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Preliminary Studies on the Isolation and Metabolism of an Intermediate in Aromatic Biosynthesis: Chorismic Acid

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It has been suggested (Gibson, Gibson, Doy & Morgan, 1962) that the pathway of biosynthesis of the aromatic amino acids, tryptophan, phenylalanine and tyrosine, and the aromatic growth factors, 4-aminobenzoic acid and 4-hydroxybenzoic acid, may be as outlined in Scheme 1.

This scheme was based particularly on the observations that 3-enolpyruvylshikimic acid 5-phosphate is a likely precursor for the three aromatic amino acids (Levin & Sprinson, 1960; Gibson *et al.* 1962; Rivera & Srinivasan, 1962) and that mutants have been described (Davis & Mingioli 1953) which accumulate 3-enolpyruvylshikimic acid and require for growth phenylalanine, tyrosine, tryptophan, 4-aminobenzoic acid and 4-hydroxybenzoic acid. From the latter observation it may be deduced that there is at least one further common intermediate beyond 3-enolpyruvylshikimic acid 5-phosphate. This is the 'compound *X*' of Scheme 1. If such a compound exists, a mutation affecting an enzyme concerned in converting compound *X* into prephenic acid should result in an auxotroph requiring phenylalanine and tyrosine. Such double requirements have been reported (Davis & Mingioli, 1953; Schwinck & Adams, 1959), but examination of the auxotrophs has shown that their requirements are due either to partial blocks further back than compound *X* or to two separate mutations affecting the conversion of prephenic acid into phenylpyruvic acid on the one hand and into 4-hydroxyphenylpyruvic acid on the other.

The present paper concerns the search for the hypothetical compound *X*. Study of the reactions in the region of compound *X* is complicated by the fact that blocking one reaction results in shunting of the intermediates along alternative pathways (Morgan, Gibson & Gibson, 1962). By imposing subsequent mutations affecting each pathway in turn it was hoped to obtain cell extracts that would accumulate compound *X* itself. To this end a strain of *Aerobacter aerogenes* (T17) was used, cell-free extracts of which had previously been studied for their ability to carry out the conversion of shikimic acid into anthranilic acid or the phenylpyruvic acids (Morgan *et al.* 1962; Morgan, Gibson & Gibson, 1963). In the present work this strain was irradiated and further mutants were isolated with additional metabolic lesions affecting the phenyl-

alanine and tyrosine pathways. As a result of this work a substance with the properties expected for compound *X* was isolated, identified and named 'chorismic acid', by which name it is referred to throughout the present paper. A preliminary report of some of this work has been published (Gibson & Gibson, 1963).

EXPERIMENTAL

Organisms. A number of different auxotrophic strains of *A. aerogenes* and *Escherichia coli* were used in the present studies. Table 1 lists the strains and some relevant information about them. All the auxotrophs isolated in this Laboratory have been isolated by the penicillin selection technique essentially as described by Davis (1948). Stock cultures are maintained on nutrient–agar slopes and subcultured monthly.

Chemicals and buffers. The chemicals used were obtained commercially and not further purified. The buffer solutions used were as described by Dawson, Elliott, Elliott & Jones (1959). Tris-HCl and sodium phosphate buffers were used.

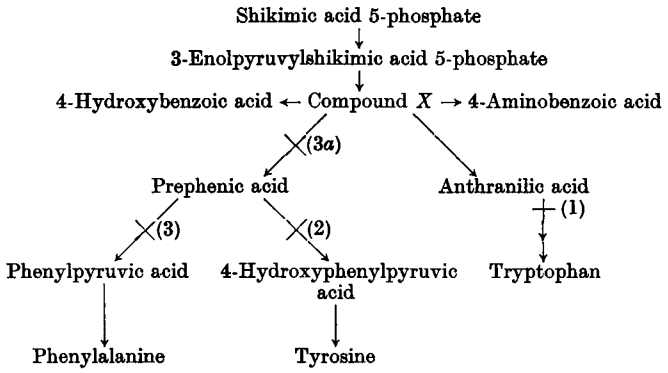
Growth experiments. Growth tests on solid media were made by preparing a suspension of cells (about 10^6 cells/ml.) and plating on the mineral salts medium described by Vogel & Bonner (1956) solidified with 2% of agar and supplemented with 0·16% (w/v) of glucose and the appropriate growth factors. Growth tests on liquid media were carried out in liquid medium (Vogel & Bonner, 1956) shaken in ⊥ tubes in a water bath at 37° or aerated in a flask. The turbidity of liquid cultures in ⊥ tubes was read as an extinction with a Spekker colorimeter and a neutral density filter. The turbidity of cultures in flasks was read in an EEL photoelectric colorimeter. An EEL galvanometer reading of 10 is equivalent to about 0·3 mg. dry wt./ml.

Unless otherwise stated, amino acids were added to give a final concentration of 0·1 mM and vitamins to give a final concentration of 1 μM.

