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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.

EXPERIMENTAL AND RESULTS

The chorismic acid used in the experiments described below was obtained in various ways. In the initial experiments solutions in organic solvents were obtained after the formation of the acid by cell extracts of strain 62–1. Experimental samples were acidified and extracted with ether or ethyl acetate. Further experiments were carried out with material obtained from ether extracts after accumulation by whole cells, and the most recent ones with barium chorismate. An experiment illustrating the isolation of barium chorismate by the best method yet available is described below and the sources of the chorismic acid for the various experiments are indicated in the text.

Isolation and analysis of barium chorismate. To each of two 1 l. volumes of medium A was added the growth from a nutrient–agar slope of strain 62–1. The cultures were incubated with shaking at 30° for 6 hr. giving a culture with an extinction at 620 mμ of 1·3. The cultures were refrigerated overnight after which the cells were centrifuged and washed once with 1 l. of 0·9 % sodium chloride. The cells were then resuspended in 2 l. of the accumulation medium B and incubated at 30° with shaking for 12 hr., and the mixture was refrigerated overnight. The cells were centrifuged, and the spectrum of the supernatant diluted 1:10 in 0·1 N-sodium hydroxide showed a peak at 274 mμ with an extinction of 1·07. After the addition of 5 ml. of 10 N-sodium hydroxide/l. the supernatant was passed under pressure through a column (2·7 cm. × 7 cm.; 25 g.) of Dowex 1 (Cl^- form). The flow rate was adjusted to about 15 ml./min. and after all the supernatant had passed through the column it was washed with 100 ml. of water. The bulk of the supernatant was held in an ice bath during the operation. The chorismic acid was then eluted with M-ammonium chloride at a rate of 8 ml./5 min. and 8 ml. fractions were collected. Fig. 1 shows the spectra of the fractions (diluted 1:300) measured against water blanks. Tube 13 showed a bright-yellow fluorescence and was discarded. Fractions 14–19 were pooled and 5 ml. of 2 M-barium acetate was added. Then 4 vol. of ethanol was added with stirring and the resulting heavy precipitate spun off. The deposit was taken up in 20 ml. of water and centrifuged, the brown deposit being discarded. To the supernatant was added 100 ml. of methanol. The precipitate was sedimented, washed with 50 ml. of methanol and then with ether, and dried over phosphorus pentoxide *in vacuo*. The yield of barium salt was 800 mg. [Found: C, 28·1; H, 3·2; Ba, 33·4. Calc. for $C_{10}H_8BaO_6,3H_2O$: C, 28·9; H, 3·4; Ba, 33·1. Loss of weight by drying at 60° (P_2O_5 *in vacuo*): 8·8 %].

Chromatography. Chorismic acid can be readily detected on paper chromatograms by using the *p*-nitroaniline reagent used usually for phenolic compounds (Smith, 1960; p. 297). Separation from breakdown products giving colours with this reagent (4-hydroxybenzoic acid and phenylpyruvic acid) was achieved by using the benzene–acetic acid–water solvent described by Smith (1960; p. 292). For successful elution of chorismic acid possessing activity as a substrate for anthranilic acid synthesis it was necessary to use a neutral solvent such as methanol–butan-1-ol–benzene–water (Mason & Berg, 1951). Such solvents, however, do not give good separations from contaminating compounds giving phenolic reactions. The mobilities and reactions of some of the compounds encountered in the present experiments are given in Table 1.

Ultraviolet-absorption spectroscopy of chorismic acid. Chorismic acid has an $\epsilon_{max.}$ at 272 mμ in aqueous solution (see Fig. 1) which shifts only slightly with change of pH. In ether the $\epsilon_{max.}$ is at 282 mμ.

As the barium chorismate isolated contained water and possibly other impurities it was necessary to determine the extinction coefficient indirectly. This has been done in two ways. The first was the conversion of chorismic acid into anthranilic acid enzymically and the second was its chemical conversion into a mixture of phenylpyruvic acid and 4-hydroxybenzoic acid. Both procedures gave

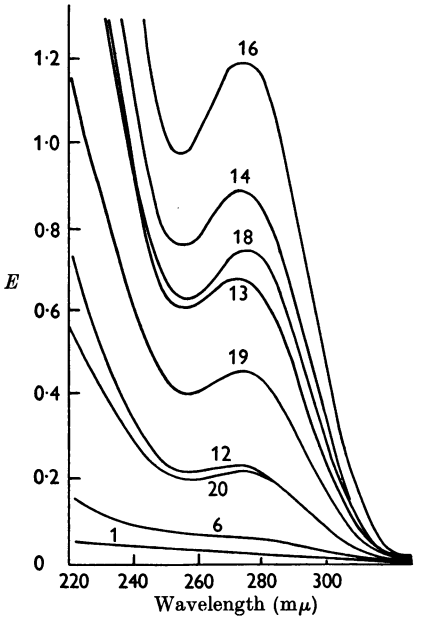


Fig. 1. Spectra of eluates (1:300 dilution in water) from the column during the purification of chorismic acid (see text for details). Fraction numbers are indicated.

