

Enzymatic Cleavage of Deoxyguanosine Triphosphate to Deoxyguanosine and Tripolyphosphate

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The enzymatic reaction reported here illustrates a unique type of hydrolysis of nucleoside triphosphates. This reaction is: deoxyguanosine triphosphate (dGTP¹) → deoxyguanosine + tripolyphosphate. The enzymatic hydrolysis of nucleoside triphosphates has been known to proceed by removal of orthophosphate or pyrophosphate (1-3), but it has not been shown previously to occur by cleavage of a tripolyphosphate group. In fact, although tripolyphosphate has been found in cell extracts, only one biochemical reaction was known for its production, namely, the reaction catalyzed by adenylate kinase (myokinase) (4): ADP + pyrophosphate → AMP + tripolyphosphate.

The enzyme that splits tripolyphosphate from dGTP hydrolyzes GTP similarly, but at a lower rate. The enzyme was discovered during the study of the deoxyribonucleic acid synthesizing system ("polymerase") of *Escherichia coli* (5), when it was found that dGTP was destroyed during incubation with certain "polymerase" fractions. Some properties of the enzyme, the identification of the reaction products, and a partial purification of the enzyme are described below.

EXPERIMENTAL

Materials and Methods—The deoxynucleoside triphosphates of guanine, adenine, cytosine, uracil, and thymine labeled with P³² in the acid-stable phosphate group were prepared by methods given elsewhere (5). GDP and GTP were purchased from the Sigma Chemical Company; the GTP was purified by ion exchange chromatography (6) by the procedure described for dGTP (5). Sodium tripolyphosphate was a product of the Victor Chemical Company, and crystalline bovine serum albumin was obtained from Armour and Company. Inorganic pyrophosphatase was a gift from Drs. M. Kunitz and G. Perlmann. Acid-labile phosphate was determined by the Fiske-Subbarow method (7) after treating the samples in 1 N HCl for 7 minutes at 100°. Protein was determined by the method of Lowry *et al.* (8). The diphenylamine reaction of Dische (9) was used to determine deoxyribose. Measurements of radioactivity were made with dried samples in a gas-flow counter. Ultraviolet absorption was measured in the Beckman model DU spectrophotometer.

Enzyme Assay—For the assay of enzyme activity, dGTP

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¹ The abbreviations used are: dGTP, deoxyguanosine triphosphate; dGTP³² for dGTP labeled with P³² exclusively in the phosphate group adjacent to the deoxyribose (acid-stable phosphate); GTP, guanosine triphosphate; GDP, guanosine diphosphate; ADP, adenosine diphosphate; AMP, adenosine 5'-monophosphate.

labeled with P³² in the acid-stable phosphate group was used as the substrate. The addition of Norit at the end of the reaction period made it possible to measure the cleavage of the dGTP at the sugar-phosphate bond, since deoxyguanosine and unreacted dGTP³² adsorb to the Norit (10), whereas the non-nucleotide phosphate remains in solution. The amount of non-nucleotide phosphate may then be determined by centrifuging the mixture and measuring the radioactivity of the supernatant fluid. The assay obviously does not indicate whether the non-adsorbed label is in tripolyphosphate, deoxyribose phosphate, or pyro- or orthophosphate.

The assay mixture was made up as follows: 20 μmoles of glycine buffer (pH 8.5), 2 μmoles of MgCl₂, 0.005 μmoles of dGTP³², and enzyme and water to a volume of 0.3 ml. The reaction tube was placed in a 37° water bath for 20 minutes. Then 0.4 ml. of a cold albumin-tripolyphosphate solution mixture (1.5 mg. of albumin and 2.1 mg. of sodium tripolyphosphate per ml.) was added, followed by 0.2 ml. of cold 0.1 N HCl and 0.1 ml. of an acid-washed Norit suspension (20 per cent by volume).² Control mixtures were made up in the same way, except that the enzyme was added after the addition of HCl. After centrifugation, 0.5 ml. of the liquid was assayed for radioactivity.

1 unit of enzyme is defined as that quantity which splits 1 μmole of dGTP in the assay period (20 minutes).

Under the assay conditions, the extent of reaction was proportional to the amount of enzyme added. For example, 0.02, 0.05, 0.10 and 0.20 ml. of a diluted enzyme solution (1.74 μg. per ml.) produced 0.17, 0.47, 0.88, and 1.5 μmoles of unadsorbed phosphate. No reaction was detectable in the absence of MgCl₂. The activities at pH 7.5, 8.0 and 8.5 were the same, and at pH 9.2 the rate was 70 per cent of this.

