anthranilic acid. No activity is demonstrable following the single addition of iron(III) to the dialyzed extracts. Minimal activity is seen upon the addition of pyridoxal phosphate with maximal response elicited upon the further addition of iron(III). The minimal reaction shown when pyridoxal phosphate is added singly is eliminated by prior treatment of all reagents with 8-hydroxyquinoline to remove traces of iron.

Further studies in aromatic metabolism with *E. coli* 0111:B4 demonstrate that the activities described here are essential to the economy of these organisms.[19]

Guirard and Snell have shown an ion requirement for the pyridoxal phosphate dependent histidine decarboxylase.[20] A general mechanism for pyridoxal phosphate-catalyzed enzyme reactions has been discussed by Metzler, Ikawa and Snell.[21] The reactions described in this paper cannot be formulated in a manner entirely analogous to the generalized reaction sequence of Metzler, *et al.*,[21b] for decarboxylation of α-amino acids. An examination of the formula of a Schiff base of pyridoxal and one of the aminobenzoates shows that decarboxylation could not occur through withdrawal of electrons from the bond to the carboxyl in the manner depicted for other B_6-catalyzed reactions.[21] A discussion of the mechanisms of the reactions described in this paper will be presented in conjunction with chemical studies of these and similar B_6-mediated reactions.[22,23]

(19) W. G. McCullough, in preparation.

(20) B. M. Guirard and E. E. Snell, THIS JOURNAL, **76**, 4745 (1954).

(21) D. E. Metzler, M. Ikawa and E. E. Snell, *ibid.*, **76**, 648 (1954); **76**, 650 (1954).

(22) W. G. McCullough, Abstracts 130th Meeting, ACS, p. 47c.

(23) W. G. McCullough, in preparation.

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[CONTRIBUTION FROM THE NATIONAL INSTITUTE OF ARTHRITIS AND METABOLIC DISEASES, NATIONAL INSTITUTES OF HEALTH, UNITED STATES PUBLIC HEALTH SERVICE]

Adenine Deaminase of *Azotobacter vinelandii*

BY LEON A. HEPPEL, JERARD HURWITZ[1] AND B. L. HORECKER

RECEIVED AUGUST 13, 1956

Adenase, which catalyzes the hydrolytic deamination of adenine to form hypoxanthine, has been purified 35-fold from extracts of *Azotobacter vinelandii*. The purified enzyme preparation is free of activity against hypoxanthine, guanine, adenosine and many related compounds. The high substrate affinity of the enzyme and lack of inhibition by other compounds make it suited for micro-assay of adenine. This can be measured spectrophotometrically by coupling the system to xanthine oxidase. By the additional use of guanase, both adenine and guanine can be determined in mixtures.

The enzymatic deamination of adenine has been observed in *E. coli*[2] and other bacteria,[3] in yeast[4] and in various invertebrate tissue preparations,[5,6] but appears to be absent in most vertebrate tissues. This distribution has been given phylogenetic significance,[5,6] although quite recently adenase has been reported to be absent in some invertebrates.[7] Very weak activity has been reported for whole blood of the rat.[8]

All of the adenine deaminase preparations so far described also exhibit deaminase activity against related compounds such as guanine or adenosine, and no decision is possible as to whether an enzyme specific for adenine is present. A protein fraction has now been partially purified from extracts of *Azotobacter vinelandii* which catalyzes the hydrolytic deamination of adenine but not of guanine, cytosine, adenosine and many other related compounds. The properties of this adenine deaminase (or adenase) have been studied. By using it in conjunction with guanase and xanthine oxidase, micro-determination of adenine and guanine in mixtures has been carried out.

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(7) A. H. Roush and R. F. Betz, *Biochim. Biophys. Acta*, **19**, 579 (1956).

