The Shikimate Pathway. Part V.[1] Chorismic Acid and Chorismate Mutase

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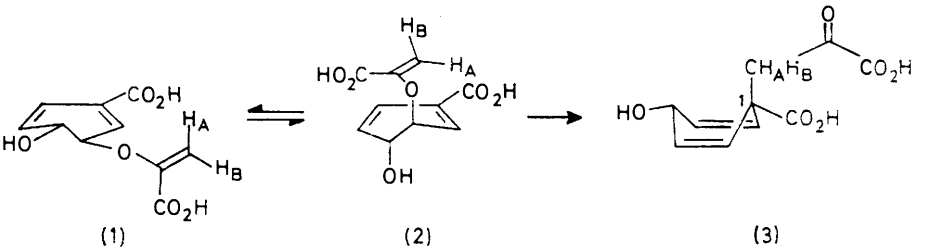
The chemistry of the biochemical intermediate chorismic acid {*trans*-3-[(1-carboxyvinyl)oxy]-4-hydroxycyclohexa-1,5-diene-1-carboxylic acid} (1) is discussed, including the formation of the fused pyrazoline (6) with diazomethane, the dihydro-derivatives (8) and (9) with di-imide, and the epoxide (10). The mode of action of the enzyme chorismate mutase (EC 1.3.1.12), which transforms chorismic acid into prephenic acid (3), is of importance in relation to general theories of enzyme action. The reaction is the sole example in primary metabolism of an enzyme-catalysed *ortho*-Claisen rearrangement, and attempts to elucidate the stereochemical features of this molecular reorganisation are outlined.

CHORISMIC ACID (1) is the first branch point intermediate in the shikimate pathway of aromatic amino-acid metabolism.[2] In addition to the synthesis of *p*-hydroxybenzoic acid and *p*-aminobenzoic acid, it may be diverted to L-tryptophan synthesis by the action of anthranilate synthetase,[3] or to L-phenylalanine and L-tyrosine synthesis by the intramolecular rearrangement to prephenic acid (3) catalysed by chorismate mutase.[4] This latter enzyme has been obtained from several organisms of widely different phylogenetic type and a range of properties relating to control by feed back inhibition, occurrence of isoenzymes, and multi-enzyme complexes, has been described.[4-8] The rearrangement of chorismic acid to prephenic acid is formally analogous to an *ortho*-Claisen transformation, and in addition to the enzyme-catalysed process the same reaction may also be brought about by heating aqueous solutions of chorismic acid (although the product then obtained is normally phenylpyruvic acid, which is the decarboxylation product of prephenic acid). Using the turnover number derived from the maximum velocity of the enzyme-catalysed reaction, Andrews, Smith, and Young[9] have calculated that chorismate mutase from *Aerobacter aerogenes* enhances the rate of reaction at pH 7.5 and 37 °C by a factor of 1.9×10^6 in comparison with the spontaneous thermal rearrangement in the absence of enzyme. It is generally agreed[10,11] that a chair-type conformation of the interacting groups is preferred for sigmatropic rearrangements of this type, although this selectivity can readily be over-ridden by steric constraints, and on the basis of this observation the reaction pathway from chorismic acid (1) to prephenic acid (3) probably involves a sequence such as (1) ⇌ (2) → (3). Implicit in this suggestion is the concept that before rearrangement the substrate must first undergo an inversion of conformation of the ring and that the enol-pyruvyl side chain must be oriented correctly. It follows that the role of the enzyme chorismate mutase is most reasonably explained in terms of Pauling's original concept,[12] that the structure of the active site of the enzyme is complementary to that of the substrate in the conformation (2) and the subsequent transition state which resembles (2), and that all the intrinsic binding energy is used to stabilise this intermediate and thus decrease the activation energy for the reaction.[13] The chorismic to prephenic acid change nevertheless remains the only authenticated example of an enzyme-catalysed Claisen rearrangement in *primary* metabolism and it therefore was of interest to discover whether the *in vivo* enzyme-catalysed and *in vitro* thermal rearrangement proceed with identical stereospecificity and whether nature has taken advantage of the lower energy transition state analogous to (2).

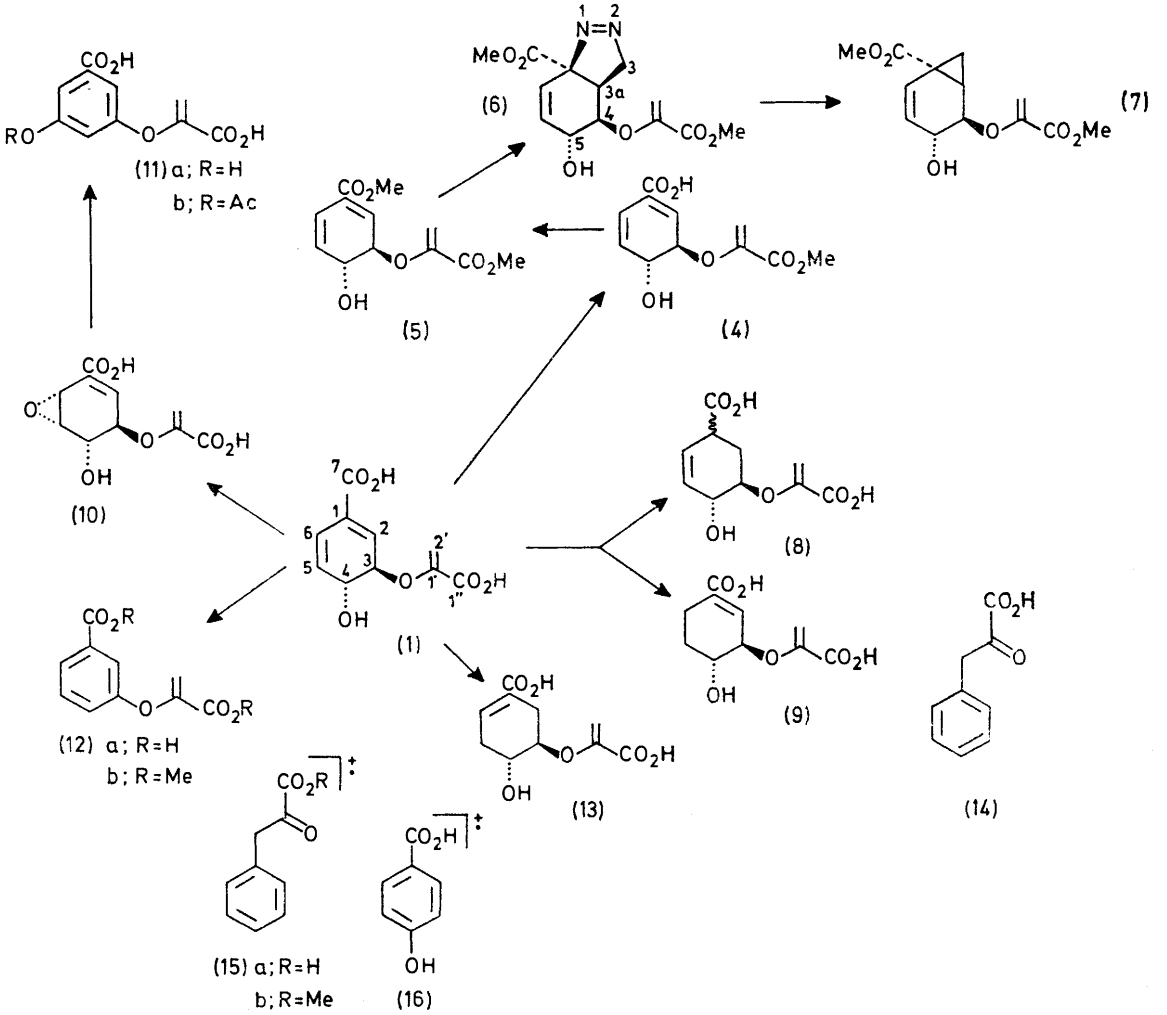
As a part of this study some chemical reactions of chorismic acid have been studied (Scheme 1). *Aerobacter aerogenes* 62—1 is a triple auxotroph which lacks both P and T chorismate mutase and *N*-phosphoribosylanthranilate synthetase activities, and a preparation of chorismic acid from glucose using a cell suspension of this mutant organism has been described.[14,15] Some improvements have been made to this preparation which have resulted in consistent and improved yields of chorismic acid; in particular the accumulation stage has been carried out under controlled aeration and at constant pH. Although Jackman and Edwards[16] have shown that catalytic reduction of (1) gives the 1,4-addition product (13), few chemical reactions of chorismic acid have been described, presumably because of its ready aromatisation.[2,11,16-19] Treatment of chorismic acid (1) with di-imide at 0—5 °C gave a mixture of the