RESULTS

Enzyme Preparation—Crude extracts of *E. coli* catalyze reactions which interfere with the assay of enzyme, for example, the stepwise removal of orthophosphate from dGTP. It was, therefore, difficult to assess the amount of splitting by the enzyme that cleaves the molecule exclusively at the sugar-phosphate bond, except in the more purified "polymerase" fractions, and these were eventually used to obtain the enzyme used in the experiments reported here.

² It was found that albumin facilitated the complete precipitation of Norit fines and that the addition of unlabeled tripolyphosphate minimized the error in the determination of Norit-nonsorbable radioactivity, inasmuch as small amounts of tripolyphosphate are adsorbed by Norit.

TABLE I
Fractionation of enzyme

Fraction	Units/ml.	Units/mg.	Yield from preceding fraction
			%
I	(0.46)		
II	(0.98)		
III	(0.92)		
V	3.1	0.6	(22)
VI	24	3.1	80
"Ethanol"	19	11	80

TABLE II
Balance study of reaction

Experiment	Time	DGTP ³²	Deoxyguano-	Tripoly-	Ortho-
		μ moles	sine	phosphate	phosphate
1	0	1.00	0.00	0.00	
	60	0.11	0.77	0.71	
	Δ	-0.89	+0.77	+0.71	
2	0	1.00	0.00	0.00	0.00
	60	0.12	0.93	0.87	0.07
	Δ	-0.88	+0.93	+0.87	+0.07

10 ml. mixtures contained 500 μ moles of glycine (pH 8.5), 50 μ moles of MgCl₂, 1 μ mole of dGTP³² and 0.9 unit of enzyme; they were incubated at 37° for 1 hour, after which aliquots were taken to determine the Norit-adsorbable radioactivity remaining (dGTP³² column). The remainder of the mixtures was adsorbed on Dowex 1 resin (chloride form, 2 per cent cross-linked, 5 \times 1 cm.²) and eluted first with 0.01 N HCl, which removed the non-radioactive, ultraviolet-absorbing product calculated as deoxyguanosine above, and the radioactive, non-ultraviolet-absorbing material calculated as orthophosphate. Then the eluant was changed to 0.02 N HCl with 0.20 N KCl, which removed the radioactive, non-ultraviolet-absorbing product identified as tripolyphosphate, as described in the text.

TABLE III

	250/260, pH 2	280/260, pH 2	250/260, pH 12	280/260, pH 12
Product	1.05	0.69	0.93	0.65
Guanosine (11)	0.99	0.70	0.89	0.63
Guanine (11)	1.37	0.84	0.98	1.13

In Table I are shown the results of assays on the "polymerase" fractions (5) and on the purified preparation of enzyme used in the experiments for the present report. Assays on the early fractions are considered unreliable, as discussed above, and values that refer to these are in parentheses in Table I. The dGTP-splitting enzyme was prepared from "polymerase" Fraction VI as follows: 1 ml. of Fraction VI, at 0°, was mixed with 1.0 ml. of potassium phosphate (0.02 M, pH 7.2) and 0.05 ml. of sodium acetate buffer (1 M, pH 4.0). Then the vessel was placed in a -15° bath and 0.5 ml. of absolute ethanol (-15°) was added, with stirring, during a 2 minute period. The temperature of the enzyme-ethanol mixture rose to -2°. After 1 minute, the mixture was centrifuged for 2 minutes, the supernatant fluid was removed, and the precipitate was dissolved in 1.0 ml. of potassium phosphate buffer (0.02 M, pH 7.2). This solution had an activity of 19 units per ml. and

11 units per mg. protein. The solution could be dialyzed for several hours at 4° (tris(hydroxymethyl)aminomethane, pH 7.4, 0.02 M) without loss in activity. A dialyzed sample was used for those experiments in which orthophosphate was determined.

Products of Reaction—A balance study of the reaction showed that the disappearance of dGTP resulted in the formation of approximately equimolar amounts of deoxyguanosine and tripolyphosphate (Table II). Large-scale mixtures incubated for 1 hour, which produced up to 0.93 μ mole of deoxyguanosine, yielded less than 0.1 μ mole of orthophosphate. The reaction products were separated by ion exchange chromatography, and their elution was followed by means of P³² analysis and measurements of ultraviolet absorption. The first product eluted contained no P³² and had the absorbancy ratios characteristic of deoxyguanosine (11) (Table III). This product (Experiment 1) contained 0.69 μ mole of deoxypentose determined by the diphenylamine reaction, and the expected value, based on the measurement of ultraviolet absorption, was 0.77 μ mole, assuming a molar extinction coefficient of 11.4 at 260 m μ for deoxyguanosine (11).

