SUMMARY

1. Experiments are described in which normal serum proteins were examined by modified cellulose chromatography after an initial electrophoretic separation.

2. Diethylaminoethylcellulose chromatography gave useful resolution of fractions obtained by starch-grain electrophoresis. Methods for the combination of chromatography with small-scale electrophoretic methods (with 0.2 ml. of serum) are described.

3. The general application of chromatography to serum protein fractionation is discussed.

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Aromatic Biosynthesis in Higher Plants

1. PREPARATION AND PROPERTIES OF DEHYDROSHIKIMIC REDUCTASE

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Davis and his co-workers (see Davis, 1955) indicated that the pathway of biosynthesis of the aromatic amino acids in *Escherichia coli* proceeded via the key intermediates dehydroquinic acid, dehydroshikimic acid and shikimic acid. The complete pathway from glucose to phenylalanine and tyrosine has since been elucidated by the recent work of Weiss & Mingioli (1956), Schwinck & Adams (1959) and Srinivasan & Sprinson (1959). Several workers have demonstrated the incorporation of labelled shikimic acid into a number of aromatic plant constituents (Brown & Neish, 1955; Eberhardt & Schubert, 1956; Underhill, Watkin & Neish, 1957; McCalla & Neish, 1959; Kosuge & Conn, 1959).

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The enzyme forming shikimic acid from its immediate precursor, dehydroshikimic acid, was demonstrated in cell-free extracts of *E. coli* by Yaniv & Gilvarg (1955) and named 'dehydroshikimic reductase'. This enzyme catalyses the reduction of dehydroshikimic acid, utilizing the reduced form of triphosphopyridine nucleotide as coenzyme. The enzyme was purified approximately ninefold, and some of its properties were studied. The presence of the enzyme was demonstrated in a number of organisms which can synthesize their own aromatic amino acids, namely *Aerobacter aerogenes*, *Saccharomyces cerevisiae*, *Euglena gracilis*, spinach leaves and peas.

This paper describes the extraction and purification of dehydroshikimic reductase from etiolated epicotyls of peas, and some of the properties of the purified enzyme. A preliminary report of this work has already been published (Balinsky & Davies, 1960).

EXPERIMENTAL

Materials. Shikimic acid was obtained from the California Foundation for Biochemical Research, and from L. Light and Co. Ltd. A small quantity of dehydroshikimic acid, synthesized by Grewe & Jeschke (1956), was kindly supplied by Dr B. D. Davis. Triphosphopyridine nucleotide (TPN) of 90–95% purity was obtained from the Sigma Chemical Co.

Enzyme assay

Yaniv & Gilvarg (1955) showed that the equilibrium constant for the reaction at 30° is given by

$$K_{\text{app.}} = \frac{[\text{SK}][\text{TPN}^+]}{[\text{DHS}][\text{TPNH}]}$$

$$= 5.7 \text{ at pH } 7.9 \text{ and } 27.7 \text{ at pH } 7.0,$$

where SK represents shikimic acid and DHS dehydroshikimic acid. Although the reaction in the direction of dehydroshikimic acid reduction is the one occurring bio-synthetically, and favoured by the equilibrium at pH 7.0 or 7.9, shikimic acid and TPN are more readily available than dehydroshikimic acid and reduced triphosphopyridine nucleotide (TPNH), therefore the reverse reaction was used to assay the enzyme.

The rate of formation of TPNH was therefore used (E at 340 m μ) to measure the activity of the enzyme. Spectrophotometric measurements were made in a Unicam SP. 500 spectrophotometer.

Procedure. Into a quartz cuvette of 1 cm. light-path were pipetted 3 μ moles of sodium shikimate, 0.5 μ mole of TPN and sufficient 0.1 M-glycine-NaOH buffer, pH 9.0, to give a final volume after addition of enzyme of 3 ml. With relatively crude preparations, a quantity of enzyme was used which gave an increase in E of not more than 0.03/min., since the plot of velocity against enzyme concentration deviated from linearity at higher rates. With more purified enzyme preparations, a rate of increase in E of up to 0.06/min. could be used, although the initial velocity declined sooner at the higher rates. To obtain a linear initial rate, five to six readings/min. over a period of 3 min. were taken for routine measurements. The blank cell contained

all the above-mentioned components except shikimate. In the latter stages of purification, when no blank reaction could be detected, TPN was also omitted from the blank cell.