[1] Part IV, M. J. Turner, B. W. Smith, and E. Haslam, *J. Chem. Soc.* 1975, 52.
[2] F. Gibson and J. Pittard, *Bacteriol. Rev.*, 1968, **32**, 465; E. Haslam, 'The Shikimate Pathway,' Butterworths, London, 1974.
[3] H. Zalkin and E. J. Henderson, *J. Biol. Chem.*, 1970, **245**, 3810.
[4] G. L. E. Koch, D. C. Shaw, and F. Gibson, *Biochim. Biophys. Acta*, 1970, **212**, 375, 387; 1971, **229**, 795, 805.
[5] H. Gorisch and F. Lingens, *Biochemistry*, 1974, **18**, 3790.
[6] J. C. Schmit and H. Zalkin, *J. Biol. Chem.*, 1970, **245**, 4019; 1971, **246**, 6002.
[7] L. G. H. Cotton and F. Gibson, *Biochim. Biophys. Acta*, 1967, **147**, 222; 1965, **100**, 76.
[8] T. I. Baker, *Biochemistry*, 1966, **5**, 2654.
[9] P. R. Andrews, G. D. Smith, and I. G. Young, *Biochemistry*, 1973, **12**, 3492.
[10] M. J. S. Dewar, *Angew. Chem. Internat. Edn.*, 1971, **10**, 761.
[11] W. von E. Doering and W. R. Roth, *Tetrahedron*, 1962, **18**, 67; 1963, **19**, 715.
[12] L. Pauling, *Scientific American*, 1948, **36**, 51.
[13] A. R. Fersht, *Proc. Roy. Soc.* 1974, **B**, **187**, 397.
[14] M. I. Gibson and F. Gibson, *Biochem. J.*, 1964, **90**, 248.
[15] F. Gibson, *Biochem. Prep.*, 1968, **12**, 94.
[16] L. M. Jackman and J. M. Edwards, *Austral. J. Chem.*, 1965, **18**, 1227.
[17] I. G. Young, F. Gibson, and C. G. MacDonald, *Biochim. Biophys. Acta*, 1969, **192**, 62.
[18] F. Gibson, *Biochem. J.*, 1964, **90**, 256.
[19] F. Lingens and G. Müller, *Naturwiss.*, 1967, **54**, 492.

dihydro-derivatives (8) and (9) in which (9) predominated. The ratio of products obtained was however sensitive to temperature; thus at −15 °C only (9) was isolable but at room temperature the major product was hydroxybenzoic acids in acid solution. Paper chromatographic analysis also showed that (9) formed some phenylpyruvic acid (14) on heating in acid. Treatment of chorismic acid (1) at −78 °C with diazomethane gave



SCHEME 1



SCHEME 2 (a) shikimate kinase; (b) 5-enolpyruvylshikimate 3-phosphate synthetase; (c) chorismate synthetase

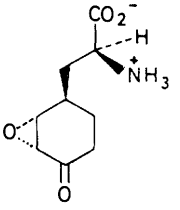
(8). The broad absorption at δ 3.36 in the ^{1}H n.m.r. spectrum of (8) due to the proton adjacent to the carboxy-group suggested that this compound was a mixture of epimers at this position. Neither reduction product [(8) or (9)] was as readily aromatised as chorismic acid itself, although both gave a mixture of 3- and 4-

two products, isolated and characterised as the pyrazoline (6) and the cyclopropane derivative (7). The Δ^1-structure was assigned to the pyrazoline on the basis of ^{1}H n.m.r. absorptions due to the C-3 methylene group at δ 4.84 and 4.38 (J_{gem} 18.0; $J_{3,3a}$ 9.5 Hz), and the presence of a weak u.v. absorption at 335 nm (ε 150) and an i.r.

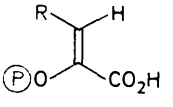
band at 1 540 cm^{-1} (-N=N-).[20,21] The problem of stereochemistry was solved by the ^1H n.m.r. spectra of the pyrazoline and its acetate, which showed $J_{4,5}$ and $J_{3a,4}$ as 7.5—8.0 and 4.5—5.0 Hz, respectively. This is consistent with addition of the diazomethane to the upper face of the molecule, leading to an equatorial–axial–quasiaxial arrangement of H-3a, H-4, and H-5 and $J_{3a,4} < J_{4,5}$. At elevated temperatures pyrazolines lose nitrogen to give a mixture of olefins and cyclopropane derivatives; in accord with this in benzene under reflux (20 min) the pyrazoline (6) was converted quantitatively into the cyclopropane (7), which was thermally stable. This observation was corroborated by the fact that the pyrazoline (6) showed no molecular ion in the mass spectrum but gave an intense peak at $M - 28$ (loss of nitrogen). Limited exposure at −78 °C (5 min) of chorismic acid to diazomethane gave the pyrazoline (6) in high yield (80%), and this conversion was later employed as a means of isolation, purification, and analysis of chorismic acid from biochemical experiments. It is interesting to compare this smooth highly stereoselective formation of (6) from (1) with the slow formation of a mixture of pyrazolines from the related metabolite, shikimic acid.[22] Both mono- and di-methyl esters of chorismic acid, (4) and (5), were derived by the action of diazomethane at −78°. The structure of the monomethyl ester (4) followed from mass spectral analysis and comparison with that of chorismic acid. Jackman and Edwards [16] proposed two principal modes of fragmentation of chorismic acid, one *via* the phenylpyruvate ion (15a) (*m/e* 164) and the other *via* the *p*-hydroxybenzoate ion (16) (*m/e* 138). In the mass spectrum of the monomethyl ester the ion (16) was still present whereas (15a) was absent and replaced by (15b) (*m/e* 178). Methylation of chorismic acid with methyl iodide–silver oxide led to aromatisation and formation of the ester (12b).[23]

Chorismic acid (1) was smoothly transformed into the epoxide (10) with *m*-chloroperbenzoic acid. The ^1H n.m.r. spectrum of (10) did not permit an unequivocal assignment of stereochemistry but the orientation shown was made on the basis of the analogy with the epoxidation of other allylic alcohols, in which it has been demonstrated that the configuration of the hydroxy-group frequently determines the preferential stereochemical mode of attack on the double bond.[24,25] Acetylation (pyridine–acetic anhydride) of the epoxide (10) gave a quantitative yield of (11b), and similar treatment of chorismic acid (1) gave the acid (12a). Treatment of the epoxide (10) with base gave 3-(1-carboxyvinyloxy)-5-hydroxybenzoic acid (11a), whose structure followed from its spectral characteristics and its conversion with acid into 3,5-dihydroxybenzoic acid. The ready conversion of chorismic acid into the epoxide (10) *in vitro* suggests that a similar transformation may be possible *in vivo*, and this may be of significance in the biosynthesis of certain natural products. Thus for example the biosynthesis of anticaps in (17), the unusual *C*-terminal amino-acid of the dipeptide bacilysin isolated from *Bacillus subtilis*, has been shown to involve shikimic acid but not L-tyrosine as an intermediate,[26-29] and a scheme of biosynthesis *via* the epoxide (10) may now be envisaged.

