RIBONUCLEOTIDE PHOSPHORYLATING ENZYMES (KINASES) IN ESCHERICHIA COLI, UNINFECTED AND INFECTED WITH RNA-BACTERIOPHAGE f_2

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Introduction

It is well known that the phosphorylation of the ribonucleoside mono-and diphosphates to the corresponding di-and triphosphates is catalysed by specific kinases, and that the incorporation of these nucleoside triphosphates into RNA is catalysed by DNA dependant RNA polymerase.

It has been shown by different laboratories that the infection of bacteria with the RNA bacteriophages initiates the synthesis of a new enzyme, which is not present in the host cells (3, 11, 17, 19). The new enzyme, called RNA-replicase (11) or RNA-synthetase (19), catalyses the incorporation of ATP, GTP, CTP and UTP into RNA.

The nucleotide kinases which are formed after infection of E.coli with the T series of DNA-bacteriophages may be divided into three groups: a)Those enzymes which are not detectable in E.coli before infection (13), b)the enzymatic activities present before infection which increase considerably after infection due to the formation of new enzymes responsible for similar reactions(5,7,8) and c)the enzymes present before infection which do not increase after infection (8).

The purpose of this report is to describe the effect of an RNA-bacteriophage f_2 on the activity of the nucleoside monophosphokinases of E.coli after infection.

Materials and Methods

Escherichia coli K-12 F^+ and the RNA bacteriophage f_2 (14) were kindly supplied by Dr.N.D.Zinder of the Rockefeller Institute.The bacteria were maintained on nutrient agar slants and

the phage were prepared and kept in bottles and tubes without any metal surface (20).Bacterial cell titers were determined from turbidity measurements with a Bausch and Lomb colorimeter, at 650 mμ.Bacteriophage titers were obtained by the agar layer technique (1).Radioactivity was measured in a thin window gas-flow counter.Radioactive compounds were purchased from the Radiochemical Center, Amersham, England.Ribonucleoside mono-di-and triphosphates, lactic dehydrogenase(crystalline from rabbit muscle, containing pyruvate kinase),phosphopyruvate,and NADH, were purchased from the Sigma Chemical Company.Protein was determined by the method of Lowry et al. (15).

Two methods were employed for the measurements of kinase activity:

I. Spectrophotometric assay: The mixture contained in μmoles: Tris buffer pH 7.4, 85, KCl 85, $MgCl_2$ 8.5, ATP 5, phosphoenolpyruvate 1.5, NADH 0.25, lactic dehydrogenase (containing pyruvate kinase), 90 μg, nucleoside monophosphate I, and enzyme (see Fig.1), in a final volume of 1 ml.The rate of the formation of nucleoside diphosphates was followed by measuring the rate of NADH oxidation, as the decrease in optical density at 334 mμ, on an Eppendorf spectrophotometer at 60 -second intervals for several minutes, at room temperature (4,16).

II. Radioactivity assay: The incubation mixture for this assay contained in μmoles: Tris buffer pH 7.4, 20, KCl 20, $MgCl_2$ 8, ATP 4, ^{14}C-labeled nucleoside monophosphates 1.1 μmole, specific activity 3 x 10^5 cpm/μmole (see (Fig.2), in a final volume of 0.6 ml.The mixture was incubated at 37° for 30 min and the reaction was stopped by adding an equal volume of cold 70% methanol.

The {^{14}C} -nucleoside di-and triphosphates(see discussion) formed from the corresponding monophosphates were separated by paper chromatography (Whatman No 3MM) for 45 hours, using isobutyric acid- 1 N NH_4OH- 0.2 M sodium ethylenediamine-tetraacetic acid (50:30:0.5 v/v) (10).The paper was dried and the di-and triphosphates were located by means of autoradiography on Kodak Medical X-ray film, Royal blue.The labeled spots were then cut out and eluted with 0.01 N HCl overnight, and a sample was plated and counted.

Growth of Bacteria, Infection and Preparation of Crude Extracts: E.coli cells were grown in tryptone broth (9) in two Baxter flasks

at 37° with vigorous aeration.When the growth reached a density of 2 x 10^8 cells per ml, the cultures were infected with f_2 bacteriophage in a ratio of five phage per bacterium. A noninfected control culture, of the same concentration, was chilled to 2°.Thirty minutes later, the aeration was discontinued, and the culture was cooled to 2° , by rotating the flask in a methanol bath (-10°).The cells were harvested at 6,000 xg for 15 minutes, and after the addition of 4 volumes of 0.05 M Tris buffer, pH 7.4, the suspended cells were disrupted by sonic oscillation (20 kc) at 50 watts for 1 minute.After centrifugation at 35,000 xg for 15 minutes the cell debris was discarded and the supernatant fluid represented the crude extract.The crude extracts were centrifuged at 105,000 xg for 1 hour.This treatment reduced the protein content by 25%.The supernatant fluid was filtered through a Sephadex G25 column (1 x 18 cm) using Tris buffer 0.05 M, pH 7.4.The activities of the infected and noninfected cells were checked in the crude extracts as well as in the eluate from the column.