A unit of activity is defined as the amount of enzyme producing a change of E at 340 m μ of 1.0/min. Specific activity is defined as the activity/mg. of protein.

Protein determinations. Protein concentration was determined by the method of Warburg & Christian (1942).

Extraction and purification of the enzyme

All manipulations were carried out at 2° unless otherwise stated. Centrifuging was carried out at 13 000–25 000g in a Servall or MSE refrigerated high-speed head.

Extraction. Chilled etiolated pea epicotyls were blended in a Waring Blendor with an equal volume of 0.2 M-potassium phosphate buffer, pH 7.4. The extract was strained through a tea-cloth.

First ammonium sulphate fractionation. To the filtrate obtained as described above, finely ground ammonium sulphate was added with stirring to 25% saturation. The mixture was well stirred for 10 min. and the precipitate formed removed by centrifuging. To the supernatant was added ammonium sulphate to 40% saturation, and the solution was stirred as described above. The precipitated protein was collected by centrifuging and redissolved in 0.2 M-potassium phosphate buffer, pH 7.4. This treatment appeared to produce considerable increase in activity, possibly owing to the removal of an inhibitor.

Heat treatment. The protein solution obtained was rapidly heated to 50° by immersion in a water bath at 60°, kept at 50° for 1 min., then rapidly cooled in an ice-salt bath. The precipitated protein was removed by centrifuging.

Second ammonium sulphate fractionation. The supernatant solution was fractionally precipitated by addition of ammonium sulphate to give 20, 25, 30 and 35% saturation. The fractions were collected as described above for the first ammonium sulphate fractionation.

Ammonium citrate fractionation. The most active fractions were combined and fractionally precipitated by

Table 1. *Purification of dehydroshikimic reductase*

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
First day						
Crude extract	300	18.0	2970	0.006	100	1.0
25–40% (NH ₄) ₂ SO ₄	34	28.0	694	0.040	156	6.7
Heated	34	25.8	558	0.046	144	7.7
Second (NH ₄) ₂ SO ₄ fractionation						
20–25%	10	6.4	69	0.093	62	{15.5
25–30%	10	4.8	47	0.102		{17.0
Second day						
20–30% mixed	20	8.0	116	0.069	44	11.5
Citrate fractionation						
35–40%	5	1.8	12	0.15	10	25.0
40–45%	5	2.0	10	0.20	11	33.3
35–45% mixed	10	3.8	22	0.17	21	28.3
Calcium phosphate-cellulose						
Unadsorbed protein	10	1.3	5.4	0.24	7.3	40.0
Eluate	5	2.1	4.5	0.47	12	78.3

addition of ammonium citrate to concentrations of 35, 40 and 45% (w/v). The precipitated proteins, collected by centrifuging, were dissolved in 0.02M-potassium phosphate buffer, pH 7.4.

Calcium phosphate-cellulose. The most active fractions were combined and adjusted to pH 6.7 with 0.2M-potassium dihydrogen phosphate. The solution was poured on to a column (4.5 cm. $\times$ 1 cm.) of calcium phosphate precipitated on cellulose (Price & Greenfield, 1954). The solution of unadsorbed protein was discarded and the column eluted with water.

Table 1 shows the results of a typical purification procedure.

RESULTS

Identification of reaction products. The oxidation of shikimate was allowed to proceed to completion in the assay procedure described above, except that potassium phosphate buffer, pH 7.4, was used because of the alkali-lability of dehydroshikimic acid

acid (Salomon & Davis, 1953). The absorption spectrum of the reaction mixture was measured and peaks were observed at 230 and 340 m μ , corresponding to the absorption maxima of dehydroshikimic acid and TPNH respectively. When the reaction was coupled with the reduction of glutathione by glutathione reductase, which reoxidizes TPNH and makes it available for further reaction, the presence of dehydroshikimic acid in the incubation mixture was shown chromatographically by comparison with the authentic acid, after development with butanol-formic acid-water (Davis & Mingioli, 1953).

