ENZYME ACTIVITY AND BACTERIOPHAGE INFECTION

I. BREAKDOWN OF ADENOSINETRIPHOSPHATE

BY ARTHUR B. PARDEE

(From the Virus Laboratory, University of California, Berkeley)

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One approach to the study of bacteriophage infection is an investigation of changes in metabolic reactions of the infected host. Experiments of this sort have been pursued with intact cells, especially by Cohen (1), Price (2, 3), and Kozloff et al. (4); however, studies in this field have not been performed with cellular extracts. Therefore, it seemed worthwhile to perform a few experiments in order to investigate such an approach.

Enzyme activities can be determined with intact cells or with enzymically active preparations. Each method has its disadvantages. It is difficult to study single reactions in intact cells; also, changes due to virus infection are likely to occur in the course of the test for enzyme activity. Use of extracts might lead to erroneous results owing to dilution of cofactors or loss of activity during preparation. Thus, it seems advisable to use both intact cells and extracts in such studies, and to provide optimum conditions, as nearly as possible (5, 6).

It was necessary to decide which of the many enzyme activities in the cell should be studied first. It seemed probable that the enzymes that are altered on bacteriophage infection would be involved in synthetic processes too complex to study by known methods. However, reactions involving transfer of energy from fermentative and oxidative reactions to synthetic reactions by means of adenosinetriphosphate (ATP) might be affected. In this connection, Price (2) found that chemicals which inhibited ATP formation also inhibited bacteriophage production, and concluded that ATP was necessary for the latter process. The enzymic activity involved in ATP metabolism which is most readily measured is apyrase.¹

Bacterial apyrase preparations can be studied to determine optimum conditions, and properties, and these can be compared with measurements on apyrase of animal tissues (7, 8). Such comparisons, and changes in bacterial cells infected with T₃ bacteriophage are reported in the present paper.

¹ In this paper the activity of a preparation causing dephosphorylation of ATP is referred to as apyrase, as contrasted to adenosinetriphosphatase which removes only the terminal phosphate of ATP.
Methods

The *E. coli*, strain B, and the T₃ bacteriophage were obtained from Dr. Dean Fraser, of this laboratory. Bacterial growth and phage infection were carried out at 40° with strong aeration in a medium containing 1.5 gm. KH₂PO₄, 3.5 gm. Na₂HPO₄, 1.0 gm. NH₄Cl, 0.1 gm. MgSO₄, 5.0 gm. bacto-casamino acids (Difeo), 0.1 ml. 1 M CaCl₂, 10 gm. glycerine, 1 ml. 1 per cent gelatin, and 0.3 ml. 5 per cent FeSO₄ per liter of tap water. This medium was derived from one suggested by Dr. Dean Fraser as suitable for production of T₃.

Bacterial concentration was measured by turbidity in a 10 × 75 mm. tube with the Klett-Summerson colorimeter, using a red filter (No. 66). Turbidity was calibrated against microscopic counts of cells using the Petroff-Hausser counting apparatus. Bacteriophage concentrations and the number of infectious particles were measured by the plaque-counting technique (9). Viable cell counts were made by plating on nutrient agar and counting colonies.

Bacterial extracts were prepared from cells in the late log phase (3 × 10⁹ cells per ml.). The cells were centrifuged for 5 minutes at 2500 × g, washed twice with distilled water, and the packed cells were ground with 3 gm. alumina¹ per gm. wet cells for 2 minutes in a mortar (10). The preparation was taken up in distilled water or buffer, and unbroken cells and alumina were removed by centrifugation for 5 minutes at 3000 × g. The extract contained 85 per cent or more of the protein of the cells, non-infected or infected, and the viable cell count was less than 0.01 per cent of the original number. The apyrase activity per gram protein was the same in this extract as in an extract containing all of the protein, which was prepared by centrifuging only fast enough to remove the bulk of the alumina (5 minutes at 500 × g).

The apyrase activity was measured by the method of DuBois and Potter (7) with modifications to be described. A cold reaction mixture was prepared containing buffer, salts, ATP, and extract. Aliquots (0.3 ml.) were removed from the reaction mixture during incubation at 40° and were pipetted into 0.02 ml. 65 per cent trichloroacetic acid (TCA) to stop the reaction. The denatured protein was removed and analyses for inorganic phosphate were made on the supernatant fluid by the method of Fiske and SubbaRow (11).

Ammonia was determined by the Conway method, followed by nesslerization (11⁴). Protein was determined by the biuret method, using the soluble reagent of Weichselbaum (12). This technique was checked by comparing the results with protein nitrogen. Protein was considered to be the precipitate remaining after heating an extract for 15 minutes at 90° in 5 per cent TCA (13). All biuret color in an extract was due to the TCA-precipitable material.

