derepression. The results, to be reported below, revealed that *L. leichmannii* reductase is derepressible, the active crude enzyme thus made available having absolute requirements for CN-B₁₂ or DBC coenzyme, ATP, Mg⁺⁺, and L(SH)₂. When extracts were freed of ribosomes, activity with CN-B₁₂ was lost and the requirement for

DBC coenzyme became absolute. It is concluded that DBC coenzyme is an essential cofactor of ribonucleotide reductase and that ribosomes are the loci of its synthesis and subsequent binding and release.

Materials and Methods.—*Reagents:* Nonradioactive nucleic acid derivatives were purchased from California Corporation for Biochemical Research; cyanocobalamin and hydroxocobalamin, Merck Laboratories; DPNH, TPN, TPNH, *dl*-lipoic acid (disulfide form), and glucose 6-phosphate, Sigma Chemical Company; bovine pancreatic ribonuclease (EC 2.7.7.16), Worthington Biochemical Corporation. Chloramphenicol was a gift of Dr. L. M. Long, Parke Davis and Company; BC and DBC coenzymes of Dr. D. Perlman, Squibb Research Institute; synthetic methylcobalamin of Dr. Roy L. Kisluk; cyanocobalamin monocarboxylic acid and monoanilide of Dr. E. L. Smith, Glaxo Research, Ltd. All C^{14} -labeled nucleotides were obtained from Schwarz BioResearch, Inc. Lipoic acid was reduced according to Gunsalus and Razzell.¹⁶ The concentration of $L(SH)_2$, pH 7.4, was adjusted to 0.12 *M* and 0.5-ml (one per day) aliquots were sealed in glass ampuls under Ar and stored in the dark at -20° . Factor B was prepared by acid hydrolysis of CN-B₁₂.¹⁷ *Crotalus adamanteus* venom was obtained from Ross Allen's Reptile Institute. Trans-*N*-deoxyriboseylase (EC 2.4.2.6) was an ammonium sulfate fraction (specific activity, 1.77) purified from *L. leichmannii*.¹⁸

Cultivation of bacteria and preparation of sonicates: The cultivation of *L. leichmannii* (ATCC 7830) has been described elsewhere.⁹ The basic medium was modified assay medium¹⁹ plus added vitamin B₁₂ (0.1 μ g/ml). This medium ordinarily contains folic acid, but folate is stored by these cells.²⁰ Therefore, folate in the medium was replaced by thymine (50 $m\mu$ moles/ml).²⁰ The thymineless state was achieved when cells were grown to log phase (turbidity, 70 Klett₆₆₀ units) in a thymine-containing "primary" culture, harvested, washed twice with saline, and resuspended in a "secondary" culture of equal volume containing thymine-free synthetic medium. Secondary cultures were further incubated at 37° for various time intervals, cells were again collected by centrifugation, washed with 0.05 *M* Tris buffer, pH 7.5, and suspended in 3 vol of the same buffer containing 0.05 *M* MgCl₂ except as noted below. Suspended cells were sonicated for 10 min in an 18–20-kc MSE ultrasonic disintegrator and extracts were centrifuged for 15 min at $25,000 \times g$. Protein was estimated by the phenol method²¹ with albumin as standard.

Ribonucleotide reductase assay: The standard incubation mixture contained: CDP-U- C^{14} or CDP-2- C^{14} (or other radioactive nucleotide as noted below), 1.25 μ moles; ATP, 4 μ moles; MgCl₂, 10 μ moles; $L(SH)_2$, 15 μ moles; Tris buffer, pH 7.3, 25 μ moles; bacterial protein, 1–2 mg; and cobamide compound as noted below in a volume of 0.5 ml. After incubation in the dark for 60 min at $37^\circ C$, the reaction was terminated with 0.5 ml of 2 *M* HClO₄. The supernatant fluid containing dCDP- C^{14} and residual CDP- C^{14} was heated in boiling water for 20 min to convert nucleoside diphosphates to monophosphates, and perchlorate was removed as KClO₄. The filtrate was assayed for dCMP- C^{14} by separation on a 1×13 -cm Dowex 50-(H⁺) column according to Reichard²² after addition of carrier dCMP. Specific activity is defined as $\Delta m\mu$ moles dCDP per hour per mg protein.