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Experimental

Material.—The compounds used in this study were commercial preparations. Each compound migrated as a single component on paper chromatography in several solvent systems; spectra agreed with published data. Adenosine 2',3'-phosphate, as obtained, showed the presence of considerable amounts of adenosine 3'-phosphate and therefore it was purified by chromatography in solvent 1 (see below). The band corresponding to adenosine 2',3'-phosphate was visualized in ultraviolet light, eluted from paper with distilled water and lyophilized. In most cases, neutralized aqueous solutions of the compounds were used. With guanine, however, 0.0025 *M* solutions were prepared in warm 0.01 *N* HCl and added to the incubation mixture with an equivalent amount of 0.5 *N* KOH.

Procedures.—For the enzymatic assay of adenase the incubation mixture contained 0.07 ml. of 0.1 *M* potassium phosphate buffer, *p*H 7.0, 0.04 ml. of 0.01 *M* adenine sulfate, enzyme and water to a total volume of 0.2 ml. After 15 minutes at 37.5° the mixture was diluted to 3.0 ml. with 0.03 *M* potassium phosphate buffer, *p*H 7.0, and the optical density at 260 mμ read in a Beckman Model DU spectrophotometer, using silica cells with a 1-cm. light path. The optical density reading was subtracted from that observed in a control incubation containing only adenine and buffer. With crude extracts an additional correction was applied for ultraviolet absorption due to the enzyme solution. At 260 mμ and *p*H 7.0, ϵ is 13.1×10^3 liters moles^{-1} cm.$^{-1}$ for adenine and 8.0×10^3 liters moles^{-1} cm.$^{-1}$ for hypoxanthine[9] and the amount of adenine deaminated was calculated using $\Delta\epsilon = 5.1 \times 10^3$ liters mole^{-1} cm.$^{-1}$. A unit of enzymatic activity is defined as that amount which catalyzes the de-

(9) E. A. Johnson in "The Nucleic Acids," Vol. I, edited by E. Chargaff and J. N. Davidson, Academic Press, Inc., New York, N. Y., 1955, p. 498.

amination of 1 μmole of adenine per hour and specific activity is units per mg. of protein.

Protein was determined by the method of Sutherland, *et al.*[10]

The reaction was also followed semi-quantitatively by applying 0.05 ml. of the incubation mixture to Whatman No. 1 or 3 MM paper, followed by descending chromatography in solvent 1.

Solvent Systems.—The solvent systems are described in the references cited below: adenine and hypoxanthine were separated in solvent 1 (isopropyl alcohol–NH_3[11]), solvent 2 (isoamyl alcohol–Na_2HPO_4[12]) and solvent 3 (isobutyric acid–NH_3[13]). Solvent 1 also was used with adenosine, adenosine 2′-phosphate, adenosine 3′-phosphate, adenosine 5′-phosphate, adenosine 2′,3′-phosphate, adenosine 5′-diphosphate and adenosine 5′-triphosphate. Solvent 4 (isopropyl alcohol–HCl[14]) was used with cytosine, cytidine and guanine.

Adenine and hypoxanthine were also separated by electrophoresis on paper as described by Markham and Smith.[11]

Preparation of the Enzyme.—*Azotobacter vinelandii* was grown and harvested as described by Grunberg-Manago, Ortiz and Ochoa.[15] The frozen cells could be stored at −15° for 3 months.

The results of the procedure are summarized in Table I. Twenty-nine grams of cells was mixed with 58 g. of alumina (A-301, 325 mesh, Aluminum Company of America) and

TABLE I

PURIFICATION OF ADENASE

	Fraction	Total units	Specific activity, units/mg. of protein
1.	Crude extract	12,700	6.5
2.	Protamine supernate	12,500	8.4
3.	Ammonium sulfate I, heated	9,520	25
4.	Acetone	6,300	123
5.	Ammonium sulfate II	6,300	225

