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Biosynthesis of 4-Aminobenzoate in *Escherichia coli*

MINTA HUANG AND F. GIBSON

Biochemistry Department, John Curtin School of Medical Research, The Institute of Advanced Studies, Australian National University, Canberra, A.C.T., Australia

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Two different mutations (*pabA* and *pabB*) affecting 4-aminobenzoate biosynthesis were obtained in strains of *Escherichia coli* lacking chorismate mutase and anthranilate synthetase activity, thus allowing study of the pathway of biosynthesis of 4-aminobenzoate by use of cell extracts of strains carrying the *pab* mutations. Two components with approximate molecular weights of 9,000 (component A) and 48,000 (component B) are concerned in the biosynthesis of 4-aminobenzoate from chorismate by *E. coli*. No diffusible intermediate compound could be detected.

Mutants requiring 4-aminobenzoate (*p*-AB) for growth have been isolated from *Neurospora crassa* (4, 7, 21) and *Escherichia coli* (13, 14; *see also* 24). Genetic analyses of the mutant strains of *N. crassa* (7) and *E. coli* (13) have shown that, in each case, mutants map at two distinct loci. The two genes concerned with *p*-AB synthesis in *E. coli* were designated the *pabA* and *pabB* genes. As the *p*-AB auxotrophs of *E. coli* require no other aromatic compounds for growth, the metabolic lesion must be between chorismate and *p*-AB (*see* 10). Cell extracts of *Aerobacter aerogenes* have been shown to convert chorismate to *p*-AB (9). The present paper describes an investigation of the metabolism of chorismate by strains of *E. coli* carrying the *pabA* or the *pabB* alleles.

MATERIALS AND METHODS

Organisms. The strains used in this work were all derived from *E. coli* K-12 and are described in Table 1. The distribution of relevant genetic markers on the chromosome is shown in Fig. 1.

Chemicals. The chemicals used were of analytical reagent grade wherever possible. Chorismic acid was isolated as described previously (8). For the assay of *p*-AB, batches of ethyl acetate were chosen which had a low fluorescence at the wavelengths of the assay.

Media and culture methods. All cultures, except for those used in conjugation and transduction experiments, were grown at 37 C in media containing the mineral salts mixture medium 56 (17). Glucose (0.5% in liquid media, 0.2% in solid media) was used as the carbon source. Supplements (proline, 1.5×10^{-3} M; arginine, 8×10^{-4} M; histidine, methionine, isoleucine, valine, leucine, each 3×10^{-4} M; phenylalanine, tyrosine, tryptophan, anthranilic acid, each 10^{-4} M; 4-aminobenzoic acid, 10^{-6} M; thiamine, 5×10^{-7} M) were added as required. The amino acids were added before the media were autoclaved, and the vitamins were added as sterile solutions after autocalving. Cultures used for conjugation and transduction experiments were grown in a nutrient broth medium described elsewhere (16).

Cultures used for the preparation of cell extracts were harvested during the late logarithmic phase of growth, and the cells were washed once with 0.9% saline.

Preparation of cell-free extracts. The washed cells were resuspended in 2 ml of tris(hydroxymethyl)-aminomethane (Tris)-hydrochloride buffer (0.05 M, *p*H 7.8) for each gram (wet weight) of cells. The cells were disintegrated in a French pressure cell or in a Sorvall Ribi cell disintegrator under a pressure of 20,000 psi. The smashed cells were centrifuged at $30,000 \times g$ for 30 min to remove cell debris, and the cell-free extracts were stored at -15 C. Protein was estimated by the method of Lowry et al. (15).

Nutritional tests. The problems of carrying out nutritional tests on *p*-AB auxotrophs have been discussed previously (12), and care was taken during the present experiments to avoid cross-feeding. Tests for *p*-AB requirements were carried out by streaking dilute cell suspensions onto the surface of suitably supplemented agar plates.

Mating conditions and transduction technique. The conditions under which matings were carried out and interrupted were essentially those described by Taylor and Thoman (22). Transduction techniques used in this work followed those described by Pittard (18).

Penicillin recycling technique. The method of Dempsey and Pachler (6) for successive penicillin treatments to concentrate mutants was used.