Preparation of cell extracts. The methods used for preparation of cell extracts were as described by Morgan *et al.* (1963). Briefly, the cells were grown in shaken flasks in glucose–mineral salts medium supplemented as required. The cells were then harvested, washed with 0·9% NaCl, suspended in 0·1 M-tris–HCl buffer, pH 7·8, and smashed in an ultrasonic disintegrator. Proteins were determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

Anthranilic acid assay methods. (*a*) Experimental samples were acidified with 0·1 ml. of N-HCl/ml. of sample and then extracted with ethyl acetate (usually 4 vol.). The ethyl acetate extract was dried briefly with anhydrous Na_2SO_4 and the spectrum was measured against an ethyl acetate blank from 250 to 360 mμ. An extinction coefficient of 4900 at 336 mμ was used to calculate the concentration of anthranilic acid.

(*b*) The fluorescence of reaction mixtures was measured with an Aminco–Bowman spectrophotofluorimeter at an activation wavelength of 325 mμ and a fluorescence wavelength of 398 mμ. Relative fluorescence was converted



Scheme 1. Proposed pathway of aromatic biosynthesis.

Table 1. *Principal strains of* Aerobacter aerogenes *used*

Details are given in the text. The growth requirements are the supplements required for optimum growth in glucose–mineral salts medium.

Strain	Growth requirements	Probable metabolic lesion	Remarks
T17	Tryptophan	*N*-(5-Phosphoribosyl)anthranilic acid ⟶ 1-(*o*-carboxyphenylamino)deoxyribulose 5-phosphate	Parent of strain 61–3
61–3	Tryptophan + tyrosine	As for strain T17 plus prephenic acid ⟶ 4-hydroxyphenylpyruvic acid	Parent of strain 62–1
62–1	Tryptophan + tyrosine + phenylalanine	As for strain 61–3 plus chorismic acid ⟶ prephenic acid	Cell extracts and whole cells form chorismic acid; strain used for assay of chorismic acid
170–44	Tryptophan + tyrosine + phenylalanine + 4-aminobenzoic acid	3-Enolpyruvylshikimic acid 5-phosphate ⟶ chorismic acid	Strain used for assay of chorismic acid

into concentrations of anthranilic acid by using a standard curve prepared under suitable conditions.

Phenylpyruvic acid assay methods. (*a*) Experimental samples were acidified and extracted with ethyl acetate as for the anthranilic acid assay. The spectrum of the ethyl acetate layer was examined immediately, and then again after incubation overnight at 30°. The development of a peak at 288 mμ was taken as an indication of the presence of phenylpyruvic acid or 4-hydroxyphenylpyruvic acid or both (Morgan *et al.* 1962, 1963). This was not an accurate assay method but was useful in detecting the phenylpyruvic acids with certainty in the presence of NAD and NADH which interfere with the following assay method.

(*b*) Experimental samples were diluted 1:10 in alkali as for the control in the prephenate assay (see below) and the extinction at 320 mμ was measured. In all experiments the spectra were measured between at least 300 and 340 mμ to check the peak. An extinction coefficient of 17500 at 320 mμ was used to calculate the phenylpyruvic acid concentration. This method was used whenever possible.

Prephenic acid assay methods. In initial experiments the technique described by Metzenberg & Mitchell (1956) was used. Two 0·4 ml. samples were taken. To one of these (the control) were added 3·2 ml. of N-NaOH and 0·4 ml. of N-HCl, and the spectrum between 300 and 340 mμ was examined as soon as possible. To the second sample (the test) was added 0·4 ml. of N-HCl and the tube incubated for 2 hr. at 37°. After the addition of 3·2 ml. of N-NaOH the spectrum was examined between 300 and 340 mμ. The difference in extinction between the control and test at 320 mμ was taken as a measure of the phenylpyruvic acid formed by acid treatment of the sample. An extinction coefficient of 17500 at 320 mμ was used for calculating the concentration of phenylpyruvic acid. In subsequent experiments the time of incubation with acid was decreased to 10 min. to avoid decomposition of chorismic acid (Gibson, 1964).

Spectroscopy. All spectra were measured with a Cary model 11 recording spectrophotometer and quartz cells of 1 cm. light-path.

Chromatography. The details of the techniques were as given by Pittard, Gibson & Doy (1961). Sprays and solvents were as described by Smith (1960; pp. 263, 264, 297).

Fluorimetry. An Aminco–Bowman spectrophotofluorimeter was used with a xenon lamp, an IP 28 detector tube, slit arrangement no. 3 and a sensitivity setting of 5.