Reversibility of reaction. The assay system described above was used. The reaction was allowed to proceed for 45 min., when the velocity had considerably slowed down. Dehydroshikimic acid was then added, and E at 340 m μ was found to decrease, owing to reoxidation of TPNH by the acid (Fig. 1). Addition of shikimic acid caused E to increase again.

Enzyme activity was measured between pH 6.0 and 10.6, potassium phosphate, 2-amino-2-hydroxy-

Fig. 1. Demonstration of the reversibility of the reaction catalysed by dehydroshikimic reductase. A purified enzyme preparation was used. Conditions of assay were as given in the text; TPN and shikimic acid (SK) were added at the points shown. Dehydroshikimic acid (DHS) was added after 65 min., and additional SK after a further 25 min.

Fig. 3. Lineweaver-Burk plot for shikimic 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 shikimic acid concentration was altered as shown.

Fig. 2. Variation of enzyme activity with pH. A purified enzyme preparation was used. Conditions of assay were as described in the text except that 0.1M-potassium phosphate buffer was used between pH 6.0 and 8.0, 0.1M-tris-HCl buffer between pH 8.0 and 9.2 and 0.1M-glycine-NaOH buffer between pH 9.0 and 10.6.

Fig. 4. Lineweaver-Burk plot for TPN. 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 TPN concentration was altered as shown.

methylpropane-1:3-diol (tris)-hydrochloric acid and glycine-sodium hydroxide buffers at a final concentration of 0.1 M being used. Activities at the same pH but with different buffers were identical, thereby demonstrating the absence of activation or inhibition by the individual buffers. The results (Fig. 2) show an optimum at pH 10.0.

Michaelis constants. The Michaelis constants (K_m) for the two substrates were obtained for several different enzyme preparations by the method of Lineweaver & Burk (1934) by plotting the reciprocal of velocity ($1/v$) against the reciprocal of substrate concentration ($1/[S]$), whilst keeping the concentrations of enzyme and one substrate constant (Figs. 3 and 4). Dilutions of substrate were used such that points along the $1/[S]$ axis were evenly spaced.

Four determinations of the Michaelis constant for shikimate at pH 9.0 gave the following values: 0.19 mM, 0.19 mM, 0.24 mM and 0.28 mM. The Michaelis constants for TPN at pH 9.0, 9.2 and 10.0 respectively were 7, 9 and $7 \mu\text{M}$. Fig. 4 shows a representative Lineweaver-Burk plot for TPN in which K_m was $7 \mu\text{M}$.

Specificity. When shikimate was replaced by quinate, protocatechuate, malate or lactate in the assay system described, no reduction of TPN occurred. Similarly, when diphosphopyridine nucleotide (DPN) (mM) replaced TPN, no formation of reduced diphosphopyridine nucleotide (DPNH) was observed in the presence of shikimate or any of the other compounds tested above.

The purified enzyme was free of dehydroquinase, which was assayed by the method of Balinsky & Davies (1961). Hence the studies of the enzyme kinetics of dehydroshikimic acid reductase were not complicated by removal of dehydroshikimic acid by dehydroquinase. The purified enzyme was shown to be free of glutathione reductase.

Metal requirement. No metal requirement could be demonstrated, since little change in enzyme activity was observed on addition of ethylenediaminetetra-acetic acid (EDTA) (10 mM) or potassium cyanide (2 mM) to the reaction mixture (Table 2).

Sulphydryl groups. The sulphydryl reagent *p*-chloromercuribenzoate was found to cause inhibition of the enzyme, which was reversed by addition of cysteine. Iodoacetate caused only slight inhibition, and this may be compared with results discussed by Boyer (1959), indicating that a number of enzymes inhibited by *p*-chloromercuribenzoate are not inhibited by iodoacetate.