The stereochemistry of prephenic acid (3) at C-1 has been previously determined,[2] and this established that the new σ-bond formed in the molecular rearrangement from chorismic acid takes place with participation of the enol methylene group from the β-face of the molecule [(1) ⇌ (2) ⟶ (3)]. However, in order to study the stereospecificity of the transformation, as it involves the carbon atom of the enolpyruvyl methylene group of (1), a prerequisite is to devise methods to replace stereospecifically one of the methylene protons (H_A and H_B) with an isotope of hydrogen and to analyse the stereochemical disposition of this same atom in the corresponding methylene group of the product (14). The analytical problem may be solved by use of the plant enzyme L-phenylalanine ammonia lyase, which stereospecifically eliminates ammonia from L-phenylalanine to give *trans*-cinnamic acid.[30,31] Thus chorismate mutase T from *A. aerogenes* poly 3, in the absence of an oxidising co-factor, converted chorismic acid (1)



(17)



(22) R = F
(23) R = Me
Ⓟ O = phosphate

into prephenic acid (3), which was 'trapped' by reduction with sodium borohydride to give (*RS*)-2-phenyllactic acid. When this reaction was carried out in deuterium oxide no incorporation of deuterium into the product was observed. The phenyl-lactic acid was then converted into DL-phenylalanine by standard procedures.

Initially it was hoped to use the biochemical synthesis

[20] C. A. Jarboe, in 'Heterocyclic Compounds,' ed. R. H. Elderfield, Wiley, New York, 1967, p. 177.
[21] J. A. Moore and R. W. Medeiras, *J. Amer. Chem. Soc.*, 1959, **81**, 6026.
[22] J. F. W. Keana and C. U. Kim, *J. Org. Chem.*, 1970, **35**, 1093.
[23] F. Lingens and B. Sprössler, *Annalen*, 1967, **709**, 173.
[24] H. O. House, 'Modern Synthetic Reactions,' Benjamin, California, 1972, p. 115.
[25] P. Chamberlain, M. L. Roberts, and G. H. Whitham, *J. Chem. Soc.* (*B*), 1970, 1374.
[26] H. J. Rogers, G. G. F. Newton, and E. P. Abraham, *Biochem. J.*, 1965, **97**, 573.
[27] H. J. Rogers, N. Lomakina, and E. P. Abraham, *Biochem. J.*, 1965, **97**, 579.
[28] J. E. Walker and E. P. Abraham, *Biochem. J.*, 1970, **118**, 557, 663.
[29] N. Neuss, B. B. Molloy, R. Shah, and N. De Lattiguera, *Biochem. J.*, 1970, **118**, 571.
[30] R. H. Wightman, J. Staunton, A. R. Battersby, and K. R. Hanson, *J.C.S. Perkin I*, 1972, 2355.
[31] R. Ife and E. Haslam, *J. Chem. Soc.* (*C*), 1971, 2818.

of chorismic acid (1) from shikimic acid and stereospecifically labelled phosphoenolpyruvate to synthesise samples of (1) in which one of the protons in the methylene group was labelled stereospecifically. The key enzyme in the biochemical synthesis of chorismic acid (1) is that which catalyses the attachment of the enolpyruvyl side chain to shikimic acid 3-phosphate (18) and for which Sprinson and Levin [32] had earlier proposed an addition–elimination mechanism, a more detailed version of which is shown in Scheme 2.

An effective enzymic method for the conversion of shikimic acid, ATP, and phosphoenolpyruvate into chorismic acid (1) was developed on the basis of the earlier work of Gibson,[14] and the product was isolated after reactions in tritiated water and in deuterium oxide. Analysis of both the free acid (1) and the pyrazoline (6) showed that the average incorporation of tritium from several experiments was 0.54 atom. Treatment of the acid (1) (^3H : ^{14}C 13.2 : 1) with acetic anhydride–pyridine and then acid gave 3-hydroxybenzoic acid (^3H : ^{14}C 0.15 : 1), demonstrating that incorporation of the isotope was limited to replacement of the protons in the enolpyruvyl group. In deuterium oxide the chorismic acid (1) formed was shown by ^1H n.m.r. analysis to incorporate the deuterium equally into both positions of the same enol methylene group. Mass spectrometric and ^1H n.m.r. analysis of the pyrazoline (6) showed that the relative incorporation of the isotope increased as the time scale of the experiment increased, and in a typical experiment the product contained 1.8 atom of deuterium after 1 h incubation. When dideuteriophosphoenolpyruvate (>90% enrichment of the methylene protons with deuterium) was utilised as substrate in ^1H_2O media the incorporation of ^1H into the methylene group of chorismic acid (1) was again time dependent (1.15 atom ^1H after 0.25 h; 1.50 atom ^1H after 1 h), but in this case the extent of isotopic incorporation did not approach equilibrium with the medium. These observations supported and extended similar results obtained during the course of this work by Sprinson and his collaborators [33] on the enzymic synthesis of 5-enolpyruvylshikimate 3-phosphate (20). Both sets of experimental data support the suggested mechanism for the mode of action of the enzyme 5-enolpyruvylshikimate 3-phosphate synthetase and they show that one or more of the steps involved in the formation or decomposition of the intermediate (21) are subject to significant kinetic isotope effects. They also suggest that partitioning of the intermediates [(19) ⇌ (21)] takes place preferentially towards the product (20).

Iron(II) inhibits chorismate synthetase activity,[34] and with the mixed enzyme system from *A. aerogenes* 62–1 inhibition was 50% at 5×10^{-3}M- and 70% at 10^{-2}M-iron(II). However in a parallel series of experiments with phosphoenolpyruvate or dideuteriophosphoenolpyruvate as substrate and water, tritiated water, or deuterium oxide as the medium this decrease in chorismate synthetase activity in the presence of 10^{-1}M-iron(II) did not lead to any significant differences in the level of incorporation of the appropriate isotope (^3H,

[32] D. B. Sprinson and J. G. Levin, *J. Biol. Chem.*, 1964, **239**, 1142.

[33] W. E. Bondinell, J. Vnek, P. F. Knowles, M. Sprecher, and D. B. Sprinson, *J. Biol. Chem.*, 1971, **246**, 6191.

[34] M. Morrell, M. J. Clark, P. F. Knowles, and D. B. Sprinson, *J. Biol. Chem.*, 1967, **242**, 82.