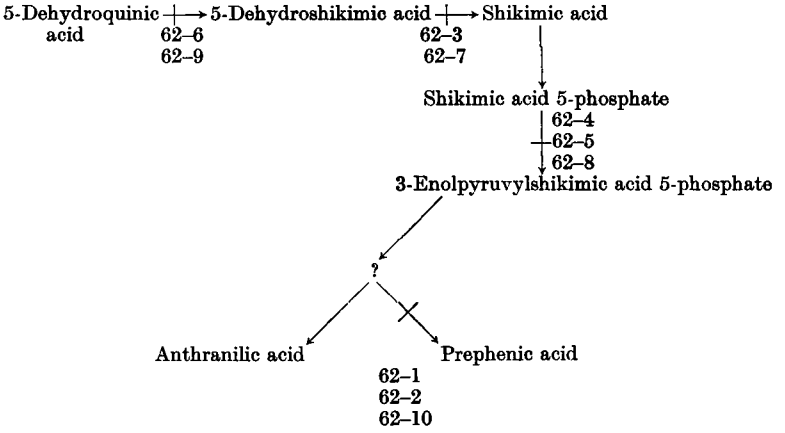
Solutions of chorismic acid. Solutions used in the various tests varied in the method of preparation during the progress of the experiments. Most of the solutions for the experiments described were prepared by allowing the chorismic acid to accumulate in the presence of whole cells or cell extracts, extracting the media with ether in the cold and evaporating the extract *in vacuo*. Buffer was added before the ether had completely evaporated and a vacuum was reapplied to remove the last traces of solvent. In later experiments the barium salt of the intermediate was used. The type of preparation used in individual experiments is indicated in the text.

Chorismic acid was assayed in one of two ways: (*a*) A solution of the acid or of the barium salt (0·1–1·5 mM) was incubated in 50 mM-tris–HCl buffer, pH 8·2, with L-glutamine (5 mM) and crude extract of strain 170–44 (1 mg. of protein/ml.) for 20 min. at 37°. After the addition of 0·1 ml. of N-HCl/ml. the solutions were extracted with ethyl acetate for anthranilic acid assay. (*b*) A solution of the acid or barium salt (0·005–0·1 mM) was incubated with L-glutamine (2·5 mM) and strain 170–44 extract (0·5 mg. of protein/ml.) for 10–15 min. at 37°. The fluorescence was then measured and the anthranilic acid concentration read from a standard curve. Assays were normally carried out in a final volume of 1 ml. Quantities of chorismic acid quoted in experiments are based on such assays.

Extracts of strain 170–44 grown in limiting indole medium were chosen for the assay, although some of chorismic acid might be converted into the phenylpyruvic acids. This strain was used to avoid the conversion of compounds such as 3-enolpyruvylshikimic acid 5-phosphate into anthranilic acid. With purified samples of chorismic acid, dialysed extracts of strain 62–1 (which had been grown in limiting tryptophan) can be used, as these give a higher yield of anthranilic acid than do extracts of strain 170–44.

RESULTS

Isolation of Aerobacter aerogenes 62–1. It was intended to isolate auxotrophs with metabolic lesions around the branch points shown in Scheme 1 to obtain mutants in which the reactions in this region would be more easily studied. Strain T17, which was unable to convert anthranilic acid into indol-3-ylglycerol phosphate, was chosen as the parent organism because the tryptophan pathway could be blocked by the omission of nitrogen from the reaction mixture. Further, the conversion of shikimic acid into anthranilic acid could be used as a test for the presence of all enzymic activities on this section of the pathway, a useful test for characterizing the multiple aromatic mutants isolated (see below). Irradiation with ultraviolet light was followed by penicillin selection with glucose–mineral salts medium plus tryptophan as 'minimal medium', with the addition of tyrosine, phenylalanine, 4-aminobenzoic acid and 4-hydroxybenzoic acid as required to demonstrate growth requirements. From this experiment a mutant requiring both tryptophan and tyrosine (strain 61–3) was isolated. This strain [unable to carry out reactions (1) and (2) of Scheme 1] was used for a further irradiation and selection with glucose–mineral salts medium plus tryptophan and tyrosine as 'minimal medium' and growth factors as for the preceding experiment. After this irradiation a number of multiple aromatic mutants were isolated. These could have been blocked in either reaction (3) or (3*a*) of Scheme 1 as a result of the last mutation, or by a single mutation anywhere along the sequence of intermediates common to the synthesis of the aromatic compounds. By testing for the accumulation of anthranilic acid by the various strains grown in limiting indole medium it was possible to detect those strains that were blocked in the common pathway. A combination of cross-feeding tests (Gibson & Jones, 1954) and nutritional



Scheme 2. Various multiple aromatic mutants isolated in a penicillin selection experiment.

tests allowed the different strains to be classified according to their metabolic lesions. The different strains isolated in this experiment are shown in Scheme 2. Only three of the strains (62–1, 62–2 and 62–10) accumulated anthranilic acid and were presumably blocked in reactions (3) or (3*a*) of Scheme 1. These three strains also differed from the others in that they did not appear to have an absolute growth requirement for phenylalanine. In growth tests on solid media, growth was merely delayed a few hours on medium without phenylalanine. This lack of an absolute requirement for phenylalanine could have reflected a partial block in these strains or the accumulation of an unstable intermediate such as prephenic acid. Strain 62–1 was chosen for further study and cell extracts were prepared.

Formation of a new substrate for anthranilic acid synthesis. The crude cell extract of strain 62–1 was incubated with the mixture of substrates for the conversion of shikimic acid into anthranilic acid with and without glutamine. Assay for anthranilic acid showed (Fig. 1) that anthranilic acid was formed in the presence of glutamine but that in the absence of glutamine the typical rising spectrum of the phenylpyruvic acids (Morgan *et al.* 1963) was not seen. Therefore strain 62–1 appeared to have the third metabolic lesion in the region expected since extracts from organisms such as strain T17 convert shikimic acid into the phenylpyruvic acids in the absence of glutamine (Morgan *et al.* 1963).