Table 1. *Mobilities and colour reactions of chorismic acid and some related compounds on paper chromatograms*

	Chorismic acid	4-Hydroxybenzoic acid	Phenylpyruvic acid	4-Hydroxyphenylpyruvic acid
R_F in benzene–acetic acid–water*	0·52	0·81	0·96	0·43
R_F in methanol–butan-1-ol–benzene–water†	0·89	0·83	—	—
Colour with *p*-nitroaniline reagent‡	Yellow turning red within a few minutes	Cherry red	Yellow	Yellow-brown

* See Smith (1960; p. 292). † See Mason & Berg (1951). ‡ See Smith (1960; p. 297).

100% conversions. As the extinction coefficients of the end products are known the extinction coefficient for barium chorismate could be calculated. The values obtained were 2700 by the first method and 2800 by the second.

Comparison of infrared-absorption spectra of chorismic acid and prephenic acid. Chorismate is readily converted into prephenate by warming (see below and Gibson & Gibson, 1964). To 50 mg. of barium chorismate in 0·5 ml. of water was added 2 N-ammonia to bring the pH to about 8·5. The solution was heated for 1½ hr. at 70° and prephenate assays were carried out to check the progress of the reaction. The heated solution was cooled in an ice bath, methanol (1 ml.) and propan-1-ol (1·5 ml.) were added and the resulting precipitate was centrifuged off. Most of the precipitate was dissolved in 0·5 ml. of water, 0·45 ml. of methanol was then added and the precipitate was discarded. To the supernatant was added 2 ml. of methanol and the resulting precipitate was centrifuged, washed with 3 ml. of ethanol then 3 ml. of ether, and dried *in vacuo* over phosphorus pentoxide. The yield was 14 mg. of a white powder that was shown spectroscopically to be readily converted by acid into phenylpyruvic acid and that was free of 4-hydroxybenzoic acid, the other known breakdown product of chorismic acid. The infrared-absorption spectra of barium chorismate and barium prephenate are compared in Fig. 2.

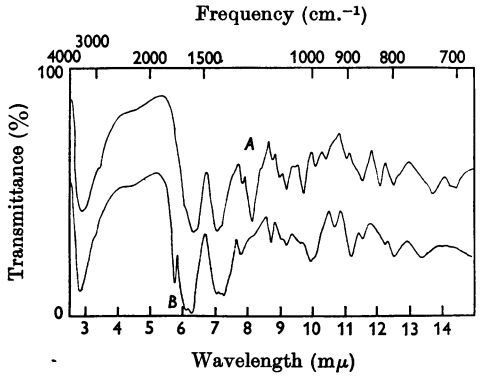


Fig. 2. Infrared-absorption spectra of barium chorismate (upper curve) and barium prephenate (lower curve). The significance of peaks *A* and *B* is discussed in the text.

Conversion of chorismic acid into phenylpyruvic acid and 4-hydroxybenzoic acid. In preliminary experiments on the conversion of shikimic acid into chorismic acid and then into anthranilic acid (Gibson & Gibson, 1964) it was noticed that heating after the first stage gave spectra typical of phenylpyruvic acid (Morgan *et al.* 1963) when assays were carried out for anthranilic acid by extraction into ethyl acetate. This observation was extended and it was found that heating at 100° under acid conditions gave a mixture of phenylpyruvic acid and 4-hydroxybenzoic acid. The spectra of the ether extracts of a reaction mixture containing chorismic acid before and after heating in acid were compared with those of phenylpyruvic acid and 4-hydroxybenzoic acid, and a mixture of the two, treated in the same way (Fig. 3). The presence of phenylpyruvic acid and 4-hydroxybenzoic acid and the disappearance of chorismic acid was confirmed by paper chromatography in benzene–acetic acid–water solvent (Smith, 1960; p. 292).

