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*ENZYMATIC SYNTHESIS OF DEOXYRIBONUCLEOTIDES, VIII. THE EFFECTS OF ATP AND dATP IN THE CDP REDUCTASE SYSTEM FROM E. COLI**

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The enzymatic formation of deoxyCDP from CDP in *Escherichia coli* B requires the participation of four different protein fractions.[1-3] Two of these (thioredoxin[2] and thioredoxin reductase[3]) have been obtained in an essentially pure form while the remaining two (enzymes B1 and B2) have hereto been available only in a relatively crude state.[1] Our present understanding of the interplay of these fractions in the formation of dCDP is summarized in Figure 1.

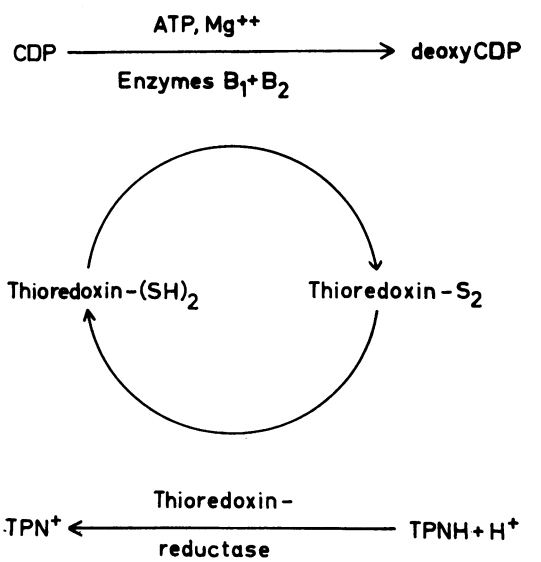

FIG. 1.—The CDP reductase system from *E. coli* B.

Two of the early, puzzling aspects of the mechanism of dCDP formation were the requirement for ATP in both microbial and animal systems, and the inhibition of the reaction by certain deoxyribonucleoside triphosphates in chick embryo extracts.[4] In the present paper we wish to present evidence indicating that ATP functions as an "activator" of the enzyme B complex, rather than by forming an "activated" intermediate with CDP, and that the inhibition by dATP depends on a competition between the deoxynucleotide and ATP. Our experiments depended on the availability of preparations of enzymes B1 and B2 essentially free from contaminating pyrophosphatase and kinase activities. The enzymes were purified from *E. coli* B and from a partially derepressed strain [5] of the thymine-requiring mutant *E. coli* B3. Purification was aided by the development of a new assay method utilizing the stoichiometric disappearance of TPNH during dCDP formation (cf. Fig. 1).

The enzymes were also obtained from *E. coli* B grown in the presence of Co^{60}-labeled vitamin B_{12}. After extensive purification, neither enzyme B1 nor enzyme B_2 contained vitamin B_{12} as measured by radioactivity or by a microbiological assay with *Lactobacillus leichmannii* 313.

Materials and Methods.—All materials were obtained from the sources mentioned previously,[1-3] unless stated otherwise in the text.

Protein was determined from the absorbance at 280 mμ, assuming an extinction value of 1 for a solution containing 1 mg/ml and a light path of 1 cm.

Standard conditions for enzyme incubation: The following substances were incubated at room temperature (22–24°) for 10 min in a final volume of 0.13 ml: 0.2 μmole of ATP, 2 μmoles of $MgCl_2$, 0.5 μmole of Tris-HCl buffer, pH 8.0, 0.06 μmole of TPNH, 0.1 μmole of EDTA, 0.05–0.1 μmole of H^3-CDP, 0.05–1 μmole of dithioerythrol (from enzyme B1), 2 μg of thioredoxin, 3 μg of thioredoxin reductase, and enzymes B1 and B2. The reaction was stopped by the addition of 1 ml *M* $HClO_4$, and the amount of dCDP formed was determined.[1] One unit of enzyme activity is defined as the amount of enzyme forming 1 mμmole of dCDP under the above conditions. Specific activity is defined as units per mg of protein. Enzyme B1 was determined in the presence of a large excess of enzyme B2 (TEAE fraction), and vice versa.

The reaction could also be followed by the disappearance of TPNH (absorbance at 340 mμ) in a Gilford multiple-sample absorbance recorder adapted with microcuvettes. In this case, incubation was first carried out for 10 min without CDP in order to establish the amount of "background oxidation" of TPNH. The reduction was then started by the addition of 3 μl of CDP, and the amount of TPNH consumed in the reaction could be calculated from the difference between the slope obtained after addition of substrate and the "background" slope (Fig. 2). A reasonably good stoichiometric relationship was observed between the disappearance of TPNH and the formation of dCDP.