Previous experiments (Gibson *et al.* 1962) had shown that it was possible, by using extracts of suitable mutants and the appropriate substrates, to synthesize anthranilic acid in two stages. During incubation of the first stage a compound with the properties of 3-enolpyruvylshikimic acid 5-phosphate was formed. After destruction of the enzyme by heating, bacterial extracts of a different

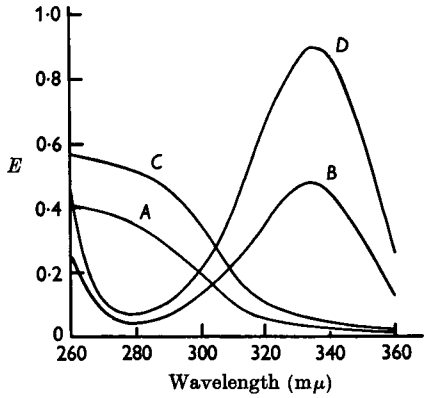


Fig. 1. Metabolism of shikimic acid by a cell extract of *A. aerogenes* strain 62–1. An extract of cells of strain 62–1 grown in limiting tryptophan (2 mg./l.) was incubated in the following mixture: shikimic acid (1 mM); ATP (1 mM); NAD (1 mM); $MgCl_2$ (5 mM); ribose 5-phosphate (2·5 mM); tris–HCl buffer, pH 8·2 (50 mM). L-Glutamine (5 mM) was added where indicated. The final volume of each mixture was 2 ml. At 30 min. and 60 min., 1 ml. samples were acidified with 0·1 ml. of N-HCl. After extraction with 4 ml. of ethyl acetate the organic layer was incubated overnight at 30°. Spectra were then examined. *A*, After incubation for 30 min. without glutamine; *B*, after incubation for 30 min. with glutamine; *C*, after incubation for 60 min. without glutamine; *D*, after incubation for 60 min. with glutamine. Spectra *B* and *D* are similar to the spectrum of synthetic anthranilic acid.

auxotroph were used to convert the ether into anthranilic acid. A similar technique was used to look for any new substrate for anthranilic acid formation which strain 62–1 might have been able to form. In this experiment the first stage was carried out with strain 62–1 in the absence of glutamine. After heating of the first stage, an

extract of strain 170–44, which was blocked after 3-enolpyruvylshikimic acid 5-phosphate, was added together with glutamine. After further incubation the test was assayed for anthranilic acid. A small amount of anthranilic acid was formed, indicating that strain 62–1 was capable of forming a substrate for anthranilic acid synthesis. As the substrate was converted into anthranilic acid by strain 170–44, it was possibly the compound *X* of Scheme 1.

It was observed in the experiment of Fig. 1 that in the ethyl acetate extract from the tube without glutamine there was some absorption in the 270 mμ region not present in the tube in which anthranilic acid was formed. Such absorption was not previously found in various experiments on anthranilic acid synthesis and it was thought possible that the substrate for anthranilic acid synthesis might be extractable into ethyl acetate. Accordingly, an extract of strain 62–1 was incubated with the appropriate substrate, and then the reaction mixture was acidified and extracted with ethyl acetate. The ethyl acetate layer was evaporated to dryness at room temperature under vacuum and the residue taken up in tris–hydrochloric acid buffer and incubated with an extract of strain 170–44 and glutamine. Samples were taken at zero time and after incubation. The spectra of the ethyl acetate layers obtained in the anthranilic acid assay are shown in Fig. 2. The material absorbing in the region of 270 mμ disappeared during incubation with glutamine and the yield of anthranilic acid was much higher than in the two-stage experiment, suggesting that the substrate was heat-labile and extractable into ethyl acetate.

The apparent new substrate (chorismic acid) for anthranilic acid synthesis was examined by paper chromatography of ethyl acetate concentrates. Briefly, the material absorbing at 272 mμ when chromatographed in neutral solvents, could be detected by the *p*-nitroaniline reagent (Smith, 1960; p. 297), and when eluted was active as a substrate for anthranilic acid synthesis.

Assay of chorismic acid. Chorismic acid was readily assayed by conversion into anthranilic acid by using dialysed cell extracts of strain 62–1. The general method used is described above. An experiment in which the rate of conversion was measured is given in Fig. 3. Barium ions did not inhibit the reaction.

Concurrent formation of anthranilic acid and pyruvic acid. If an enolpyruvic acid residue is present in chorismic acid (Gibson, 1964) one of the reaction products during anthranilic acid formation should be pyruvic acid. An experiment was carried out in which anthranilic acid was formed by an extract of strain 170–44 under the conditions described for Fig. 2. Assay for anthranilic acid and for pyruvic acid showed that 0·26 μmole of anthranilic acid was formed together with 0·27 μmole of pyruvic acid measured by the toluene-extraction method of Friedemann & Haugen (1943).

