ruled out. Fig. 4 is a diagram of the possible attachment sites (drawn in solid lines) on the enzyme surface.

SUMMARY

1. A number of aromatic and hydro-aromatic compounds of similar structure to the natural substrates of dehydroshikimic reductase, shikimic acid and dehydroshikimic acid, were examined for possible inhibition.

2. Several of the compounds found to cause inhibition were examined further, and were shown to inhibit competitively.

3. The inhibitor constants for these competitive inhibitors were calculated.

4. Since these compounds presumably inhibit by occupying the site normally serving for attachment of shikimic acid and dehydroshikimic acid, a theory of the mode of attachment of the substrates to the enzyme is discussed.

5. It is suggested that shikimic acid and dehydroshikimic acid are attached to the enzyme by means of the carboxyl and 3- and 4-hydroxyl groups.

The authors wish to express their gratitude to Professor T. A. Bennet-Clark, in whose Department this research was carried out. Our thanks are due to Dr L. Crombie for helpful discussions on the conformation of shikimic acid. One of us (D.B.) wishes to acknowledge with thanks the award of a King's College Tutorial Studentship and subsequently of a Witwatersrand Council of Education Scholarship.

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Aromatic Biosynthesis in Higher Plants

3. PREPARATION AND PROPERTIES OF DEHYDROQUINASE

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The preparation and purification of dehydroshikimic reductase, the enzyme converting dehydroshikimic acid into shikimic acid, was described by Balinsky & Davies (1961*a*). The present

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paper describes the preparation and purification of dehydroquinase, the enzyme catalysing the inter-conversion of dehydroquinic acid (I) and dehydroshikimic acid (II), as shown below.

A preliminary account has been published (Balinsky & Davies, 1960).

The presence of this enzyme was first demonstrated in cell-free extracts of *Escherichia coli* by Davis & Weiss (1953). It was purified eightfold by

Mitsuhashi & Davis (1954), who studied some of its properties and demonstrated its occurrence in a number of organisms that can synthesize their own aromatic amino acids, namely *Aerobacter aerogenes*, *Saccharomyces cerevisiae*, *Euglena gracilis*, spinach leaves and peas.

EXPERIMENTAL

Materials. Dehydroquinic acid, synthesized by Grewe & Jeschke (1956), was kindly supplied by Dr R. Grewe. A small quantity of dehydroshikimic acid, synthesized by Grewe & Jeschke, was obtained by the courtesy of Dr B. D. Davis.

Assay procedure. Mitsuhashi & Davis (1954) showed that the equilibrium constant for the reaction at pH 7.4, neglecting the water term, was

$$K = \frac{[\text{dehydroshikimic acid}]}{[\text{dehydroquinic acid}]} = 15 \pm 1,$$

which shows that the reaction is favourable for the formation of dehydroshikimic acid, hence the reaction was assayed in that direction. The assay is based on the absorption of dehydroshikimic acid at 234 m μ ; dehydroquinic acid has a negligible absorption at this wavelength. For routine measurements during purification procedures, the change in *E* at 240 m μ was measured in preference to that at 234 m μ . Since the enzyme appeared to be active over a wide range of pH (Fig. 2), a pH of 7.4 was chosen, this being the pH at which Mitsuhashi & Davis (1954) assayed dehydroquinase activity. Moreover, both dehydroquinic acid and dehydroshikimic acid are stable at this pH.

Procedure. Into a quartz cuvette of 1 cm. light-path were pipetted 3 ml. of 0.1M-potassium phosphate buffer, pH 7.4, 0.1 ml. of 3 mM-sodium dehydroquininate and an appropriate amount of enzyme. Changes of *E* were measured in a Unicam SP. 500 spectrophotometer against a blank cell containing 3.1 ml. of buffer and the same amount of enzyme as the test cell. It was shown that the rate of change of *E* was proportional to the enzyme concentration. The reaction rate was linear for at least 5 min. A unit of activity is defined as the amount of enzyme producing a change of *E* of 1.0/min. at 240 m μ (or 234 m μ) under the conditions of the assay procedure. Specific activity is defined as the activity/mg. of protein.

Protein measurements. The protein concentration was determined by the method of Warburg & Christian (1942).

Extraction and purification of dehydroquinase

The enzyme was found to lose one-third to one-half of the original activity on overnight storage at +3° or -15°. The purification procedure adopted was based on the need to obtain in 1 day a purified enzyme preparation of fairly high specific activity and to use it at once for studies of the properties of the enzyme.