^2H, or ^1H) into chorismic acid (1) in comparison with the experiments described earlier. Thus although Levin and Sprinson [32] originally demonstrated the reversibility of the enzymic synthesis of (20) from (18), these observations suggest that under the conditions employed little equilibration between (18) and (20) took place.

Stubbe and Kenyon [35,36] have described the synthesis and use of (*Z*)-3-fluoro- and (*Z*)-3-methylphosphoenolpyruvate [(22) and (23)] to determine stereochemical features of the pyruvate kinase reaction. Both substrates (22) and (23) were investigated as substrates for the enzyme 5-enolpyruvylshikimate 3-phosphate synthetase with the possibility that the increased steric size due to substitution of fluorine or methyl for hydrogen might prevent free rotation in the intermediates analogous to (19) ⇌ (21) and thus to stereoselectivity in the enzymic synthesis. However both substrates were inactive in the standard assay procedure. When (*Z*)-3-fluorophosphoenolpyruvate (22) was used in ten-fold excess some incorporation was observed and in a large scale preparation chorismic acid was isolated and identified after conversion into the pyrazoline (6). The ^1H n.m.r. spectrum of (22) showed the presence of up to 5% of unfluorinated material. Repeated recrystallisation failed to remove this contaminant, which was shown to arise (g.l.c. analysis) from traces of ethyl acetate in the commercially available ethyl fluoroacetate. In the light of these observations it may be desirable to reinvestigate the position of (22) as a pseudo-substrate for the pyruvate kinase reaction,[36] since the observed rate of reaction was only 0.23% of that of phosphoenolpyruvate.

If the activity of the enzyme chorismate mutase derives from its ability to stabilise the conformation (2) and the subsequent transition state which resembles (2) in its geometry with respect to the ground state conformation (1) of chorismic acid, then some information on the nature of the binding at the enzyme active site may be derived by the use of structural analogues of the normal substrate. Chorismate mutase T was obtained from the multiple aromatic auxotroph *A. aerogenes* poly 3,[4] and the derivatives (4), (8), (9), and (10) were tested as substrate analogues. None of these compounds, however, displayed any tendency to rearrange with the enzyme. The inability of the enzyme to catalyse rearrangement of the monomethyl ester (4) suggested that at least one (and possibly both) of the carboxylate groups of (1) was involved in binding to the active site. However various monocarboxylic acids (0.3—3.0mM concentration in the assay procedure)—acetic, quinic, and shikimic acids and glycine—and various di- and tri-carboxylic acids—malonic, succinic, glutaric, adipic, glutamic, citric, maleic, and 3-carboxyvinyloxybenzoic acids—showed no capacity to inhibit chorismate mutase T activity. The structural analogues monomethyl chorismate (4) and 1,2-dihydrochorismic acid (8) were similarly inactive as inhibitors of chorismate mutase T activity, but the epoxide (10) and 5,6-dihydrochorismic acid (9) showed kinetic behaviour typical of that of competitive reversible inhibitors. Thus (9) and (10) gave K_i values of 4.6 and 6.9 $\times 10^{-3}$M, respectively, to be compared with the K_m value of 1.2×10^{-3}M obtained for chorismic acid, and both, like chorismic acid itself, protected chorismate mutase T against irreversible inhibition by iodoacetamide [37] and acetylimidazole. The implication of these results in relation to the binding of the substrate (1) at the active site is that the presence of the 1,2-double bond and the carboxy-group of the enolpyruvyl side chain are important factors in this process. In addition the inability of the enzyme to effect any detectable rearrangement of the derivatives (9) and (10), although these bind to the active site, may be an indication that binding of the hydroxy-group at C-4 also takes place in the transition state complex.

Further work is in progress to establish the stereochemical features of this enzymic transformation.

EXPERIMENTAL

Chorismic Acid.—Medium A (3 × 1 l) [15] was inoculated with *Aerobacter aerogenes* 62–1 (1 ml of L broth containing a 20 h culture) and incubated at 30 °C for 6 h. The cell growth was removed by centrifugation (5 000 g; 10 min; 5 °C) and resuspended in medium B[15] (3 l) in a NBS microfermentor. The solution was stirred and aerated (500 rev. min^{-1}; 2 l air min^{-1}) at 30 °C and the pH was monitored and maintained constant by automatic titration (0.1N-sodium hydroxide). The accumulation of chorismic acid in the medium was followed by taking samples and measuring the absorbance at 274 nm after removing the cells by centrifugation and dilution (× 25; 0.1N-sodium hydroxide). After 14 h the cells were removed (5 000*g*; 10 min; 5 °C) and the supernatant was applied under pressure to an Amberlite CG400 column (Cl^- form; 50 g; 15 × 3.5 cm), held at 5 °C in a cold room, at *ca.* 15 ml min^{-1}. The column was washed with water (150 ml) and eluted (2 ml min^{-1}) with ammonium chloride solution (1M; pH 8.5). Fractions (10 ml) were collected and those with an absorbance (after 300 × dilution) greater than 0.1 at 274 nm were combined, acidified (2N-hydrochloric acid), and extracted with ether. The extract was dried ($MgSO_4$) and evaporated to *ca.* 30 ml. To this solution was added an equal volume of light petroleum (b.p. 60—80 °C) and the whole was cooled to −78 °C. The crude product was filtered off and recrystallised without heating from ethyl acetate–light petroleum (b.p. 60—80 °C) to give chorismic acid as needles (1.6 g), m.p. 147—148° (decomp.) [lit.,[15] 148—149° (decomp.)] (Found: C, 49.0; H, 5.3. Calc. for $C_{10}H_{10}O_6,H_2O$: C, 49.2; H, 5.0%); δ_H [$(CD_3)_2CO$; 100 MHz] 6.90 (1 H, m), 6.31 (1 H, dt, *J* 10.0, 2.5, and 1.5 Hz), 6.01 (1 H, dd, *J* 10.0 and 2.5 Hz), 5.47 (1 H, d, *J* 3.0 Hz), 4.91 (1 H, d, *J* 3.0 Hz), 5.02 (1 H, dd, *J* 3.0 Hz), and 4.70 (1 H, dt, *J* 11.5, 2.5, and 2.5 Hz); δ_C [$(CD_3)_2CO$; 25.15 MHz] 70.5 and 81.2 (C-3 and -4), 97.0 (C-2′), 121.9, 133.6, and 134.1 (C-2, -5, and -6), 130.0 (C-1), 150.3 (C-1′), and 164.4 and 165.9 (C-1″ and -7).

[35] J. A. Stubbe and G. L. Kenyon, *Biochemistry*, 1971, **10**, 2669.

[36] J. A. Stubbe and G. L. Kenyon, *Biochemistry*, 1972, **11**, 339.

[37] G. L. E. Koch, D. C. Shaw, and F. Gibson, *Biochim. Biophys. Acta*, 1972, **258**, 1719.