Chromatography of 2,4-dinitrophenylhydrazones of keto acids in butan-1-ol–ethanol–water (7:1:2, by vol.) showed that the keto acid formed during anthranilic acid synthesis was indistinguishable from synthetic pyruvic acid.

Chorismic acid as a metabolic precursor of the phenylpyruvic acids. If chorismic acid were in fact the compound *X* of Scheme 1 then it should be a precursor of the phenylpyruvic acids. This possibility was examined by the general techniques described by Morgan *et al.* (1963). Extracts of strain 170–44, when incubated with a solution of chorismic acid in 50 mM-tris–hydrochloric acid buffer, pH 7–8, or 50 mM-sodium phosphate buffer, pH 7·8, readily form phenylpyruvic acids. Chromatography showed the presence of both phenylpyruvic acid and 4-hydroxyphenylpyruvic acid, and this finding was supported by the absorption spectra (Morgan *et al.* 1963).

Identity of the substrate for anthranilic acid formation with that for phenylpyruvic acid formation. The new substrate was incubated in the absence of glutamine with an extract of strain 170–44 for various times to allow phenylpyruvic acid formation, then glutamine was added and the mixture further incubated to allow anthranilic acid forma-

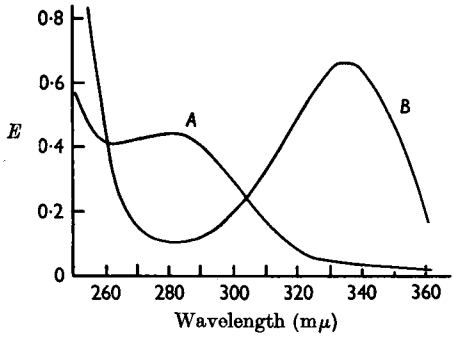


Fig. 2. Extraction of chorismic acid into ethyl acetate. A dialysed extract of *A. aerogenes* strain 62–1 (2·3 mg. of protein/ml.) was incubated at 37° in a final volume of 4 ml. of 50 mM-tris–HCl buffer, pH 8·2, with the mixture of substrates given for Fig. 1. After 45 min. 0·4 ml. of N-HCl was added and the solution extracted with 20 ml. of ethyl acetate. The organic layer was separated, and 5 ml. was taken to measure spectrum *A* and 14 ml. evaporated to dryness at room temperature under vacuum. The residue was taken up immediately in 0·5 ml. of the following mixture: tris–HCl buffer, pH 8·2 (50 mM); L-glutamine (5 mM); crude extract of strain 170–44 (2 mg. of protein/ml.). After incubation for 1 hr. at 37°, 0·1 ml. of N-HCl was added, the reaction mixture was extracted with 4 ml. of ethyl acetate and the spectrum of the organic layer was measured (*B*).

tion. With an extract that had been derepressed along the tryptophan pathway the addition of glutamine allowed most of the remaining substrate to be converted into anthranilic acid. The results are shown in Table 2. The amount of substrate available for anthranilic acid synthesis decreases after the formation of phenylpyruvic acid. This experiment also illustrates the removal of phenylpyruvic acids by cell extracts previously noted by Morgan *et al.* (1962).

Conversion of chorismic acid into prephenic acid. Cell extracts prepared from wild-type *A. aerogenes* were tested for the presence of enzymes capable of synthesizing prephenic acid, and thence phenylpyruvic acid, from the intermediate (see Scheme 1). An extract, dialysed to prevent prephenate-dehydrogenase activity which would convert prephenic acid into 4-hydroxyphenylpyruvic acid, was incubated with chorismic acid with and without phenylalanine. As phenylalanine inhibits the activity of the prephenate-aromatizing enzyme which gives phenylpyruvic acids (C. H. Doy, personal communication), this could be used to prevent utilization of prephenic acid. The results in Fig. 4 show that wild-type *A. aerogenes* does appear to contain both enzymes. Phenylalanine inhibits phenylpyruvic acid formation and promotes the accumulation of prephenic acid. Strain 62–1 does not form prephenic acid or phenylpyruvic acid from chorismic acid but can readily convert prephenic acid into phenylpyruvic acid. In these experiments controls without enzyme extract were necessary to measure the relatively small amounts of prephenic acid formed by the non-enzymic conversion of chorismic acid.

Chorismic acid as a metabolic precursor of 4-hydroxybenzoic acid. By prolonged incubation of strain 62–1 with chorismic acid itself or with the reaction mixture for the formation of chorismic acid from shikimic acid, 4-hydroxybenzoic acid could be detected by spectroscopy and chromatography. As the latter compound is also formed chemically from chorismic acid (Gibson, 1964), suitable controls were necessary. The enzymic formation of 4-hydroxybenzoic acid is stimulated by NAD but the enzyme(s) concerned have yet to be studied in detail.