Conversion of chorismic acid into prephenic acid. When an accumulation mixture, from which the cells had been removed, was stored for a week at −15°, the small amount of chorismic acid present disappeared and a substance with the properties of prephenic acid appeared. This observation suggested that chorismic acid was readily converted into prephenic acid. Prephenate together with 4-hydroxybenzoate is formed when solutions of chorismate are heated at an alkaline pH. At pH values less than 6 the prephenate formed is converted into phenylpyruvic acid. The conversion of chorismic acid into prephenic acid is appreciable at 37°, which is of importance in the study of the accumulation of chorismic acid by cells (Table 2). The conditions under which the conversion has normally been carried out is at pH 8 in 50 mM-sodium phosphate buffer for 1 hr. at 70°. The rate of conversion of chorismate to prephenate is shown in Fig. 4. As chorismic acid is not stable to acid

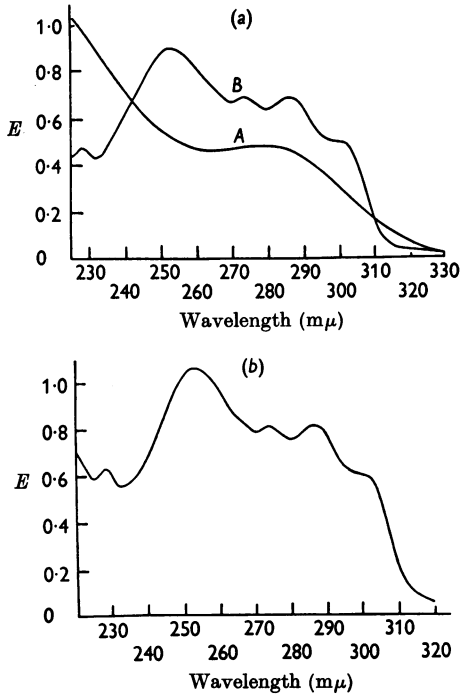


Fig. 3. Formation of phenylpyruvic acid and 4-hydroxybenzoic acid from chorismic acid. Chorismic acid was formed in two tubes containing an extract of strain 62–1 (2 mg. of protein/ml.) in 50 mM-tris–HCl buffer, pH 8·2, in the following mixture of substrates: shikimic acid (1 μmole); ATP (1 μmole); NAD (1 μmole); $MgCl_2$ (5 μmoles); ribose 5-phosphate (3 μmoles). The final volume in each tube was 1 ml. After incubation for 1 hr. at 37°, 0·1 ml. of N-HCl was added to both tubes and one tube placed in a boiling-water bath for 3 min. Both tubes were extracted with 4 ml. of ethyl acetate and the spectra were measured (Fig. 3*a*). *A*, Unheated; *B*, heated. A mixture of 0·6 μmole of phenylpyruvic acid and 0·2 μmole of 4-hydroxybenzoic acid was heated, extracted as above and the spectrum measured (Fig. 3*b*). A number of different mixtures of the acids was used. The spectrum of the above mixture closely resembled that given by the treated chorismic acid.

Table 2. *Conversion of chorismic acid into prephenic acid at 37°*

Chorismic acid, obtained from ethereal extracts of accumulation by whole cells, was incubated at 37° in 5 ml. (final vol.) of 50 mM-sodium phosphate buffer, pH 8·0. Samples were taken at intervals for phenylpyruvic acid assay and chorismic acid assay. The latter was assayed by extraction into ether and subsequent conversion into anthranilic acid.

Time (hr.)	Concn. of product (μmole/ml.) Chorismic acid	Prephenic acid
0	0·96	0·1
4½	0·52	0·31
24	0·10	0·87

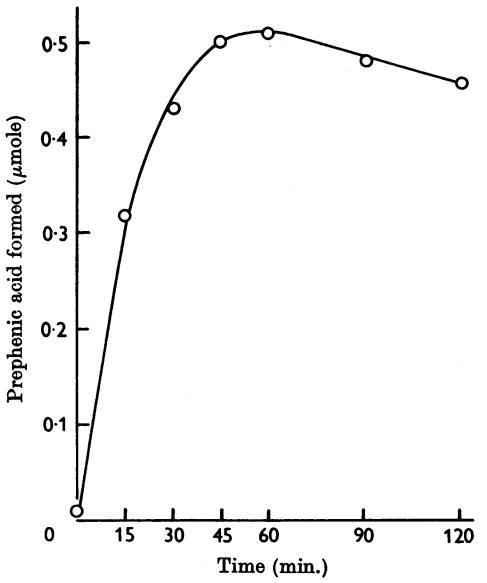


Fig. 4. Rate of conversion of chorismic acid into prephenic acid at 70°. Chorismic acid was prepared as described in Fig. 3 except that the final volume was 10 ml. After extraction with ether the chorismic acid was transferred to 100 mM-sodium phosphate buffer, pH 8·0. The solution was heated at 70° and samples for prephenic acid assays were taken at intervals.