Decomposition of Chorismic Acid and its Derivatives.—Chorismic acid or its derivative (5 mg) was dissolved in the solution under examination (1 ml) and heated at 100 °C for 0.25 h. The solution was cooled to room temperature and extracted with ethyl acetate (2 × 20 ml) after acidification to pH 1.0. The organic extract was examined by paper chromatography on Whatman No. 2 paper in system A [benzene–acetic acid–water (125 : 72 : 3)] or B [sodium formate (5% w/v) in water–formic acid (200 : 1)]. Chromatograms were dried overnight at room temperature, examined under u.v. light, and then sprayed with diazotised *p*-nitroaniline [38] or Gibbs reagent.[39] R_F Values in solvent systems A and B and the colour reaction with diazotised *p*-nitroaniline are as follows: chorismic acid, 0.38, 0.90, yellow to red; 5,6-epoxychorismic acid, 0.24, 0.94, brown; 2,5-dihydroxybenzoic acid, 0.35, 0.94, brown; 2,3-dihydroxybenzoic acid, 0.35, 0.94, brown; 3-(1-carboxyvinyloxy)benzoic acid, 0.79, 0.86, pink; 3,4-dihydro-3,4-dihydroxybenzoic acid 0.25, 0.85, orange to brown; phenylpyruvic acid, 0.85, 0.85, yellow; 2-hydroxyphenylpyruvic acid, 0.88, 0.53, brown; 3-hydroxyphenylpyruvic acid, 0.29, 0.76, yellow to brown; 4-hydroxyphenylpyruvic acid, 0.39, 0.84, yellow to brown; 4-hydroxyphenyl-lactic acid, 0.21, 0.69, purple; 3-hydroxybenzoic acid 0.66, 0.71, orange to red; 4-hydroxybenzoic acid 0.66, 0.63, red to purple; 3,5-dihydroxybenzoic acid, 0.20, 0.63, yellow to orange; 3-hydroxy-5-(1-carboxyvinyloxy)benzoic acid, 0.30, 0.80, orange to red.

Acetylation of Chorismic Acid.—A solution of the acid (0.09 g) in pyridine (1 ml) containing acetic anhydride (1 ml) after 12 h at room temperature gave 3-(1-carboxyvinyl)benzoic acid (0.08 g), m.p. 179—180° (from methanol–ether) (lit.,[17] 180—181°) (Found: C, 57.4; H, 3.6%; $M^{+\cdot}$, 208.054. Calc. for $C_{10}H_8O_5$: C, 57.7; H, 3.8%; *M*, 208.079); δ_H [$(CD_3)_2CO$; 100 MHz] 5.21 (1 H, d, *J* 2.0 Hz), 5.87 (1 H, d, *J* 2.0 Hz), 7.06 (1 H, dq, *J* 8.0, 2.0, and 2.0 Hz), 7.62 (1 H, dd, *J* 2.0 and 2.0 Hz), 7.47 (1 H, t, *J* 8.0 and 8.0 Hz), 7.77 (1 H, 2t, *J* 8.0, 2.0, and 2.0 Hz), and 9.46 (2 H, m).

Methylation of Chorismic Acid.—Silver oxide (1.80 g) was added to a stirred solution of chorismic acid (0.7 g) in dimethylformamide (20 ml) containing methyl iodide (3.7 g) and the mixture was left at room temperature for 48 h. The silver salts were removed by filtration and the product isolated by preparative t.l.c. (R_F 0.9 on silica in ethyl acetate–ethanol, 3 : 2) as an oil (0.3 g), b.p. 140 °C at 0.6 mmHg (lit.,[23] 123° at 0.15 mmHg) (Found: C, 61.1; H, 5.2%; $M^{+\cdot}$ 236.0830. Calc. for $C_{12}H_{12}O_5$: C, 61.0; H, 5.1%% *M*, 236.0785). Methyl 3-(1-methoxycarbonylvinyl)benzoate showed δ_H ($CDCl_3$; 100 MHz) 3.81 (3 H, s), δ 3.90 (3 H, s), 5.81 and 5.01 (two 1 H, d, *J* 2.0 Hz), 7.24 (1 H, m, *J* 8.0, 2.0, and 2.0 Hz), 7.41 (1 H, t, *J* 8.0 Hz), 7.70 (1 H, m, *J* 2.0, 2.0, and 1.0 Hz), and 7.82 (1 H, m, *J* 8.0, 2.0, and 2.0 Hz), $\nu_{max.}$ (film) 1 740 and 1 725 cm^{-1}.

Reaction of Chorismic Acid with Diazomethane.—(*a*) Chorismic acid (0.12 g) in ether (5 ml) was treated at −78 °C for 3 h with an excess of ethereal diazomethane (*ca.* 9 mmol). Removal of the solvent gave a gum which was separated by preparative t.l.c. (silica in 99 : 1 chloroform–methanol) into two components, R_F 0.30 and 0.48. The former crystallised from methanol–ether to give *methyl* 3a,4,5,7a-*tetrahydro*-5α-*hydroxy*-4β-(1-*methoxycarbonylvinyloxy*-3H-*indazole*-7aα-*carboxylate* (6) as needles (0.06 g), m.p. 85° (decomp.) (Found: C, 49.9; H, 5.8; N, 8.9. $C_{13}H_{16}N_2O_6,H_2O$ requires C, 49.7; H, 5.7; N, 8.9%); δ_H ($CDCl_3$; 100 MHz) 3.17 (1 H, td, *J* 9.5, 9.0, and 4.0 Hz), 3.80 and 3.89 (two 3 H, s), 4.36 (2 H, m), 4.38 (1 H, dd, *J* 18.0 and 9.5 Hz), 4.86 (1 H, dd, *J* 18.0 and 9.0 Hz), 5.02 (1 H, d, *J* 3.0 Hz), 5.51 (1 H, d, *J* 3.0 Hz), 5.81 (1 H, dd, *J* 10.0 and 1.5 Hz), and 6.20 (1 H, d, *J* 10.0 Hz), $\nu_{max.}$ (Nujol) 1 730 and 1 623 cm^{-1}. The *acetate*, prepared in acetic anhydride–pyridine, crystallised from ethyl acetate–light petroleum (b.p. 60—80 °C) as needles, m.p. 56—58° (Found: C, 53.3; H, 5.4; N, 8.5. $C_{15}H_{18}N_2O_7$ requires C, 53.3; H, 5.3; N, 8.3%); δ_H ($CDCl_3$; 100 MHz) 6.29 (1 H, dd, *J* 10.0 and 2.0 Hz), 5.74 (1 H, dd, *J* 10.0 and 3.0 Hz), 5.50 (1 H, d, *J* 2.5 Hz), 5.42 (1 H, dt, *J* 7.5, 3.0, and 2.0 Hz), 4.97 (1 H, d, *J* 2.5 Hz), 4.90 (1 H, dd, *J* 18.0 and 9.0 Hz), 4.52 (1 H, dd, *J* 7.5 and 5.2 Hz), 4.51 (1 H, dd, *J* 18.0 and 8.3 Hz), 3.86 (3 H, s), 3.75 (3 H, s), 3.14 (1 H, td, *J* 9.0, 8.3, and 5.2 Hz), and 2.03 (3 H, s); $\nu_{max.}$ (Nujol) 1 730 and 1 620 cm^{-1}.

The less polar component, *methyl* 4α-*hydroxy*-5β-(1-*methoxycarbonylvinyloxy*)*norcar*-2-*ene*-1α-*carboxylate* (7) was obtained as a gum (0.03 g) (Found: C, 58.3; H, 5.9%; $M^{+\cdot}$, 268.0924. $C_{13}H_{16}O_6$ requires C, 58.2; H, 5.9%; *M*, 268.0946); δ_H ($CDCl_3$; 100 MHz) 1.21 (1 H, dd, *J* 7.0 and 4.5 Hz), 1.61 (1 H, dd, *J* 9.0 and 4.5 Hz), 2.34 (1 H, m), 3.70 (3 H, s), 3.80 (3 H, s), 5.02 (1 H, d, *J* 3.0 Hz), 5.49 (1 H, d, *J* 10.0 Hz), 5.59 (1 H, d, *J* 3.0 Hz), and 6.41 (1 H, d, *J* 10.0 Hz), $\nu_{max.}$ (film) 1 725 cm^{-1}.