Isolation of mutants. Ultraviolet irradiation and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) were used for the isolation of mutants. Cultures to be irradiated were grown to early logarithmic phase, diluted to 10^6 to 10^7 cells/ml in half-strength medium 56, and irradiated with ultraviolet light to give about a 99.7% kill. The irradiated cells (5 ml) were incubated overnight in a suitably supplemented medium to allow phenotypic expression. The method of Adelberg, Mandel, and Chen (1) for the use of NTG was followed.

Fractionation of p-AB synthesizing system in cell-

TABLE 1. *Strains of E. coli K-12 used*

Strain no.	Sex	Genotype[a]
AB1446	F′ ♂	*his-4 pro-2 trp-15 str*^{R}
AB2882	F$^-$	*his-4 pro-2 arg-3 ilv-7 xyl*$^-$ *str*^{R} *tsx*^{R}
AB3292	F$^-$	*pabA1 his-4 pro-2 arg-3 ilv-7 xyl*$^-$ *str*^{R} *tsx*^{R}
AB3294	F$^-$	*pabB3 his-4 pro-2 arg-3 ilv-7 xyl*$^-$ *str*^{R} *tsx*^{R}
AN1	F$^-$	*his-4 proA2 argE3 pheA1 tyrA4 trp-401*
AN2	F$^-$	*his-4 proA2 argE3 pheA1 tyrA4*
AN3	F$^-$	*pabA1 his-4 proA2 argE3 pheA1 tyrA4 trp-401 str*^{R}
AN4	F$^-$	*pabB404 argE3 ilvC7 pheA1 tyrA4 trp-403*
AN6	F$^-$	*pabB404 his-4 proA2 argE3 ilvC7 leu-351 tyrA4 trp-356*
AN10	F$^-$	*pabB404 his-4 argE3 ilvC7 tyrA4 trp-356 str-750*
AN15	F′ ♂	*proA2 F-arg*$^+$*/argE3 pheA1 tyrA4 trp-401*
AN16	F$^-$	*his-4 proA2 argE3 ilvC7 leu-351 tyrA4 trp-356*
AN58	F$^-$	*proA2 argE3 pheA1 tyrA4 trp-401*
AT2022	F$^-$	*pheA1 his-4 proA2 argE3*
AT2471	Hfr	*tyrA4 thi*$^-$

[a] The genetic nomenclature is that proposed by Taylor and Trotter (23).

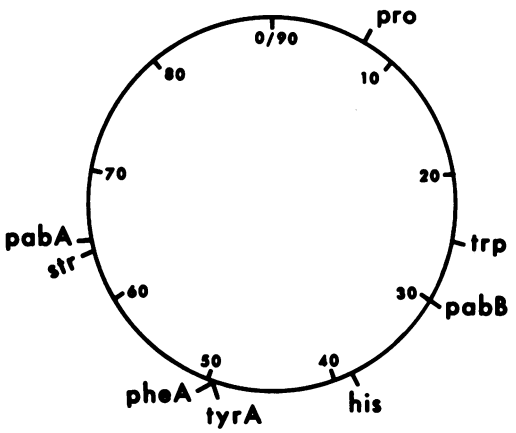

FIG. 1. *Map of E. coli K-12 chromosome, showing genetic markers relevant to this work. The map is according to the conventions of Taylor and Trotter (23).*

free extracts. The conditions used for the swelling of Sephadex gels and the packing of columns were those recommended by the manufacturer. Cell extracts (4 ml) containing 80 to 100 mg of protein were applied to a Sephadex G-100 column (2 × 38.5 cm) and eluted with the column buffer (0.05 M Tris-hydrochloride buffer, *p*H 7.8). The column was eluted at 10 to 12 ml/hr, and 3-ml fractions were collected. The columns were run at 4 C, and the fractions were transferred to an ice bath as soon as possible after collection.

Synthesis of p-AB by cell-free extracts. The reaction mixture for the synthesis of *p*-AB contained chorismic acid (0.2 μmole) Tris-hydrochloride buffer, *p*H 8.2 (50 μmoles), L-glutamine (80 to 120 μmoles), and crude cell-free extract (containing 5 to 8 mg of protein) in a final volume of 1 ml. After incubation at 37 C for 40 min, 1 N HCl (0.2 ml) was added and *p*-AB was extracted into ethyl acetate (4 ml). After the organic layer had been dried with anhydrous Na_2SO_4, the extracted *p*-AB was assayed fluorimetrically [activation, 296 nm; fluorescence, 340 nm; uncorrected (9)] on an Aminco-Bowman spectrophotofluorometer. The concentration of *p*-AB was determined from a standard curve obtained after extraction of *p*-AB (1 to 100 μM) from aqueous solution (Tris-hydrochloride, *p*H 8.2; 50 mM) by the procedure described above.

