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Metabolism of Radioactive Para-Aminobenzoic Acid in an *Escherichia coli* Mutant.* (22199)

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While metabolism of para-aminobenzoate (PAB) has been extensively investigated in the mammalian body(1,2), its function and biochemical transformations in microorganisms are still obscure. Conversion of PAB into folic acid and the citrovorum factor occurs in some organisms(3,4) although there is some question whether these are the active forms of PAB. A number of other routes of metabolism have been reported. Davis(5,6) has implicated PAB in the metabolism by *Escherichia coli* of tryptophane, tyrosine and other aromatic compounds, as well as vit. B_{12}. Ratner *et al.*(7) isolated a glutamic acid peptide of PAB from yeast, and Flynn *et al.*(8) have found in 2 strains of streptococci an antibiotic containing PAB and cytosine among its components. Sloane *et al.*(9) identified aniline and p-aminophenol as degradation products in *Mycobacterium smegmatis.* The present studies had their inception in the isolation by Lampen, Roepke, and Jones(10) of an X-ray mutant of *E. coli* which required exogenous PAB for growth but apparently did not utilize it in producing folic acid. Since the wild strain of this organism normally has no requirement for preformed PAB, it seemed of interest to investigate the fate of this compound labeled with C^{14} in such a mutant.

Materials and methods. PAB used was labeled in the carboxyl group with carbon 14 and had a specific activity of 0.037 mC/mg.‡ The mutant of *E. coli* (ATCC No. 9723a) was grown on basal medium modified from that of Cohen and Arbogast(11) by use of 0.4% glucose. The medium of Lampen *et al.*(10) contained asparagine as well, but this substance was not necessary for maximum growth, and was omitted to facilitate isolations. Organisms were grown in 3 liter batches with aeration for 24 hours at PAB concentration of 0.01 μg/ml. Experiments in which PAB level was 0.05 μg/ml gave similar results. Bacterial suspensions were filtered on Buechner funnel using Celite as filter aid. The filtrate contained less than 20% of total radioactivity. The cells, plus filter-aid, were suspended in water and made up to 60% acetone-water (v/v) by addition of acetone. The residue was filtered off, re-extracted, and extracts, containing approximately half the total cell radioactivity, were combined. Subsequent extractions of cells with hot acetone, chloroform,

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TABLE I. R_f Values of Various PAB Derivatives.

Compound or extract	Solvent 1	Solvent 2
60% acetone extract	.15, .22, .48	.87–.95
PAB	.78	.69
Folic acid		.31, (.19)*
N^{10}-formyl folic acid	.36, .55, .75	.36, .55, .74 (.23, .55, .70)*
Natural citrovorum factor†	.55, .71	.46, .64
Leucovorin	.36, .55, .71	.49, .64, (.65)*
Pteroic acid		(.04)*
Pteroylglutamyl-alpha-glutamic acid		(.17)*
Pteroylglutamyl-gamma-glutamic acid		(.35)*
Pteroylglutamyl-gamma-glutamyl-gamma-glutamic acid		(.16, .34)*
p-aminobenzoylglutamic acid	.68	.90
p-aminobenzoylaspartic acid	.62	.88

* These figures from Wieland *et al.*(12).
† Obtained through the courtesy of Dr. K. Schwarz, N. I. H.

and petroleum ether removed practically no radioactivity. An additional 30-40% of the original total radioactivity of cells could be extracted with water. Extracts were lyophilized and kept at –20°C until they were analyzed.

Results. Chromatographic separation of fractions. The bacterial extracts were subjected to ascending paper chromatography on Whatman No. 1 filter paper to investigate the nature of radioactive components. The behavior in the systems investigated led to the conclusion that PAB, folic acid, citrovorum factor, and several other related compounds were not among the bacterial metabolites, although good resolution of radioactive spots was never attained. The R_f values of various substances obtained in 2 systems, including results observed by Wieland *et al.*(12) are indicated in Table I. The solvent systems were 1) methanol, *iso*-butanol, water (1:1:1, v/v) and 2) 5% ammonium citrate buffer of pH 9 saturated with *iso*-amyl alcohol. Folic acid and its derivatives were located by ultraviolet absorption; PAB and its amino acid derivatives by the Bratton-Marshall aromatic amine reaction(13). Several of the folic acid compounds give more than one spot in certain solvents. Detection of the unknown was solely on the basis of direct radioactive assay of the paper strips, since there was insufficient material present for chemical identification or for spectrum analysis.

Because of the low recovery of radioactivity and poor definition of spots on the paper strips, column chromatographic separations were undertaken. Radioactivity was too tightly bound on Dowex 1 anion exchange resin, but chromatography on Dowex 50 cation exchange resin (10% cross-linkage, 200-400 mesh) provided separation. For elution 0.1 M ammonium acetate buffers were selected because they lent themselves to easy removal. The columns finally used were 100 cm by 0.9 cm, and were initially equilibrated with pH 3.32 buffer. Fractions of about 5 ml were taken and suitable aliquots were radioassayed. Three large radioactive peaks, denoted as B, C, and D in Fig. 1, were detected in the 60% acetone extract. The water extract contained mainly an additional peak, marked as fraction A, along with small amounts of B, C, and D. Fig. 1 shows typical elution patterns obtained at different pH values, PAB has never been seen in more than trace amounts and is eluted in pH 5.9 buffer. The supernatant medium was analyzed in the same chromatographic system and radioactivity was found to consist chiefly of PAB.

