The purification and molecular properties of the tryptophan-sensitive 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase from *Neurospora crassa*

Gillian A. NIMMO and John R. COGGINS

Department of Biochemistry, University of Glasgow, Glasgow G12 8QQ, Scotland, U.K.

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Neurospora crassa contains three isoenzymes of 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase, which are inhibited by tyrosine, tryptophan and phenylalanine respectively, and it was estimated that the relative proportions of the total activity were 54%, 14% and 32% respectively. The tryptophan-sensitive isoenzyme was purified to homogeneity as judged by polyacrylamide-gel electrophoresis and ultracentrifugation. The tyrosine-sensitive and phenylalanine-sensitive isoenzymes were only partially purified. The three isoenzymes were completely separated from each other, however, and can be distinguished by $(\text{NH}_4)_2\text{SO}_4$ fractionation, chromatography on DEAE-cellulose and Ultrogel AcA-34 and polyacrylamide-gel electrophoresis. Polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate indicated that the tryptophan-sensitive isoenzyme contained one type of subunit of molecular weight 52000. The molecular weight of the native enzyme was found to be 200000 by sedimentation-equilibrium centrifugation, indicating that the enzyme is a tetramer, and the results of cross-linking and gel-filtration studies were in agreement with this conclusion.

3-Deoxy-D-arabino-heptulosonate 7-phosphate synthase [7-phospho-2-keto-3-deoxy-D-arabino-heptonate D-erythrose 4-phosphate-lyase (pyruvate-phosphorylating), EC 4.1.2.15] catalyses the formation of 3-deoxy-D-arabino-heptulosonate 7-phosphate and inorganic phosphate from D-erythrose 4-phosphate and phosphoenolpyruvate. This is the first committed step of the pathway by which the three aromatic amino acids, tyrosine, tryptophan and phenylalanine, are synthesized. The enzyme represents an important site for the regulation of the pathway, and various methods for regulating this step have been found in different groups of organisms (Jensen *et al.*, 1967; Jensen & Nasser, 1968). It has been shown that *Neurospora crassa* belongs to the group of organisms that contain three isoenzymes of 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase, each of which is subject to feedback inhibition by one of the three aromatic amino acids (Jensen & Nasser, 1968; Hoffmann *et al.*, 1972).

The three 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase isoenzymes from *N. crassa* have been separated (Jensen & Nasser, 1968; Doy, 1968; Gaertner & De Moss, 1970; Hoffmann *et al.*, 1972),

and the tyrosine-sensitive and phenylalanine-sensitive isoenzymes (Hoffmann *et al.*, 1972) and the tryptophan-sensitive isoenzyme (Ip & Doy, 1979) have been partially purified. No tryptophan-sensitive 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase from any source has hitherto been purified to homogeneity.

The present paper describes the purification to homogeneity of the tryptophan-sensitive 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase from *N. crassa* as a preliminary to the study of its properties and regulation.

Materials and methods

Materials

Rabbit muscle fructose bisphosphate aldolase (EC 4.1.2.13), *Escherichia coli* β -galactosidase (EC 3.2.1.23), rabbit muscle pyruvate kinase (EC 2.7.1.40), rabbit muscle lactate dehydrogenase (EC 1.1.1.27), pig heart malate dehydrogenase (EC 1.1.1.37), phosphoenolpyruvate and D-erythrose 4-phosphate were obtained from Boehringer Corp., Lewes, Sussex, U.K. Bistrispropane {1,3-bis[tris(hydroxymethyl)methylamino]propane},

bovine serum albumin (fraction V), dithiothreitol, ovalbumin (grade V) and phenylmethanesulphonyl fluoride were obtained from Sigma Chemical Co., Kingston upon Thames, Surrey, U.K. Benzamidine hydrochloride was obtained from Aldrich Chemical Co., Gillingham, Dorset, U.K. Tyrosine, tryptophan and phenylalanine were obtained from BDH Chemicals, Poole, Dorset, U.K. DEAE-cellulose (DE-52) and phosphocellulose (P-11) were obtained from Whatman Biochemicals, Maidstone, Kent, U.K. Ultrogel AcA-34 was obtained from LKB Instruments, South Croydon, Surrey, U.K. Sephadex G-25 was obtained from Pharmacia, London W.5, U.K.