Release of chorismic acid by whole cells. It was found that whole cells of strain 62–1 either as cultures or used as washed-cell suspensions would release the new substrate into the suspending medium. The results of an experiment in which the

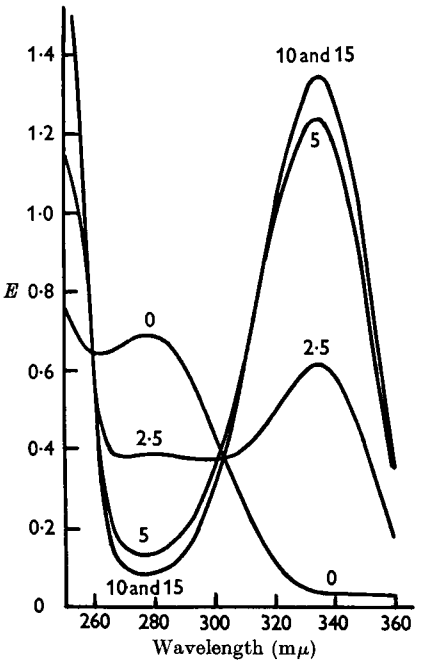


Fig. 3. Rate of conversion of chorismic acid into anthranilic acid. Chorismic acid (1 mM) in 50 mM-tris–HCl buffer, pH 8·2, was incubated with L-glutamine (5 mM) and dialysed cell extract of *A. aerogenes* strain 62–1 (2 2 mg. of protein/ml.). The total volume of the reaction mixture was 7 ml. At the intervals indicated (in minutes) 1 ml. samples were acidified with 0·1 ml. of N-HCl and extracted with 4 ml. of ethyl acetate. The organic phase was dried with anhydrous Na_2SO_4 and the spectra were then measured.

Table 2. *Identity of substrate for the formation of phenylpyruvic acids and anthranilic acid*

The new intermediate, chorismic acid, was prepared as described in Fig. 2. A sample in 3·6 ml. of 100 mM-tris–HCl buffer, pH 8·2, was incubated at 37° with 0·23 ml. of 50 mM-NAD and 1·3 ml. of strain 170–44 crude extract (16 mg. of protein/ml.). Portions (0·3 ml.) of the incubation mixture were taken at the times shown (stage 1) and transferred to 0·1 ml. of N-HCl for the assay of phenylpyruvic acids by method (*a*) (described in the Experimental section) and to 0·1 ml. of 50 mM-L-glutamine to allow conversion of the remaining chorismic acid into anthranilic acid. This test mixture (stage 2) was incubated at 37° for 20 min. and assayed for anthranilic acid by method (*a*) (described in the Experimental section) by acidification and ethyl acetate extraction. The spectra were measured immediately and after incubation overnight at 30°. The treatment of known concentrations of synthetic phenylpyruvic acids allowed determination by measurement of extinction at 288 mμ.

Time of incubation in stage 1 (min.)	Phenylpyruvic acids formed in stage 1 (μmole)	Anthranilic acid formed in stage 2 (μmole)
0	0·06	0·31
10	0·31	0·18
30	0·22	0·06
60	0·06	0 06

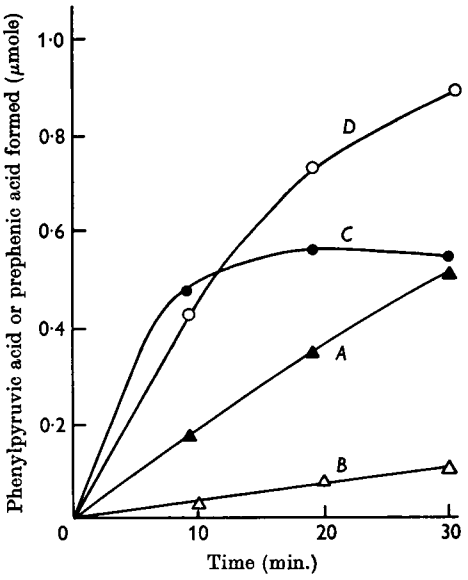


Fig. 4. Formation of phenylpyruvic acid and prephenic acid by wild-type *A. aerogenes*. The barium salt of chorismic acid (1·3 mM) was incubated at 37° in 50 mM-tris–HCl buffer, pH 8·2, with a crude extract of *A. aerogenes* NCW (1 mg. of protein/ml.) with and without L-phenylalanine (2 mM). The final volume of each mixture was 4 ml. Samples were taken at intervals for prephenic acid and phenylpyruvic acid assays. *A*, Phenylpyruvic acid formed; *B*, phenylpyruvic acid formed in the presence of phenylalanine; *C*, prephenic acid formed; *D*, prephenic acid formed in the presence of phenylalanine.

