The Shikimate Pathway. Part IV.[1] The Stereochemistry of the 3-Dehydroquinate Dehydratase Reaction and Observations on 3-Dehydroquinate Synthetase

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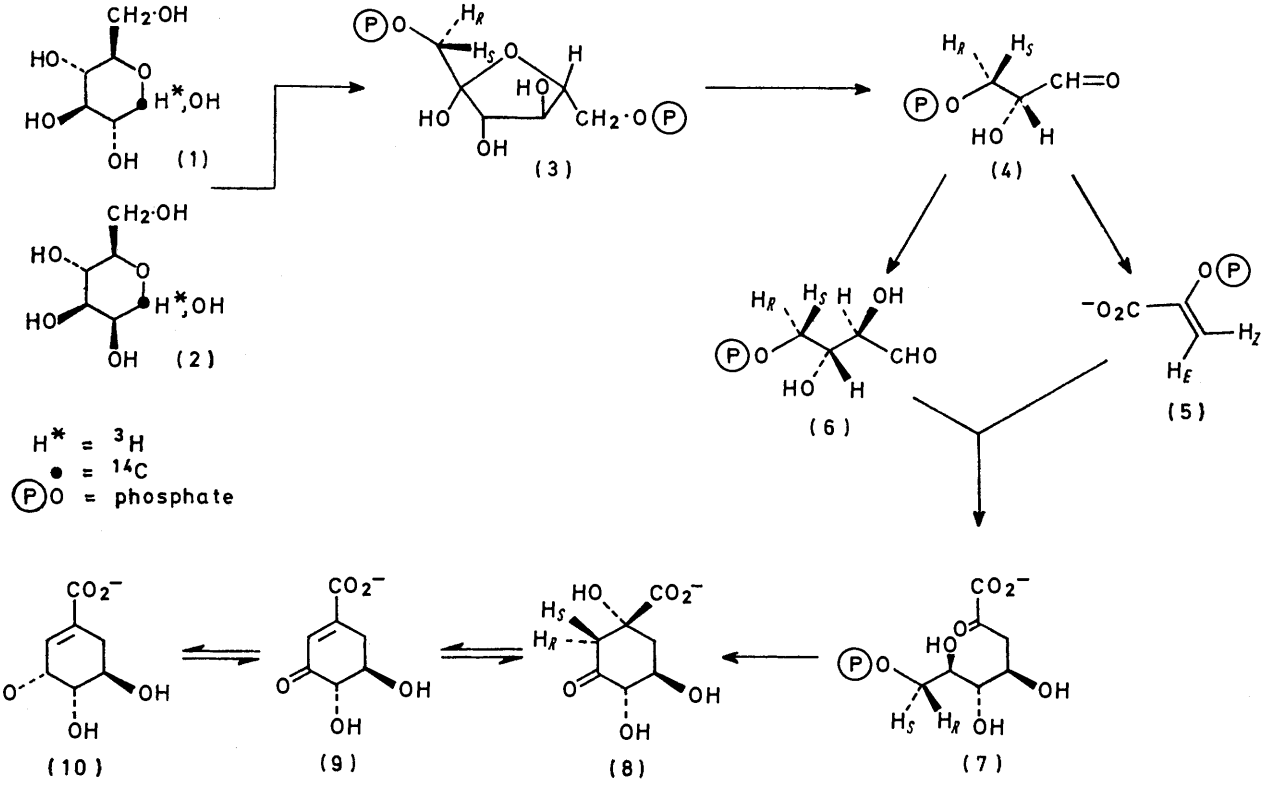
The conversion of 3-dehydroquinate (1,3,4-trihydroxy-5-oxocyclohexanecarboxylate) (8) into 3-dehydroshikimate (4,5-dihydroxy-3-oxocyclohex-1-enecarboxylate) (9) involves the *syn*-elimination of the elements of water; this observation confirms earlier work on the reaction. Studies on the 3-dehydroquinate synthetase reaction show it to possess a stereospecificity with respect to the C-7 protons of the substrate 3-deoxy-D-*arabino*-heptulosonic acid 7-phosphate (7).