at 37° it was necessary to re-examine the conditions for prephenic acid assays. In the initial experiments the method of Metzenberg & Mitchell (1956) was used, but this involved incubation for 2 hr. in acid solution, a time which seemed long when the short half-life described for the compound is considered. Accordingly assays for phenylpyruvic acid were carried out during acid treatment of chorismic acid as for the prephenic acid assay and on the same sample of chorismic acid after it had been heated at 70° for 1 hr. and converted into prephenic acid (Fig. 5). The formation of phenylpyruvic acid from chorismic acid is much slower than from the compound provisionally identified as prephenic acid. In subsequent experiments the time of acid treatment was decreased to 10 min.

DISCUSSION

Previous studies on chorismic acid have shown it to be a labile compound converted enzymically and chemically into various compounds found on the pathway of biosynthesis of aromatic compounds

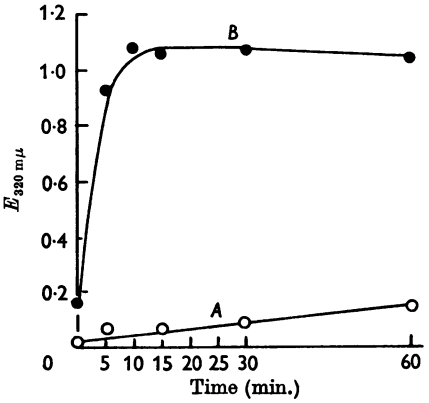
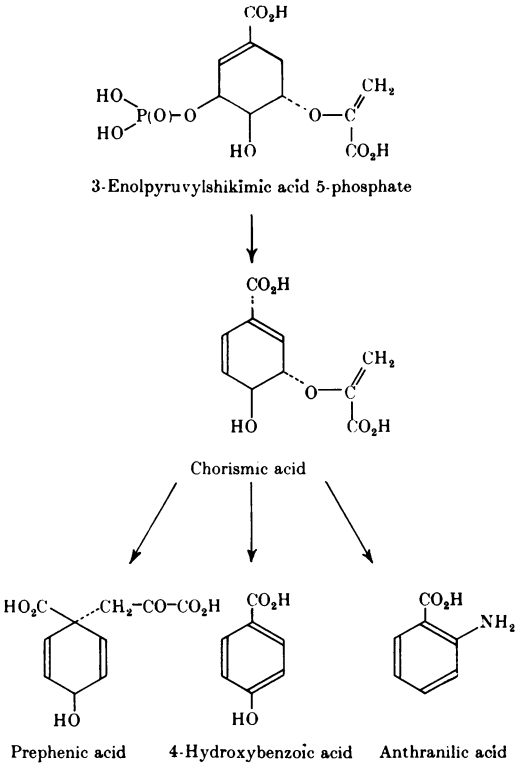


Fig. 5. Rate of phenylpyruvic acid production from chorismic acid and prephenic acid by acid treatment. Part of a solution of barium chorismate (0·77 mM) in 100 mM-sodium phosphate buffer, pH 8·0, was heated at 70° for 1 hr. to give prephenate solution. The chorismate and prephenate solutions were then incubated with an equal volume of N-HCl for various times, alkali was added as for the prephenate assay, and spectra between 250 mμ and 340 mμ were measured as soon as possible. *A*, Chorismic acid; *B*, prephenic acid.



Scheme 1

(Gibson & Gibson, 1962, 1964). The present work shows that barium chorismate may be readily obtained provided that washed cell suspensions are used to accumulate the acid (see also Gibson & Gibson, 1964). The lability of chorismic acid under physiological conditions means that prolonged experiments with an organism accumulating chorismic acid would result in the formation of prephenic acid. With strain 62–1 the presence of tryptophan during the accumulation is essential to repress the formation of enzyme(s) converting chorismic acid into anthranilic acid. The ability of strain 62–1 to form such enzymes is useful, however, because extracts of cells grown under conditions of limiting tryptophan may be used to assay chorismic acid by conversion into anthranilic acid. However, if extracts of strain 62–1 are used, any 3-enolpyruvylshikimic acid 5-phosphate present will also yield anthranilic acid in the experimental system used. This can be avoided by using *A. aerogenes* 170–44 (Gibson & Gibson, 1964) but the assay gives a low result possibly due to diversion of chorismic acid along the prephenic acid pathway.