Results.—*Derepression of ribonucleotide reductase by thymine withdrawal:* When cells grown to log phase in a medium optimal in thymine were harvested, washed, and resuspended in medium containing no thymine, the time-course of reductase activity in whole sonicates assayed in mixtures containing 2 $m\mu$ moles of CN-B₁₂ (see below) was as shown in Figure 1. In the absence of thymine, specific activity increased steadily for 5 hr; in contrast, there was a negligible increase in a control secondary culture containing optimal thymine. Thus, at 5 hr a 12-fold increase in specific activity had occurred in the thymineless medium. Activities decreased when thymine starvation was continued longer. It is of interest that the maximum specific activity attained in derepressed *E. coli*_{15T} sonicates was 4 when CN-B₁₂ or DBC coenzyme was present in the incubation mixture;¹⁵ specific activity in derepressed *L. leichmannii* was 40–85 when assayed with CN-B₁₂, and 100–190 with DBC coenzyme.

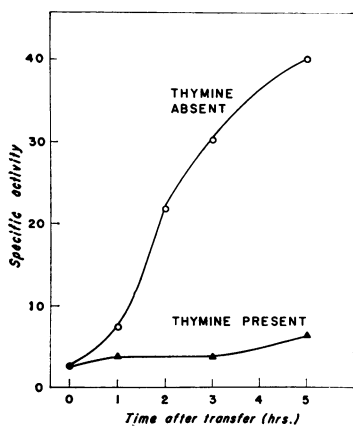


FIG. 1.—Time-course of enzyme activity following transfer from thymine-containing primary culture to secondary cultures containing no thymine, O, and 50 μ moles/ml of thymine, \blacktriangle . Folate was omitted from all cultures.

The ATP requirement was also absolute (Fig. 2D). Activity was not increased by additions to the complete system of 1 μ mole each of TPNH or of glucose 6-phosphate alone or with TPN (Table 2). L(SH)₂ could not be replaced by 2-mercaptoethanol. In other experiments, 1 μ mole of added DPNH had no effect and activity was 75 per

To determine if the increase in reductase activity following thymine deprivation requires prior protein synthesis, cells grown in a thymine-containing primary culture were transferred to thymine-free media containing chloramphenicol. Table 1 summarizes the results. The increase in enzyme activity following thymine deprivation is blocked by chloramphenicol, indicating that thymine withdrawal derepresses enzyme synthesis.

Requirements for dCDP formation: Figure 2 shows the effects on reductase activity of variations in the concentrations of added CN-B₁₂, DBC coenzyme, L(SH)₂, Mg⁺⁺, and ATP. There were absolute requirements for a vitamin B₁₂ derivative, L(SH)₂, and ATP, and a relative requirement for Mg⁺⁺. Activity with DBC coenzyme was approximately twice that obtained with CN-B₁₂. Figure 2C reveals a sharp activity maximum at a Mg⁺⁺ concentration of 0.02 M.