For complementation studies, an appropriate amount of a cell extract to be tested (crude or partially purified) was incubated with a crude cell extract of one of the mutant strains (AN3 or AN4) containing 5 to 8 mg of protein, in the presence of substrates as described above.

Ultrafiltration. Reaction mixtures to be ultrafiltered were chilled in an ice bath and transferred to a prechilled Diaflo ultrafiltration cell (model 50) fitted with a UM-10 membrane which filters off compounds having molecular weights greater than 10,000. Filtration was carried out with continuous stirring at 4 C under a pressure of 50 psi of helium.

RESULTS

Strains carrying the *pabA* and *pabB* mutations were used in the present work. The isolation and genetic analysis of strain AB3292 (*pabA1*) have been reported previously (13). The strain (AB-3299) carrying the *pabB3* mutation described previously (13) was not suitable for the present experiments (see below). After NTG treatment of strain AN16, a new *p*-AB auxotroph, strain AN6, was isolated which carried a *pab* mutation mapping, by interrupted mating, at 8.5 min anticlockwise from *his* (M. Huang, *unpublished data*). The *pab* mutation in strain AN6 was therefore in the same region of the chromosome as the *pabB* mutation in strain AB3299 and in the related strain AB3294 (13). In two-factor crosses with a strain derived from AN6 and strain AB3294, Pab$^+$ transductants were detected at frequencies

of 2% or less of the frequencies of cotransduction of unlinked markers, indicating that the *pab* mutations in the two strains were probably allelic. The *pab* mutation in strain AN6 was therefore designated *pabB404*.

Isolation of a triple mutant blocked in phenylalanine, tyrosine, and tryptophan biosynthesis. To study the enzymatic conversion of chorismate into *p*-AB, it is necessary to use cell extracts from a strain unable to metabolize chorismate towards the three major end products derived from chorismate, namely, phenylalanine, tyrosine, and tryptophan. Such a mutant of *A. aerogenes* has been described previously (11) and used for studies on *p*-AB biosynthesis (9).

A triple aromatic auxotroph of *E. coli* K-12, strain AN1, was isolated as follows: a bacteriophage P1-mediated transduction was carried out to transduce the *tyrA* mutation from strain AT-2471 into a phenylalanine auxotroph, strain AT2022 (*pheA1*). To increase the proportion of desired transductants (*pheA1*, *tyrA1*) the phage-treated cells were subjected to three successive cycles of penicillin treatment and selecting for *Tyr*$^-$ transductants. Such organisms could be either Phe$^+$ or Phe$^-$ as the *pheA* and *tyrA* alleles are 50% cotransducible (19). Therefore, a further penicillin selection was made for transductants which had retained the *pheA* mutation. After penicillin treatment followed by growth in a suitably enriched medium, single colonies were tested to detect Phe$^-$ Tyr$^-$ auxotrophs. Nine such strains were found among 144 tested. One of these, strain AN2, was shown to lack chorismate mutase activity (5) and was used as the parent strain for the isolation of a triple aromatic auxotroph.

Strain AN2 was irradiated with ultraviolet light and subjected to three successive cycles of penicillin treatment and selecting for Trp$^-$ mutants. Cells from 6 colonies of 60 tested were found to be tryptophan auxotrophs responding to anthranilate. Cell extracts of one of these triple auxotrophs was shown to lack anthranilate synthetase (11), and this strain (AN1) was used for subsequent experiments.

p-AB synthesis by cell-free extracts of strain AN1 and AB2882. Cell-free extract (0.2 ml containing 5 to 6 mg of protein) of the triple mutant strain AN1 was incubated in the reaction mixture for the synthesis of *p*-AB. Fluorimetric assay showed that about 5 to 6 nmoles of *p*-AB per mg of protein per 40 min was formed.

Under similar conditions, a cell extract of a strain (AB2882) not containing mutations affecting chorismate metabolism towards the amino acids formed no detectable *p*-AB (<0.25 nmole per mg of protein per 40 min).

