able. It was isolated from the remainder of the 24-hr. digest by paper chromatographic methods previously described.[14] The isolated compound migrated on paper as one homogeneous spot and at a rate intermediate between those for maltotriose and panose. According to French and Wild[8] an oligosaccharide with α-D-(1 → 3) and α-D-(1 → 6) linkages would possess this type of mobility. Hydrolysis of the oligosaccharide should yield nigerose and isomaltose as the two disaccharide fragments and glucose as the monosaccharide fragment. Three reducing compounds were produced on hydrolysis as indicated by paper chromatography with R_f values (three ascents of the solvent) 0.74, 0.58, 0.38. Under similar conditions R_f values of glucose, nigerose and isomaltose were 0.75, 0.60 and 0.39. In consideration of these findings and the mechanism of action of the *A. oryzae* transferring enzyme, it is likely that the new oligosaccharide is O-α-D-glucopyranosyl-(1 → 6)-O-α-D-glucopyranosyl-(1 → 3)-D-glucose.

Enzymolysis of Glucose-C^{14} and Nigerose.—A solution of 5 mg. of nigerose and 2 mg. of glucose-C^{14} (total radioactivity, 31,900 c.p.m.) in 0.1 ml. of phosphate buffer (*p*H 6.5 and 0.1 *M*) was treated with 0.1 ml. of enzyme (total activity, 4.4 units). Samples of 0.05 ml. were taken at 0 and 24 hr. and analyzed for reducing and radioactive components by the methods of the previous sections. The radioactivities of the compounds in the 24-hour sample were as follows: glucose, 30,150 c.p.m.; nigerose, 420 c.p.m.; maltose, 180 c.p.m. and isomaltose, 1460 c.p.m.

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[CONTRIBUTION FROM THE DEPARTMENT OF MICROBIOLOGY, WAYNE STATE UNIVERSITY, COLLEGE OF MEDICINE]

Enzymatic Decarboxylation of the Aminobenzoates[1]

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From *E. coli* 0111:B4 cell-free enzyme preparations have been obtained which decarboxylate *p*-aminobenzoic acid and anthranilic acid to aniline. These preparations do not metabolize *m*-aminobenzoate. Dialysis of the enzyme preparations against 0.2 *M* acetate buffers at *p*H 5.0 results in a complete loss of the ability to mediate these chemical transformations. Restoration of the decarboxylating activity is obtained upon the addition of pyridoxal phosphate and iron(III), but the addition of either singly causes no reconstitution. These studies show that the above reactions are mediated by a pyridoxal phosphate-dependent enzyme(s) and that a metallic ion is an obligatory requirement of the system.

Introduction

During studies of *p*-aminobenzoic acid (PABA) metabolism in *Mycobacterium* species, Sloane, *et al.*,[3] demonstrated the conversion of PABA to aniline and to *p*-aminophenol. Small yields of the end-products were obtained and no detection of the liberation of carbon dioxide was possible. With *in vitro* preparations of *Mycobacterium* species Sloane[4] was able to demonstrate that the tetracycline antibiotics would block the conversion of aniline to *p*-aminophenol. The observed inhibition of the hydroxylating system was reversed by a PABA-metabolite which was obtained in crystalline form. The metabolite did not influence the antibiotic action of the tetracycline compounds in the standard antibiotic assay procedures. Studies of the acquisition of resistance by *E. coli* 0111:B4[5] toward the action of chlorotetracycline led us to the observation that the incorporation of pyridoxine and of the aminobenzoates into a chemically defined medium resulted in a partial reversal of the antibiotic action of chlorotetracycline.[6] *In vitro* studies of the relationship between vitamin B_6 and the aminobenzoates have demonstrated the existence in these cells of a pyridoxal phosphate dependent enzyme(s) which decarboxylates PABA and anthranilic acid. Experiments delineating this relationship are described in this paper.

Experimental

Production of Cells.—Cells of *E. coli* 0111:B4 were produced in a chemically defined medium[6] under aerobic conditions, harvested by centrifugation, washed three times by centrifugation with 0.067 *M* phosphate buffer of *p*H 6.0, and stored at 4° in the same buffer of such volume that each ml. of suspension contained 8 mg. of bacterial nitrogen.

Preparation of Cell-free Extracts.—For cell disruption a quantity of cells equivalent to 16 mg. of bacterial nitrogen was suspended in 5.0 ml. of 0.067 *M* phosphate buffer at *p*H 6.0 and was shaken with 10 g. of small glass beads for one minute at 4° in a bacterial disintegrator operating at a speed of approximately 2500 cycles per minute. This machine has been described in other studies.[7] Under these conditions essentially 100% of the aminobenzoate decarboxylase activity of the intact cells may be extracted in the *p*H range of 6.0 to 7.0. The homogenized mixture was decanted from the beads. The beads were washed with a small volume of buffer and the washing added to the mixture. The latter was then centrifuged at 3,000 *g* for five minutes and the supernatant was used as the extract. Microscopic examination revealed no whole cells and repeated attempts to culture these preparations failed.