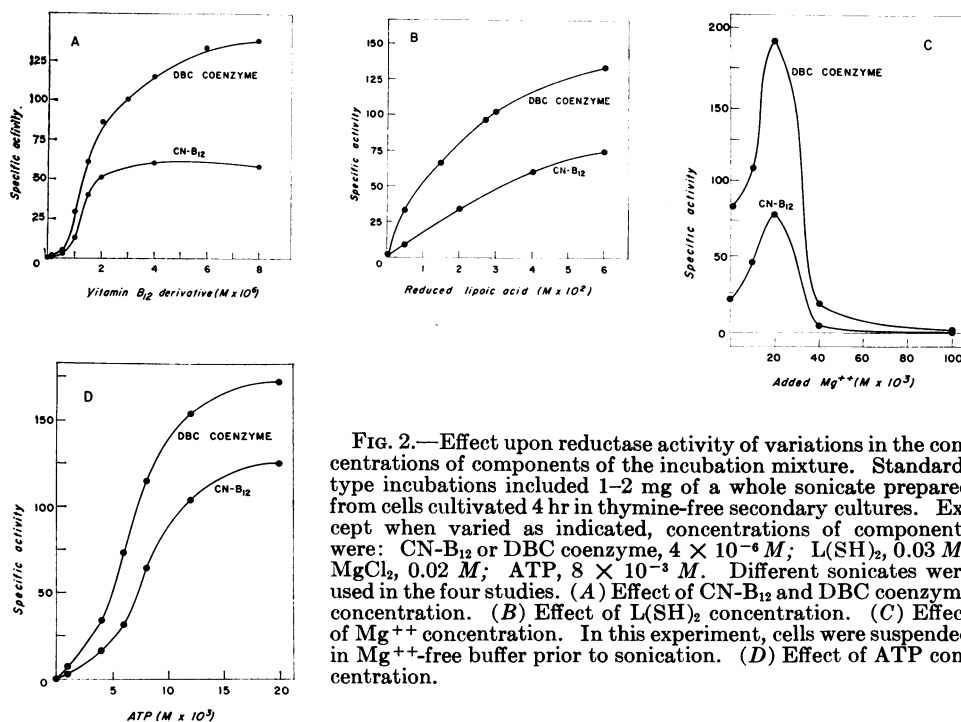


FIG. 2.—Effect upon reductase activity of variations in the concentrations of components of the incubation mixture. Standard-type incubations included 1–2 mg of a whole sonicate prepared from cells cultivated 4 hr in thymine-free secondary cultures. Except when varied as indicated, concentrations of components were: CN-B₁₂ or DBC coenzyme, 4×10^{-8} M; L(SH)₂, 0.03 M; MgCl₂, 0.02 M; ATP, 8×10^{-3} M. Different sonicates were used in the four studies. (A) Effect of CN-B₁₂ and DBC coenzyme concentration. (B) Effect of L(SH)₂ concentration. (C) Effect of Mg⁺⁺ concentration. In this experiment, cells were suspended in Mg⁺⁺-free buffer prior to sonication. (D) Effect of ATP concentration.

TABLE 1
EFFECT OF CHLORAMPHENICOL ON *L. leichmannii* REDUCTASE
ACTIVITY 4 HR AFTER TRANSFER TO THYMINE-FREE MEDIUM

Chloramphenicol	Δ dCDP ($m\mu$ moles/mg/hr)	
	Transferred to -T medium	Transferred to +T medium
Absent	174.5	28.5
Present	28.5	12.3

Cells were harvested and assayed for ribonucleotide reductase (substrate, CDP-2-C¹⁴) after cultivation for 4 hr in secondary cultures containing thymine (50 $m\mu$ moles/ml) and chloramphenicol (0.2 μ mole/ml) as noted. Reductase was assayed in standard incubations containing 2 $m\mu$ moles of DBC coenzyme.

TABLE 2
REQUIREMENTS OF RIBONUCLEOTIDE REDUCTASE SYSTEM

System	Δ dCDP ($m\mu$ moles/mg/hr)
Complete	191.0
Minus DBC coenzyme	0.1
Minus L(SH) ₂	0.8
Minus L(SH) ₂ plus 2-mercaptoethanol	3.2
Minus ATP	0.0
Minus Mg ⁺⁺	47.5
Plus TPNH	188.5
Plus glucose 6-phosphate	187.0
Plus glucose 6-phosphate plus TPN	192.5

The complete system contained (in $m\mu$ moles): CDP-2-C¹⁴, 1,250 (specific radioactivity, 4,530 cpm/ μ mole); DBC coenzyme, 2; ATP, 4; MgCl₂, 10; L(SH)₂, 15; Tris buffer, pH 7.3, 25; and 1.2 mg of *L. leichmannii* sonicate prepared from cells that had been cultivated in a thymineless secondary culture for 4 hr and suspended in Mg⁺⁺-free buffer prior to sonication. Volume of the incubation mixture, 0.5 ml. The amounts of other additions were (in μ moles): TPNH, 1; TPN, 1; glucose 6-phosphate, 1; 2-mercaptoethanol, 15.

cent lower with CMP as substrate than with CDP. The pH optimum of derepressed enzyme is 7.3. Lineweaver-Burk plots of reaction velocities and CDP concentrations at saturating cofactor concentrations yielded K_m values of 9.38×10^{-4} for derepressed enzyme and 9.50×10^{-4} for repressed enzyme.

Comparison of the activities of various cobamide derivatives (Table 3) revealed maximal reductase activity with BC and DBC coenzymes. CN-B₁₂ was half as effective, OH-B₁₂ slightly less so, and the other forms tested were virtually inert. Cobamide participation was catalytic in nature, approximately 100 moles of CDP being reduced per mole of coenzyme added (Fig. 2A and Table 3).