The P³²-containing product eluted by stronger salt did not absorb ultraviolet light, and its phosphate was completely acid-labile. An aliquot of this solution was compared with a sample of authentic tripolyphosphate by chromatographing a mixture of the two (84,000 c.p.m. mixed with 23.3 μ moles of sodium tripolyphosphate) on Dowex 1-resin, as described before (12). The radioactivity and acid-labile P were eluted from the column in parallel in 17 fractions (Fig. 1). The total P³² in these fractions was 93 per cent of the radioactivity adsorbed on the column, whereas 94 per cent of the acid-labile P was recovered in the same eluates.

Specificity and Some Kinetic Properties of Reaction—The cleavage of the P³²-labeled triphosphates of deoxyuridine, deoxyadenosine, deoxycytidine, and thymidine was also investigated. Under the assay conditions, 5 μ moles of these triphosphates produced 0.07, 0.04, 0.20, and 0.02 μ mole of nonadsorbable P³², respectively. dGTP³² tested simultaneously produced 3 μ moles, and its splitting was not inhibited by

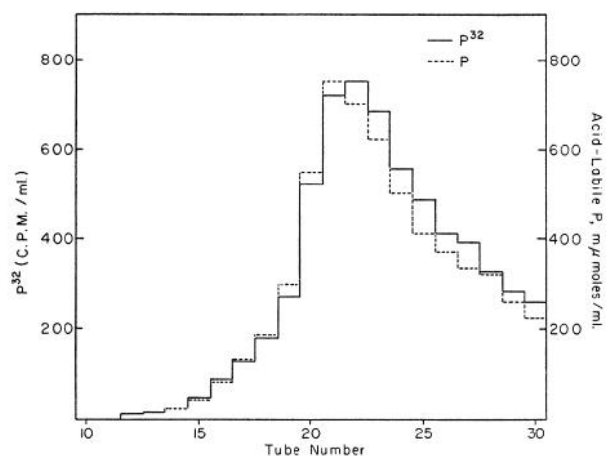


FIG. 1. Chromatogram of tripolyphosphate and Norit-nonadsorbable split-product of dGTP³². The P³²-containing Norit-nonadsorbable split-product of dGTP³² (84,000 c.p.m.) was mixed with 23.3 μ moles of sodium tripolyphosphate and adsorbed on a Dowex 1 resin column (chloride form, 2 per cent cross-linked, 5 \times 1 cm.²), and was eluted with 0.02 N HCl containing 0.20 N KCl.

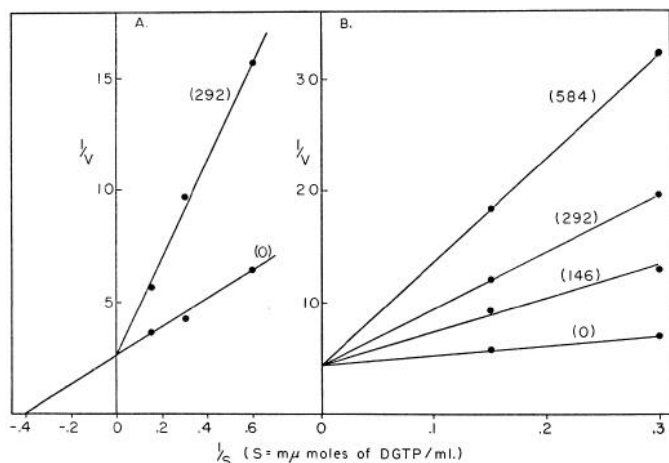


FIG. 2. Determination of K_m for dGTP and K_I for GTP. Numbers in parentheses on each curve indicate concentrations of GTP in $\mu\text{moles per ml}$.

the presence of any one of the above triphosphates. The enzyme produced no Norit-nonadsorbable phosphate from GDP³ (1 to 3×10^{-4} M GDP in the presence of 0.04 unit of enzyme). GDP inhibited the reaction on dGTP³²; 3.3×10^{-4} M GDP produced a 50 per cent inhibition, and 6.6×10^{-4} M GDP produced a 60 per cent inhibition. The concentration of dGTP³² in both experiments was 1.7×10^{-6} M.