ground with a mortar and pestle for about 10 minutes at room temperature. The resulting paste was treated with 118 ml. of water and the mixture centrifuged for 10 minutes at 13,000 *g*. The supernatant fluid was collected and the residue re-extracted with 59 ml. of water. All subsequent steps were at 0–2° except as noted. The combined supernatant fluid (crude extract, 170 ml.) was diluted with an equal volume of water and treated with 13.6 ml. of protamine sulfate solution (Nutritional Biochemicals Corp., 20 mg. per ml. in 0.2 *M* acetate buffer, *p*H 5.0). After centrifugation, 325 ml. of supernatant solution was obtained. This was treated with 155 g. of solid ammonium sulfate and the mixture centrifuged. The precipitate was dissolved in 89 ml. of water to give 96.5 ml. of solution. This was warmed to 55° over a period of 2 minutes and held at this temperature for 5 minutes. A precipitate formed which was separated by centrifugation and extracted with water. The combined supernatant solutions had a volume of 133 ml. (ammonium sulfate I, heated).

The protein concentration at this stage was 2.5 mg. per ml. The solution (133 ml.) was diluted to 219 ml. and then fractionated with acetone as follows. An equal volume of acetone (cooled to −10°) was added rapidly to the enzyme solution (which was at 0°) and the mixture centrifuged for 3 minutes at 13,000 *g*. It was sufficient to use a centrifuge kept in a cold room at 2°. The supernatant solution (415 ml.) was treated with 276 ml. of acetone in the same way. The precipitate was collected and dissolved in water (step 4, acetone, 7.8 ml.). The solution was treated with 5.2 ml. of ammonium sulfate solution (saturated at room temperature and neutralized to *p*H 7.4). The precipitate was removed by centrifugation and to the supernatant solution (12.3 ml.) was added 8.9 ml. of the same saturated ammonium sulfate solution. The mixture was again centrifuged, and the precipitate dissolved in water (ammonium sulfate II, 1.5 ml.). This procedure can be carried out in one day, but it may be interrupted after step 3 and the heated ammonium sulfate fraction stored for weeks at −15°.

The over-all purification was 35-fold with a 50% yield. The purified enzyme has a specific activity 100 times greater than that reported for an ammonium sulfate fraction from adenine-adapted yeast.[4]

Properties of the Enzyme

Stability.—Fractions corresponding to step 3 or step 5 (Table I) were stable for at least 2 months when stored at −15°. There was no loss in activity after dialysis for 24 hours against 0.9% KCl. In this way ammonium sulfate can be removed from the enzyme preparations. At *p*H 6.5, 80% of the activity was destroyed after 60 minutes at 55°.

The following studies were carried out with purified enzyme (step 5).

Nature of the Reaction.—The enzyme catalyzes the anaerobic conversion of adenine to a compound with the spectral properties and chromatographic and electrophoretic behavior of hypoxanthine. At *p*H 7, $\epsilon_{250}/\epsilon_{260}$ was found to be 1.32, $\epsilon_{280}/\epsilon_{260}$ was 0.092, and $\epsilon_{290}/\epsilon_{260}$ was 0.01. In addition, the compound is oxidized by xanthine oxidase at the same rate as authentic hypoxanthine. The reaction catalyzed is therefore a hydrolytic deamination of adenine to form hypoxanthine.

Specificity.—Extracts of *Azotobacter vinelandii* contain adenosine deaminase activity, but this was no longer detectable following the ammonium sulfate-heating step. The purified enzyme appears to be quite specific for adenine and does not act on the following compounds: guanine, cytosine, guanosine, cytidine, adenosine, adenosine 2′-phosphate, adenosine 3′-phosphate, adenosine-2′,-3′-phosphate, adenosine 5′-phosphate, adenosine 5′-diphosphate, adenosine 5′-triphosphate and guanosine 5′-phosphate. This was determined by paper chromatography and, where feasible, by spectral analysis. In all cases the rate of deamination was less than 1/200 of that for adenine. Hypoxanthine oxidase activity is also absent.

Effect of Metals and *p*H.—There was no stimulation by $MgCl_2$ or $CaCl_2$ (Table II), and 0.01 *M* $MnCl_2$ caused nearly complete inhibition of adenase activity. The *p*H optimum was broad, extending from about *p*H 6.7 to 7.7.