Results and Discussion

When GMP kinase was compared in the extracts of uninfected cells and cells infected for 30 minutes with f_2 bacteriophage, no difference in the activity was detected.The results are shown in Figures 1 and 2.In Fig.2 the kinase activity was estimated on the basis not only of GDP but both GDP and GTP, since with these crude extracts GDP was also partially converted to GTP.

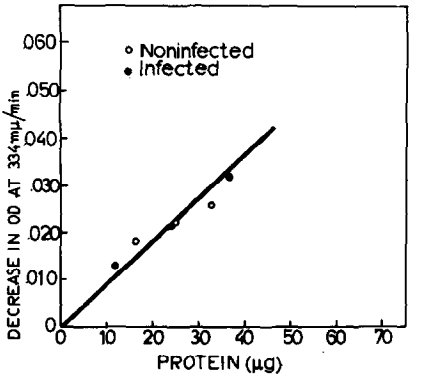

Fig.1.Rate of oxidation of NADH as a function of enzyme concentration in standard optical assay,(see "Methods"), with GMP as substrate.

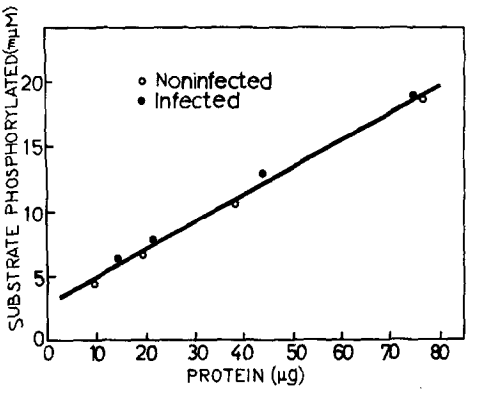

Fig.2. Effect in increasing amounts of enzyme on the amount of GMP phosphorylated.
Activity was measured with the radioactivity assay (see "Methods").

The kinases activity was also measured in the eluates obtained after filtration through Sephadex G25, and was found to be the same as in the crude extracts. Similar results were obtained with CMP, UMP and AMP kinases, as measured by both methods.

In order to eliminate the possibility of incomplete infection of the cells, a culture was infected and aliquots were withdrawn at 5, 15 and 30 minutes, and the concentration of the nonabsorbed phage was determined, as described by Adams (1). After 5 minutes, only 9% of the phage had remained. To exclude the possibility of the formation of an inhibitor in the infected cell extracts, the activities of the kinases were measured in a mixture of uninfected and infected extracts. From Table 1, it can be seen that the activity of the mixture is equal to the sum of the individual extracts.

Table 1. EFFECT OF MIXED EXTRACTS ON GUANOSINE MONOPHOSPHATE PHOSPHORYLATION.

Extracts	Protein added (μg)	Δ_{OD}/min. at 334 mμ
Control	16.6	0.017
Infected	12.2	0.012
Control	16.6	
+Infected	12.2	0.028(0.029)*

* This figure is the calculated value assuming no interaction of the extracts.

In order to determine whether the kinase activity after infection was due to the same protein existing in normal cells or to a newly synthesized kinase having the same specific activity as in normal cells, chloramphenicol , an inhibitor of protein synthesis, was used.The results shown in Fig.3 indicate that no decrease in guanilic kinase activity occured in the culture containing chloramphenicol (100 μ g/ml), in aliquots withdrawn immediately and at 15 and 30 minutes after infection, and it was found unchanged in aliquots withdrawn from a normal culture at the same time intervals.

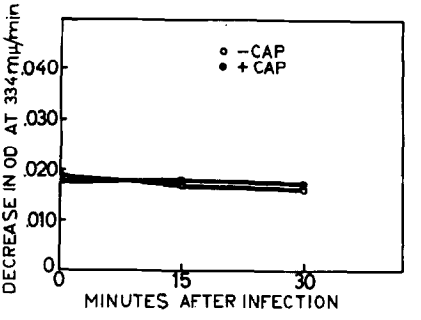

Fig. 3. Effect of Chloramphenicol (CAP)(100 μg/ml) on GMP-kinase formation.

Moreover, pH-activity profiles of both sets of kinases with GMP as substrate, suggest that the kinases of the phage-infected cells are similar in this respect the non-infected cells as shown in Fig. 4.