HANSON and ROSE [2] were the first to study the enzyme-catalysed conversion of 3-dehydroshikimate (9) into 3-dehydroquinate (8), and showed that this reaction follows an unusual stereochemical course [3] leading to a reaction which forms part of the shikimate pathway and the forward reaction in the direction of biosynthesis has now been studied by using the stereospecifically labelled substrates (8; $H_S = {}^2H$) and (8; $H_R = {}^2H$).[4]



syn-addition of the elements of water. The conversion (9) ⟶ (8) is the reverse of the normal biosynthetic

The substrates (2*S*)-2-deuterio-3-dehydroquinic acid [8; $H_S = {}^2H$ (75%)] and the (2*R*)-isomer [8; H_R =

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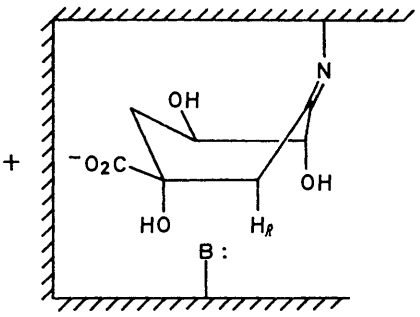
^2H (70%)] were prepared as previously described.[5] Incubation of the diastereoisomeric deuterio-acids with a preparation of 3-dehydroquinate dehydratase (syn. 3-dehydroquinate hydrolase EC 4.2.1.10) isolated [6] from *Escherichia coli* 83—24 and coupled with the cyclic generation of NADPH from $NADP^+$ by isocitrate dehydrogenase [7] and isocitrate gave samples of (—)-shikimic acid (10) (*ca.* 60%). The latter were examined by ^1H n.m.r.[8] and, as their methyl ester triacetates,[9] by mass spectrometry. The signal due to the vinyl proton at C-2 in (10) and the molecular ion peak of the methyl ester triacetate showed that loss of deuterium had occurred with (8; $H_R = {}^2H$) as substrate and retention with (8; $H_S = {}^2H$). This procedure therefore provides, when (8; $H_S = {}^2H$) is used as substrate, a useful method for preparing (—)-[2-^2H]shikimic acid.

The results also confirm the previous observations of Hanson and Rose [2] on the stereochemistry of 3-dehydroquinate dehydratase but they contrast with the normal acid- or base-catalysed loss of water from (8) to give (9). Treatment of both substrates with acid or base under mild conditions gave samples of 3-dehydroshikimic acid (9) which were isolated and converted enzymically into (—)-shikimic acid (10). Deuterium analysis showed that only the product (10) derived from (8; $H_R = {}^2H$) retained significant amounts of deuterium under these conditions. The deuterium retentions (base: 0·55; acid: 0·45 atom deuterium) observed also indicated that some non-specific chemical exchange of the protons at C-2 also occurred during these reactions. Support for this view was obtained when the base-catalysed elimination was carried out in deuterium oxide and the deuterium retention from (8; $H_R = {}^2H$) was found as 0·65 atom. The chemical conversion (8) ⟶ (9) therefore proceeds predominantly by the normal *anti*-elimination of the elements of water.

In order to rationalise their observations on the enzymic reaction (8) ⟶ (9), Hanson and Rose [2] suggested that it proceeds *via* the enolate form of the substrate (8) and that the relative disposition of an acidic and a basic group on the enzyme active site was responsible for the overall stereochemical result. Recent evidence [10] suggests that the substrate (8) first forms a Schiff's base (11) with an amino-group on the enzyme. Chemical considerations also indicate that if the oxygen of the carbonyl group [2] or the nitrogen of the Schiff's base [10] is to participate in the enolisation step and subsequent elimination of OH then the reaction should take place from a boat or twist-boat form of the substrate [*e.g.* (11)]. In such a conformation, stabilisation of the incipient carbanion formed by loss of the 2-*pro*-*R*-proton is then possible *via* the heteroatom. The group B could then also function in both steps of the reaction—as a general base in the enolisation step and as a general acid in the elimination of OH. This would then satisfactorily explain the overall stereochemical features of the enzymic reaction.