TABLE II

EFFECT OF METAL SALTS ON ADENASE ACTIVITY

The enzymatic assay was carried out as described under "Procedures," except for the additions listed in the table. Purified enzyme (step 5) was used.

Addition	Concn.	Enzyme activity, units/ml.
None	..	306
$MgCl_2$	0.01	304
$CaCl_2$	.01	284
$MnCl_2$	.01	7

Substrate Affinity.—The substrate affinity of adenine for the enzyme was so high that an accurate estimate of the Michaelis–Menten constant, K_m,[16] was not possible with the assay method used. K_m was less than 10^{-6} mole/liter.

(10) E. W. Sutherland, C. F. Cori, R. Haynes and N. S. Olsen, *J. Biol. Chem.*, **180**, 825 (1949).

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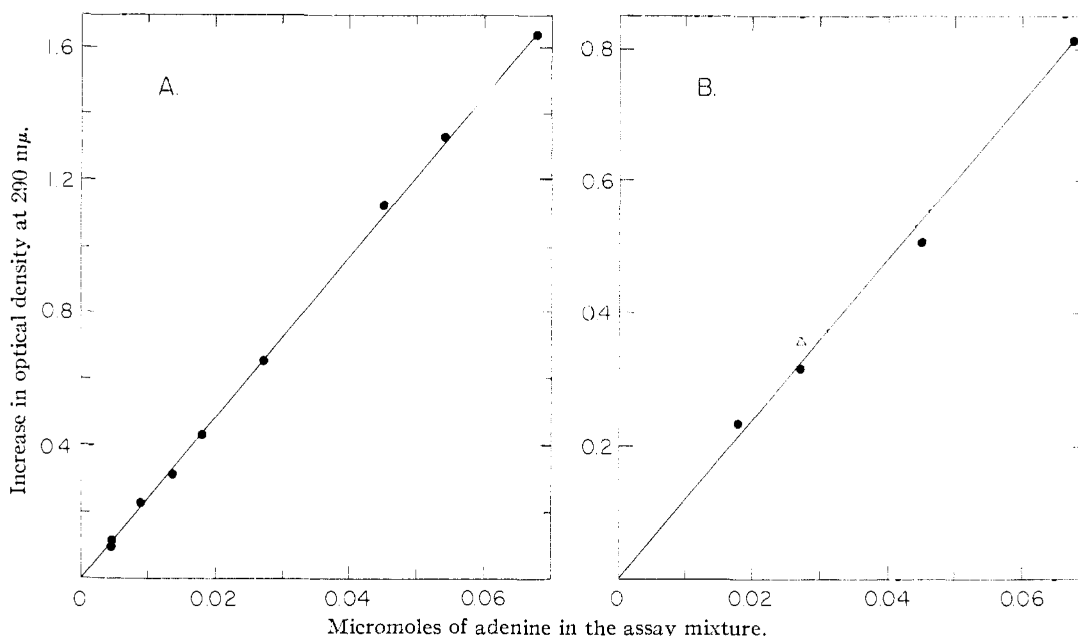

Fig. 1.—Recovery of adenine when present alone (A) and when mixed with three other bases (B). The incubation mixture for (A) contained 15 μmoles of potassium phosphate buffer, *p*H 7, adenine sulfate as indicated, 60 γ of xanthine oxidase, 60 γ of purified adenase and water up to 0.5 ml. For (B) the mixture contained adenine as shown, 0.05 μmole of each of guanine, uracil and cytosine, 60 γ of xanthine oxidase, 60 γ of adenase, 30 μmoles of potassium phosphate buffer, *p*H 7 and water up to 1.0 ml. (Δ) represents an assay exactly comparable to the others shown in (B) but with the other bases left out.