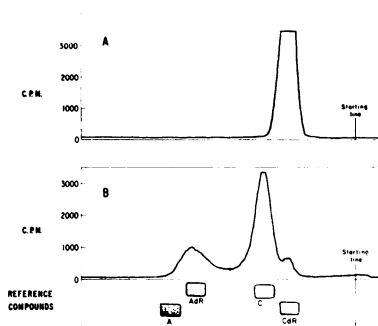
Proof of ribosyl reduction: A standard-type incubation was run in which the substrate was uniformly labeled CDP-C¹⁴. As outlined in Figure 3, product

TABLE 3
ACTIVITY OF VARIOUS VITAMIN B₁₂ DERIVATIVES
IN RIBONUCLEOTIDE REDUCTASE SYSTEM

Addition	Δ dCDP ($m\mu$ moles/mg/hr)
None	0.0
BC coenzyme	192.0
DBC coenzyme	184.5
Cyanocobalamin	61.5
Hydroxocobalamin	45.6
Methylcobalamin	34.0
Vitamin B ₁₂ anilide	11.1
Vitamin B ₁₂ monocarboxylic acid	1.1
Factor B (cobinamide)	0.0

Ribonucleotide reductase was assayed in the standard incubation mixture described in the text. Vitamin B₁₂ derivatives were added in concentrations of 4×10^{-4} M. Incubations and other operations were conducted in the dark.

FIG. 3.—Evidence that ribosyl moiety of CDP-U-C¹⁴ is directly reduced to deoxyribosyl. Substrate was uniformly labeled CDP-C¹⁴ (1,310 cpm/m μ mole) plus 2 m μ moles of DBC coenzyme and 1.5 mg of a crude sonicate prepared from cells that had been cultivated for 3 hr in a thymine-free secondary culture (specific activity with CN-B₁₂, 50.1). Carrier dCMP was omitted after acid hydrolysis. Chromatography yielded 60.2 m μ moles of dCMP-C¹⁴ (1,043 cpm/m μ mole). This was lyophilized, dephosphorylated with snake venom,¹⁵ and reappplied to a Dowex-50-(H⁺) column. Residual dCMP-C¹⁴ eluted with 0.2 N acetic acid contained only 1,340 cpm. Further elution with 0.5 N HCl yielded 57.6 m μ moles of CdR-C¹⁴ (885 cpm/m μ mole). Of this, 5 μ moles were lyophilized, taken up in 0.5 ml Tris maleate, pH 5.9, and incubated with 5 μ moles of non-radioactive adenine and 1 mg of trans-*N*-deoxyribosylase in a volume of 1.0 ml. After 0 and 60 min at 37°, the pH was adjusted to 7, mixtures were heated, filtrates were dried and separated by descending paper chromatography in *n*-butanol-water (86:14, v/v).²³ (A) Radioactivity scan of trans-*N*-deoxyribosylase reaction mixture at zero time. (B) Scan after incubation for 60 min.



dCDP-C¹⁴ was converted to CdR-C¹⁴. To determine if the -dR was labeled, the CdR-C¹⁴ was incubated with nonradioactive adenine and trans-*N*-deoxyribosylase. The appearance of radioactive AdR (Fig. 3) is evidence that the deoxyribosyl moiety had originally been formed by reduction of the radioactive ribosyl of CDP.

Effect of removing ribosomes from whole sonicates: An earlier report from this laboratory²⁴ demonstrated that *L. leichmannii* and certain other vitamin B₁₂-requiring lactobacilli can bind far more vitamin B₁₂ than is required to satisfy nutritional requirements. The bulk of the added vitamin B₁₂ is bound in the ribosomes, and purified ribosomes bind vitamin B₁₂ *in vitro*.²⁵ A ribosomal vitamin B₁₂-binding substance with characteristics of an acidic glycoprotein is reversibly released in concentrated salt solutions.²⁶ Their relative resistance to ribonuclease digestion suggests that vitamin B₁₂-binding ribosomes constitute a specific class within the ribosome population.²⁷

It was therefore of interest to determine the effect upon reductase activity of removing ribosomes from whole sonicates. This experiment had two notable results (Fig. 4): (1) the reductase system of ribosome-free 144,000 \times *g* super-