GTP, however, was split by the enzyme and this reaction was investigated with unlabeled GTP.³ The maximal rate of splitting was $3.5 \mu\text{moles}$ in 20 minutes per ml. of an enzyme preparation which split $8.9 \mu\text{moles}$ of dGTP per ml. in 20 minutes. The evidence that triphosphosphate is also split from GTP is the following. A 5 ml. reaction mixture containing $0.70 \mu\text{mole}$ of GTP and 0.16 unit of enzyme produced $0.74 \mu\text{mole}$ of Norit-nonadsorbable phosphate (all of which was acid-labile) and no measurable orthophosphate (less than $0.01 \mu\text{mole}$) during a 1 hour incubation period. The action of inorganic pyrophosphatase (13) on this product formed no measurable orthophosphate. As a check on the activity of this preparation of pyrophosphatase, it was determined that the enzyme produced no measurable orthophosphate from commercial triphosphosphate, but it formed the theoretical amount of orthophosphate ($0.23 \mu\text{mole}$) when sodium pyrophosphate ($0.12 \mu\text{mole}$) was acted on in the presence of the GTP split-product.

Fig. 2 is a plot, according to Dixon's treatment (14) of the Lineweaver-Burk plot (15), for the determination of the K_m for dGTP. It can be calculated from Experiment A that the K_m is 2.5×10^{-6} M. Extrapolation of the curve without inhibitor in Experiment B gives the value 2.2×10^{-6} M. Fig. 2 also shows the competitive inhibition by GTP; K_I values of 1.3×10^{-4} and 0.73×10^{-4} M were calculated from Experiments A and B, respectively, by the formula (15): $1/V_I = 1/V_{\text{max}} (K_s + (K_s)(I)/K_I)1/s + 1/V_{\text{max}}$. The K_m for GTP determined by the plot (14) in Fig. 3 was 1.5×10^{-4} M. From the similarity of the K_I and K_m values for GTP, it would appear that dGTP and GTP are split by the same enzyme.

³ 1 ml. assay mixtures were used in the proportions given for the usual assay. The reaction was allowed to run for 1 hour and was stopped by adding 0.4 ml. of 0.1 N HCl and 0.1 ml. of Norit. After centrifugation, 1 ml. of the supernatant fluid was used to determine acid-labile phosphate.

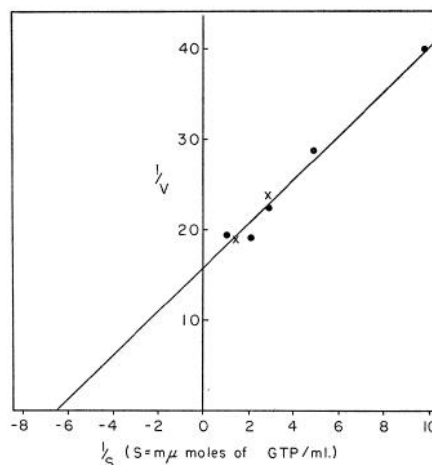


FIG. 3. Determination of K_m for GTP. X—X, Experiment A; ●—● Experiment B.

DISCUSSION

The cleavage of nucleoside triphosphate to a nucleoside and triphosphosphate by the enzyme described here is remarkable for its uniqueness. It is also of considerable interest that the enzyme shows such a high degree of specificity for deoxyguanosine triphosphate. None of the other deoxynucleoside triphosphates (with the possible exception of deoxycytidine triphosphate) is split at a significant rate, and, whereas guanosine triphosphate is a substrate, its affinity for the enzyme is only about 2 per cent of that of the deoxynucleotide. The physiological implications of this reaction are not immediately apparent, and much additional information may be necessary before its relevance to other metabolic events becomes clear. For example, it will be of considerable interest to determine the equilibrium of the reaction and particularly to investigate the possibility of exchange reactions with ribo- and deoxyribonucleosides, or with pyro- or polyphosphates.

Whether similar enzymatic cleavages of other nucleoside triphosphates are to be found in *E. coli* or other cells is not yet known. It should be pointed out that the rather widespread occurrence of triphosphatase and the abundance of alternative pathways for releasing nucleosides from nucleoside triphosphates may obscure and complicate the detection of the type of cleavage described in this study.

SUMMARY

An enzyme was partially purified from *Escherichia coli* extracts which catalyzes the reaction: deoxyguanosine triphosphate \rightarrow deoxyguanosine + triphosphosphate. The products of the reaction have been isolated and identified. Guanosine triphosphate is split in a similar way but at approximately 40 per cent of the rate of deoxyguanosine triphosphate cleavage. The K_m for the ribonucleotide is 1.5×10^{-4} M compared with 2.5×10^{-6} M for the deoxynucleotide. The enzyme does not act upon guanosine diphosphate and reacts little or not at all with the deoxynucleoside triphosphates of adenine, uracil, cytosine and thymine.

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