(*b*) Treatment of chorismic acid (0.1 g) with an excess of ethereal diazomethane at −78 °C for 5 min gave, after crystallisation from methanol–ether, the indazole (6) (0.08 g), m.p. and mixed m.p. 85°.

(*c*) Treatment of chorismic acid (0.1 g) in ether (10 ml) with an excess of ethereal diazomethane at −78 °C for 30 s gave, after preparative t.l.c., the pyrazoline (6) (0.02 g), m.p. and mixed m.p. 85°, and *dimethyl chorismate* as an oil (0.06 g), R_F 0.45 on silica in 99 : 1 chloroform–methanol (Found: $M^{+\cdot}$, 254.0803. $C_{12}H_{14}O_6$ requires *M*, 254.0790); δ_H [$(CD_3)_2CO$; 100 MHz] 6.83 (1 H, m), 6.31 (1 H, dt, *J* 10.0, 2.5, and 1.5 Hz), 6.05 (1 H, dd, *J* 10.0 and 2.5 Hz), 5.00 (1 H, dd, *J* 11.5 and 3.0 Hz), 4.68 (1 H, dt, *J* 11.5, 2.5, and 2.5 Hz), 5.46 (1 H, d, *J* 3.0 Hz), 4.91 (1 H, d, *J* 3.0 Hz), 3.80 (3 H, s), and 3.73 (3 H, s), $\nu_{max.}$ (Nujol) 1 720 and 1 620 cm^{-1}.

(*d*) Chorismic acid (0.2 g) in ether (10 ml) at −78 °C was treated dropwise with 1 mol. equiv. of diazomethane in ether over 10 min. After a further 5 min the solvent was removed and the product separated by preparative t.l.c. (silica; 90 : 10 chloroform–methanol) to give, after crystallisation from ethyl acetate–light petroleum (b.p. 60—80 °C), trans-4-*hydroxy*-3-(1-*methoxycarbonylvinyloxy*)*cyclohexa*-1,5-*diene*-1-*carboxylic acid* (4) as a white solid (0.05 g), m.p. 68—72° (decomp.), R_F 0.55 (Found: C, 51.0; H, 5.6%; $M^{+\cdot}$, 240.063 80. $C_{11}H_{12}O_6,H_2O$ requires C, 51.2; H, 5.4%. $C_{11}H_{12}O_6$ requires *M*, 240.063 36); δ_H [$(CD_3)_2CO$; 100 MHz] 6.90 (1 H, m), 6.33 (1 H, dt, *J* 10.0, 2.5, and 1.5 Hz), 6.05 (1 H, dd, *J* 10.0 and 2.5 Hz), 5.48 (1 H, d, *J* 3.0 Hz), 5.05 (1 H, dd, *J* 11.5 and 3.0 Hz), 4.98 (1 H, d, *J* 3.0 Hz), 4.72 (1 H, dt, *J* 11.5, 5.2, and 2.5 Hz), and 3.77 (3 H, s), $\nu_{max.}$ (Nujol) 1 720 and 1 620 cm^{-1}.

Reaction of Chorismic Acid with m-*Chloroperbenzoic Acid.*—Chorismic acid (1.00 g, 4.4 mmol) in ether (25 ml) at

[38] K. Randerath, 'Thin Layer Chromatography,' Verlag Chemie–Academic Press, Weinheim and London, 1963, p. 223.

[39] R. S. Thompson, D. Jacques, E. Haslam, and R. J. N. Tanner, *J.C.S. Perkin I*, 1972, 1387.

0 °C was treated with *m*-chloroperbenzoic acid (0.74 g, 4.5 mmol). After 72 h the product which had separated was isolated and recrystallised from methanol–ether or acetone–light petroleum (b.p. 60—80 °C) to give the 5,6-*epoxide* (10) as prisms (0.93 g), m.p. 149—152° (decomp.) (Found: C, 49.3; H, 4.4%; $M^{+\cdot}$, 242.0434. $C_{10}H_{10}O_7,H_2O$ requires C, 49.6; H, 4.1%. $C_{10}H_{10}O_7$ requires *M*, 242.0426); δ_H [$(CD_3)_2CO$; 100 MHz] 6.88 (1 H, t, *J* 2.0 and 2.0 Hz), 5.51 (1 H, d, *J* 3.0 Hz), 4.90 (1 H, d, *J* 3.0 Hz), 4.62 (1 H, dd, *J* 8.0 and 2.0 Hz), 4.21 (1 H, dd, *J* 8.0 and 1.0 Hz), 3.65 (1 H, dd, *J* 4.2 and 1.0 Hz), and 3.97 (1 H, dd, *J* 4.2 and 2.0 Hz).

The epoxide (0.05 g) in pyridine (1 ml) and acetic anhydride (1 ml) was left at room temperature for 24 h. The acetate, isolated in the usual way, was an oil (0.05 g) (Found: $M^{+\cdot}$, 266.0382. $C_{12}H_{10}O_7$ requires *M*, 266.0426), $\nu_{max.}$ (film) 1 720 and 1 700 cm^{-1}, δ_H [$(CD_3)_2CO$] 2.27 (3 H, s), 5.37 (1 H, d, *J* 2.0 Hz), 5.97 (1 H, d, *J* 2.0 Hz), 7.07 (1 H, t, *J* 2.0 Hz), 6.51 (2 H, d, *J* 2.0 Hz), and 9.07 (2 H, m).

3-(1-*Carboxyvinyloxy*)-5-*hydroxybenzoic Acid* (11a).—The 5,6-epoxychorismic acid (0.075 g) in sodium hydroxide (1N; 7.5 ml) was heated at 100 °C for 20 min. After cooling to room temperature the solution was acidified (HCl) to pH 1.5 and extracted with ethyl acetate (3 × 80 ml). After drying ($MgSO_4$) and removal of the solvent the *product* was crystallised from ethyl acetate–light petroleum (b.p. 60—80 °C) to give white needles (0.05 g), m.p. 175—176° (Found: C, 53.2; H, 3.8%; $M^{+\cdot}$ 224.0324. $C_{10}H_8O_6$ requires C, 53.6; H, 3.6%; *M*, 224.0321); δ_H [$(CD_3)_2CO$] 5.34 (1 H, d, *J* 2.0 Hz), 5.96 (1 H, d, *J* 2.0 Hz), 6.82 (1 H, t, *J* 2.5 Hz), 7.23 (1 H, t, *J* 2.5 Hz), and 7.37 (1 H, t, *J* 2.5 Hz).

The acid (0.03 g) was heated in hydrochloric acid (1N; 7.5 ml) at 100 °C for 1 h. Extraction (ethyl acetate) and crystallisation of the product from ethyl acetate–light petroleum (b.p. 60—80 °C) gave 3,5-dihydroxybenzoic acid (0.01 g), m.p. and mixed m.p. 236°.