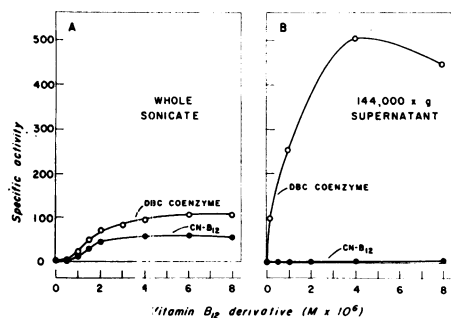


FIG. 4.—Effect of ribosome removal from whole sonicate upon activity of reductase system with CN-B₁₂ and DBC coenzyme. Incubations were of standard type. *Abscissas*, concentrations of CN-B₁₂ and DBC coenzyme; *ordinates*, specific reductase activity. (A) Activity in whole sonicate. (B) Activity in 144,000 \times *g* supernatant fraction.

natant was active only with DBC coenzyme, CN-B₁₂ being inert as a cofactor; and (2) activity of the supernatant with DBC coenzyme was substantially higher than that of whole sonicate. These results suggested that ribosomes are the locus of the conversion of CN-B₁₂ (or OH-B₁₂) to DBC coenzyme; that coenzyme synthesized in the ribosome is held there until released in accordance with undetermined de-

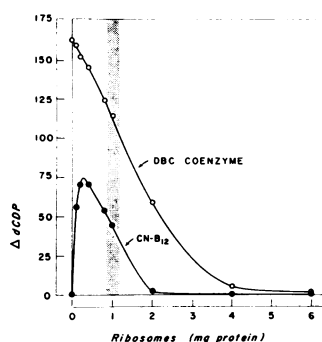


FIG. 5.—Effects upon reductase activity of variations in ribosome content. Ribosomes were obtained from whole derepressed sonicate by centrifugation for 4 hr at $144,000 \times g$. An aliquot of the upper two thirds of the remaining supernatant fraction containing 1 mg protein was used in each incubation. Incubations also contained 2 μ moles of CN-B₁₂ or DBC coenzyme and the indicated quantities of ribosomes. The shading denotes the amount of ribosomes relative to 1 mg of supernatant that occurs in an ordinary whole sonicate.

sorption equilibria or other regulatory influences; and that ribosomes also bind exogenously added coenzyme, thereby making it unavailable to reductase apoenzyme.

These conclusions were supported by a comparison of reductase activity with DBC coenzyme and CN-B₁₂ in ribosome-free supernatant alone and supernatant supplemented with ribosomes in varying quantities (Fig. 5). In the absence of ribosomes, activity was maximal in the presence of DBC coenzyme and absent with CN-B₁₂. With the addition of ribosomes, activity with DBC coenzyme decreased progressively. However, the capacity to utilize CN-B₁₂ increased as ribosomes were added until a maximum was reached. With further additions of ribosomes, binding activity appeared to exceed activating activity.

The conclusion that ribosomes bind DBC coenzyme was supported by an analysis of the upper curve of Figure 5 in terms of Freundlich's adsorption isotherm. A detailed analysis of adsorption kinetics will be reported in a later paper.

Evidence of coenzyme production by ribosomes: The experiment in Figure 6 gives more direct evidence of ribosomal synthesis of active coenzyme (presumably DBC coenzyme). Whole sonicate was preincubated with CN-B₁₂ and the other ingredients usually present in the standard reductase system. At various times, the ribosomes were removed by centrifugation, CDP-C¹⁴ was added, and CDP reductase was assayed in the $144,000 \times g$ supernatant. The results indicate that (1) active coenzyme remained behind when ribosomes were removed; (2) the amount of coenzyme formed was an approximately linear function of the duration of the preincubation for the first hour; (3) ATP and L(SH)₂ were each essential for the activation occurring during the preincubation; and (4) 2-mercaptoethanol could