Attempts to derepress the p-AB-synthesizing system. It has been reported (25) that some sulfonamide-resistant strains of *Staphylococcus aureus* produced *p*-AB in much larger amounts than the parental cell type. Isolation of sulfonamide-resistant derivatives of strain AN1 was made in an attempt to derepress the *p*-AB-synthesizing system in strain AN1. Two strains, sulfadiazine-resistant, were isolated. One was resistant to 8 μg of sulfadiazine/ml of medium and the other was resistant to 12 μg of sulfadiazine/ml of medium. Cell extracts of either of these strains grown in the presence or absence of sulfadiazine did not synthesize *p*-AB more actively than extracts from sulfonamide-sensitive cells.

Transfer of pab alleles to triple mutant. Enzymatic studies of the conversion of chorismate to *p*-AB in *p*-AB auxotrophs required that the *pabA* and *pabB* mutations were in mutants such as strain AN1.

The *pabA* mutation from strain AB3292 was transferred into strain AN1 by cotransduction. Strain AN1 was streptomycin-sensitive, and strain AB3292 (*pabA*), streptomycin-resistant; the *pabA* allele was cotransducible with *str* at a frequency of about 30% (13). Thus, a P1-mediated transduction was carried out with strain AB3292 as the donor strain and AN1 as recipient. Selection was made for Str^R transductants, and of the 40 transductants tested 10 were shown to have an absolute growth requirement for *p*-AB. One of these (strain AN3) was purified and used for subsequent work.

Unlike the *pabA* allele, there is no known marker with which the *pabB* allele is cotransducible (13). Attempts were made to transfer the *pabB3* mutation from strain AB3294 into the triple mutant (strain AN1), but without success. Therefore the incorporation of the *pabB404* mutation into a suitably blocked strain was achieved by a series of conjugation experiments. The strain which carried the *pabB404* mutation (strain AN6) also carried *tyrA* and *trp-356* mutations, and therefore the incorporation of a *pheA* mutation would give the desired genotype. A suitable male strain carrying the *pheA* mutation was obtained by making a His$^+$ derivative of the triple mutant strain AN1 (AN58), into an F′ male by conjugation with the F′ male AB1446. The resulting strain was designated AN15 and was *trp*$^-$, *phe*$^-$, *tyr*$^-$, and *pro*$^-$.

Strain AN15 was then crossed with a *tyr*$^-$, *trp*$^-$, *pabB*$^-$, *his*$^-$ strain (AN10) derived from strain AN6, and selection was made for His$^+$, Pro$^+$ recombinants. Screening of such recombinants allowed the isolation of a strain (AN4) containing *phe*$^-$, *tyr*$^-$, *trp*$^-$, and *pabB*$^-$ alleles.

Strains AN3 and AN4 therefore contained the

pabA⁻ and *pabB*⁻ alleles, respectively, and also lacked active enzymes converting chorismate along the pathways to the aromatic amino acids.

Enzymatic complementation studies. When a cell extract of strain AN3 (*pabA*) or AN4 (*pabB*) was incubated with the substrates necessary for the synthesis of *p*-AB, no significant amount of *p*-AB was formed, as determined by (i) fluorimetric assay and (ii) growth tests with *p*-AB auxotrophs. However, when similar tests were carried out with mixtures of cell extracts (total protein, 12 mg/ml) from the two strains, *p*-AB was formed. The *p*-AB-synthesizing activity in such an incubation mixture was about 1.5 nmole per mg of protein per 40 min.

Attempts to demonstrate the presence of a diffusible intermediate. The isolation of two classes of *p*-AB auxotrophs which were genetically distinct, and the successful complementation by extracts of the two mutants, suggested that there could be at least two reactions involved in the conversion of chorismate to *p*-AB. Experiments were carried out to test for a diffusible intermediate in *p*-AB synthesis formed from chorismate by extracts of strains carrying either *pabA* or *pabB* mutations. Such an intermediate would be likely to be converted to *p*-AB by the mutant which could not form the intermediate.

Cell extracts of strains AN3 (*pabA*) and AN4 (*pabB*) were incubated separately with chorismate and Tris-hydrochloride buffer in the presence and absence of glutamine (final total volume, 5 ml). After incubation at 37 C for 40 min, each reaction mixture was filtered through a Diaflo filter (see Materials and Methods). Suitable samples of the filtrate were incubated with the crude cell extract of the second strain in the presence and absence of glutamine, and *p*-AB was assayed fluorimetrically. No significant amount of *p*-AB was detected in any of the systems.

