The Properties of Adenosine Deaminase and Adenosine Nucleoside Phosphorylase in Extracts of Escherichia coli*

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It has been known for a number of years that there are two principal processes whereby Escherichia coli acts on adenosine (1, 2): deamination to inosine and cleavage to adenine and sugar. Later work (3–7) indicated that the cleavage was phosphorolytic in nature, as had been shown by Kalckar (8) to be the case with a mammalian enzyme.

In E. coli there appears to be a number of phosphorylases acting on purine ribonucleosides and a number acting on deoxyribonucleosides (6). These various activities may be differentiated by changes in the pH optima caused by adding various purine analogues. There is, however, no reason for assuming that common enzymes are involved in the cleavage of the ribose and deoxyribose series of nucleosides, although this may well be the case. On the other hand, it appears reasonable to assume that only one enzyme is involved in deaminating both adenosine and deoxyadenosine for the two substrates are cleaved at a comparable rate (6), and the purified mammalian enzyme (9) acts on both substrates.

It was found (6, 7) that the amount of adenosine deaminase activity in individual extracts of bacteria is extremely variable. Some extracts were completely devoid of activity and others displayed a great deal. In those extracts not containing deaminase, the adenosine nucleoside phosphorylase activity could be measured by isolating the reaction product, adenosine. This could not be done in the presence of deaminase activity because of the concomitant production of hypoxanthine and inosine.

The present study was undertaken to investigate the occurrence of deaminase and phosphorylase acting on deoxyadenosine. Extracts of E. coli grown under a variety of conditions were extracted in various ways. It is now possible to achieve the resolution of deaminase and phosphorylase in crude extracts as well as to understand some of the factors leading to the biogenesis of both.

**EXPERIMENTAL**

*Preparation of Cell-free Extracts—E. coli strain B, was grown with aeration at 37°. Media used were synthetic lactate (L medium) (10), mineral salts-glucose (M-9 medium) (11), and lactate supplemented with adenine, hypoxanthine, caffeine, and theophylline at 2.16 mM (12). In one experiment, the sulfate was increased 100-fold with Na₂SO₄, and in another, 0.1 mg. per l. of MgSO₄ was the only added source of sulfate.

Growth was followed by nephelometry with the use of the Spectronic “20” colorimeter at 400 μm. A standard curve, relating optical density to the protein nitrogen of cultures, was used. The protein nitrogen was determined with the Folin phenol method (13). The latter was standardized against Kjeldahl determination of bacterial nitrogen. For a culture of bacteria growing exponentially in M-9 medium, 1 mg. per ml. of protein corresponds to 6.7 × 10⁶ viable bacteria per ml.

The bacteria were harvested, washed, and ground as previously described (3, 6). The abrasives used were Alumina A-301, washed, powdered Pyrex, and HCl-treated washed sand. In all cases the amount used was twice the wet weight of the cells. The extracting fluid was 0.01 M Tris¹ buffer at pH 7.5. Phosphate or pyrophosphate at the same pH and molarity were used alternatively. The ratio of extracting solution to original wet weight of cells was 5. The extracts were clarified by centrifugation for 30 minutes at 30,000 × g in the Servall centrifuge. All operations were carried out in the cold.

**Adenosine Deaminase Activity—**Kalckar’s differential spectroscopic method (14) was modified by balancing a spectrophotometer against solutions of optical density such that readings would be obtained in the more accurate range of the instrument. This permitted direct analysis of the reaction at higher substrate concentrations than would have otherwise been possible. This modification was made possible by the availability of a photomultiplier attachment for the Beckman DU spectrophotometer. At pH 7.5 the change in optical density of a solution containing 1 mM deoxyadenosine upon conversion to deoxyinosine is 7.7 at 265 μm and is 7.0 at 260 μm. The same change in optical density was also used for adenosine and adenosine 3'-phosphate. The following assay system was employed. One ml. of 0.01 M buffer, 80 μg. of deoxyadenosine, and the bacterial extract were combined in a final volume of 3.2 ml. The optical density changes were followed at either 260 μm or 265 μm. One unit of enzyme activity is defined as the amount of enzyme that will convert 1 μmole of deoxyadenosine to deoxyinosine per hour in the above system when the buffer is 0.01 M Tris at pH 7.5 and at room temperature of 25–27°. The temperature coefficient of the reaction is quite small, as indicated below, so that precise temperature control is not required.