Chorismic acid is converted by mild heating under alkaline conditions into prephenic acid, which is recognized by its conversion into phenylpyruvic acid enzymically or by acid treatment. The lability of chorismic acid means that the preparation and purification of derivatives is difficult and therefore studies on structure have been largely dependent on spectroscopic evidence. The presence of an absorption peak at 274 mμ with an extinction coefficient of about 2700 is consistent with a cyclohexadienecarboxylic acid structure. Comparison of the infrared-absorption spectra gives a clear indication that an enol ether band at 1221 cm.$^{-1}$ (peak *A* in Fig. 2) is present in barium chorismate, and that this band disappears on conversion into prephenate and that the expected band due to a keto group at 1730 cm.$^{-1}$ (peak *B* in Fig. 2) (Bellamy, 1959) appears. Other peaks which could be attributed to $\rangle C{=}CH_2$ (e.g. 694 cm.$^{-1}$) also disappear during the conversion. The evidence therefore suggested a conjugated hexadiene with the carbon skeleton of 3-enolpyruvylshikimic acid 5-phosphate. Tests for phosphate on barium chorismate (Feigl, 1960) were negative. Elimination of phosphoric acid from 3-enolpyruvylshikimic acid 5-phosphate could lead to a structure for chorismic acid (Scheme 1). The relative stereochemistry is deduced from that of shikimic acid and prephenic acid (Plieninger, 1962). A model of such a structure shows that $C_{(3)}$ of the enolpyruvic acid side chain lies in close proximity to $C_{(1)}$ of the ring which would presumably facilitate conversion into prephenic acid. Direct confirma-

tion of the structure shown for chorismic acid was obtained by examination of a deuterium oxide solution of barium chorismate by nuclear-magnetic-resonance spectroscopy (Gibson & Jackman, 1963). Chorismic acid can be formulated as the 3-enol-pyruvic ether of *trans*-3,4-dihydroxycyclohexa-1,5-dienecarboxylic acid.

SUMMARY

1. Chorismic acid, a new intermediate in aromatic biosynthesis, is the 3-enolpyruvic ether of trans-3,4-dihydroxycyclohexa-1,5-dienecarboxylic acid.

2. Although chorismic acid itself is a fairly labile compound the barium salt is readily isolated and has been examined.

3. The ultraviolet- and infrared-absorption spectra of chorismate are given together with details of chromatography.

4. Chorismate is readily converted, by warming, into a mixture of prephenate and 4-hydroxybenzoate. Under acid conditions the prephenic acid is further converted into phenylpyruvic acid.

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Ketohexokinase, Isoenzymes of Glucokinase and Glycogen Synthesis from Hexoses in Neonatal Rat Liver

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The activities of many enzymes in the liver are known to change during foetal and post-natal development of the mammal, and such changes reflect important alterations in the physiological functions of the liver. The urea cycle (Kennan & Cohen, 1959), the glucuronide-conjugating system (Brown & Zeulzer, 1958) and the activities of several respiratory enzymes (Dawkins, 1959) have been measured during liver development. In previous reports (Ballard & Oliver, 1962, 1963) evidence was presented for the view that mechanisms of glyconeogenesis develop in the rat liver only after birth of the animal. These results showed that the foetal liver is solely dependent upon blood hexose for the synthesis of its glycogen store.

In the present work, the utilization of glucose, fructose and galactose for the synthesis of liver glycogen has been investigated during neonatal development in the rat. To this end, the incorporation of ^{14}C-labelled hexoses into hepatic glycogen and the activities of the liver enzymes, ATP–D-fructose 1-phosphotransferase (ketohexokinase, EC 2.7.1.3) and ATP–D-glucose 6-phosphotransferase (glucokinase, EC 2.7.1.2) have been measured. The results obtained in the glucokinase assays suggested the existence of different forms of the enzyme. The Michaelis kinetics and the variation of liver-glucokinase activity with pH indicate the existence of two forms of the enzyme, one found in both the foetal and the adult liver and a second