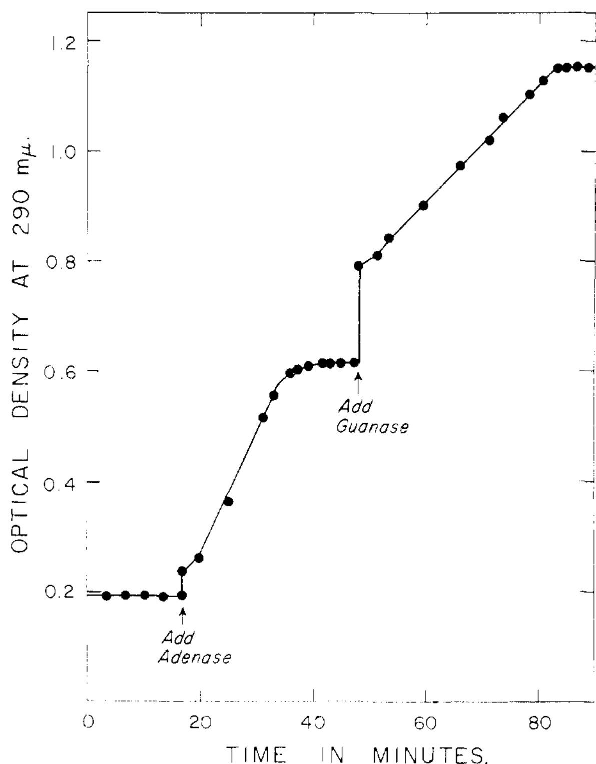

Fig. 2.—Assay of a mixture of adenine (0.03 μmole/ml.) and guanine (0.064 μmole/ml.), using xanthine oxidase followed, in turn, by adenase and guanase. Experimental details are given in the text. Addition of adenase and guanase causes an instantaneous increase in optical density due to ultraviolet absorbing components of the preparations, followed by a progressive increase due to the enzymatic reaction.

Effect of Other Bases and of Nucleotides.—The rate of deamination of adenine was not diminished by the presence, in equimolar concentration, of guanine, cytosine and uracil. The reaction proceeded to completion at a constant rate. No kinetic studies were carried out in the presence of nucleotides, but semi-quantitative observations indicated rapid and complete splitting of 0.01 to 0.02 *M* adenine in the presence of 0.02 *M* adenosine triphosphate, adenosine diphosphate, adenosine 5′-phosphate or uridine 5′-phosphate.

Coupling to Xanthine Oxidase for Micro Assay of Adenine and Guanine.—By including xanthine oxidase in the reaction mixture, one obtains a coupled sequence of reactions. Adenine is converted to hypoxanthine by adenase, which in turn is oxidized to uric acid by xanthine oxidase. This results in a large increase in optical density at 290 mμ, so that 0.005 μmole (0.7 γ) of adenine may be determined with an accuracy of 10%. This is shown in Fig. 1, which also indicates that other bases which might be present in a perchloric acid digest of ribonucleic acid do not interfere with the assay. Xanthine oxidase was prepared according to Horecker and Heppel[17] from raw cream; the purification was carried only through their ammonium sulfate step.

If both adenine and guanine are present each of them may be determined quantitatively by the change in absorption following the addition, first of adenase, then of guanase. Guanase was prepared by extraction of rabbit liver acetone powder with 0.1 *M* borate, *p*H 8.6, followed by fractionation with ammonium sulfate.[18] For the assay, a silica

(17) B. L. Horecker and L. A. Heppel, *J. Biol. Chem.*, **178**, 683 (1949).

(18) H. M. Kalckar, *ibid.*, **167**, 461 (1947).

cell (1 cm. light path, 1.75-ml. capacity) contained 50 μmoles of potassium phosphate buffer, pH 7, 60 γ of xanthine oxidase, guanine, adenine and water in a total volume of 1.525 ml. The optical density at 290 mμ was measured and found to be stable. Purified adenase (0.005 ml., 60 γ protein) was then added. This resulted in a linear increase in density which came to a halt in 10–20 minutes. Finally, 0.005 ml. of guanase (0.25 mg. protein) was added and the increase in optical density at 290 mμ was again measured. The results of one such experiment are plotted in Fig. 2. From the density change (corrected for enzyme blank) the concentration of base was calculated according to Kalckar.[19] Recovery figures are given in Table III. It was Kalckar who introduced this general technique for spectrophotometry of purines. However, at that time he was not able to apply the method for the determination of adenine and mixtures of adenine and guanine, because a specific adenase was not available.