Growth of bacteria: Escherichia coli B was grown on a nutrient broth medium as described elsewhere.[1] In some experiments, 15 μc of Co^{60}-vitamin B_{12} (1.86 μc/μg of vitamin B_{12}, obtained from the Radiochemical Centre, Amersham, England) were added to 15 liters of medium.

The derepressed bacteria were obtained by growing a 500-ml culture of *E. coli* B3 overnight at 37° on Davis' minimal medium supplemented with 1 mg of thymine. The bacteria were collected by centrifugation, washed twice with physiological saline, and used for the inoculation of 15 liters of fresh Davis medium containing 1.8 mg of thymine. The bacteria were then grown at 37° with vigorous aeration for about 6 hr, at which time they had reached the stationary phase and were collected by centrifugation.

Results.—Purification of enzymes: The preparation of fraction B and the separation of enzymes B1 and B2 was carried out as described elsewhere.[1] Identical procedures were used for the purification of enzymes from *E. coli* B and *E. coli* B3. The apparent specific activity of crude extracts from the derepressed bacteria was about ten times that of those from the wild strain, confirming the results of Biswas

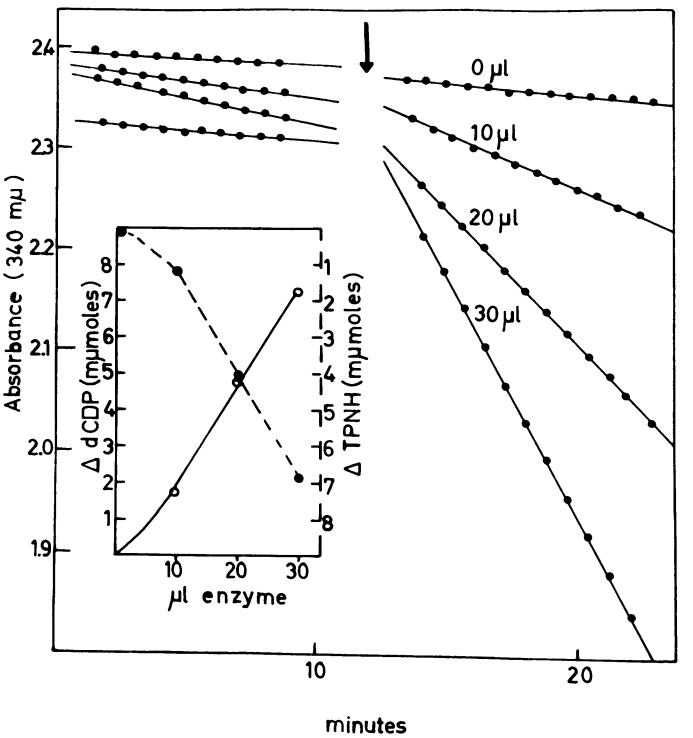

FIG. 2.—Assay of activity of fraction B by TPNH oxidation. Increasing aliquots of an enzyme solution (6 mg/ml) were incubated under standard conditions and the absorbance at 340 mμ was measured with a Gilford multiple-sample absorbance recorder attached to a Zeiss PMQ2 spectrophotometer. H^3-CDP was added at the arrow. After 10 min, 0.1 ml of the incubation mixture was used for the determination of dCDP formed.[1] The small diagram shows the stoichiometric relationship between the amount of TPNH consumed and the amount of dCDP formed.

et al.[5] Fraction B from the derepressed strain represented a 30–50-fold purification of enzyme activity as compared to the crude extract.

Enzyme B1: Further purification was achieved by chromatography on hydroxylapatite and TEAE-cellulose. It was found that enzyme B1 activity was stabilized by SH-groups. Dithioerythrol (Cyclo Chemical Corp., Los Angeles, Calif.) at 0.005–0.01 *M* concentrations was included in all buffers used.

In a typical experiment, crude enzyme B1 (89 mg), obtained after the C_γ-alumina step,[1] was adsorbed to a column (2 × 10 cm) of Hypatite (Clarkson Chemical Co., Williamsport, Pa.). A peak of inactive protein was eluted with 150 ml of 0.07 *M* phosphate buffer pH 7.0. Enzyme activity was then eluted in a second protein peak with 34 ml of 0.20 *M* phosphate buffer, pH 7.0. The enzyme solution was concentrated to a final volume of 3 ml by ultradialysis (filters from Membrangesellschaft, Göttingen, Germany) against 0.02 *M* Tris-HCl buffer, pH 7.70.