The *arom* multienzyme complex was purified from *N. crassa* by the method of Lumsden & Coggins (1977). Phosphorylase was purified from rabbit muscle by the method of Fischer & Krebs (1958) and was recrystallized three times. 3-Deoxy-D-arabino-heptulosonate 7-phosphate was prepared and purified by the method of Herrmann & Poling (1975).

Growth of cells

Neurospora crassa strain 74-OR23-1A (F.G.S.C. no. 987, obtained from the Fungal Genetics Stock Center, Humboldt State University Foundation, Arcata, CA, U.S.A.) was grown, harvested and then stored in the form of powdered freeze-dried cells at -20°C as described by Lumsden & Coggins (1977).

Purification of tryptophan-sensitive 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase

All steps were performed at 4°C , and all solutions of phenylmethanesulphonyl fluoride and dithiothreitol were prepared just before use. The pH of the buffers was adjusted at 4°C .

Extraction. A 200 g batch of powdered *N. crassa* cells was added to 3 litres of 100 mM-Tris/HCl buffer, pH 7.5, containing 5 mM-EDTA, 0.4 mM-dithiothreitol and 1.2 mM-phenylmethanesulphonyl fluoride and stirred for 1 h. The suspension was centrifuged at 20000 g for 30 min in an MSE 18 centrifuge.

$(\text{NH}_4)_2\text{SO}_4$ precipitation. Benzamidine was added to the supernatant to give a concentration of 1 mM, and $(\text{NH}_4)_2\text{SO}_4$ was added to give a 40%-saturated solution (Dixon, 1953). The mixture was stirred for 15 min, then centrifuged at 20000 g for 30 min. Further $(\text{NH}_4)_2\text{SO}_4$ was added to the supernatant to give a 50%-saturated solution. The mixture was stirred and centrifuged as before, and the pellet was resuspended in 100 ml of buffer A (50 mM-Tris/HCl buffer, pH 7.5, containing 0.4 mM-dithiothreitol and 1.2 mM-phenylmethanesulphonyl fluoride) and dialysed overnight against 2×2 litres of the same buffer. The supernatant was used to prepare the other two 3-deoxy-D-arabino-heptulos-

onate 7-phosphate synthase isoenzymes as described below.

Chromatography on DEAE-cellulose. The dialysed material was loaded on to a column of DEAE-cellulose (bed volume 150 ml) that had been equilibrated with buffer A. The column was washed with buffer A until the A_{280} of the eluate was less than 0.1. The synthase was eluted with a linear gradient of 0–250 mM-KCl in buffer A (total volume 3 litres). The flow rate was 200 ml/h, and 12 ml fractions were collected. The fractions containing tryptophan-sensitive 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase were pooled (Fig. 1), and fresh phenylmethanesulphonyl fluoride was added to give a concentration of 1.2 mM.

Chromatography on phosphocellulose. The mixture was loaded on to a column of phosphocellulose (bed volume 40 ml) that had been equilibrated in buffer B (10 mM-potassium phosphate buffer, pH 6.5, containing 0.4 mM-dithiothreitol and 1.2 mM-phenylmethanesulphonyl fluoride). The column was washed with buffer B at a flow rate of 100 ml/h until the A_{280} of the eluate was zero. Tryptophan-sensitive 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase was eluted with 1 mM-phosphoenolpyruvate in buffer B; the flow rate was lowered to 50 ml/h. Those fractions that had a constant specific activity were pooled (Fig. 2) and concentrated by dialysis against 100 ml of 50 mM-Bistrispropane/HCl buffer, pH 7.4, containing 50% (v/v) glycerol, 0.4 mM-dithiothreitol, 1 mM-benzamidine and 0.1 mM-phosphoenolpyruvate. The enzyme was stored at -20°C .

Partial purification of the phenylalanine-sensitive and tyrosine-sensitive 3-deoxy-D-arabino-heptulosonate 7-phosphate synthases

These isoenzymes were prepared from the supernatant obtained after the precipitation of the tryptophan-sensitive isoenzyme with 50%-saturated $(\text{NH}_4)_2\text{SO}_4$.

$(\text{NH}_4)_2\text{SO}_4$ fractionation. Phosphoenolpyruvate (0.1 mM) was added to the supernatant, and $(\text{NH}_4)_2\text{SO}_4$ was added to give a 58%-saturated solution. The mixture was stirred and centrifuged as described above. Then $(\text{NH}_4)_2\text{SO}_4$ was added to the supernatant to give a 68%-saturated solution, and the precipitated material was collected in the same way as before. The 50–58%-satn. $(\text{NH}_4)_2\text{SO}_4$ and 58–68%-satn. $(\text{NH}_4)_2\text{SO}_4$ pellets were dialysed overnight against 2×2 litres of buffer A containing 0.1 mM-phosphoenolpyruvate.