For comparison a series of pure compounds were subjected to the same separation procedure. Para-aminobenzoylglutamate and aspartate both emerge at the position of fraction C, but were definitely distinguishable from C

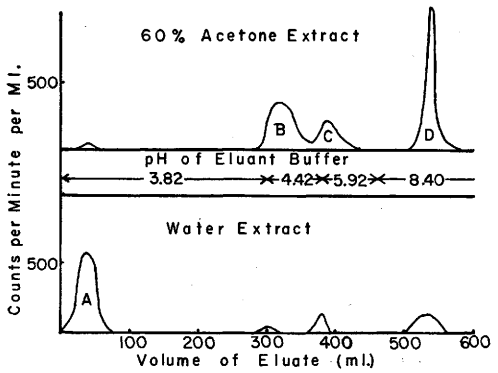

FIG. 1. Elution patterns of PAB metabolites.

by paper chromatography in a solvent of concentrated acetic acid, *iso*-propanol, concentrated ammonium hydroxide (3:2:1, v/v). Fraction C has an R_f of 0.30, while the 2 amides have R_f values of 0.78 and 0.74 respectively. Folic acid, formylfolic acid, and leucovorin are eluted in the general range of fraction D but again have been eliminated as possible metabolites by paper chromatography in the above solvent system, where D has an R_f of 0.30 while the 3 folic acid derivatives have values of 0.55, 0.80, and 0.64 respectively.

Acidic hydrolysates of all 4 metabolic fractions, prepared by refluxing with equal parts of concentrated hydrochloric and formic acids for 16 hours, and the corresponding basic hydrolysates, resulting from boiling with 1 M sodium hydroxide for 2 hours, were rechromatographed on the columns. In every case, PAB was identified as a major component of the hydrolysate.

It seems probable that the 4 metabolic fractions are closely related to each other, perhaps as degradation products, for on rechromatographing each separate fraction, some of the other fractions were formed. During this subsequent treatment fraction D was always formed in largest amount, and may represent the simplest compound.

The addition of methionine, asparagine or a mixture of other amino acids to the growth medium did not alter the elution pattern of the bacterial products, in spite of the observation of Lampen *et al.*(10) that some of these compounds spared a large portion of the PAB requirement of the mutant.

Growth of the mutant with PAB substitutes. A number of other compounds were tested for their ability to promote growth of the mutant. Growth was measured turbidimetrically at 430 mμ. It was felt that extension of such studies, initiated by Lampen *et al.* (10), might facilitate identification of the 4 fractions. Certain of these compounds have been implicated with PAB metabolism in other organisms, either by replacing its requirement for the compound or as metabolites. Some of these data are seen in Table II.

In confirmation of Lampen *et al.*(10), the folic acid-like compounds were only weakly effective. Most other substances studied were without effect except for those in which PAB was coupled with an amino acid, or where the amino group was acylated. However since higher concentrations were necessary for comparable growth, it seems likely that these compounds become effective only after hydrolysis. The most active compound, other than PAB, was p-aminosalicylic acid. Delay in growth seen with this substance suggests that it is further metabolized before utilization, and indeed growth in the presence of p-aminosalicylic acid is inhibited by sulfadiazine as if it were PAB. Para-aminosalicylic acid, however, was not eluted from the columns at the position of any of the labeled fractions. The results of Lampen *et al.*(10) with regard to the PAB sparing action by amino acids and purines on growth of the mutant as well as inhibition by sulfadiazine were confirmed. The latter substance inhibits completely at a concentration of 1 μg/ml in the presence of 0.0005 μg/ml of PAB whereas 4 other metabolic inhibitors, A-methopterin, desoxypyridoxine, 8-azaguanine and 6-mercaptopurine, were without effect at 10 times this level. The fact that A-methopterin did not inhibit growth

TABLE II. Growth Promotion of *E. coli* Mutant by Various Substances.

Drug		Conc. for half max growth—40 hr (μg/ml)
PAB		.001
p-aminosalicylic acid		.001*
p-aminobenzoylglutamic acid		.1
p-aminobenzoyl aspartic acid		.1
p-formamido benzoic acid		.1
p-formamido salicylic acid		.1
N-formyl-p-aminobenzoylglutamic acid		.1
N-formyl-p-aminobenzoylaspartic acid		.1
Folic acid		1.0
Formylfolic acid		1.0
p-aminohippuric acid		5.0
p-acetamidobenzoic acid		10
p-acetamidosalicylic acid		10
Pantothenic acid	No growth,	5
Aniline	*Idem*	5
Vit. B_{12}	"	5
p-hydroxybenzoic acid	"	5
3-hydroxy-4-aminobenzoic acid	"	5
p-aminophenol	"	5
N-acetyl-p-aminophenol	"	5
Pyridoxine	"	10
Leucovorin	"	1000

* Delayed.