The concentrated solution (30 mg of protein) was adsorbed to a column (1 × 10 cm) of TEAE-cellulose (Serva Laboratories, Heidelberg, Germany). The column was eluted with a linear gradient of phosphate buffer, pH 7.0 (30 ml each of 0.05 *M* and 0.2 *M* buffer) at a rate of 1.8 ml per 30 min. The upper part of Figure 3 il-

lustrates a chromatogram of enzyme B1 from wild-type *E. coli* B. Enzyme activity was eluted early in the chromatogram and was separated from vitamin B_{12}.

The two purification steps resulted in an 8–12-fold purification of enzyme B1 activity with an over-all yield of 30–40 per cent. The specific activity of the enzyme from *E. coli* B was 100–200, that of the enzyme from the derepressed *E. coli* B3 was 250–500.

Enzyme B2: The crude enzyme solution (130 mg of protein in 26 ml) obtained by elution from C_γ-alumina[1] was diluted with 13 ml of water and adsorbed to a column (2 × 8 cm) of Hypatite. The enzyme was then eluted with 80 ml of 0.04 *M* phosphate buffer, pH 7.0, and concentrated by ultradialysis against 0.05 *M* Tris buffer, pH 7.0, to a final volume of 3 ml. All buffers used during the purification procedure contained 0.001 *M* EDTA.

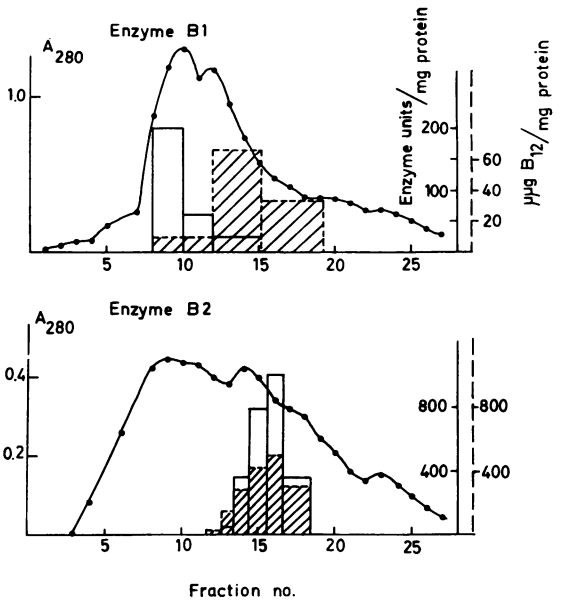

FIG. 3.—Chromatography of enzymes B1 and B2 on TEAE-cellulose. Conditions are given in the text. The continuous line shows absorbance at 280 mμ; the open bar diagram, enzyme activity; the shaded bar diagram (broken line), the amount of vitamin B_{12}.

The concentrated solution (28 mg of protein) was adsorbed to a column of TEAE-cellulose (1.3 × 10 cm) and eluted with a linear phosphate gradient (0.1–0.3 *M*) achieved with two chambers, each containing 50 ml of buffer, pH 7.0. Two-ml fractions were collected at 30-min intervals. The lower part of Figure 3 shows that enzyme B2 activity and vitamin B_{12} emerged together in the latter part of the chromatogram.

However, the enzyme could be completely separated from vitamin B_{12} by electrophoresis on polyacrylamide gel (Fig. 4). In this experiment, 3.4 mg of enzyme B2 (after TEAE chromatography), obtained from Co^{60}-vitamin B_{12} bacteria, was subjected to electrophoresis under the conditions described by Racusen and Calvanico.[6] A voltage of 300 v was applied over the column (1.5 × 3 cm) resulting in a current of 5 ma. Fractions of 1 ml were collected at 20-min intervals.

The two first purification steps resulted in a 10–15-fold purification of enzyme B2 with a final yield of about 50 per cent. The specific activity of the enzyme from *E. coli* B was 300–1000, that of the enzyme from *E. coli* B3 was 4000–6000.

All further experiments described below were done with enzymes from derepressed *E. coli* B3.