Chromatography on DEAE-cellulose. Each pellet was loaded on to a column of DEAE-cellulose, which was run in the same manner as that used for the purification of the tryptophan-sensitive isoenzyme, except that all buffers contained 0.1 mM-

phosphoenolpyruvate. The elution profiles are shown in Fig. 1. The fractions containing the tyrosine-sensitive and phenylalanine-sensitive isoenzymes obtained from the 58–68%-satn. $(\text{NH}_4)_2\text{SO}_4$ pellet were pooled separately (Fig. 1) and concentrated in an Amicon ultrafiltration cell (PM 30 membrane). Each enzyme was further purified by gel filtration.

Gel filtration of tyrosine-sensitive 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase. The enzyme was loaded on to a column (44 cm $\times$ 2.2 cm) of Ultrogel AcA-34 equilibrated in buffer C (50 mM-Tris/HCl buffer, pH 7.5, containing 0.1 mM-phosphoenolpyruvate, 1 mM-benzamidine and 0.4 mM-dithiothreitol) containing 0.1 mM-phenylalanine. The flow rate was 20 ml/h, and 10 min fractions were collected. The fractions containing the tyrosine-sensitive isoenzyme were pooled and concentrated, and rechromatographed on the Ultrogel AcA-34 column equilibrated with buffer C containing 0.1 mM-tyrosine. The fractions containing the tyrosine-sensitive isoenzyme were pooled and concentrated.

Gel filtration of phenylalanine-sensitive 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase. The phenylalanine-sensitive isoenzyme was loaded on to the column of Ultrogel AcA-34, described above, equilibrated in buffer C. The fractions containing the phenylalanine-sensitive isoenzyme were pooled and concentrated, and rechromatographed on the Ultrogel AcA-34 column equilibrated in buffer C containing 0.1 mM-phenylalanine. The fractions containing the phenylalanine-sensitive isoenzyme were pooled and concentrated.

Both preparations of enzyme were stored at -20°C in 50 mM-Bis-trispropane/HCl buffer, pH 7.4 containing 50% (v/v) glycerol, 0.4 mM-dithiothreitol, 1 mM-benzamidine and 0.1 mM-phosphoenolpyruvate.

Cross-linking with dimethyl suberimide

Dimethyl suberimide dihydrochloride was prepared by the method of Davies & Stark (1970), and stock solutions (0.1 M) were prepared immediately before use (Lumsden & Coggins, 1977). Tryptophan-sensitive 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase was dialysed into 20 mM-Bis-trispropane/HCl buffer, pH 8.0, containing 0.4 mM-dithiothreitol, 1 mM-benzamidine and 0.1 mM-phosphoenolpyruvate. Cross-linking was performed at 20°C in a mixture containing 0.21 mg of tryptophan-sensitive 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase/ml, 0.1 mM-phosphoenolpyruvate, 1 mM-benzamidine, 0.4 mM-dithiothreitol, 0.25 M-triethanolamine hydrochloride/NaOH, 25 mM-dimethyl suberimide, 50 mM-NaCl and 12 mM-Bis-trispropane/HCl buffer, pH 8.0. After 1 h 1% (w/v) sodium dodecyl sulphate and 0.1%

(v/v) 2-mercaptoethanol were added, and the mixture was boiled for 3 min. The solution was dialysed overnight against 10 mM-sodium phosphate buffer, pH 7.0, containing 0.1% (v/v) 2-mercaptoethanol and 0.1% (w/v) sodium dodecyl sulphate before polyacrylamide-gel electrophoresis.

Cross-linked ovalbumin (Carpenter & Harrington, 1972), *arom* complex, bovine serum albumin and fructose biphosphate aldolase were prepared in a similar manner and were used as molecular-weight markers (Lumsden & Coggins, 1977).