Extraction. Fresh cauliflower heads were thoroughly washed in water, cooled to 2° and the tips of the buds removed with a sharp knife. A 1:1 (w/v) extract of these buds was prepared by blending in 0.2M-potassium phosphate buffer, pH 6.5, in a Waring Blender. The extract was strained through a cloth and excess of cell debris removed by centrifuging for 10 min. at 25 000 *g*.

First ammonium sulphate fractionation. Finely ground ammonium sulphate was added very gradually with stirring to the crude extract to 30% saturation. The mixture was stirred for 10 min. and the precipitate formed removed by centrifuging for 10 min. at 25 000 *g*. Ammonium sulphate was added to the supernatant solution to 55% saturation, the solution stirred and the precipitate collected by centrifuging as described above. The precipitate formed was dissolved in 0.2M-potassium phosphate buffer, pH 6.5, and dialysed for 1 hr. against 4 l. of 5 mM-potassium phosphate buffer, pH 6.5 (flow dialysis).

Second ammonium sulphate fractionation. Finely ground ammonium sulphate was added to the dialysed protein solution to concentrations of 25, 30, 35, 40, 45 and 50% saturation. The fractions were collected and dissolved as described above. The most active fractions obtained were mixed and used for studies of the properties of the enzyme, after dialysis for 1 hr. against 4 l. of 5 mM-potassium phosphate buffer, pH 7.4.

Results of a typical purification procedure are summarized in Table 1.

RESULTS

Identification of reaction product. In the assay system described, the reaction was allowed to proceed to completion, and the absorption spectrum of the reaction mixture measured. A peak was observed at 234 m μ corresponding to the absorption

Table 1. *Purification of dehydroquinase*

Enzyme activity was assayed as described in the text.

Fraction	Volume (ml.)	Total activity (units)	Total protein (mg.)	Specific activity (unit/mg.)	Recovery (%)	Purification factor
Crude extract	200	15.0	12 400	0.012	100	1.0
First ammonium sulphate fractionation						
30-55%	60	13.2	876	0.015	88	1.3
Second ammonium sulphate fractionation						
0-25%	10	0.6	37.5	0.016	4	1.3
25-30%	5	0.2	16.8	0.012	1	1.0
30-35%	5	2.5	30.0	0.083	17	6.9
35-40%	5	2.0	22.0	0.091	13	7.6
40-45%	5	2.2	27.5	0.078	15	6.5
45-50%	5	2.0	28.8	0.070	13	5.8

maximum of dehydroshikimic acid (Salomon & Davis, 1953). The presence of dehydroshikimic acid was demonstrated chromatographically by comparison with synthetic acid in a butan-1-ol-formic acid solvent (Davis & Mingioli, 1953).

Reversibility of the reaction. Although the reaction is more favourable to the formation of dehydroshikimic acid, the reverse reaction could be shown by incubating the enzyme with dehydroshikimic acid and measuring the decrease of E .

Into a quartz cuvette of 1 cm. light-path were pipetted 3 ml. of 0.1 M-potassium phosphate buffer, pH 7.4, and 0.1 ml. (0.063 μ mole) of dehydroshikimic acid. After 1 min. 0.1 ml. of purified enzyme was added and the reaction followed at 234 m μ in the spectrophotometer against a blank cell containing no dehydroshikimic acid. Fig. 1 shows that there was a decrease in E , indicating at equilibrium a 19% conversion of dehydroshikimic acid into dehydroquinic acid. This value is higher than that obtained by Mitsuhashi & Davis.

Measurement of pH optimum. In the assay procedure described, the variation of enzyme activity at different pH values was measured, potassium phosphate, 2-amino-2-hydroxymethylpropane-1:3-diol (tris)-HCl (0.1 M) and glycine-NaOH (0.1 M) buffers being used. Activities were measured for at least one identical value of two buffers when changing from one buffer to another. Fig. 2 shows that the enzyme was active over a broad range of pH, with no marked peak between pH 7.0 and 9.0. At pH 10.0 there was a marked decrease in enzyme activity.

Michaelis constant. The Michaelis constant (K_m) for dehydroquinic acid was obtained by the method of Lineweaver & Burk (1934) (Fig. 3). The value at pH 7.4 was 61 μ M.