Enzyme Resolution.—The cell-free extracts were dialyzed against 0.2 *M* acetate buffer at *p*H 5.0 and room temperature for 24 hours. A small amount of insoluble protein was removed by centrifugation and the extracts were adjusted to *p*H 6.0. These preparations are water-clear.

Incubation. The various cell preparations were incubated with the substrates at 35° on a rotary shaker[8] under aerobic conditions or in an atmosphere of nitrogen.

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(1) A preliminary report of this work was presented at the 128th National American Chemical Society Meeting at Minneapolis, September, 1955. Supported in part by a grant from The Upjohn Company, Kalamazoo, Michigan.

(2) Abstracted in part from theses submitted to Wayne University in partial fulfillment of the requirements for the degree of Master of Science, 1955 (J. T. P.) and 1956 (I. J. D.).

(3) N. H. Sloane, C. Crane and R. L. Mayer, *J. Biol. Chem.*, **193**, 453 (1951).

(4) N. H. Sloane, THIS JOURNAL, **75**, 6352 (1953).

(5) F. Kauffmann, *J. Immunology*, **57**, 71 (1947).

(6) W. G. McCullough, J. T. Piligian and I. J. Daniel, unpublished data; the cells used in this study were produced by incubating the cultures at 35° for 20 hr. on a rotary shaker (*cf.* ref. 8). The initial *p*H of the medium was 6.8 and 50 ml. of medium were contained in a 250-ml. erlenmeyer flask. The composition of the medium per liter is: glucose, 10 g.; $K_2C_6H_6O_7 \cdot H_2O$, 10 g.; NH_4Cl, 2 g.; K_2HPO_4, 6 g.; KH_2PO_4, 6 g.; $MgSO_4 \cdot 7H_2O$, 50 mg.; $MnSO_4$, 2.4 mg.; NaCl, 10 mg.; $FeSO_4$, 5 mg.; KNO_3, 300 mg.; and *l*-cystine, 40 mg. Incorporation of the aminobenzoates into this medium does not stimulate growth and does not affect the production of the aminobenzoate decarboxylase(s)

(7) L. M. Corwin, L. J. Schroeder and W. G. McCullough, THIS JOURNAL, **78**, 1372 (1956).

(8) New Brunswick Scientific Co., New Brunswick, N. J.

Chemical Analyses.—Following incubation of the reaction mixtures, samples were deproteinized by the addition of an equal volume of 5% trichloroacetic acid and were clarified by centrifugation at 3,000 *g*. The clear solutions were diluted so that from 6 to 20 μg. of amine was contained in 5 ml. Aliquots (5 ml.) of the alkalinized solutions were extracted with 5 ml. of ether. The ether extracts were washed with 1 ml. of aqueous 10% HCl and the aniline hydrochloride was subjected to diazotization at 0° with subsequent coupling of the diazonium salt with 5 ml. of N,N-dimethyl-1-naphthylamine reagent[9] (this reagent was prepared by dissolving 1 g. in 250 ml. of 95% alcohol). The azo dye derivative was read spectrophotometrically[10] at 540 mμ and the quantity of aniline was estimated with the aid of concentration *vs.* optical density curves prepared for this derivative of authentic aniline.[11]

From larger scale experiments, further identification of aniline was afforded by subjecting the azo dye to neutral reduction with $Na_2S_2O_4$[12] and preparation of the benzoyl derivative[13] of the primary amine thus obtained.

For a determination of the amounts of unmetabolized aminobenzoates, aniline was first extracted and aliquots of the acidified reaction mixtures were subjected to the procedure described for aniline with the same coupling reagent being employed. The *p*-aminobenzoic acid derivative was read spectrophotometrically at 530 mμ, the anthranilic acid derivative at 535 mμ.

An estimation of the purity of the azo derivatives of the aminobenzoates was obtained by chromatographic procedures.[14] The azo dyes were extracted with ether. The acidic dyes were then extracted from ether by dilute (2%) Na_2CO_3 and brought back into fresh ether by acidification with H_3PO_4 and chromatographed on alumina.[15] Development with ether containing 2% acetic acid moved the *p*-aminobenzoic acid dye down the column as a sharp yellow ring. The anthranilic acid dye was eluted by ether containing 20% acetic acid. The dyes were extracted from ether with 20% aqueous HCl and examined spectrophotometrically. The quantities of aminobenzoates calculated from this procedure agreed with those obtained by direct analyses without chromatography.[16]

Results

When intact cells of *E. coli* 0111:B4 are incubated with *p*-aminobenzoic acid under aerobic conditions, the aniline produced is further metabolized through *p*-aminophenol.[3,17] Aging cells in 0.067 *M* phosphate buffer of *p*H 6.0 at 4° for four to six weeks diminishes their ability to metabolize aniline unless catalytic quantities of *p*-aminobenzoic acid are added in confirmation of the report by Sloane.[4] The cells employed in these studies were stored at 4° for no longer than 72 hr. An examination of the data presented in Tables I and II shows that little aniline is found under aerobic conditions although significant quantities of *p*-aminobenzoic acid or of anthranilic acid have been metabolized. However, the amount of aniline detected parallels the concentration of the substrate.