7%-polyacrylamide-gel electrophoresis

Electrophoresis was performed by the method of Davis (1964). The gels were maintained at 5°C during electrophoresis and were pre-electrophoresed for 30 min before the enzyme samples were applied. Gels were stained for protein with Coomassie Brilliant Blue G-250. Gels to be stained for 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase activity were washed twice for 15 min at 25°C in 100 mM-glycine/KOH buffer, pH 10.0. They were then incubated at 25°C in 100 mM-glycine/KOH buffer, pH 10.0, containing 8 mM- CaCl_2 , 0.1 mM-phosphoenolpyruvate and 0.1 mM-D-erythrose 4-phosphate until a white band of precipitated calcium phosphate had formed. The gels were then rinsed and stored at 5°C in 100 mM-glycine/KOH buffer, pH 10.0, containing 8 mM- CaCl_2 .

5%- and 3%-polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate

Electrophoresis was performed by the method of Shapiro & Maizel (1969). Gels were stained for protein with Coomassie Brilliant Blue G-250 and were scanned at 600 nm in a Gilford spectrophotometer fitted with a model 252 gel scanner. The following cross-linked proteins were used as molecular-weight markers: rabbit muscle fructose biphosphate aldolase (subunit mol.wt. 40 000; Castellino & Barker, 1968), bovine serum albumin (mol.wt. 68 000; Tanford *et al.*, 1967), *arom* multi-enzyme complex from *N. crassa* (subunit mol.wt. 165 000; Lumsden & Coggins, 1977) and ovalbumin (mol.wt. 43 000; Castellino & Barker, 1968). A standard curve was constructed by the method of Weber & Osborn (1969).

Determination of molecular weights by gel filtration on Ultrogel AcA-34

The column (44 cm $\times$ 2.2 cm) of Ultrogel AcA-34 was equilibrated at 5°C in 50 mM-Tris/HCl buffer, pH 7.5, containing 0.1 mM-phosphoenolpyruvate, 1 mM-benzamidine and 0.4 mM-dithiothreitol with the additions indicated in the text. The column was run at 20 ml/h, and 10 min fractions were collected. The column was calibrated with the following molecular-

weight markers: pig heart malate dehydrogenase (mol.wt. 70000; Andrews, 1965), rabbit muscle phosphorylase (mol.wts. 200000 and 400000; Cohen *et al.*, 1971), bovine serum albumin (mol.wts. 68000 and 136000; Tanford *et al.*, 1967), rabbit muscle lactate dehydrogenase (mol.wt. 140000; Castellino & Barker, 1968), rabbit muscle pyruvate kinase (mol.wt. 237000; Steinmetz & Deal, 1966), rabbit muscle fructose bisphosphate aldolase (mol.wt. 160000; Kawahara & Tanford, 1966) and *E. coli* β -galactosidase (mol.wt. 540000; Ullmann *et al.*, 1968). Blue Dextran was used to measure the void volume, and a calibration curve was constructed by the method of Andrews (1965).

Ultracentrifugation studies

Sedimentation-equilibrium centrifugation was performed at 20°C in a Beckman model E ultracentrifuge fitted with a monochromator/scanner sensor. The 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase was dialysed into 50 mM-Bis-trispropane/HCl buffer, pH 7.4, containing 0.1 mM-dithiothreitol, 0.1 mM-phosphoenolpyruvate and 0.5 mM-benzamidine before centrifugation. The densities of the buffer and of the protein solution were measured with a Precision DMA 02C density meter (Anton Paar) and the partial specific volume $\bar{v}$ was calculated to be 0.7047 ml/g.

The same enzyme solution was used for sedimentation-velocity centrifugation studies. It was sedimented through 250 mM-KCl in 50 mM-Bis-trispropane/HCl buffer, pH 7.4, containing 0.1 mM-dithiothreitol at 60000 rev./min at 20°C. In some runs 0.2 mM-D-erythrose 4-phosphate, 0.2 mM-phosphoenolpyruvate, 0.1 mM-tryptophan, 20 mM-potassium phosphate, 0.2 mM-3-deoxy-D-arabino-heptulosonate 7-phosphate or combinations of these were included in the buffer.

Assay of 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase activity

Continuous assay method. The synthase activity was routinely assayed by the method of Schoner & Herrmann (1976). The standard assay mixture (1 ml) contained 50 mM-Bis-trispropane/HCl buffer, pH 7.4, 0.4 mM-D-erythrose 4-phosphate, 0.4 mM-phosphoenolpyruvate and enzyme. Either enzyme or D-erythrose 4-phosphate was added last to start the reaction, and the rate of decrease in absorbance at 232 nm was monitored at 25°C.