TABLE III

THE ENZYMATIC DETERMINATION OF ADENINE AND GUANINE IN SOLUTIONS OF THE MIXED BASES[a]

Sample	ΔE_{290} after adenase	Adenine found, μmoles/ml.	Adenine added, μmoles/ml.	ΔE_{250} after guanase	Guanine found, μmoles/ml.	Guanine added, μmoles/ml.
1	0.726	0.059	0.059	0.097	0.0155	0.016
2	.715	.058	.059	.180	.030	.032
3	.378	.031	.030	.362	.060	.064

[a] The experimental procedure is described under "Coupling to Xanthine Oxidase." ΔE_{290} is the net change in optical density at 290 mμ after subtraction of an enzyme blank.

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[CONTRIBUTION FROM THE NORTHERN UTILIZATION RESEARCH BRANCH[1]]

Phytin Elimination in Soybean Protein Isolation

BY ALLAN K. SMITH AND JOSEPH J. RACKIS

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Phytin accounts for about 70% of the phosphorus in soybean meal. In extracting proteins from the meal, the phytin reacts with the proteins to form complex products of varying composition. In the water extract of the meal, which has pH of about 6.6, the reaction is limited but increases as the pH is lowered for precipitation of the protein. It has been demonstrated that phytin can be eliminated from water extract of soybean meal by a combination of dialysis and treatment with the anionic-exchange resin Dowex-1-X10. Electrophoretic studies have shown that one minor component of the acid-precipitated protein is a protein–phytin reaction product, and two other minor components are affected by the presence of phytin. Removing the phytin raises the isoelectric point of the acid-precipitated protein by 0.8 unit, and increases the pH range of complete dispersibility on the acid side of its isoelectric point.

It has been reported by many investigators[2,3] that the principal form for storage of phosphorus in seeds is phytin, the calcium–magnesium–potassium salt of phytic acid. Other phosphorus compounds known to be present are phospholipids, inorganic phosphorus and nucleic acids.

Earle and Milner[4] studied the distribution of phosphorus in a single variety of soybeans, Dunfield. They accounted for 92% of the total phosphorus and found that about 11% was phosphatide phosphorus, 4.5% inorganic phosphorus and 71% phytin phosphorus, with 5.5% of unknown composition remaining in the residue. According to Earle and Milner, the petroleum ether extracted approximately 0.5% of the total phosphorus, showing that hexane-defatted meal contains more than 99% of the original phosphorus of the beans. Recently DiCarlo, Schultz and Kent[5] found that defatted and dehulled soy flour contains 1.3% ribonucleic acid. Their results indicate that one or more of the proteins of the soybean may belong to the class of nucleoproteins.

The presence of various phosphorus compounds in the soybean meal introduces a series of complicating factors to the problem of isolation and purification of homogeneous proteins from the soybean. For example, it has been demonstrated by Fontaine, *et al.*,[6] that the solubility of the phosphorus compounds in the water extract of soybean meal varies with the pH of the solution, in a manner similar to the solubility of the major protein components. Smiley and Smith[7] have shown that the acid-precipitated protein of the soybean is low in nitrogen as compared to proteins extracted with salt solutions. McKinney, Sollars and Setzkorn[8] showed that phosphorus compounds in the acid-precipitated protein are largely responsible for the low nitrogen values.

Bourdillon[9] obtained a crystalline protein–phytic acid complex from *Phaseolus vulgaris*, whereas Barré, *et al.*,[10–12] have reported a reaction between phytin or phytic acid and the basic groups of proteins, the extent of the reaction being controlled by the pH of the system. Smith, Schubert and

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