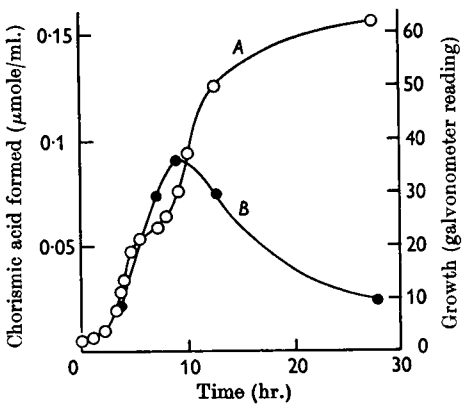


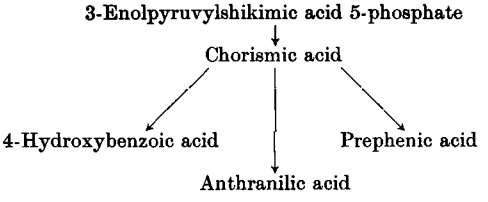
Fig. 5. Growth of *A. aerogenes* strain 62–1 on limiting phenylalanine and the accumulation of chorismic acid. To 200 ml. of glucose–mineral salts medium supplemented with L-tryptophan (0·1 mM), L-tyrosine (0·1 mM) and limiting L-phenylalanine (25 μM) were added cells from a nutrient–agar slope of strain 62–1 to give an initial population of about 2×10^6 cells/ml. The culture was incubated at 37° and aerated through a sintered-glass filter. Turbidity was measured at intervals and assays for chorismic acid were carried out by acidification and extraction with ether. The ether was evaporated off over tris–HCl buffer and the chorismic acid assayed by conversion into anthranilic acid. *A*, Growth; *B*, concentration of chorismic acid formed.

cells were grown in a limiting phenylalanine medium are shown in Fig. 5. After exhaustion of the phenylalanine there is only a short period of delay before growth is resumed, probably as a result of chemical conversion of chorismic acid into phenylpyruvic acid. For the maximum production of chorismic acid cell suspensions were used, and the details are given by Gibson (1964). For accumulation by whole cells of strain 62–1 it is important that excess of tryptophan is present throughout the experiment to repress the formation of the enzyme(s) that convert chorismic acid into anthranilic acid.

Effect of tryptophan on anthranilic acid formation. As the conversion of chorismic acid into anthranilic acid is the most likely point of action of tryptophan in end-product inhibition, the effect of tryptophan and related amino acids on this conversion was tested. The results of one such experiment in which anthranilic acid formation was followed fluorimetrically are shown in Fig. 6. The results indicate that L-tryptophan is a potent inhibitor whereas phenylalanine and tyrosine at higher concentrations do not inhibit the reaction.

DISCUSSION

From these experiments it appears that cell extracts and whole cells of strain 62–1 are capable of forming a new intermediate which is a precursor for several aromatic compounds. As whole cells of auxotrophs often accumulate more than one substance on a biosynthetic pathway it was possible that we were dealing with a mixture of substrates. However, the evidence (see also Gibson, 1964) leaves little doubt that we are dealing with a substance that can probably be metabolized by several enzymes leading to different pathways. For this reason we propose the trivial name 'chorismic acid' (chorismic = separating) for the new intermediate. It would seem that chorismic acid occupies a position in the pathway of synthesis as follows:



Whether each of the reactions shown is in fact a single step is unknown. The ready chemical conversion of chorismic acid into 4-hydroxybenzoic

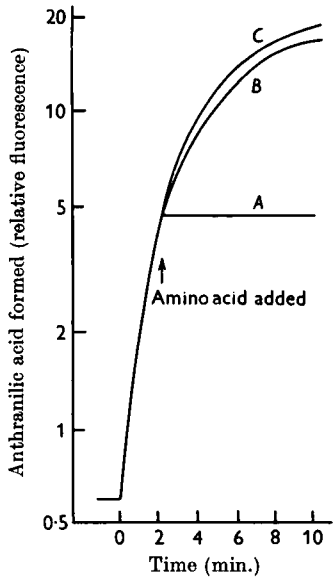


Fig. 6. Effect of aromatic amino acids on the conversion of chorismic acid into anthranilic acid. To crude extract of *A. aerogenes* strain 170–44 (0·15 mg. of protein/ml.) in 50 mM-tris–HCl buffer, pH 8·2, containing L-glutamine (2·5 mM) was added chorismic acid (0·05 μmole) at zero time. The fluorescence of anthranilic acid formed was measured at 30 sec. intervals, and amino acids were added at the time indicated by the arrow. *A*, After the addition of L-tryptophan (0·01 mM); *B*, after the addition of L-phenylalanine (0·2 mM) and no addition; *C*, after the addition of L-tyrosine (0·2 mM). Separate tubes with a final volume of 1 ml. were used for each test.

acid and prephenic acid might suggest single enzymic steps. By mixing cell extracts of several mutants unable to convert chorismic acid into anthranilic acid it has not been possible to detect more than one class of mutant in this sequence. It was predicted (Gibson *et al.* 1962) that 4-aminobenzoic acid should also be formed from chorismic acid. Preliminary experiments have not indicated such a conversion but we still consider that it is likely and that it may be shown by a more detailed examination of the problem. Auxotrophs blocked in the region of tryptophan biosynthesis just before anthranilic acid release various phenolic compounds (Pittard *et al.* 1961; Pittard, Gibson & Doy, 1962). These compounds have been detected on paper chromatograms during the present experiments but not enough is known yet to determine their position in the scheme given above.