Requirements of the CDP-reductase system: Table 1 demonstrates that optimal formation of dCDP required all four purified proteins. In contrast to earlier experience[3] with less pure enzymes, the addition of thioredoxin reductase was found to have a stimulating effect. Dithioerythrol can partially replace reduced thioredoxin. This probably explains the small amounts of dCDP formed in the

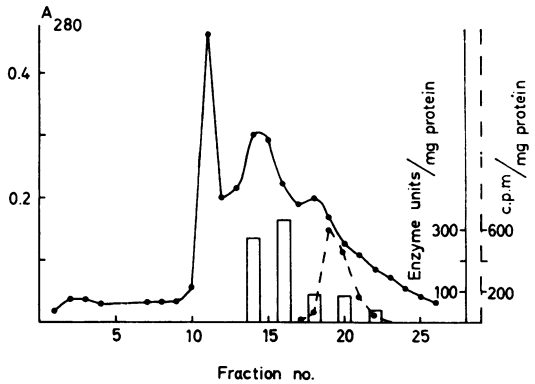

FIG. 4.—Preparative electrophoresis of enzyme B2 on polyacrylamide gel. The continuous line shows the absorbance at 280 mμ; the broken line, Co^{60} activities; the bar diagram, the enzyme activity. The enzyme B2 preparation was not identical with the one illustrated in Fig. 3. The recovery of enzyme activity was 35% in this step, with no change in the specific activity. The specific activity of vitamin B_{12} was 4.8×10^5 cpm/μg.

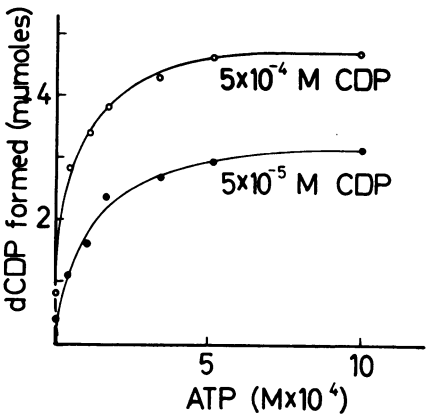

FIG. 5.—Stimulation of dCDP formation by increasing concentrations of ATP. Each tube contained 18 μg of enzyme B1 and 9 μg of enzyme B2, and was incubated under standard conditions except as noted.

absence of TPNH or thioredoxin. The enzyme preparations contained only very small amounts of pyrophosphatases or kinases. CTP or CMP could not replace CDP as substrate.

Effects of ATP: The influence of the concentration of ATP on dCDP formation was investigated at two different concentrations of CDP (Fig. 5). The ATP requirement was not absolute and was more pronounced at the lower CDP concentration. Apparent binding constants for ATP of 1.1×10^{-4} (5×10^{-5} *M* CDP) and of 0.75×10^{-4} *M* (5×10^{-4} *M* CDP) can be calculated from the data.[7]

The stoichiometry of the ATP requirement was investigated in a large-scale experiment. C^{14}-ATP (0.27 μmole, 1.6×10^6 cpm/μmole) was incubated with the CDP reductase system under standard conditions (0.67 μmole of H^3-CDP) with 0.15 mg each of enzymes B1 and B2 in a final volume of 1.2 ml. An identical mixture without TPNH served as control. After 30 min the reaction was stopped by boiling, and nucleotides were separated by chromatography on Dowex-1 formate.[8]

A total of 0.155 μmole of deoxycytidine phosphates was formed in the complete experiment, while the "control" contained 0.021 μmole. In both cases only 0.015

TABLE 1

REQUIREMENTS OF THE CDP REDUCTASE SYSTEM

Experimental conditions	dCDP formed (mμmoles)
Complete system	7.5
Omit enzyme B1	0.06
Omit enzyme B2	0.06
Omit thioredoxin	0.48
Omit thioredoxin reductase	2.1
Omit ATP	1.8
Omit CDP, add CTP	0.56
Omit CDP, and CMP	0.19

In the complete system, incubations were carried out under standard conditions with 15 μg of enzyme B1 and 9 μg of enzyme B2 at 5×10^{-4} *M* CDP.

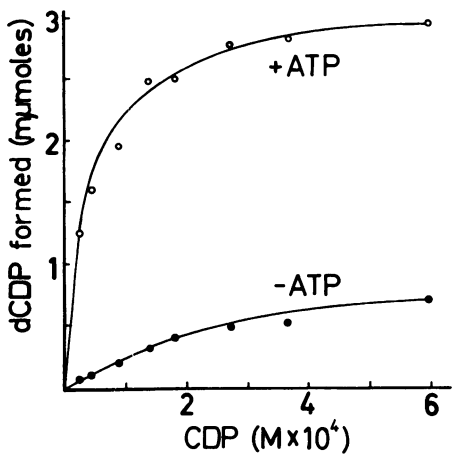

FIG. 6.—Influence of ATP on the dependence of the reaction on CDP concentration. Conditions were as in Fig. 5.