B = basic group

(11)

Knowledge of the stereochemical features of the 3-dehydroquinate dehydratase reaction has been utilised in a preliminary examination of the cyclisation which leads from 3-deoxy-D-*arabino*-heptulosonic acid 7-phosphate (DAHP) (7) to 3-dehydroquinate (8). This reaction is catalysed by an enzyme system which has been described by Sprinson and his collaborators.[11] Its mechanism has been the subject of some speculation.[12] Some observations have now been made on the fate of the 7-*pro*-*R*- and 7-*pro*-*S*-protons in DAHP (7) in the conversion. The *E. coli* mutant 170—27 (blocked after 3-dehydroquinate) was grown on a minimal medium supplemented in one case with [1-^3H, 1-^{14}C]-D-glucose (1) and in another with [1-^3H, 1-^{14}C]-D-mannose (2) and the accumulated metabolite, 3-dehydroquinic acid (8), was isolated. The latter was then converted into (—)-shikimic acid (10) as described above. In a further series of experiments *E. coli* 83—24 (blocked after shikimate) was grown on the same isotopically labelled media and both metabolites [(8) and (10)] were isolated.

^3H : ^{14}C Ratios of (—)-shikimic acid and 3-dehydroquinic acid

	3-Dehydroquinic acid	(—)-Shikimic acid
E. coli 170—27		
D-Glucose *	42·2 : 1	33·8 : 1
D-Mannose †	8·68 : 1	2·03 : 1
E. coli 83—24		
D-Glucose *	41·0 : 1	34·7 : 1
D-Mannose †	8·66 : 1	3·35 : 1

* ^3H : ^{14}C = 50 : 1. † ^3H : ^{14}C = 10 : 1.

The measured ^3H : ^{14}C ratios of the various products are shown in the Table.

Sprinson [12] has demonstrated that DAHP (7) is

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derived from carbohydrate by condensation of a four-carbon intermediate, D-erythrose 4-phosphate (6), originating from the pentose phosphate pathway, and a three-carbon fragment, phosphoenol pyruvate (PEP) (5), derived from the glycolytic pathway. Although some randomisation of the isotopic tracers would be expected, the general fate of the tritium located at C-1 in (1) and (2) in the formation of (5) and (6) may be predicted in terms of the routes delineated by Sprinson and the previously derived knowledge of the stereochemical mode of action of the enzymes involved.[13-16] The key enzymes are the initial isomerases [15,16] which mediate the conversion of the aldose [(1) or (2)] into fructose 1,6-diphosphate (3; H_R or $H_S = {}^3H$). Subsequent steps lead from the triose phosphate (4) to PEP [(5; $H_Z = {}^3H$ from D-glucose); (5; $H_E = {}^3H$ from D-mannose)].[13,14] The triose phosphate is also an intermediate in the formation of the D-erythrose phosphate [(6; $H_S = {}^3H$ from D-glucose); (6; $H_R = {}^3H$ from D-mannose)].

An unexpected feature of the results was the predominance of the tritium label found in the four-carbon fragment from the pentose phosphate pathway, but if we assume the pathways outlined to be broadly followed the observations in the Table show that the cyclisation proceeds in a stereospecific manner in relation to the C-7 methylene protons of DAHP (7). The results with D-mannose (2) as substrate further indicate that the 2-*pro*-*R*-proton of 3-dehydroquinate (8) is derived from the 7-*pro*-*R*-proton of DAHP (7) and show that the formation of a methyl group from C-7 of DAHP during this transformation [12] is improbable.

EXPERIMENTAL

Paper chromatography was carried out as previously described.[5] Mass spectra were recorded with A.E.I. MS9 and MS12 instruments.

Enzyme-catalysed Conversion of 3-Dehydroquininic Acid into Shikimic Acid.—3-Dehydroquinic acid (0·20 g), sodium (±)-isocitrate (5·0 g), and $NADP^+$ (0·075 g) were dissolved in potassium phosphate buffer (M/30; pH 7·4; 300 ml) and a cell-free extract of *E. coli* (83—24) (300 ml; 0·6 units per ml of 3-dehydroquinate dehydratase activity [6]) was added. After 20 h at 20°, acetic acid was added to bring the solution to pH 2·0 and the precipitated protein was removed by centrifugation. The supernatant liquid was concentrated at 30° to *ca.* 30 ml and methanol (270 ml) was added. After removal of further protein by centrifugation the methanol was evaporated off and the residue dissolved in water (30 ml) and applied to a column of Amberlite C.G. 400 (1 × 30 cm; acetate form). The column was washed with distilled water (1000 ml) and eluted with acetic acid of increasing strength (gradient elution [5] with a reservoir of 2N-acetic acid). Fractions (10 ml) were collected and analysed [5] and 21—28 were combined to give (−)-shikimic acid (0·15 g), m.p. 184°. The sample was repurified by the same procedure to yield (−)-shikimic acid (0·12 g), a small sample (0·07 g) of which was converted [11] into methyl tri-*O*-acetylshikimate, isolated by t.l.c. (silica; chloroform; R_F 0·70) as a colourless gum, $M^{+\cdot}$ 314 at 12 eV (relative intensities $M^{+\cdot}$ 1·00, $M + 1$ 0·181, $M + 2$ 0·0536).