¹ The abbreviation used is: Tris, tris(hydroxymethyl)amino-methane.
Nucleoside Phosphorylase—As we have already noted, the presence of deaminase can seriously affect the assay of phosphorylase in methods which depend on the isolation of adenine. The presence of deaminase would also invalidate differential spectroscopic methods.

Methods that depend on the production of reducing sugar should be independent of the deaminase if both deoxyadenosine and deoxyinosine are good substrates for the enzyme. The Nelson (15) arsenomolybdate method is complicated because of the interference of deoxyadenosine with color development (4, 6). In effect, the assay is rendered more sensitive when deoxyadenosine is the substrate than when some other nucleoside which does not influence color development is employed, because the enzyme produces not only reducing sugar but also removes the chromophore inhibitor, deoxyadenosine.

In this work we have used a micro modification of Nelson's method. A 0.2-ml. sample and 0.2 ml. of combined copper reagent (15) are heated in a boiling water bath for 80 minutes. After cooling has taken place, 0.2 ml. of arsenomolybdate is added, and 10 minutes later the sample is diluted to 4.0 ml. and read at 640 mμ in a spectrophotometer. It is important to keep the sample and reagents in the original proportions during reduction of the copper and during chromophore development, but after the chromophore has been formed it may be diluted since it obeys Beer’s law and is stable indefinitely. Under these conditions, the blank value is markedly reduced and is less variable than if standard amounts (10 times more) of the reagents are used. Twenty minutes is enough for complete reduction of the copper with glucose but, as observed by Manson and Lampen (16), a much longer time is required for maximal yield from deoxyribose.

The following components were mixed for the assay system employed: 0.1 ml. of deoxyadenosine (10 mg. per ml.), 0.1 ml. of 0.2 m, pH 5.0 arsenate buffer, enzyme preparation, and water to a final volume of 0.5 ml. The reaction mixture was incubated for 30 minutes at 37°. A 0.2-ml. sample was used directly in the reducing sugar method described above. There was, however, no deproteinization step. Zero time samples were run whenever necessary. The contribution of the enzyme extract itself to the observed color was in no case more than one sixth the color produced by enzymatic action in the 30-minute incubation period.

With the use of this assay system, approximate linearity is achieved between the increment of optical density and the amount of enzyme used (see below). We have, therefore, arbitrarily defined a unit of activity for this enzyme as that amount of enzyme that gives rise to an increment of optical density of 1.0 in 30 minutes.

RESULTS AND DISCUSSION

Occurrence and Extraction of Enzymes

Table I shows the results of the determination of the two enzyme activities present in extracts prepared in various ways from bacteria grown under a variety of conditions. Of the different abrasives tried, alumina gives by far the greatest percentage of protein extracted and also the greatest yield of both enzymes, although the specific activity of extracts obtained with the other two agents is not greatly different. These findings suggest that the abrasives differ in their ability to disrupt the cell wall, but do not differentially influence the appearance of these two enzyme activities in the extract.

The percentage of extracted protein varies from 9 to 28 in various experiments in which alumina is used as the abrasive. This range would be 9 to 22 per cent if we considered only cells grown on lactate medium. This variation reflects variation in the grinding and preparation procedure, but only to a minor degree, the phase of growth of the cells at the time of harvest. It must be noted, however, that merely increasing the grinding time does not proportionally increase the extraction of protein. As is seen in the case of Preparation 12, grinding and extracting with Tris buffer yielded 9.4 per cent protein; regrinding and re-extracting gave only an additional 2 per cent.

It was noted before (6) that some extracts are devoid of de-
aminase activity. Wang et al. (17) also failed to find any adenosine deaminase in their extract, and one preparation, No. 8, in this series was completely devoid of activity. The probable explanation for the absence of the deaminase activity is that the deaminase is extremely sensitive to heavy metals (see below). Previously, on the basis of data accumulated at that time (6), it was suggested that the active material was not soluble in phosphate buffer. However, it is seen from Table I that this is not the case. Phosphate buffer may be somewhat less effective as an extracting agent, but this does not account for the complete absence of the enzyme.

