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 (dGTP$^{32}$) → 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 triphosphophate group. In fact, although triphosphophate has been found in cell extracts, only one biochemical reaction was known for its production, namely, the reaction catalyzed by adenylyl 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 P$^{32}$ 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°.

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, since deoxyguanosine and unreacted dGTP$^{32}$ 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 dGTP$^{32}$, 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). 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.

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-nonadsorbable radioactivity, inasmuch as small amounts of tripolyphosphate are adsorbed by Norit.
After 1 minute, the mixture was centrifuged for 2 minutes, pH 7.2. This solution had an activity of 19 units per ml. and was placed in a -15° bath and 0.5 ml. of absolute ethanol was added, with stirring, during a 2 minute period. The temperature of the enzyme-ethanol mixture rose to -2°.

Fractionation of enzyme

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Units/ml</th>
<th>Units/mg.</th>
<th>Yield from preceding fraction</th>
</tr>
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<tbody>
<tr>
<td>I</td>
<td>0.46</td>
<td></td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>0.98</td>
<td></td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>0.92</td>
<td></td>
<td></td>
</tr>
<tr>
<td>V</td>
<td>0.1</td>
<td>0.6</td>
<td>80</td>
</tr>
<tr>
<td>VI</td>
<td>24</td>
<td>3.1</td>
<td>80</td>
</tr>
<tr>
<td>&quot;Ethanol&quot;</td>
<td>19</td>
<td>11</td>
<td>80</td>
</tr>
</tbody>
</table>

<table>
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<tr>
<th></th>
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</thead>
<tbody>
<tr>
<td>Guanosine (11)</td>
<td>0.99</td>
<td>0.70</td>
<td>0.89</td>
<td>0.63</td>
</tr>
<tr>
<td>Guanine (11)</td>
<td>1.37</td>
<td>0.84</td>
<td>0.98</td>
<td>1.13</td>
</tr>
</tbody>
</table>

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 triphosphate (Table II). Large-scale mixtures incubated for 1 hour, which produced up to 0.93 pmole of deoxyguanosine, yielded less than 0.1 pmole 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 pmole of deoxypentose determined by the diphenylamine reaction, and the expected value, based on the measurement of ultraviolet absorption, was 0.77 pmole, assuming a molar extinction coefficient of 11.4 at 260 m\(\mu\) 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 triphosphate by chromatographing a mixture of the two (84,000 c.p.m. mixed with 23.3 pmoles 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, deoxyxycytidine, and thymidine was also investigated. Under the assay conditions, 5 pmoles of these triphosphates produced 0.07, 0.04, 0.20, and 0.02 pmole of nonadsorbable split-product of dGTP32, respectively. dGTP32 tested simultaneously produced 3 pmoles, and its splitting was not inhibited by

![Fig. 1. Chromatogram of tripolyphosphate and Norit-nonadsorbable split-product of dGTP32.](http://www.jbc.org)
the presence of any one of the above triphosphates. The enzyme produced no Norit-nonadsorbable phosphate from GDP
(1 to 3 $\times 10^{-4}$ m GDP in the presence of 0.04 unit of enzyme). GDP inhibited the reaction on dGTP; 3.3 $\times 10^{-4}$ m GDP produced a 50 per cent inhibition, and 6.6 $\times 10^{-4}$ m GDP produced a 60 per cent inhibition. The concentration of dGTP in both experiments was 1.7 $\times 10^{-4}$ 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 $\mu$moles in 20 minutes per ml. of an enzyme preparation which split 8.9 $\mu$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 $\mu$ mole of GTP and 0.16 unit of enzyme produced 0.74 $\mu$ mole of Norit-nonadsorbable phosphate (all of which was acid-labile) and no measurable orthophosphate (less than 0.01 $\mu$ 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 tripolyphosphate, but it formed the theoretical amount of orthophosphate (0.23 pmole) when sodium pyrophosphate (0.12 pmole) 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 $\times 10^{-5}$ M. Extrapolation of the curve without inhibitor in Experiment B gives the value 2.2 $\times 10^{-4}$ M. Fig. 2 also shows the competitive inhibition by GTP; $K_I$ values of 1.3 $\times 10^{-4}$ and 0.73 $\times 10^{-4}$ M were calculated from Experiments A and B, respectively, by the formula (15): $1/V_f = 1/V_{\text{max}} (K_i + [GTP])/(D/K_i)1/s + 1/V_{\text{max}}$. The $K_m$ for GTP determined by the plot (14) in Fig. 3 was 1.5 $\times 10^{-4}$ 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.

\(^{161}\)
REFERENCES