Fig. 1. Reversibility of the reaction catalysed by dehydroquinase. Quartz cuvettes of 1 cm. light-path contained 3 ml. of 0.1 M-potassium phosphate buffer, pH 7.4, and 0.063 μ mole of dehydroshikimic acid. A portion (0.1 ml.) of purified enzyme was added as indicated to both the blank and experimental cuvettes and the decrease in E at 234 m μ was measured in a Unicam SP. 500 spectrophotometer.

Specificity. The α -hydroxy acids malic and citric are dehydrated to the corresponding $\alpha\beta$ -unsaturated acids fumaric and aconitic by fumarase and aconitase respectively. Since these reactions resemble the reaction catalysed by dehydroquinase,

Fig. 2. Variation of enzyme activity with pH. Conditions of assay were as described in the text except that the pH of the buffer was varied and the enzyme activity was measured at 234 m μ . 0.1 M-Potassium phosphate buffer was used between pH 6.5 and 8.0, 0.1 M-tris-HCl buffer between pH 7.0 and 9.0 and 0.1 M-glycine-NaOH buffer between pH 9.0 and 10.0. (A) 0-80% Ammonium sulphate precipitate of the crude extract, dissolved in 0.1 M-potassium phosphate buffer, pH 7.4. (B) Purified enzyme, prepared as described in the Experimental section.

Fig. 3. Lineweaver-Burk plot for dehydroquinic acid. The position of the reciprocal of the Michaelis constant ($1/K_m$) is indicated. A purified enzyme preparation was used. Conditions of assay were as described in the text except that E at 234 m μ was measured and dehydroquinic acid concentration was altered as shown.

malate and citrate were tested as substrates of the purified dehydroquinase preparation. The molar-extinction coefficients of fumarate and *cis*-aconitate at 240 m μ are 2110 and 3540 respectively (Ochoa, 1951). Fumarase and aconitase activities were therefore measured at this wavelength. In the assay procedure described above, 3 μ moles of malate and citrate in turn replaced the dehydroquinic acid. 3-Deoxy-2-oxoheptonic acid 7-phosphate, a precursor of dehydroquinic acid in *Escherichia coli* (Srinivasan & Sprinson, 1959), was also examined for its ability to produce dehydroshikimic acid.

Fumarase and aconitase activities were not observed, and 3-deoxy-2-oxoheptonic acid 7-phosphate did not appear to form dehydroshikimic acid.

It was shown chromatographically that the purified enzyme could not interconvert the closely related quinic acid and shikimic acid.

Inhibition studies. Compounds of similar structure to dehydroshikimic acid and dehydroquinic acid examined for possible competitive inhibition were shikimic acid, quinic acid, 3-deoxy-2-oxoheptonic acid 7-phosphate and benzoic acid. The aromatic hydroxy acids and polyphenols tested in an analogous experiment with dehydroshikimic reductase (Balinsky & Davies, 1961*b*) could not be used because of their strong absorption at 234 m μ . A metal-chelating agent, ethylenediaminetetraacetic acid and two sulphhydryl reagents, iodoacetate and *p*-chloromercuribenzoate, were also tested. In these experiments the dehydroquinic acid concentration was decreased to 10 μ M and the 'inhibitor' was added to a concentration of 0.1 mM.

No inhibition or activation by any of these substances was observed. Dialysis for 1 hr. against 4 l. of 5 mM-potassium phosphate buffer, pH 6.0 or 7.4, slightly enhanced the activity of the enzyme, possibly owing to the removal of an inhibitor. The fact that activity was not lost on dialysis indicates that there is no requirement for a dialysable cofactor.

DISCUSSION

Dehydroquinase has been extracted from cauliflower buds and has been purified 7.6-fold. This enzyme has some similar properties to the dehydroquinase purified from extracts of *Escherichia coli* by Mitsuhashi & Davis (1954). Both enzymes were active between pH 6.5 and 9.0, the bacterial enzyme showing a slight activity peak at pH 8.0 which was even less marked for the plant enzyme. The Michaelis constants for dehydroquinic acid for the two enzymes were very similar, namely 61 μ M and 41 μ M for the plant and bacterial enzymes respectively at pH 7.4. The two enzymes differed considerably with respect to stability, the bacterial enzyme retaining its activity for at least 6 months

at -15° , whereas the plant enzyme was considerably inactivated on overnight storage at this temperature.