TABLE III. Relative Growth Promotion by PAB Metabolites for 3 Microorganisms.

Compound or metabolite	mμg/ml of medium for one-half maximal growth		
	E. coli (ATCC 9723a)	*S. lactis* R. (ATCC 8043)	*L. citrovorum* (ATCC 8081)
PAB	1	* (100)	* (100)
Folic acid	1000	.2	* (10)
Leucovorin	* (1000)	.2	.1
Fraction A†	3	* (65)	* (140)
B†	* (50)	* (100)	* (200)
C†	* (50)	16	36
D†	5	4	8

* No growth observed at highest concentration tested; concentration given in parentheses.
† Weights of PAB computed from radioactive assay and converted to equivalents of folic acid or leucovorin.

of mutant indicated that folic derivatives might not be closely involved in the anabolic metabolism.

Bacterial growth studies with metabolic fractions. Ability of each of the 4 fractions to support growth of the mutant was then investigated. The possible similarity to folic acid and citrovorum factor was also studied by the ability to promote multiplication of *Streptococcus lactis* and *Leuconostoc citrovorum,* using commercial Difco media(14) for these microbiological assays. Suitable controls were run with mixtures of the appropriate growth factors and the 4 fractions, in order to demonstrate that none of the 4 fractions contained a growth inhibitor. Table III gives the relative concentrations of each fraction for growth of these 3 organisms.

It was possible that the growth stimulation seen in Table III by certain fractions might have resulted from the presence of unlabeled folic acid or leucovorin present as a contaminant. Therefore paper chromatograms of all 4 fractions were prepared under aseptic conditions in 2 solvent systems and the dried sheets were placed on agar plates seeded with *S. lactis* or *L. citrovorum* but devoid of the limiting growth factors. Growth of the organisms was seen only with fractions C and D and was localized at the site of radioactivity. Contaminating folic acid or leucovorin were apparently absent from the fractions, because no growth could be seen where these substances would have been located on the paper strip.

Discussion. While insufficient material has prevented chemical identification of the metabolites of PAB in this mutant of *E. coli,* the evidence suggests considerable similarity exists between certain of the metabolites and the citrovorum factor. It is of interest that Nichol *et al.*(15) recently reported synthesis from folic acid of various labile precursors and conjugates of citrovorum factor by pigeon liver and *S. faecalis.* Failure to stimulate growth of the mutant by the fractions at concentrations quite comparable to those at which PAB is active might suggest that all fractions are derived from some highly active metabolite degraded as a result of the isolation procedure or that one or more of the fractions consists of a mixture of active and inactive metabolites. The evidence suggests that the substances are quite labile. It remains for further purification and isolation of much larger amounts to elucidate their structure and possible function.

Summary. Radioactive p-aminobenzoic acid has been incubated with an x-ray mutant of *Escherichia coli* which requires this substance as a growth factor. By column chromatography 4 soluble radioactive fractions have been isolated from the cell bodies of the organism, none of them consisting of the original substrate. Two of the fractions possess citrovorum factor activity, but none of them are identical with folic acid, citrovorum factor or certain of its derivatives.

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Effects of Guinea Pig Serum on 6C3HED and AKRL1 Lymphoma Cells Implanted in Various Hosts.* (22200)

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Cells of Lymphoma 6C3HED promptly begin to die when serum from normal guinea pigs is injected into C3H mice in which they are growing(1); yet the lymphoma cells remain viable when they are held in contact with guinea pig serum during several hours at 37°C *in vitro*(1,2). The facts suggest that the animal host somehow participates in the process whereby these lymphoma cells are killed *in vivo,* perhaps by supplying special conditions essential for the process, or by furnishing some substance—an isoantibody, for example, or one or another of the components of complement—that might work with guinea pig serum in producing the effect(1,2). A number of questions related to the facts just mentioned are raised by a more recent observation—namely that cells of Lymphoma AKRL1 continue to proliferate without restraint when normal guinea pig serum is given to AKR mice carrying them(3). Do the AKR mice fail to supply such conditions as might be required for the effectiveness of guinea pig serum *in vivo,* or are they devoid of some essential host factor? Is there an intrinsic difference in cells of Lymphoma 6C3HED and those of Lymphoma AKRL1, the former being susceptible to the effects of guinea pig serum *in vivo* while the latter are insusceptible? The findings of several experiments, now to be given, bear on these questions.

Results. The experiment of Fig. 1 shows that the cells of Lymphoma 6C3HED were inhibited quite conspicuously when guinea pig serum was given intraperitoneally to AKR mice into which 10 million lymphoma cells had been implanted in each of 2 subcutaneous sites by a technic already described(1), and the finding was confirmed in a subsequent experiment done in precisely the same way. Furthermore, in the experiment of Fig. 1 the inhibitory effect of guinea pig serum was even more marked in the alien and resistant AKR hosts than in the C3H hosts of the sort in which this lymphoma had originated (and which had been implanted with 1 million 6C3HED lymphoma cells in each of 2 subcutaneous situations). This result suggests that the effects of guinea pig serum might depend upon, or be enhanced by, some resistance manifested by the animal

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