Discontinuous assay method. In crude extracts the synthase activity was assayed by a modification of the method of Doy & Brown (1965). Enzyme samples were desalted, just before assay, on a small column of Sephadex G-25 to remove dithiothreitol, which interferes with this assay method. The assay mixture contained 50 mM-Bis-trispropane/HCl buffer, pH 7.4, 0.4 mM-phosphoenolpyruvate, 0.4 mM-D-

erythrose 4-phosphate and enzyme in a volume of 0.2 ml. The mixture was incubated at 25°C for 10 min. The rate of reaction was proportional to the enzyme concentration and was linear with time. The reaction was initiated by the addition of D-erythrose 4-phosphate and terminated by the addition of 70 μ l of ice-cold 10% (w/v) trichloroacetic acid. The assay mixture was left in ice for 5 min, and denatured protein was removed by centrifugation. A 200 μ l sample of the supernatant was warmed to 37°C, mixed with 100 μ l of prewarmed 0.2 M-NaIO₄ in 9 M-H₃PO₄ and incubated for 5 min at 37°C. The reaction was stopped by the addition of 500 μ l of 0.8 M-NaAsO₃ and 0.5 M-Na₂SO₄ in 0.1 M-H₂SO₄. The mixture was left for at least 5 min at room temperature before the addition of 3 ml of 0.6% (w/v) thiobarbituric acid in 0.5 M-Na₂SO₄. The mixture was boiled for 5 min and immediately cooled in iced water. This gave a cloudy pink solution, which was mixed with 2.5 ml of cyclohexanone. The two layers were separated by centrifugation, and the absorbance of the cyclohexanone layer was measured at 549 nm. The assay was calibrated by using a sample of purified enzyme of known activity measured by the continuous assay method described above.

To distinguish between the three isoenzymes of 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase, assays were performed with and without the addition of 1 mM-tyrosine, 1 mM-tryptophan or 1 mM-phenylalanine.

One unit of activity is defined as the amount of enzyme that catalyses the conversion of 1 μ mol of substrate/min.

Determination of protein

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as the standard.

Results and discussion

Estimation of the relative proportions of the three isoenzymes in crude extracts

There are three 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase isoenzymes present in *N. crassa* (Jensen & Nasser, 1968; Hoffmann *et al.*, 1972). When the three isoenzymes had been separated from each other and partially purified, as described below, the tryptophan-sensitive enzyme was inhibited only by tryptophan, the phenylalanine-sensitive enzyme was inhibited only by phenylalanine and the tyrosine-sensitive enzyme was inhibited 11% by phenylalanine, 14% by tryptophan and 88% by tyrosine (Table 1). However, in agreement with the results obtained by Jensen *et al.* (1967), it was observed that the inhibitions of 3-deoxy-D-arabino-heptulosonate 7-

Table 1. Separation of *N. crassa* 3-deoxy-D-arabino-heptulosonate 7-phosphate synthases and purification of the tryptophan-sensitive isoenzymeStarting material was 200g of freeze-dried *N. crassa* cells. For full experimental details see the text.

Fraction	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Purifi- cation (fold)	% of activity with 1 mM-amino acid		
							Tyr	Phe	Trp
Extract	2605	17400	56.4*	0.003	100	1	45	70	46
40–50%-satn. (NH ₄) ₂ SO ₄	110	2845	13.0*	0.005	23	2	100	85	5
DEAE-cellulose	393	102	10.5†	0.103	19	32	128	142	11
(Trp-sensitive peak)									
Phosphocellulose	3.6	1.25	10.1†	8.1	18	2530	100	103	15
(Trp-sensitive enzyme)									
50–58%-satn. (NH ₄) ₂ SO ₄	90	2237	6.5*	0.003	11		56	67	78
58–68%-satn. (NH ₄) ₂ SO ₄	81	1432	37.0*	0.026	66		29	71	100
DEAE-cellulose	241	349	28.2†	0.081	50		75	24	94
(Phe-sensitive peak)									
Ultrogel ± Phe	16	0.60	2.7†	4.5	5		103	2	98
(Phe-sensitive enzyme)									
DEAE-cellulose	319	325	30.3†	0.093	54		17	92	99
(Tyr-sensitive peak)									
Ultrogel ± Tyr	24	2.13	0.57†	0.27	1		12	89	86
(Tyr-sensitive enzyme)									

* Measured by using the discontinuous assay method.

† Measured by using the continuous assay method.

phosphate synthase activity in the extract by each amino acid when present separately added up to 139% (Table 1), yet the inhibition observed when all three amino acids were present together was only 90%. Therefore it was not possible to determine the relative proportions of the three isoenzymes from measurements of the crude extract.