ATP was obtained from the Sigma Chemical Company. Phosphate analyses indicated that it contained 90 per cent ATP, and 10 per cent adenosinediphosphate (ADP) plus inorganic phosphate. ADP was obtained from the same source, and muscle adenylic acid (AMP) was obtained from Nutritional Biochemicals Corporation. Other chemicals were commercial products of reagent grade.

¹ Adolph Buehler, 1557AB levigated alumina.
² Umbreit, Burris, and Stauffer (11), 156.
EXPERIMENTAL RESULTS

Optimum Conditions.—Since extracts from a bacterial source were used in the present work, it was desirable to check the experimental conditions set down by DuBois and Potter (7), which were obtained with animal tissues. Alumina extracts of uninfected E. coli were used for these experiments. The data, which will be discussed below, are shown in Table I.

Although apyrase activity was proportional to the amount of tissue when rat homogenate was used (7), this was not the case with bacterial extracts or with intact bacteria. The phosphate released by increasing amounts of extract was not proportional to the amount of extract as can be seen from Fig. 1. Further, if the phosphate released was plotted against time a similar picture was obtained (Fig. 2). It is of course preferable to have a linear relation between extract and activity, to simplify the determination. However, no changes of conditions were found which would accomplish this. Enzyme instability was not responsible for the falling off of the rate because activity was retained for several days at 0° or for 30 minutes at 40°. To choose a quantitative measure of apyrase activity, use was made of the principle that the amount of enzyme is proportional to the reciprocal of the time required for a fixed amount of reaction. An examination of Fig. 2 shows that this is the case when the amount of reaction is taken as the release of phosphate equal to the terminal phosphorus of the added ATP.

The decrease in activity with time was not due to insufficient substrate. If can be seen from Table I that a final concentration of 0.0029 M ATP was adequate. The rate of production of phosphate from a higher level of ATP was

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It was desired to check the experimental conditions set down by DuBois and Potter (7), which were obtained with animal tissues. Alumina extracts of uninfected E. coli were used for these experiments. The data, which will be discussed below, are shown in Table I.
Fig. 1. Effect of amount of extract on phosphate released from ATP in 15 minutes. The conditions of the experiment with bacterial extract are described in Table I, those for the experiment with rat liver are taken from reference 7. The amount of tissue represents the milliliters of 2 per cent rat liver homogenate per 0.65 ml. total volume, or of extract containing 4.9 mg. protein per ml. in 0.45 ml. total volume.

Fig. 2. Rate of release of phosphate by various amounts of bacterial extract. The experimental conditions are described in Table I. The inset is a plot of the reciprocal of the time required to break down 100 per cent of the third phosphorus of ATP vs. amount of extract.
less at the beginning of the reaction and slightly greater late in the reaction than when the standard concentration was used.

The optimum pH was 9.0 to 9.5, the value found for animal tissues (7). The activity decreased rapidly at higher pH and rather slowly at lower pH (Table I). In the region pH 8.0 to 9.0 barbital and borate buffers gave closely similar results, indicating that the anion did not affect the activity. In spite of the observed optimum, a more physiological pH was chosen and the experiments were carried out in 0.05 M barbital at pH 8.1.

The bacterial extracts required Mg~2~ rather than Ca~2~+, a fact noted by Utter and Werkman (14). Ca~2~+ was required in animal experiments, but was inhibitory to the apyrase of bacterial extracts; for example, 0.005 M Ca~2~+ reduced the activity 60 per cent. Other metal ions (Mn~2~+, Fe~2~+, Zn~2~+, Al~3~+) were also inhibitory. Half the activity of extracts dialyzed against 0.15 M KCl overnight was lost; however, it was completely restored by addition of Mg~2~+. A final concentration of 8 X 10~4~ M MgCl~2~ was used as routine (Table I).

Other Reactions.—Phosphate was released from ADP at one-third the rate observed with ATP. The rate when both ATP and ADP were added to the reaction mixture was similar to the rate when only ATP was added at the same total concentration. This shows that the decrease in rate (Fig. 2) is not caused by formation of ADP which then competes with ATP for the enzyme.

Phosphate was released from AMP at too slow a rate to measure. Addition of AMP to ATP caused the initial rate to be slower, decreasing the activity by 25 per cent when 0.0029 M AMP was added. It is possible, therefore, that the observed falling off in rate when ATP was used as the substrate was in part due to inhibition by AMP formed during the reaction.

No measurable deamination of ATP and ADP was observed to occur in a period during which 30 per cent of the labile phosphate was released by the bacterial extracts.

