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

ARTHUR L. KOC'H AND GERALD VALLEE

From the Department of Biochemistry, College of Medicine, University of Florida, Gainesville, Florida

(Received for publication, October 24, 1958)

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 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, adenine. 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 NaSO₄ 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 Tris³ 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.

³ The abbreviation used is: Tris, tris(hydroxymethyl)aminomethane.

* Aided by a Grant C-3255 from the United States Public Health Service. A preliminary report of this work was presented at the meeting of the American Chemical Society's 134th meeting, Chicago, Illinois, September 1958.
## 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 the 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 procedures, but not in 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 aden-
sine deaminase in their extract, and one preparation, No. 8, in
this series was completely devoid of activity. The probable ex-
planation for the absence of the deaminase activity is that the
deeaminase 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 hypox-
anthine, 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 prep-
parations, 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 modi-
fied by the presence of purine bases in the growth medium. It
should be mentioned, however, that the enzyme is strongly in-
hibited by sulfate ions and that the latter are present in inhibi-
tory 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 ac-
curate measurement of the course of the deamination pro-
cess (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

\(^2\) A. L. Koch, unpublished method.

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 16.5 \(\mu\)moles per mg. per hour and for \(K_m\) are 29 and 31.1
\(\mu\)M, respectively.

The kinetics of two other preparations were investigated. \(K_m\)
appeared to be 30 \(\mu\)M for No. 9 and 32.4 \(\mu\)M for No. 17. It
should be noted that the initial substrate concentration utilized
in obtaining data in Table I is 100 \(\mu\)M 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 \(\mu\)M
for all preparations, and therefore, all data have been computed
in Table I 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 concentra-
tions, on the assumption of a \(K_m\) of 30 \(\mu\)M 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
deeamination adenine (1, 2, 22). The rate at which adenine was
deeaminated by such suspensions was increased by the addition of
small amounts of the nucleoside (2) or inorganic phosphate (1).
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_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>

FIG. 2. Temperature dependence of adenosine deaminase activity

This, of course, suggested that adenine deamination was a 3-step process whereby adenine is converted to adenosine, which is then deaminated and finally cleaved. The process for adenosine formation could conceivably be a transamination, a transglycosylation, or a nucleoside phosphorylase action. The phosphate effect suggests the latter possibility. An attempt to 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. 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 10⁻⁶ 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, 10⁻⁶ M ions giving complete inhibition on preincubation in the absence of substrate for 3 minutes. The inhibition is prevented by the presence of other metals 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
slightly more active in arsenate or in Tris than in the others, but not by more than 10 per cent increase in rate.

Temperature Coefficient—The activity of the enzyme is only slightly influenced by temperature within the stability range. The $Q_{10}$ between 20° and 30° is only 1.14 (Arrhenius $E = 642$ calories per mole), and between 30° and 40° the apparent $Q_{10}$ value is 1.03 (Fig. 2).

**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 mp 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 $1.25 \times 10^{-4}$ M Ag⁺, Hg⁺, or p-chloromercuribenzoate for 30 minutes at 37° caused complete inhibition. At a concentration of $1.25 \times 10^{-5}$ 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; $Q_{10}$ 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, chloride, 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 $4 \times 10^{-3}$ M, and is 65 per cent at $4 \times 10^{-2}$ 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 deoxyribose 1-phosphate which does not reduce the Cu⁺⁺ or from an inhibition of the enzyme by phosphate or deoxyribose 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 arsenolytic 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**

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