The suggested position of the third metabolic block in strain 62–1 is confirmed by the observation that, though lacking the ability to convert chorismic acid into prephenic acid, this strain has the enzyme that converts prephenic acid into phenylpyruvic acid. Both enzymes are present in wild-type cells grown under similar conditions.

The conversion of chorismic acid through prephenic acid into phenylpyruvic acid by an extract of wild-type *A. aerogenes* is noteworthy because of the marked accumulation of prephenic acid in the absence of phenylalanine. This accumulation has been noticed with auxotrophs that can carry out both reactions, and on further incubation prephenic acid disappears to be replaced by phenylpyruvic acid. One factor responsible for this accumulation in the absence of phenylalanine may be that the condition under which the cells were grown (excess of phenylalanine) repressed the formation of the prephenate-aromatizing enzyme. Also, the removal of NAD by dialysis stops one of the reactions (to 4-hydroxyphenylpyruvic acid) for which prephenic acid is normally a substrate.

The isolation of chorismic acid has made possible the study of several new enzymes. No experimental evidence is available yet as to the number of enzymes involved in the various steps, and it would be unwise to assign names to any of these enzymes until such evidence has been obtained.

End-product control of anthranilic acid formation has been described previously (Moyed, 1960; Morgan *et al.* 1962, 1963). These experiments involved a number of steps in aromatic synthesis and the precise point of action of tryptophan was not defined. The present experiments provide more definite evidence than available hitherto that tryptophan inhibits the first reaction specific to the tryptophan pathway.

The chemical structure of chorismic acid and its chemical relationship to other aromatic precursors is discussed by Gibson (1964).

SUMMARY

1. A new intermediate in aromatic synthesis has been sought and found. The trivial name 'chorismic acid' is suggested for this compound.

2. The isolation of the auxotroph of *Aerobacter aerogenes* which accumulates this compound is described.

3. Chorismic acid has been shown to be converted enzymically into anthranilic acid, prephenic acid, phenylpyruvic acid, 4-hydroxyphenylpyruvic acid and 4-hydroxybenzoic acid.

4. Chorismic acid is formed by cell extracts from shikimic acid and is also accumulated by whole cells.

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Chorismic Acid: Purification and some Chemical and Physical Studies

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A multiply blocked auxotroph of *Aerobacter aerogenes* (strain 62–1) has been isolated which forms a new compound, chorismic acid, from which several aromatic compounds may be formed enzymically. Preliminary studies on the metabolism of chorismic acid have been described (Gibson & Gibson, 1962, 1964), and the present paper is concerned with the preparation and isolation of chorismic acid and various observations leading to the elucidation of its structure. The conversion of chorismic acid non-enzymically into several known products of metabolism has also been studied. Some of this work has been briefly reported (Gibson & Gibson, 1962).

MATERIALS AND METHODS

Organism. The multiply blocked aromatic auxotroph of *A. aerogenes* (strain 62–1) has been described by Gibson & Gibson (1964). It was maintained by serial subculture on nutrient–agar slopes. Methods for the preparation of cell extracts and determination of protein have been given by Morgan, Gibson & Gibson (1963).

Media used for accumulation of chorismate. Medium A (for growth of cells). To 20 ml. of the mineral salts base described by Vogel & Bonner (1956) was added 40 ml. of 5% (w/v) Difco yeast extract, 40 ml. of 5% (w/v) Difco casein hydrolysate and 41 mg. of DL-tryptophan. The volume was made up to 1 l. and the mixture autoclaved at 15 lb./in.^{2} for 15 min. To the sterile solution was added 10 ml. of sterile 16% (w/v) glucose solution.

Medium B (for accumulation of chorismic acid). This consisted of the following reagents made up in 1 l. of water: Na_2HPO_4, 12·8 g.; KH_2PO_4, 1·36 g.; glucose, 18 g.; NH_4Cl, 2·7 g.; $MgCl_2$, 2 ml. of 50 mM solution; L-tryptophan, 1 ml. of 10 mM solution. The mixture (final pH 7·8) was not sterilized.

Chromatography. The solvents and methods were as described by Pittard, Gibson & Doy (1961).

Spectroscopy. Ultraviolet spectroscopy was carried out with a Cary model 11 recording spectrophotometer with quartz cells of 1 cm. light-path. Infrared spectroscopy was carried out with a Perkin–Elmer Infracord with KCl disks.

Assay for prephenic acid. This was as described by Gibson & Gibson (1964) and depends on the conversion of prephenic acid into phenylpyruvic acid under acid conditions and subsequent measurements of the 320 mμ peak of the latter compound in alkali. In all experiments the spectra were measured at least from 290 mμ to 340 mμ.

Assay for phenylpyruvic acid. Method (*b*) given by Gibson & Gibson (1964) was used.

Chemicals. The chemicals used were the purest obtainable commercially. They were not further purified. The Dowex 1 (X4) resin used was in the Cl^- form and 100–200 mesh. The buffer solutions were as described by Dawson, Elliott, Elliott & Jones (1959).

Analyses. Microanalyses were carried out by the Australian Microanalytical Service, Chemistry Department, University of Melbourne.