Several compounds were tested for inhibitory properties, and the following per cent inhibitions were found: 0.01 M citrate, 40 per cent; 0.01 M oxalate, 15 per cent; 0.01 M fluoride, no inhibition.

Apyrase of Injected Cells.—E. coli, strain B, were grown to 3 X 10~9~ cells per ml. and were mixed with approximately 5 X 10~7~ bacteriophages per cell. The infected cells were aerated vigorously at 40°. Turbidity was followed in order to determine the time of lysis. At desired times samples for enzyme assay were mixed with approximately 5 X 10~7~ bacteriophages per cell. The infected cells were washed with ice water in the cold, and an extract was prepared by grinding with alumina. Although the treatment was intended to prevent changes during preparation, the appearance of the samples indicated that some cellular lysis had occurred in samples taken at times close to lysis. Other samples were pipetted into cold medium, and were diluted and plated for viable cells, and for infectious particles, or were centrifuged and plated for free bacteriophage.
Fig. 3. Apyrase activity, phage in solution, turbidity, and viable cells vs. time after bacteriophage infection. The experimental conditions and method of calculation are given in Table I, and under Methods. Results of three experiments are given for the apyrase activity of extracts, while representative curves are shown for the other data.

The data are shown in Fig. 3. Under the experimental conditions, approximately two phages were adsorbed per bacterium, and over 99 per cent of the bacteria were infected. Apyrase increased sixfold in the intact cells, and a 30 per cent increase was noted when extracts were used, followed by a sharp drop just before lysis.
DISCUSSION

Two differences between animal and bacterial apyrases were noted. One was the stimulation of animal apyrases and inhibition of bacterial apyrase by Ca++. The other was the linear relation between amount of extract and activity in animal preparations, and the absence of such a relation in bacterial extracts. Apparently the enzyme reactions are qualitatively different.

The apyrase activity of intact cells was approximately one-third that of the extract per milligram of protein, a result which could be attributed to low permeability of the intact cells to ATP, or to other causes discussed below.

It is of interest to compare apyrase activity with oxygen uptake because the former is involved in breakdown of ATP and the latter in its formation. Calculation of apyrase activity of extracts from the initial slopes of plots of phosphate release vs. time gives 13 μM P per mg. dry weight per hour. Measurements of O2 uptake in the Warburg apparatus on intact cells in the growth medium yield 13 μM O2 per mg. dry weight per hour. The number of phosphates esterified per O2 is not known for E. coli. In animals 5 to 6 phosphates are esterified per O2 taken up (15, 16). It is concluded that apyrase could break down a considerable fraction of the high energy phosphate if it were present in intact cells at the same activity as it is present in extracts.

Infection by bacteriophage brought about a sixfold increase in apyrase activity as measured in the intact cell; however, this can probably be attributed to a change in permeability to ATP shortly before lysis. The experiments with extracts showed a small initial decrease at the time of infection, then a 30 per cent increase in apyrase activity during the period of intracellular phage growth, followed by a sharp decrease at lysis. These changes are greater than the average error of duplicate determinations, which was ± 4 per cent. An increase in enzymic activity is in contradiction to the observation that infected cells did not produce adaptive enzymes (17), and to the observation that infection did not change O2 uptake or CO2 output (18). The increase in activity might be the result of an actual increase in the amount of enzyme, or of the appearance of a new reaction in which ATP is used to phosphorylate some compound which is then broken down, releasing phosphate (19), or of the removal of a natural inhibitor or control mechanism (20, 21). The observed activities are calculated on the basis of phosphate released per gram of protein. Therefore, the results might be attributed to leakage of protein low in apyrase from the cell until lysis, whereas changes in permeability might permit leakage of large amounts of all soluble proteins. In this connection, Price (reference 3, and unpublished data) has noted a release of nucleic acid and protein from the cell before lysis. Many experiments on a diversity of enzymes will have to be performed before the significance of the change in apyrase activity can be appraised.
The author wishes to thank Dr. W. H. Price for helpful suggestions and for his interest in the work.

SUMMARY

Experiments have been performed on the apyrase activity of E. coli, strain B. Although the dependence on pH and substrate is similar to that of rat tissue, the bacterial extracts are inhibited by Ca ++ and stimulated by Mg ++. In bacterial extracts the rate of phosphate release decreases in the course of the reaction, possibly owing to product inhibition. With multiple bacteriophage infection, the apyrase activity of the intact cells increased several fold, and the activity of extracts increased about 30 per cent. It is suggested that the changes could be attributed to an increase in the amount of enzyme although other alternatives cannot be precluded at present.

BIBLIOGRAPHY