(2*S*)-2-Deuterio-3-dehydroquinic acid (65% $^2H_1$) gave (−)-shikimic acid with deuterium content of 60% ($^2H_1$). ^1H N.m.r. showed the deuterium to be located at C-2 [τ 2·84 (m)] in (−)shikimic acid. (2*R*)-2-Deuterio-3-dehydroquinic acid (70% $^2H_1$) similarly gave (−)-shikimic acid (6% $^2H_1$).

Chemical Conversion of 3-Dehydroquinic Acid into 3-Dehydroshikimic Acid.—(a) *With base.* A solution of 3-dehydroquinic acid (0·5 g) in water or deuterium oxide (30 ml) was adjusted to pH 7·0 with solid potassium carbonate. After 21 days at 25° the solution was chromatographed on Amberlite C.G.400 (50 ml slurry; acetate form) and fractions (10 ml) were collected by gradient elution (reservoir of 2N-acetic acid [5]). Fractions 50—65 gave 3-dehydroshikimic acid (0·07 g) as needles, m.p. and mixed m.p. 139—141° (from ethyl acetate).

(b) *With acid.*[17] An aqueous solution (100 ml) of 3-dehydroquinic acid (0·3 g) was maintained at 100° for 1 h in the presence of a strongly acidic resin, Amberlite IR-120 (0·5 g). The filtrate was applied to a column of Amberlite C.G.400 resin as above and 3-dehydroshikimic acid (0·05 g) was isolated, m.p. and mixed m.p. 139—141°.

Deuterium analysis of 3-dehydroshikimic acid was carried out by ^1H n.m.r. analysis [C-2 proton at τ (D_2O) 2·81 (d)] or by conversion into (−)-shikimic acid as described above. The results are discussed on p. 53.

Growth of E. coli *Mutants and Accumulation of Metabolites.*—Strains of both *E. coli* mutants 83—24 and 170—27 were grown on minimal medium A [18] with the addition of six aromatic supplements (per 488 ml of solution: L-tyrosine, 0·005 g; L-phenylalanine, 0·10 g; L-tryptophan, 0·0025 g; *p*-aminobenzoic acid, 5×10^{-6} g; *p*-hydroxybenzoic acid, 5×10^{-6} g; and 2,3-dihydroxybenzoic acid, 5×10^{-6} g). A sterile glucose solution (20%; 12·5 ml) including the radioactive substrate ([1-^{3}H]-D-glucose, 1·25 mCi, [1-^{14}C]-D-glucose, 0·025 mCi; [1-^{3}H]-D-mannose 0·25 mCi, [1-^{14}C]-D-mannose, 0·025 mCi) was added and the growth medium inoculated with 1 ml of a freshly grown (5—7 h) broth culture of the appropriate mutant. Growth was maintained at 37° for 60 h with shaking. The medium was then centrifuged (20 min; 5000*g*) to remove bacterial cells, and carrier [(−)-shikimic acid (0·25 g) and/or 3-dehydroquinic acid (0·25 g)] was added as appropriate. The solution was passed through a cation-exchange column (Zeokarb 225; H^+ form; 30 × 1 cm) and then placed on an anion-exchange column (Amberlite C.G.400; acetate form; 30 × 1 cm). The column was washed with water (500 ml) before gradient elution with acetic acid (0—6·0N). Fractions (10 ml) were collected and analysed by paper chromatography. Typically (−)-shikimic acid occurred in fractions 15—25, and 3-dehydroquinic acid in fractions 50—75. The acids were isolated and crystallised [17,19] to give a constant ^3H : ^{14}C ratio.

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Samples of 3-dehydroquinic acid (0·10 g) obtained as above were converted into (−)-shikimic acid by using a cell-free extract of *E. coli* 83—24 as described above. The $^{3}H : ^{14}C$ ratios obtained are shown in the Table.

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