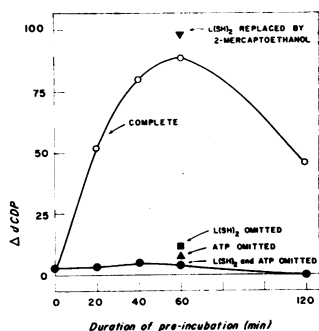


FIG. 6.—Evidence of coenzyme production by ribosomes. Whole derepressed sonicate was preincubated at 37° for the time intervals shown with CN-B₁₂, $4 \times 10^{-6} M$, plus or minus ATP, Mg⁺⁺, and L(SH)₂ in the usual concentrations (see Fig. 2). After the preincubation, ribosomes were removed by ultracentrifugation in the cold in 2-ml Spinco tubes. CDP-2-C¹⁴ was then added to the supernatant fractions and dCDP production was determined after 60 min. Cofactors omitted during preincubation were added with the CDP-2-C¹⁴. The following additions and omissions refer to the preincubation during which ribosomes were present and CDP-2-C¹⁴ absent: ○, complete system; ●, L(SH)₂ and ATP omitted; ▲, ATP omitted; ■, L(SH)₂ omitted; ▼, L(SH)₂ replaced by 2-mercaptoethanol, 0.03 M.

replace L(SH)₂ in the activation reaction, although it could not do so in the reductase reaction itself (Table 2).

Discussion.—Although BC coenzyme and DBC coenzyme were equally active in the ribonucleotide reductase system (Table 3), DBC coenzyme is apparently the major naturally occurring vitamin B₁₂ derivative in *L. leichmannii*.^{28, 29} It appears therefore that reductase requires DBC coenzyme *in vivo*. Current attempts to prove that ribosomes make DBC coenzyme include efforts to characterize the product and to demonstrate its activity with diol dehydrase and methylmalonyl CoA isomerase, enzymes known to require DBC coenzyme.^{30, 31}

The data indicating that ribonucleotide reductase of *L. leichmannii* is repressible—the system apparently being controlled by an intracellular thymine derivative—parallel our observations of *E. coli*_{15T}.^{14, 15} Although the agent governing the repressor mechanism is not identifiable from the data at hand, presumably it is the same in the two species. Preliminary results have shown that vitamin B₁₂ deficiency also derepresses *L. leichmannii* reductase, perhaps by lowering the intracellular concentration of the same deoxyribonucleotide.⁸ It may be supposed that repressor control of the reductase system is of widespread occurrence and of potential importance in determining the rate of synthesis of DNA precursors.

The similarity of the reductase control systems of *L. leichmannii* and *E. coli*_{15T} does not appear to extend to the enzyme systems themselves. Reductase specific activities are higher in *L. leichmannii* extracts and the *K_m* of the former enzyme is twice that of the latter. It has recently been suggested that the favored substrate of *L. leichmannii* may be CTP rather than CDP,³² although differences between the two are slight in crude extracts. Finally, little evidence implicates vitamin B₁₂ in the reductase systems of *E. coli*_{15T},¹⁵ *E. coli* B,¹³ and Novikoff rat hepatoma.¹⁴ Cobamide requirements for these reductases have not been ruled out, however.

Our results suggest the following model. CN-B₁₂ enters the cell from the medium and is converted to OH-B₁₂,²⁸ which is rapidly collected in (or on) a special class of ribosomes²⁷ by a ribosomal vitamin B₁₂-binding glycoprotein—the collection of vitamin B₁₂ to an activation site by a ribosomal glycoprotein bearing a certain resemblance to the collection of vitamin B₁₂ to an absorption site by the mammalian glycoprotein, intrinsic factor. Bound OH-B₁₂ is converted on the ribosome to cobamide coenzyme (presumably DBC coenzyme) by enzymes requiring L(SH)₂ or 2-mercaptoethanol and ATP. Cobamide coenzyme is then slowly released to serve as a cofactor for reductase apoenzyme in the soluble phase of the cell.

The evidence in Figure 1 for derepression of the reductase system could conceivably be accounted for by derepression of the ribosomal activating system, since these assays were performed with CN-B₁₂. However, experiments have shown a comparable time-course of reductase activity in 144,000 × *g* supernatants assayed with DBC coenzyme after transfer of cells to thymine-free media. Preliminary experiments, nevertheless, do suggest that ribosomal binding and activation of CN-B₁₂ may increase in extracts of thymine-starved cells.²⁸ (See *Note added in proof.*)

Note added in proof: Since this paper was submitted, Blakely has reported higher specific activities than those obtained earlier.¹¹ In the modified system, L(SH)₂ is superior to mercaptoethanol as reductant in the reductase reaction [*J. Biol. Chem.*, **240**, 2173 (1965)].

Abbreviations used: AdR, deoxyadenosine; BC, benzimidazolylcobamide; CdR, deoxycytidine;

CN-B₁₂, cyanocobalamin; DBC, 5,6-dimethylbenzimidazolylcobamide; L(SH)₂, dihydrolipoic acid; OH-B₁₂, hydroxocobalamin; T, thymine.