DISCUSSION

Dehydroshikimic reductase, which has been purified 78-fold from extracts of pea epicotyls, has several properties in common with the corresponding enzyme isolated from extracts of *Escherichia coli* by Yaniv & Gilvarg (1955). Both enzymes are TPN-specific and do not catalyse the interconversion of the closely related quinic acid and dehydroquinic acid. The Michaelis constants for shikimic acid and TPN for the two enzymes are of the same order of magnitude. Michaelis constants of 55 and $31 \mu\text{M}$ at pH 8.0 were obtained for shikimic acid and TPN respectively by Yaniv & Gilvarg, compared with values of 0.19–0.28 mM and $7 \mu\text{M}$ respectively at pH 9.0 in the present studies. The pH optima for the two enzymes differ somewhat. The bacterial enzyme showed a pH optimum of 8.5, with activity declining sharply above pH 9.5, whereas the plant enzyme showed a pH optimum of 10.0. The difference in pH optima may conceivably be due to the fact that the bacterial enzyme was not fully saturated with substrate, since TPN was used at a concentration of only 1.4 times the value of the Michaelis constant at pH 8.0. The purified bacterial enzyme appears to be more stable than the purified plant preparation obtained in this investigation.

The low Michaelis constants for shikimic acid and TPN and the substrate specificity suggest that these are natural substrates of the enzyme. Unfortunately insufficient dehydroshikimic acid and TPNH were available to examine the Michaelis constants of these substances.

SUMMARY

1. A triphosphopyridine nucleotide-linked enzyme catalysing the interconversion of dehydroshikimic acid and shikimic acid was extracted and purified from etiolated epicotyls of pea seedlings. An overall purification of 78-fold was obtained.

2. The pH optimum for the oxidation of shikimic acid was found to be 10.0.

3. The Michaelis constant for shikimic acid at pH 9.0 was 0.19–0.28 mM. The Michaelis constant for triphosphopyridine nucleotide at pH 9.0 was $7 \mu\text{M}$.

4. The purified enzyme preparation appeared to have no multivalent-ion requirement.

Table 2. *Effect of various compounds on enzyme activity*

Enzyme activity was measured as described in the text.

Compound added	Concentration (mM)	Activity (% of original)
None	—	100
EDTA	10	108
Potassium cyanide	2	92
Cysteine	10	100
<i>p</i> -Chloromercuribenzoate	2.5	8
<i>p</i> -Chloromercuribenzoate + cysteine	10	84
Iodoacetate	10	84

5. The enzyme was inhibited by *p*-chloro-mercuribenzoate with reversal of inhibition by cysteine. Iodoacetate was slightly inhibitory.

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Aromatic Biosynthesis in Higher Plants

2. MODE OF ATTACHMENT OF SHIKIMIC ACID AND DEHYDROSHIKIMIC ACID TO DEHYDROSHIKIMIC REDUCTASE

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The extraction of dehydroshikimic reductase from etiolated epicotyls of pea seedlings and the purification of the enzyme have been described by Balinsky & Davies (1961). In order to determine the mode of attachment of the substrates shikimic acid and dehydroshikimic acid to the enzyme, various compounds of similar structure to these substrates were examined for possible competitive inhibition. A preliminary report of this work has already been published (Balinsky & Davies, 1960).

EXPERIMENTAL

Special chemicals. Shikimic acid was obtained from L. Light and Co. Ltd. Triphosphopyridine nucleotide was obtained from the Sigma Chemical Co.

Enzyme preparation. A purified enzyme preparation was obtained as described by Balinsky & Davies (1961).

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Measurement of enzyme activity. The enzyme was assayed as described by Balinsky & Davies (1961), except that the shikimic acid concentration was altered as indicated in Tables 1 and 2 and in Figs. 1-3. In addition, the compound being examined for inhibition was added at the concentrations shown in the Tables.

Some difficulty was experienced when testing pyrogallol, catechol, resorcinol, gallic acid and resorcylic acid. These compounds are readily oxidized by atmospheric or dissolved oxygen, and the increase in extinction at 340 mμ due to this oxidation masked any increase due to enzyme activity. In order to overcome this, all solutions were prepared in oxygen-free water and covered with a layer of toluene. Buffer, enzyme and substrates were pipetted into the cuvette and a few drops of toluene were added before addition of the polyphenol or hydroxy acid. Under these conditions, there was only a very slight blank reaction due to the added compound.

RESULTS

Several of the compounds tested were inhibitory (Table 1). Some of these compounds were more extensively studied to determine whether inhibi-