The above results suggested the absence of a free intermediate in the synthesis of *p*-AB from chorismate, and further experiments were carried out in which cell extracts of the two mutant strains were kept in two compartments separated by a dialysis membrane.

A cell extract of each of the mutant strains was incubated in the presence of chorismate and glutamine. One reaction mixture (3 to 4 ml) was held in a dialysis bag immersed in the second reaction mixture (4 ml), and the tube was incubated at 37 C. Samples from inside and outside the dialysis bag were removed at intervals and examined fluorimetrically for the presence of *p*-AB. The results were negative. As a control, a cell extract of strain AN1, which had *p*-AB-synthesizing activity, was placed in one of the compartments, with a second reaction mixture containing an extract of one of the mutant strains in the other compartment; *p*-AB was detected in the latter compartment within 40 min.

Fractionation of p-AB synthesizing activity. The isolation of two classes of *p*-AB auxotrophs, exhibiting enzymatic complementation in the synthesis of *p*-AB from chorismate, offered a convenient method of examining the fractionation of the enzymes concerned in cell extracts of *E. coli* wild type with respect to this pathway. Cell extracts of strain AN1 were filtered through Sephadex G-100 as described in Materials and Methods. None of the fractions collected showed any *p*-AB-synthesizing activity (Fig. 2). However, when such fractions were assayed in the presence of an extract of either mutant strain, two distinct peaks of activities were observed (Fig. 2). The first peak resulted from complementation of column fractions with an extract of strain AN4 (*pabB*), a strain which had functional *pabA* gene; this peak is labeled component B in Fig. 2. The second peak was due to complementation with an extract of strain AN3 (*pabA*), a strain which had functional *pabB* gene; this activity is labeled component A.

A cell extract of each of strains AN3 (*pabA*) and AN4 (*pabB*) was chromatographed separately on the same Sephadex G-100 column, and column fractions were assayed for the ability to complement with a cell extract of the other mutant strain. The distribution of the two activities is shown in Fig. 2b and 2c. It can be seen that component B, from a cell extract of either the strain AN1 or the mutant strain, AN3 (*pabA*), was eluted in the same fractions. Similarly, the peaks of component A fractionated from the wild-type cell extract or from strain AN4 (*pabB*) coincided.

The A and B components fractionated from a cell extract of either the triple mutant or the appropriate *p*-AB auxotroph (strain AN3 or AN4) were unstable compared with the corresponding activities in the appropriate crude cell-free extracts. The instability of component A was more pronounced; on standing at 4 C for 4 to 5 hr, its activity steadily decreased to about 50% of the initial level determined within 2 hr of collection of column fractions. Component B was more stable. Its activity, assayed within 6 to 8 hr after fractionation, showed no appreciable change. However, standing at 4 C over 24 hr brought about a 25 to 30% loss of activity. Freezing and thawing accelerated the inactivation of both components.

When the peak fractions from the A and B components were mixed and incubated in the substrate mixture for *p*-AB synthesis, *p*-AB was formed (Table 2). Thus, the wild-type *p*-AB-synthesizing system in *E. coli* can be separated into two components. However, when component