5,6-*Dihydrochorismic Acid* (9).—Chorismic acid (250 mg) methanol (10 ml) was stirred at 0 °C under nitrogen, and potassium azodiformate (700 mg) was added, followed by acetic acid (0.5 ml). After a further 1 h at 0 °C the solution was kept at −5 °C overnight, the solvent was removed, and the product was dissolved in water (5 ml). Acidification to pH 1.5 at 0 °C (HCl) and extraction with ether (2 × 25 ml) gave, after removal of the ether and crystallisation from acetone–light petroleum (b.p. 60—80 °C), the *dihydro-derivative* as needles (140 mg), m.p. 151—152° (Found: C, 52.6; H, 5.4%; $M^{+\cdot}$ 228.0642. $C_{10}H_{12}O_6$ requires C, 52.7; H, 5.3%; *M*, 228.0634); δ_H [$(CD_3)_2CO$] 6.8 (1 H, d, *J* 2.0 Hz), 5.53 (1 H, d, *J* 3.0 Hz), 5.0 (1 H, d, *J* 3.0 Hz), 4.68 (1 H, dd, *J* 7.0 and 2.0 Hz), 4.0 (1 H, m), and 2.4—1.9 (4 H, m), $\nu_{max.}$ (Nujol) 1 700, 1 620, and 1 200 cm^{-1}.

1,2-*Dihydrochorismic Acid* (8).—Chorismic acid (250 mg) in methanol (10 ml) was treated as above with potassium azodiformate (700 mg) and acetic acid (0.5 ml). Stirring under nitrogen was continued for 3 h but the solution was allowed to reach room temperature. The product was isolated as above to give, after crystallisation from acetone–light petroleum (b.p. 60—80 °C), 1,2-*dihydrochorismic acid* as needles (145 mg), m.p. 144—145° (Found: C, 52.3; H, 5.7%; $M^{+\cdot}$ 228.0638); δ_H [$(CD_3)_2CO$] 5.8br (2 H, s), 5.4 (1 H, d, *J* 3.0 Hz), 5.18 (1 H, d, *J* 3.0 Hz), 4.25 (2 H, m), 3.36 (1 H, m), and 2.4—1.8 (2 H, m, *J* 14.0 Hz), $\nu_{max.}$ (Nujol) 1 725, 1 685, 1 620, and 1 200 cm^{-1}.

Chorismate Mutase T from Aerobacter aerogenes *poly* 3.—Cells of the organism were grown as previously described and cell extracts were prepared by ultrasonification in Tris buffer (pH 7.5) containing L-tyrosine, EDTA, and dithiothreitol (1mM). Cell extracts were treated with protamine sulphate (1 volume; 2% w/v) to remove nucleic acid and then with ammonium sulphate (20.5 g per 100 ml). After 0.5 h the suspension was centrifuged (23 000*g*; 0.25 h) and further ammonium sulphate (8.4 g per 100 ml) was added. The suspension was centrifuged after 0.5 h (23 000*g*; 0.25 h) and the precipitate resuspended in the buffer solution used for extraction (40 ml per 10 g of cells). Ammonium sulphate (15 g) was added and the enzyme solution stored at 0—4 °C until required.

The activity of the enzyme was assayed in the presence or absence of inhibitors by the following procedure. A sample (1 ml) of a solution of chorismic acid [20 mg in Tris buffer (0.1M; pH 9.0; 100 ml) containing EDTA (10^{-3}M)] was placed in a cuvette, followed by a sample (1 ml) of the same buffer containing any inhibitor and EDTA (10^{-3}M). The cuvettes were placed in a u.v. spectrophotometer with a thermostatted cell compartment at 30 °C, and the absorbance at 278 nm was followed at 60 s intervals after addition of enzyme solution (1 ml). Initial rate data were obtained in the usual way. The Michaelis constant for the samples of chorismate mutase used was determined by the procedure of Gorisch and Lingens:[5] K_m (chorismate) 1.2×10^{-3}M. Inhibitor constants, K_i, were determined from the Lineweaver–Burk plot of the kinetic data: 5,6-epoxychorismic acid, 6.9×10^{-3}M; 5,6-dihydrochorismic acid, 4.6×10^{-3}M.

DL-*Phenylalanine from Chorismic Acid*.—Chorismic acid (100 mg) in Tris buffer (0.1M; pH 9.0; 50 ml) containing EDTA (10^{-3}M) was treated at 30 °C for 1 h with a solution of chorismate mutase T (10 ml). The solution was then cooled to 0 °C and treated with aqueous sodium borohydride (0.2%; 5 ml). After 0.5 h the solution was acidified (HCl) to pH 1.5 and incubated at 30 °C for 1 h. Extraction with ethyl acetate (3 × 50 ml) and evaporation of extract gave, after crystallisation from ethyl acetate–light petroleum (b.p. 60—80°), (*RS*)-2-hydroxy-3-phenylpropionic acid (35 mg), m.p. and mixed m.p. 92—94°. When this reaction was carried out in media containing not less than 90% deuterium oxide the product contained no detectable amount of deuterium (^{1}H n.m.r. and mass spectroscopy). The acid (5.0 g) was converted into (*RS*)-2-bromopropionic acid (b.p. 138—140° at 0.2 mmHg) and thence into DL-phenylalanine (0.8 g) by standard procedures.[40,41]

Diethyl Bromofluoro-oxalacetate.—Diethyl oxalate (27.7 g) was added to a stirred suspension of sodium ethoxide 12.9 g) in benzene followed by ethyl fluoroacetate (20 g), and the mixture was stirred for a further 24 h at room temperature. The precipitate was filtered off and washed with benzene and ether to give the enolate salt of diethyl fluoro-oxalacetate (37.7 g). This was suspended in benzene (500 ml) and bromine (27.2 g) was added to the stirred solution at such a rate that decolourisation balanced the rate of addition (2 h). After a further 6 h the solution was filtered under suction and the filtrate washed [10% sodium sulphite (30 ml) and water (20 ml)] and dried (Na_2SO_4). Removal of the solvent left an oil which was distilled to give diethyl bromofluoro-oxalacetate (36.6 g, 75%), b.p. 114° at 0.5 mmHg (Found: C, 33.9; H, 3.8; Br, 28.0. Calc. for $C_8H_{10}BrFO_5$: C, 33.7; H, 3.6; Br, 28.1%).

[40] J. Eck and C. S. Marvel, *Org. Synth.* 1943, **11**, 74.
[41] C. S. Marvel, *Org. Synth.*, 1955, **111**, 705.

Cyclohexylammonium (Z)-3-*Fluorophosphoenolpyruvate*.—Bromofluoropyruvic acid (b.p. 74—78° at 0.7 mmHg) (1.85 g) in ether (25 ml) was added to trimethyl phosphite (1.86 g) at 0 °C, and the mixture was stirred at room temperature for 48 h. Water (25 ml) and cyclohexylamine (0.99 g) were added to the oil obtained on removing the solvents and reagents, and the solution was kept for 3 h at 0 °C and for 72 h at room temperature. Removal of the solvents left a slurry which was filtered; the solid was crystallised from methanol–ether (1 : 1) to give the product (1.1 g), m.p. 139—140° (lit.,[36] 139—141°) (Found: C, 38.2; H, 6.1; N, 5.2. Calc. for $C_9H_{17}FNO_6P$: C, 37.9; H, 6.0; N, 4.9%).