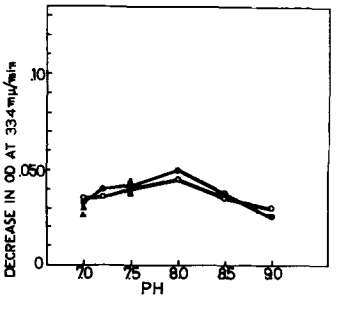

Fig. 4. pH optima with GMP as substrate.Kinase activity was measured with the spectrophotometric assay (see "Methods"), in noninfected (o and Δ)and in infected (• and ▲) cells, with two buffers:Tris 0.2 M (o and •) and phosphate buffer 0.5 M (Δ and ▲).About 50 μg of protein was added to the reaction mixture.

The buffer effect on the specific activity of the kinases was checked using two different buffers: 0.2 M Tris and 0.5 M phosphate buffer.

These results agree with the results obtained after infection of E.coli with the DNA-bacteriophages, T_3 and T_7 (8). It is possible that the activity of the kinases in the uninfected E.coli cells is already high enough to catalyze the phosphorylation of all the nucleoside monophosphates necessary for the synthesis of the new RNA.These findings also agree with the complementation studies with the related RNA-phage, R17 (18), in which the total number of genes likewise may be three.One of the genes codes for the RNA-synthesizing enzyme, the RNA replicase, the second for the coat protein and the third remains unknown, although there are suggestions that this last gene is involved in adsorption of the virus to the host cell.Thus it may be suggested that the RNA molecule, which is the genetic material of the virus, can not provide the information for the synthesis of a new kinase.

The above results are in contrast with the results obtained with E.coli after infection with the DNA-bacteriophages T_2, T_4, T_5, T_6 where 10-20 fold increases in nucleoside monophosphokinases was observed (8).However, when the nucleoside diphosphokinases were measured in the same system, they were found to be similar before and after infection (6).

Results in our laboratory indicate, that after infection of E.coli with f_2 phage, the nucleoside diphosphokinases are similar in activity to those of the uninfected cells (2).

During the course of this work, it was reported that similar results were obtained after infection of E.coli with the bacteriophage, MS2 (12).

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REFERENCES

1. Adams, M.H., in J.H.Comroe, Jr. (Editor), Methods in Medical Research, Vol.II, Yearbook Publishers, Inc.Chicago,(1950) , p.I.
2. Argyrakis, M.P., In Preparation.
3. August, J.T., Cooper, S., Shapiro, L., and Zinder, N.D., Cold Spring Harbor Symp.Quant.Biol. 28,95 (1963).

4. Bello, L.J., and Bessman, M.J., J.Biol.Chem., 238, 1777 (1963).
5. Bello, L.J., Van Bibber, M.J., and Bessman, M.J., J.Biol.Chem. 236, 1467 (1967).
6. Bello, L.J., and Bessman, M.J., Biochim.Biophys.Acta, 72, 647 (1963).
7. Bessman, M.J., Herriott, S.T., Van Bibber Orr, M.J., J.Biol.Chem 240,439 (1965).
8. Bessman, M.J., J.Biol.Chem., 234, 2735 (1959).
9. Cooper, S., and Zinder, N.D., Virology, 18,405 (1962).
10. Firschein, W., J.Bacteriol., 90, 327 (1965).
11. Haruna, I., Nozu, K., Ohtaka, Y., and Spiegelman, S., Proc.Natl. Acad.Sci.U.S., 49, 407 (1963).
12. Hiraga, S., and Sugino, Y., Biochim.Biophys.Acta, 114, 416 (1966).
13. Kornberg, A., Zimmerman, S.B., Kornberg, S.R., and Josse, J., Proc.Natl.Acad.Sci.U.S.,45, 772 (1959).
14. Loeb, T., and Zinder, N.D., Proc.Natl.Acad.Sci.U.S.47, 282 (1961).
15. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R. J., J.Biol.Chem.,193, 265 (1951).
16. Maley, F., and Ochoa, S., J.Biol.Chem.233, 1538 (1958).
17. Ochoa, S., Weissmann , C., Borst, P., Burdon, R.H., and Billeter, M.A., Federation Proc., 23, 1285 (1964).
18. Watson, J.D., Personal Communication, Third Advanced Study Institute of Molecular Biology, Spetsai, Greece (1966).
19. Weissmann, C., Simon, L., and Ochoa, S., Proc.Natl.Acad. Sci.U.S. 49 , 407 (1963).
20. Yamamoto, N., Hiatt, C.W., and Haller, W., Biochim.Biophys. Acta, 91, 257 (1964).