The amount of deaminase is increased markedly in bacteria grown in the presence of the purine bases, adenine and hypoxanthine, and, to a lesser extent, when caffeine is present. There is no increase when theophylline is present in the growth medium.

The adaptive nature of the deaminase seems clear although it is of some interest in that its formation is induced by a substance akin to its reaction product.

From the data of Table I, something about the constancy of the occurrence and extraction of this enzyme can be inferred. This can be done by comparing the deaminase activity of preparations, prepared under substantially identical conditions, viz. cells grown in lactate medium, harvested in the late exponential phase of growth, ground with alumina, and extracted with Tris buffer. In these preparations the activity ranges from 2 to 3 and does not correlate with the percentage of protein extracted. Lack of correlation can be taken to indicate that the deaminase is liberated from the bacteria neither much more nor much less readily than is the bulk protein of the cell. It may be well to note in passing that only very little (approximately 1.4 per cent) of the total deaminase in Preparation 13 could be found asso-
ciated with the resuspended particulate fraction after grinding. The same argument appears valid for the nucleoside phosphoryl-
ase activity, although here the variation in enzyme activity ap-
ppears somewhat larger.

The activity of the phosphorylase does not appear to be modified by the presence of purine bases in the growth medium. It should be mentioned, however, that the enzyme is strongly inhibited by sulfate ions and that the latter are present in inhibitory concentration in the synthetic L medium, but that either greatly increasing or decreasing the amount of sulfate in the growth medium does not influence the level of enzyme.

Properties of Adenosine Deaminase

Kinetics—The differential spectroscopic method allows the accurate measurement of the course of the deamination process (Fig. 1). The results obtained indicate that the course of the reaction may be predicted from the integrated form of the Michaelis-Menten equation

\[(S_0 - S) + K_m \ln \frac{S_0}{S} = V_{\text{max}} t\]

where \(S\) is substrate concentration, \(t\) is the time, \(K_m\) is the Michaelis-Menten constant, and \(V_{\text{max}}\) is maximal velocity. From the experimental data values for estimates of \(K_m\) and of \(V_{\text{max}}\) may be obtained in several ways (18, 19)\(^2\) each method of calculation giving concordant results.

The validity of the integrated Michaelis-Menten expression is apparent, since the reaction is essentially irreversible and is measured at low initial substrate concentration thus reducing the possibility of product inhibition.

This is confirmed by the observation that the values for \(K_m\) are independent of initial substrate and enzyme concentration. Thus, in the four runs shown in Fig. 1 the average values for \(V_{\text{max}}\) at the higher and lower initial substrate concentrations are 18.2 and 18.6 μmoles per mg. per hour and for \(K_m\) are 29 and 31.1 μM, respectively.

The kinetics of two other preparations were investigated. \(K_m\) appeared to be 30 μM for No. 9 and 32.4 μM for No. 17. It should be noted that the initial substrate concentration utilized in obtaining data in Table I is 100 μM and that no curvature in the plots of optical density against time was found for the first one-third of the reaction. \(K_m\) must be much less than 100 μM for all preparations, and therefore, all data have been computed on the basis of zero order kinetics. However, the reaction rates reported in Table I are 23 per cent less than the maximal rate obtainable with large initial substrate concentrations, on the assumption of a \(K_m\) of 30 μM for all preparations.

Specificity—Deaminases isolated from various sources have proved to be very variable in their specificity. The mammalian enzyme attacks both adenosine and deoxyadenosine (9, 14). In Lactobacillus helveticus a deaminase specific for deoxyadenosine (20) is found, whereas the enzyme isolated from Aspergillus oryzae (21) has a broad specificity and will act on a variety of adenosine derivatives substituted in the ribose moiety at either the 3' or 5' position.

None of these enzymes can attack adenine, nor, in general, do extracts of E. coli. However, suspensions of resting E. coli can deaminate adenine (1, 2, 22). The rate at which adenine was deaminated by such suspensions was increased by the addition of small amounts of the nucleoside (2) or inorganic phosphate (1).