The enzyme does not appear to be identical with fumarase or aconitase, since malate and citrate did not appear to be dehydrated by the purified dehydroquinase preparation. The observation that 3-deoxy-2-oxoheptonic acid 7-phosphate does not give rise to dehydroshikimic acid indicates that the enzyme or enzymes converting this compound into dehydroquinic acid were inactive or absent in this preparation.

No inhibition was observed by any of the compounds tested which had similar structures to dehydroquinic acid and dehydroshikimic acid. This indicates that the site occupied by these acids is specific for these two compounds. It is possible that the carbonyl group is essential for attachment of the substrates to the enzyme, since the presence of this group in place of CH(OH) in the 5-position of the ring is the only structural difference between the natural substrates and their hydrogenated analogues, quinic acid and shikimic acid.

SUMMARY

1. An enzyme catalysing the interconversion of dehydroquinic acid and dehydroshikimic acid was extracted from cauliflower buds and purified 7.6-fold by ammonium sulphate fractionation.

2. The enzyme was found to be active over a broad range of pH, with no marked peak between pH 7.0 and 9.0.

3. The Michaelis constant for dehydroquinic acid at pH 7.4 was 61 μ M.

4. The enzyme did not catalyse the dehydration of malate or citrate.

5. No inhibition was observed by sulphhydryl reagents or by compounds related in structure to the natural substrates.

6. There appeared to be no dialysable cofactor.

The authors wish to express their gratitude to Professor T. A. Bennet-Clark, in whose Department this research work was carried out. One of us (D.B.) wishes to acknowledge with thanks the award of a King's College Tutorial Studentship and subsequently of a Witwatersrand Council of Education Scholarship.

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Studies on Carbohydrate-Metabolizing Enzymes

6. THE ACTION OF POTATO PHOSPHORYLASE (P-ENZYME) ON STARCH-TYPE POLYSACCHARIDES*

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Although the synthetic action of P-enzyme (potato phosphorylase) has been widely studied (for review see Whelan, 1958), observations on the degradation of amylose, amylopectin and glycogen by this enzyme are limited, and the results are often conflicting. For example, Katz, Hassid & Doudoroff (1948) claimed that potato amylose was almost completely converted into α -D-glucosyl phosphate by P-enzyme, whereas Peat, Thomas & Whelan (1952) found only 70% conversion. Baum, Gilbert & Scott (1956) have suggested that the incomplete phosphorolysis was due to oxidative modification of the substrate during the preparation from starch; other explanations, are, however, possible.

The action of P-enzyme on amylopectin, like that of β -amylase, is confined to the exterior chains, but the extent of degradation (see Table 1) is uncertain, and there appears to be no constant difference between the phosphorolysis and β -

amyolysis limits. The extent of arsenolysis of amylopectin (see equation 2) was found by Meyer, Weil & Fischer (1952) to be the same as the β -amyolysis limit.

This would suggest that P-enzyme can, in fact, degrade α -1 $\rightarrow$ 4-glucosidic linkages which are separated by only two or three glucose residues from an α -1 $\rightarrow$ 6-glucosidic inter-chain linkage. However, the β -amyolysis limits reported by Meyer *et al.* (1952) are about 10% higher than those found by other workers.

The extent of P-enzyme action on glycogen is not known. Swanson (1948) found that a sample of rabbit-liver glycogen had a phosphorolysis limit of

* Part 5: Gunja & Manners (1960).

Table 1. *Extent of degradation of amylopectins by P-enzyme and β -amylase*

Source of amylopectin	Phosphorolysis (%)	Arsenolysis (%)	β -Amylolytic (%)	Reference
Corn	40*	—	60	Swanson (1948)
Potato	59†	54	About 55	Katz, Hassid & Doudoroff (1948)
Potato	39†	—	53	Bourne, Sitch & Peat (1949)
Potato‡	48†	—	59	Peat, Thomas & Whelan (1952)
Potato	—	70	70	Meyer, Weil & Fischer (1952)
Tapioca	57†	—	—	Katz <i>et al.</i> (1948)
Tapioca	—	62	62	Meyer <i>et al.</i> (1952)
Waxy-maize starch	—	70	70	Meyer <i>et al.</i> (1952)

* Calculated from residual non-dialysable polysaccharide.

† Conversion into α -D-glucosyl phosphate.

‡ This sample was a sub-fraction and was considered to possess unusual properties.