*This investigation was supported by grant CA 03728 from the National Cancer Institute, National Institutes of Health, USPHS.

- ¹ Beck, W. S., *New Eng. J. Med.*, **266**, 708, 765, 814 (1962).
- ² Kitay, E., W. S. McNutt, and E. E. Snell, *J. Biol. Chem.*, **177**, 993 (1949).
- ³ Downing, M., and B. S. Schweigert, *J. Biol. Chem.*, **177**, 993 (1949).
- ⁴ Wacker, A., S. Kirschfeld, and L. Trager, *Z. Naturforsch.*, **14b**, 145 (1959).
- ⁵ Spell, W. H., Jr., and J. S. Dinning, *J. Am. Chem. Soc.*, **87**, 3804 (1959).
- ⁶ Manson, L. A., *J. Biol. Chem.*, **235**, 2955 (1960).
- ⁷ Williams, A. M., J. J. Chosy, and R. F. Schilling, *J. Clin. Invest.*, **42**, 670 (1963).
- ⁸ Beck, W. S., M. Goulian, and S. Hook, *Biochim. Biophys. Acta*, **55**, 470 (1962).
- ⁹ Beck, W. S., S. Hook, and B. H. Barnett, *Biochim. Biophys. Acta*, **55**, 455 (1962).
- ¹⁰ Beck, W. S., *Medicine*, **43**, 715 (1964).
- ¹¹ Blakley, R. L., and H. A. Barker, *Biochem. Biophys. Res. Commun.*, **16**, 391 (1964).
- ¹² Moore, E. C., and P. Reichard, *J. Biol. Chem.*, **239**, 3453 (1964).
- ¹³ Reichard, P., *J. Biol. Chem.*, **237**, 3513 (1962).
- ¹⁴ Biswas, C., M. Goulian, J. Hardy, and W. S. Beck, *Federation Proc.*, **23**, 532 (1964).
- ¹⁵ Biswas, C., J. Hardy, and W. S. Beck, *J. Biol. Chem.*, in press.
- ¹⁶ Gunsalus, I. C., and W. E. Razzell, in *Methods in Enzymology*, ed. S. P. Colowick and N. O. Kaplan (New York: Academic Press, Inc., 1957), vol. 3, p. 941.
- ¹⁷ Armitage, J. B., J. R. Cannon, A. W. Johnson, L. F. J. Parker, E. L. Smith, W. H. Stafford, and A. R. Todd, *J. Chem. Soc.*, 3849 (1953).
- ¹⁸ Beck, W. S., and M. Levin, *J. Biol. Chem.*, **238**, 702 (1963).
- ¹⁹ *The Pharmacopeia of the United States of America (U.S. Pharmacopeia): Fifteenth Revision* (Easton: Mack Publishing Co., 1955), p. 885.
- ²⁰ Goulian, M., and W. S. Beck, to be published.
- ²¹ Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
- ²² Reichard, P., *Acta Chem. Scand.*, **12**, 2048 (1958).
- ²³ Markham, R., and J. D. Smith, *Biochem. J.*, **45**, 294 (1952).
- ²⁴ Kashket, S., J. T. Kaufman, and W. S. Beck, *Biochim. Biophys. Acta*, **64**, 447 (1962).
- ²⁵ *Ibid.*, p. 458.
- ²⁶ Kashket, S., P. A. Eliashof, and W. S. Beck, to be published.
- ²⁷ Kashket, S., and W. S. Beck, *Biochem. Z.*, in press.
- ²⁸ Kashket, S., and W. S. Beck, unpublished observations.
- ²⁹ Tamao, Y., T. Kato, S. Shimizu, and S. Fukui, *Bitamin*, **30**, 471 (1964).
- ³⁰ Stadtman, E. R., P. Overath, H. Eggerer, and F. Lynen, *Biochem. Biophys. Res. Commun.*, **2**, 1 (1960).
- ³¹ Abeles, R. H., and H. A. Lee, Jr., *J. Biol. Chem.*, **236**, 2347 (1961).
- ³² Abrams, R., Presentation at the 49th Annual Meeting of the Federation of American Societies for Experimental Biology (American Society of Biological Chemists), Atlantic City, New Jersey, April 12, 1965.