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\(^2\) A. L. Koch, unpublished method.
Enzymes from E. coli Acting on Deoxyadenosine

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TABLE II
Effect of substrate on kinetic constants

<table>
<thead>
<tr>
<th>Compound</th>
<th>( V_{\text{max}} )</th>
<th>( K_m )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenosine</td>
<td>3.41</td>
<td>130</td>
</tr>
<tr>
<td>Deoxyadenosine</td>
<td>2.41</td>
<td>32.4</td>
</tr>
<tr>
<td>Adenosine 3'-phosphate</td>
<td>4.87</td>
<td>66</td>
</tr>
</tbody>
</table>

This experiment was carried out to determine whether we could explain in this fashion the fact that one preparation in the previous series had been found to attack adenine (6). All other preparations (including those grown in the presence of adenine) have shown only extremely small deaminase activity on adenine.

Other adenine-containing compounds are also not attacked by the enzyme. These include adenosine 2'-phosphate, adenosine 5'-phosphate, adenosine diphosphate, adenosine triphosphate, adenosine monophosphate, and diphosphopyridine nucleotide, triphosphopyridine nucleotide, and coenzyme A. The enzyme preparations act on adenosine and deoxyadenosine and most rapidly on adenosine 3'-phosphate (Table II). Deamination occurs without lag, indicating that it is not the case that adenosine 3'-phosphate is first cleaved by a phosphorylase and then the resultant adenosine attacked by the deaminase.

Heavy Metal Inactivation—Adenosine deaminase from E. coli, like that from mammalian sources (9, 23), is inhibited by heavy metals such as Ag\(^{+}\), Cu\(^{++}\), and Cr\(^{+++}\). Hg\(^{++}\), Ag\(^{+}\), Zn\(^{++}\), and p-chloromercuribenzoate at 10\(^{-4}\)M decrease the initial rate of reaction significantly. After 3 to 4 minutes the enzyme action completely stops for all the inhibitors except Zn\(^{++}\). This finding suggests that preincubation of the metal ion with the enzyme in the absence of substrate would be more effective. This was found to be the case, 10\(^{-4}\)M ions giving complete inhibition on preincubation in the absence of substrate for 3 minutes. The inhibition is prevented by the presence of other agents forming metal complexes such as ethylenediaminetetraacetic acid, cysteine, and reduced glutathione. These agents themselves have no influence on the enzyme action. Furthermore, the addition of gelatin does not affect the rate of reaction. Once the enzyme is inactivated by preincubation with Hg\(^{++}\), it cannot be reactivated by the addition of the tetraacetate. Because of the apparent irreversible inactivation by heavy metals, it is impossible to rule out trace heavy metal contamination during enzyme preparation as the causative agent for the occasional absence of enzyme in this and previous work.

Although precautions had been taken during enzyme preparation, it appeared to be worthwhile to add ethylenediaminetetraacetate to the solutions used in the extraction procedures. Enzyme Preparation 17 was prepared with Tris containing 0.003 per cent of ethylenediaminetetraacetate. The enzyme preparation obtained was slightly less active but was considerably more stable.

Effect of Sulfhydryl Reagents—Although the enzyme is sensitive to very small amounts of heavy metals, it does not appear that this results from heavy metal interaction with free —SH groups, because other —SH inhibitors, iodosobenzoate and iodoacetate, do not decrease activity.

Thermal Denaturation—With enzyme extracts prepared in the absence of chelating agents, the activity decreases to about one-half the initial value during a period of several minutes and then remains quite constant over a period of hours at 38°. A relatively slow drop is seen at 0°. With the enzyme prepared in buffers containing ethylenediaminetetraacetate, the activity is stable for hours at 38°. It is clear from experiments described above that heavy metal inactivation occurs more slowly in the presence of substrate. All this points to some combination of a heavy metal contaminant with the enzyme that can be prevented by keeping the enzyme cold, by chelation, or by keeping substrate present.

At higher temperatures thermal denaturation of the enzyme takes place, with the protein becoming quite rapidly denatured at 55°. The enzyme is inactivated by repeated freezing and thawing.

pH Optimum—The pH optimum was determined by both differential spectrophotometry and paper chromatography to be in the range of pH 7.5 to 8. The latter method has the advantage over the differential spectrophotometric method in that the difference spectrum of deoxyadenosine and deoxyinosine is pH-dependent. The optimum is broad and activity can be demonstrated at pH 5.