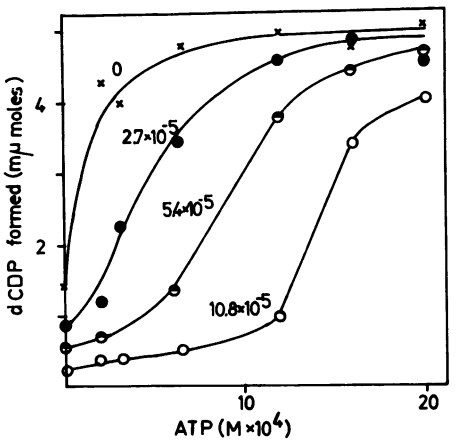

FIG. 7.—Influence of different concentrations of dATP on the dependence of the reaction rate on ATP concentration. Each tube contained 10 μg of enzyme B1 and 9 μg of enzyme B2 under standard conditions (4 × 10^{-4} *M* CDP). The concentrations of dATP are given with the individual curves.

μmole of C^{14}-ADP and no measurable amounts of C^{14}-AMP were formed. Thus, this experiment shows that only very small and nonstoichiometric amounts of ATP were broken down by the enzyme system during the formation of dCDP.

The above experiment excluded the possibility that ATP was required for the formation of "activated" CDP. Instead, it was found that ATP strongly influenced the dependence of the reaction rate on the concentration of CDP (Fig. 6). Thus, addition of ATP both increased the maximal rate of the reaction (3–4-fold) and the affinity of the enzyme(s) for the substrate. The apparent K_m values for CDP were 2.9 × 10^{-4} *M* in the absence of ATP, and 3.7 × 10^{-5} *M* with 7 × 10^{-4} *M* ATP.

The lack of specificity of the ATP requirement is demonstrated in Table 2.

TABLE 2

EFFECT OF DIFFERENT NUCLEOTIDES ON THE CDP REDUCTASE SYSTEM

Nucleotide	mμMoles	dCDP Formed (mμmoles) Without ATP	With ATP (7 × 10^{-4} *M*)
None	—	0.46	2.22
ADP	150	0.38	—
UTP	150	1.35	—
GTP	150	0.70	—
CTP	150	0.72	—
dCTP	150	1.02	—
dGTP	30	1.08	1.05
	75	0.83	0.59
TTP	1	1.61	—
	15	2.15	2.10
	40	2.20	1.48
dATP	15	0.06	0.70
	45	0.06	0.15
dADP	15	0.39	—
	45	0.17	1.05

Incubations were carried out under standard conditions (6 μg of enzyme B1 + 7 μg of enzyme B2 at 3 × 10^{-4} *M* CDP) except as stated.

While ADP could not substitute for ATP, several nucleoside triphosphates produced various degrees of stimulation. Thus, TTP was effective at lower concentrations than ATP, whereas UTP, CTP, GTP, and dGTP always gave less stimulation than ATP. However, addition of dGTP (and TTP) to the ATP-stimulated reaction resulted in an inhibition of dCDP formation. Finally, dATP and, to a lesser degree, dADP inhibited the reaction both in the presence and absence of ATP.

A competition between dATP and ATP is illustrated by the results shown in Figure 7. When the dependence of the reaction rate on ATP concentration was studied in the presence of dATP, the addition of increasing amounts of dATP converted the Michaelian hyperbolic rate-concentration curve of the uninhibited reaction into a family of more or less S-shaped curves. In all cases, high concentrations of ATP reversed the inhibition by dATP. Increasing the CDP concentration did not affect the inhibition.

Discussion and Summary.—Our results with highly purified enzymes of the CDP reductase system from *E. coli* B and B3 seem to have established two points:

(1) Vitamin B_{12} is not involved in the reaction. This is in contrast to the ribotide reductase system from *Lactobacillus leichmannii*[9, 10] which in many other respects (requirements for ATP, Mg^{++}, and for a dithiol as the hydrogen donor) is similar to the enzyme system from *E. coli* B.

(2) ATP does not directly "activate" CDP as suggested earlier.[1] Instead, ATP probably brings about changes in the physical state of the enzyme(s) resulting *inter alia* in a higher affinity for CDP. The inhibition of the reaction by dATP seems to be connected with the ATP effect since it can be reversed by increasing the ATP concentration. Several different types of models can be visualized for the action of ATP and dATP. Thus, the data could be explained if enzymes B1 or B2 contained one or several allosteric sites[11] capable of binding an activator (ATP) or an inhibitor (dATP). According to more complex hypotheses, the function of ATP may be connected with the requirement for two enzymes (enzymes B1 and B2) for the reduction of CDP.

The complexity of the situation is also indicated by the finding that several nucleotides (TTP, dGTP) stimulate dCDP formation in the absence of ATP, but act as inhibitors in the presence of ATP.

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