Cyclohexylammonium [3-$^2H_2$]*Phosphoenolpyruvate*.—Diethyl bromo-oxalacetate (5.0 g) was treated with 38% deuterium chloride in deuterium oxide (25 ml) for 2 h at 80 °C and then for 24 h at room temperature. The excess of reagent was removed and the residue treated with ether (50 ml) and water (50 ml). The organic extract was dried ($MgSO_4$) and evaporated to yield starting material (1.1 g); evaporation of the aqueous layer gave [3-$^2H_2$]bromopyruvic acid (>90% $^2H_2$; 2.00 g). The latter in ether (20 ml) was converted by published procedures[42] into *cyclohexylammonium* [3-$^2H_2$]*phosphoenolpyruvate* (0.8 g), m.p. 142—143° (decomp.) (Found: C, 39.9; H, 6.5; N, 5.2. $C_9H_{16}{}^2H_2NO_6P$ requires C, 40.2; H, 6.7; N, 5.2%). Occasionally the product crystallised as the *biscyclohexylammonium salt*, m.p. 144—146° (decomp.) (Found: C, 48.7; H, 8.5; N, 7.6. $C_{15}H_{29}{}^2H_2N_2O_6P$ requires C, 49.0; H, 8.4; N, 7.6%).

Enzymic Synthesis of Chorismic Acid.—(a) *Cell extract*. Growth media [4 × 1 l, containing L-phenylalanine (20 mg), L-tyrosine (20 mg), and L-tryptophan (2 mg) and minimal salt base (20 ml) (quantities per litre)] were inoculated with an overnight culture (14 ml; L broth) of *Aerobacter aerogenes* 62–1. The solutions were shaken overnight at 30 °C and the cells were collected by centrifugation (5 000*g*; 10 min; 5 °C), resuspended in 0.9% w/v sodium chloride solution and collected by centrifugation at 5 °C. The cells (6.0 g) were stored at −15 °C prior to use. Cells (1.5 g) were dispersed in Tris buffer (pH 8.2; 5 ml) and ruptured in a French press. The cell debris was removed by centrifugation (20 000*g*; 30 min; 5 °C) and the supernatant solution was dialysed (3—16 h) against Tris buffer (pH 8.2; 500 ml; 25mM) containing dithioerythritol (75 mg) at 5 °C. After dialysis the solutions were stored at 0 °C.

(b) NADH (barium salt; 1.13 g) was dissolved in water (15 ml) at 5 °C and sodium sulphate solution (2M) was added dropwise until precipitation was complete. Barium sulphate was removed by centrifugation and to the supernatant solution Tris buffer (pH 8.2; 60 ml), ATP (575 mg), magnesium chloride hexahydrate (640 mg), shikimic acid (150 mg), and cell extract (38.4 ml, from 11.5 g of cells) were added. The pH was adjusted to 8.5 by addition of Tris solution (0.2M) and the solution was incubated at 37 °C for 30 min. Phosphoenolpyruvate [sodium salt; in water at pH 8.5, prepared from monocyclohexyl phosphoenolpyruvate (250 mg)] was added and the incubation was continued for a further 1 h. The mixture was cooled in ice, acidified to pH 1.5 with hydrochloric acid and extracted with cold freshly distilled ethyl acetate (3 × 500 ml). The extract was dried ($MgSO_4$) and evaporated to give a gum which after chromatography (as above) was crystallised to yield chorismic acid (0.05 g), m.p. 147—148° (decomp.), or treated directly with diazomethane to give the indazole (6) (0.065 g), m.p. 85° (decomp.).

(c) Reactions in deuterium oxide were carried out by the following procedure. Tris buffer (50mM; pH 8.2; 100 ml) was evaporated to dryness; the residue was equilibrated with deuterium oxide (2 × 5 ml) and made up to 100 ml with deuterium oxide. A cell-free extract of *Aerobacter aerogenes* 62–1 was prepared in Tris buffer (pD 9.0; 30 ml; 50 mM) containing dithioerythritol (45 mg). After freeze-drying, deuterium oxide (30 ml) was added with minimal exposure to the atmosphere. A solution of shikimic acid (550 μmol), ATP (550 μmol), NADH (650 μmol), and magnesium chloride (2 mmol) was made up in 50mM-Tris buffer (pD 8.2; 20 ml) and evaporated to dryness; the residue was equilibrated with deuterium oxide (2 × 5 ml) and finally made up to 30 ml with deuterium oxide. More Tris buffer (pD 8.2; 30 ml) was added and the whole was added to the cell extract. After incubation for 30 min at 37 °C, solid phosphoenolpyruvate (85 mg; K^+ salt) was added and the incubation continued for 1 h. Chorismic acid was isolated as its adduct (6) with diazomethane (0.035 g), m.p. 85—86° (decomp.); δ_H 5.51 (0.11 H) and 5.02 (0.11 H); the $M^{+\cdot} - 28$ ion showed *P* (3%), *P* + 1 (17%), and *P* + 2 (80%) peaks.

(d) With dideuteriophosphoenolpyruvate (PEP) as substrate the following results were obtained on mass spectrometric analysis of the diazomethane adduct ($M - 28$); incubation times after addition of PEP are shown in parentheses: (0.25 h) *P* (40.6%), *P* + 1 (34.1%), *P* + 2 (25.4%); (0.5 h) *P* (57.8%), *P* + 1 (26.9%), *P* + 2 (15.3%); (1 h) *P* (60.5%), *P* + 1 (27.0%), *P* + 2 (12.5%). Incubations in the presence of added iron(II) sulphate (5 μmol Fe^{2+} ml^{-1}) showed the following deuterium retentions: (0.33 h) *P* (48.5%), *P* + 1 (33.5%), *P* + 2 (18.2%); (1.33 h) *P* (62.0%), *P* + 1 (26.9%), *P* + 2 (11.2%).

(e) In media containing tritiated water the following procedure was used. A solution was made up in Tris buffer (50mM; pH 8.2; 7.1 ml) containing [U-^{14}C]shikimic acid[43] (15 mg; 3.23 × 10^4 disint. min^{-1} μmol^{-1}), magnesium chloride hexahydrate (66 mg), ATP (54 mg), NADH (2.4 ml of standard solution), *A. aerogenes* 62–1 extract (2.4 ml), and tritiated water (0.1 ml; 500 mCi). The pH of the mixture was then adjusted to 8.2 with 0.2M-Tris solution. The mixture was incubated for 27 min at 37 °C, phosphoenolpyruvate was added (0.6 ml; 0.1M-solution of the sodium salt) and the incubation was continued for a further 1 h. The solution was cooled in ice, acidified (2N-HCl), and extracted with ethyl acetate (3 × 50 ml). The chorismic acid was isolated as above after chromatography or converted directly into its adduct (6) with diazomethane. The ^3H : ^{14}C ratio found in the product was 13.1 : 1 (^3H incorporation 27.7% of 2 atoms).

In further experiments the above procedure was carried out in the presence of 10^{-3}, 5 × 10^{-3}, and 10^{-2}M-iron(II) (sulphate) and the derived ^3H : ^{14}C ratios found in the product chorismic acid were 13.2 : 1, 13.2 : 1, and 12.0 : 1, respectively.

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[42] V. M. Clark and A. J. Kirby, *Biochem. Prep.*, 1966, **11**, 101.
[43] P. M. Dewick and E. Haslam, *Biochem. J.*, 1969, **113**, 537.