Buffer Effects—There appear to be relatively minor buffer effects. The enzyme has activity in Tris, citrate, pyrophosphate, phosphate, and arsenate buffers at pH 5. The enzyme may be...
Properties of Nucleoside Phosphorylase

Kinetics—As mentioned in the experimental section, the analytical method used here leaves much to be desired, owing to the influence of the substrate employed in chromophore development. In addition to this phenomenon and the possible influence of the deaminase on phosphorylase assay mentioned earlier, there is the known inhibitory action of free purine bases, which are reaction products, on the enzyme (4, 6, 7). All of these factors limit the reliability of the assay and preclude accurate kinetics. Under the conditions of the assay employed here, there is a continuous increase in the difference between the optical density at 640 μm of the sample and that obtained at zero time upon varying the following parameters: initial concentration of substrate, length of time of incubation, amount of enzyme added. None of the curves obtained indicates exact proportionality.

The assay, however, is quite reproducible. Data accumulated over a period of a month indicate that individual determinations have a standard deviation from the mean of 7 per cent.

Inhibition by Heavy Metals—Preincubation of the enzyme with 1.25 × 10⁻⁴ M Ag⁺, Hg²⁺, or p-chloromercuribenzoate for 30 minutes at 37° caused complete inhibition. At a concentration of 1.25 × 10⁻⁵ M no inhibition was found. The addition of this concentration of any of the three inhibitors completely inactivates the deaminase without influencing the phosphorylase.

Thermal Denaturation—The enzyme is not detectably inactivated by heating to 45° for 30 minutes. At 55° 74 per cent of the activity is lost, and 100 per cent is lost at 60° during the same period of time.

Temperature Coefficient—The logarithm of the activity of the enzyme gives a linear plot against the reciprocal of absolute temperature over the range of 20-50°. The calculated energy of activation is 3500 calories; Q₁₀ is 2.2 between 20-30° (Fig. 3).

Inhibition by Anions—The nucleoside phosphorylase is strongly inhibited by sulfate ions. Molybdate, pyrophosphate, succinate, acetate, fluoride, chloride, iodide, sulfate, and nitrate do not influence the reaction when present in the enzyme assay system at 0.1 M. The inhibition by sulfate is complete at 4 × 10⁻³ M, and is 95 per cent at 4 × 10⁻⁴ M. The effect appears to be one of reversible inhibition because activity can be restored by dialysis or dilution.

Phosphate ions cause a significant decrease in the amount of reaction. This probably results either from the formation of deoxynucleoside 1-phosphate which does not reduce the Cu⁺⁺ or from an inhibition of the enzyme by phosphate or deoxynucleoside 1-phosphate.

The sulfate effect appears to be unrelated to the phenomenon found to occur by Lampen and Wang (24) with the enzymes acting on nucleosides in Lactobacillus pentosus. In this organism, the predominating type of enzyme appears to be hydrolytic in character. Nonetheless, the pyrimidine-specific enzyme activity was found to be enhanced by the presence of phosphate, arsenate, or sulfate. On the other hand, phosphate or arsenate decreased the activity on purine ribonucleosides. These agents appeared to affect the stability of the enzymes and not their catalytic activity. The enzymes acting on deoxyribonucleosides in E. coli are completely arsenate- or phosphate-dependent (6, 7), and the sulfate seems to affect the activity but not the stability of the enzyme.

SUMMARY

Extracts of a number of cultures of Escherichia coli strain B were examined for ability to deaminate deoxyadenosine and to catalyze the asemyolytic or phosphorolytic cleavage of deoxyadenosine. Variation of the conditions of growth and of enzyme extraction indicated no major variation in enzyme content with the exception that deaminase activity is greatly augmented when certain purine compounds are present in the growth medium.

The deaminase is very sensitive to heavy metals and reacts with them irreversibly. It does not appear to be a sulfhydryl-dependent enzyme. The phosphorylase activity is relatively insensitive to heavy metal but is inhibited by sulfate ions quite specifically.

REFERENCES

The Properties of Adenosine Deaminase and Adenosine Nucleoside Phosphorylase in Extracts of *Escherichia coli*
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