Data from a comparable experiment with anthranilic acid as substrate are depicted in Table II.

TABLE I

DECARBOXYLATION OF PABA BY CELLS OF *E. coli* 0111:B4[a]

p-Aminobenzoic acid, μmoles		Aniline, μmoles
Initial	Final	
0.0	0.0	0.0
40.0	36.0	0.86
100.0	44.6	2.15

[a] 8 mg. bact. N, *p*H 6.0, 0.067 *M* phosphate buffer, 25 ml. reaction mixture, 2 hr., 35°, aerobic.

TABLE II

DECARBOXYLATION OF ANTHRANILIC ACID BY CELLS OF *E. coli* 0111:B4

Anthranilic acid, μmoles		Aniline, μmoles
Initial	Final	
0.0	0.0	0.0
40.0	35.4	1.5
100.0	87.0	4.1

To minimize the metabolism of aniline the reaction mixtures were gassed with N_2 prior to incubation. The data set forth in Tables III and IV were secured under these conditions.

TABLE III

DECARBOXYLATION OF PABA BY CELL-FREE EXTRACTS OF *E. coli* 0111:B4

Enzyme[a]	PABA, μmoles		Aniline, μmoles
	Initial	Final	
Non-dialyzed	100	69	25.5
Dialyzed	100	99	0.8
Dialyzed + Fe(III)[b]	100	99	0.7
Dialyzed + B_6[b]	100	96.5	3.2
Dialyzed + B_6 + iron(III)[b]	100	70.2	24.7

[a] Equiv. 8 mg. bact. N, *p*H 6.0, 25 ml. reaction mixture, 3 hr., 35°, anaerobic. [b] Pyridoxal phosphate,[18] 0.4 μmoles; iron(III), 1.0 microatom.

TABLE IV

DECARBOXYLATION OF ANTHRANILIC ACID BY CELL-FREE EXTRACTS OF *E. coli* 0111:B4

Enzyme (as in Table III[a])	Anthranilic acid, μmoles		Aniline, μmoles
	Initial	Final	
Non-dialyzed	100	45	53
Dialyzed	100	98.2	1.2
Dialyzed + Fe(III)[a]	100	98.2	1.1
Dialyzed + B_6[a]	100	92	7.4
Dialyzed + B_6 + iron(III)[a]	100	47	51

No decarboxylation of either substrate is seen at the temperature and *p*H employed in these studies in the absence of an active enzyme with extended incubation periods (12 hours). When the enzyme preparations are inactivated at "zero time" added substrates and product are completely recoverable within the limits of the methods employed. No metabolism of *m*-aminobenzoate was observed with any of the preparations described.

No appreciable diminution in aminobenzoate decarboxylase activity is observed upon storage of cell-free extracts in 0.067 *M* phosphate buffer of *p*H 6.0 at 4° for periods of 7 days.

Discussion

The data summarized in Tables III and IV demonstrate resolution and reconstitution of the decarboxylase activity for *p*-aminobenzoic acid and

(9) A. C. Bratton and E. K. Marshall, Jr., *J. Biol. Chem.*, **128**, 537 (1939).

(10) A Coleman spectrophotometer, model 6A, was employed.

(11) Aniline (Eastman) was distilled prior to use.

(12) L. Fieser, "Experiments in Organic Chemistry," D. C. Heath and Co., New York, N. Y., 1941, p. 211.

(13) S. M. McElvain, "The Characterization of Organic Compounds," The Macmillan Co., New York, N. Y., 1947, pp. 203, 206.

(14) R. Lemberg, D. Tandy and N. E. Goldsworthy, *Nature*, **157**, 103 (1946).

(15) Alumina, Adsorption, Fisher Scientific Co.

(16) When the aminobenzoates are employed as substrates at concentrations 10 and 20 times those used here, other diazotizable substances are obtained. Experiments to identify these compounds are in progress.

(17) W. G. McCullough, unpublished data.

(18) Pyridoxal phosphate was purchased from the California Foundation for Biochemical Research, Los Angeles, Calif.

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.

DETROIT, MICHIGAN

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

Adenine Deaminase of *Azotobacter vinelandii*

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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.[5]

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.

(1) Fellow in Cancer Research of the American Cancer Society.

(2) C. Lutwak-Mann, *Biochem. J.*, **30**, 1405 (1936).

(3) W. Franke and G. E. Hahn, *Z. physiol. Chem.*, **301**, 90 (1955).

(4) A. H. Roush, *Arch. Biochem. Biophys.*, **50**, 510 (1954).

(5) G. Duchateau-Bosson, M. Florkin and G. Frappez, *Compt. rend. soc. biol.*, **133**, 274 (1940).

(6) G. Duchateau-Bosson, M. Florkin and G. Frappez, *ibid.*, **133**, 433 (1940).

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

(8) M. B. Blauch, F. C. Koch and M. E. Hanke, *J. Biol. Chem.*, **130**, 471 (1939).

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.