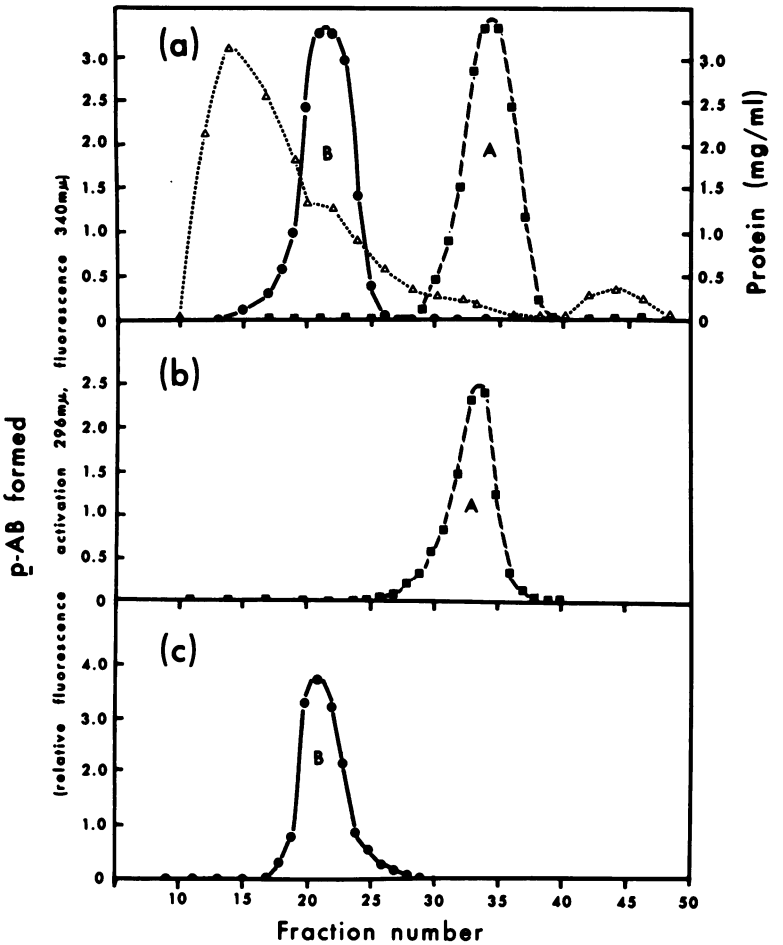

FIG. 2. *Chromatography of cell-free extracts of (a) strain AN1, (b) strain AN4 (pabB), and (c) strain AN3 (pabA) on Sephadex G-100. Fractions (0.4-ml samples) were assayed for p-AB formation in the presence of unfractionated cell extracts (0.2 ml, about 5 mg of protein) of (●) strain AN4 (pabB) or (■) strain AN3 (pabA). Details of reaction mixture in Materials and Methods. Protein, △.*

A and component B from the Sephadex columns were used in experiments similar to those described earlier in which the components were separated by a dialysis membrane, no *p*-AB was formed.

Estimation of the molecular weights of A and B components. The chromatographic properties of the A and B components on a Sephadex G-100 column were consistent, and the two peaks of activity were well separated. By calibrating the column with a number of compounds of known molecular weights (3), estimates of the molecular weights of the A and B components were determined.

The compounds used as markers were bovine serum albumin (molecular weight of monomer, 71,000; of dimer, 142,000) ovalbumin (molecular weight, 41,000) and cytochrome *c* (molecular weight, 12,400). The elution volume of each marker compound was determined under the same conditions as those used for the fractionation of the cell extracts. A calibration curve correlating the elution volumes of various markers versus the logarithm of the corresponding molecular weight is shown in Fig. 3. The elution volumes of the A and B components, when the same column was used, were 103.5 and 64.5 ml, respectively (Fig. 3), and the corresponding approximate molecular weights, as determined on the calibration curve, were 9,000 for component A and 48,000 for component B.

DISCUSSION

Little is known about the conversion of chorismate into *p*-AB. Weiss and Srinivasan (24)

TABLE 2. *Reconstitution of the p-AB-forming system by mixing fractions from Sephadex columns*

p-AB-synthesizing system[a]		*p*-AB formed (nmoles)	
Peak fraction	Further addition	Exp 1	Exp 2
A	Cell extract AN3 (*pabA*)	<0.1	<0.1
A	Cell extract AN4 (*pabB*)	4	2.8
B	Cell extract AN3 (*pabA*)	5.6	6
B	Cell extract AN4 (*pabB*)	<0.1	<0.1
A	Peak fraction B	3	2

[a] The conditions for synthesis of *p*-AB were as described in Materials and Methods except that 0.4 ml of column fractions from the experiment shown in Fig. 2a were used and 0.2 ml (5 to 6 mg of protein) of cell extract of each mutant.

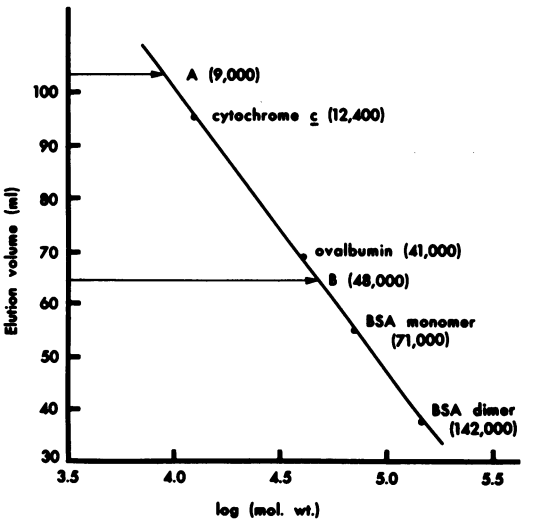

FIG. 3. *Molecular weights of components A and B as determined by chromatography on a Sephadex G-100 column calibrated with proteins of known molecular weight as shown. For details of chromatography see Materials and Methods. BSA = bovine serum albumin. Molecular weights are shown in parentheses.*

showed that shikimate 5-phosphate plus glutamine can be converted to *p*-AB by a yeast preparation and, using isotopically labeled glutamine (20), showed that the nitrogen of *p*-AB came from the amide nitrogen of glutamine. Gibson, Gibson, and Cox (9), using cell extracts of the triple mutant *A. aerogenes* 62-1, established the conversion of chorismate plus glutamine to *p*-AB. The genetic mapping of *p*-AB auxotrophs of *N. crassa* (7) and of *E. coli* (13) showed that in each case the mutants could be divided into two classes, suggesting the presence of an intermediate between chorismate and *p*-AB. Further evidence (12) suggesting such an intermediate was the observation that fractionation of cell extracts of *N. crassa* with ammonium sulfate gave two fractions, either of which is inactive but which complement each other in the formation of *p*-AB. Cross-feeding between the two classes of *Neurospora* mutant suggested that a diffusible intermediate was formed, although no cross-feeding could be demonstrated between *p*-AB auxotrophs of *E. coli* (13). It has been reported (2) that a *p*-AB auxotroph of *A. aerogenes* accumulates a compound which can be converted to *p*-AB (see below).

The present work has shown that the *p*-AB synthesizing system can be separated into two components with molecular weights of about 9,000 and 48,000, but it has not been possible to demonstrate the existence of a diffusible intermediate formed by either one of these components. The possibility that the intermediate was enzyme-bound was also investigated (M. Huang, Ph.D. Thesis, Australian National University, Canberra City, 1969) by allowing cell extracts from *E. coli* mutants carrying the *pabA* and *pabB* mutations to metabolize ^{14}C-shikimate under the conditions for *p*-AB synthesis. Early in the incubation period, the reaction mixture was passed through a column of Sephadex G-100 and the distribution of the A and B components and of radioactivity was examined. No radioactivity was associated with either the A or the B components so that an intermediate, if formed, is removed during gel filtration. Altendorf, Bacher, and Lingens (2) have briefly reported the formation, by a *p*-AB auxotroph (*A. aerogenes* 62-1AC), of a compound which could be assayed by an auxotroph of *E. coli* K-12. It was also shown that the proposed intermediate (compound A) was converted to *p*-AB by prolonged incubation at *p*H 3.5. Culture filtrates from the various *p*-AB auxotrophs used during the present work have been subjected to prolonged incubation at *p*H 3.5, but no evidence of conversion of any compound to *p*-AB has been obtained. Experiments in which culture filtrates from the various *p*-AB auxotrophs were incubated with cell extracts of *pabA* or *pabB* strains, together with glutamine and buffer, showed no evidence of the accumulation of any intermediate in *p*-AB biosynthesis.

It is not yet possible to explain the different results. Among the possible explanations, is that the pathway in *E. coli* differs from that in *A. aerogenes*, that there are more than two reactions concerned in the conversion of chorismate to *p*-AB, or that the component A and component B

described in the present work are polypeptides concerned in one (presumably the first) of the reactions in the conversion of chorismate to *p*-AB. The very low molecular weight of component A might indicate that it may function together with component B or an unknown polypeptide in one reaction in *p*-AB synthesis. The relatively small number of *p*-AB auxotrophs (4) which have been genetically mapped do not preclude the possibility of a third genetic locus affecting yet another polypeptide. On the other hand, the experiment (Table 2) in which mixtures of fractions A and B from the Sephadex column gave apparently good recoveries of the overall activity in converting chorismate to *p*-AB would argue against the existence of a third polypeptide unless it co-chromatographs with either component A or B.

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