The tryptophan-sensitive isoenzyme was separated from the other two isoenzymes by (NH₄)₂SO₄ fractionation and was found in the 40–50%-satn.-(NH₄)₂SO₄ pellet. Most of the phenylalanine-sensitive and tyrosine-sensitive activity was found in the 58–68%-satn.-(NH₄)₂SO₄ pellet. Exactly 100% of the 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase activity measured in the extract was precipitated in the 40–68%-satn.-(NH₄)₂SO₄ pellets (Table 1), and less than 3% was found in the 0–40%-satn.-(NH₄)₂SO₄ and 68–90%-satn.-(NH₄)₂SO₄ pellets. The 40–50%-satn.-(NH₄)₂SO₄, 50–58%-satn.-(NH₄)₂SO₄ and 58–68%-satn.-(NH₄)₂SO₄ pellets were fractionated further by chromatography on DEAE-cellulose. This procedure resolved all three isoenzymes, and revealed the presence of a small amount of enzyme that was not inhibited by any of the aromatic amino acids (Fig. 1). The tryptophan-sensitive isoenzyme was eluted at a conductivity of 3.5 mS, the insensitive isoenzyme at 5.6 mS and the main peaks of the phenylalanine-sensitive and tyrosine-sensitive isoenzymes at 4.7 and 6.8 mS respectively.

On the basis of the recoveries shown in Fig. 1, it

was estimated that 54% of the total 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase activity in *N. crassa* was the tyrosine-sensitive isoenzyme, 14% was the tryptophan-sensitive isoenzyme and 32% was the phenylalanine-sensitive isoenzyme. No allowance was made for the fact that the tryptophan-sensitive isoenzyme is more stable during purification than are the other two isoenzymes, nor for the small amount of insensitive isoenzyme (approx. 7% of the total activity). This may represent desensitized tyrosine-dependent isoenzyme; we have found that this isoenzyme can be desensitized to tyrosine inhibition by chromatography on phosphocellulose. The main difference between these proportions and the proportions that might be deduced from the inhibition studies of the crude extract is that the proportion of tryptophan-sensitive isoenzyme is overestimated in the latter. This seems to be caused by two factors. Firstly, the tyrosine-sensitive isoenzyme is also partially inhibited by tryptophan. Secondly, the tyrosine-sensitive and phenylalanine-sensitive isoenzymes [the 58–68%-satn.-(NH₄)₂SO₄ fraction] are activated 1.6-fold by chromatography on DEAE-cellulose (Table 1); we have assumed that this activation is caused by the removal of an endogenous non-diffusible inhibitor.

Purification of tryptophan-sensitive 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase

The purification of the tryptophan-sensitive iso-

Fig. 1. Separation of *N. crassa* 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase isoenzymes by chromatography on DEAE-cellulose

The (a) 40–50%-satn., (b) 50–58%-satn.- and (c) 58–68%-satn.-(NH₄)₂SO₄ fractions from an extract of 200 g of freeze-dried *N. crassa* cells were each chromatographed on a DEAE-cellulose column equilibrated and run as described in the Materials and methods section. Each column was assayed for 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase activity with no addition (●) and with 1 mM-tryptophan (○), 1 mM-phenylalanine (▲) and 1 mM-tyrosine (□). —, Conductivity. All the activity was bound to the column, and only the part of the elution profile that contained activity is shown. The gradient was applied at zero elution volume in each case. The fractions indicated by the bars were pooled for further purification.

enzyme is summarized in Table 1. The tryptophan-sensitive isoenzyme obtained from the DEAE-cellulose column described above (Fig. 1) was purified to homogeneity by substrate elution from a phosphocellulose column (Fig. 2). The specific activity was constant across most of the peak, and the activity of the peak fractions was unaffected by 1 mM-phenylalanine or 1 mM-tyrosine (results not shown) but was inhibited by 85% when 1 mM-tryptophan was added to the standard assay mixture (Fig. 2). The purification was rapid and simple, and 10 units of enzyme (1.25 mg of protein) were obtained from 200 g of freeze-dried cells. The final specific activity of 8 units/mg is 4-fold higher than that obtained by Ip & Doy (1979) for their partially purified preparation of tryptophan-sensitive 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase from *N. crassa*. The apparently low yield at the first stage

of the purification [(NH₄)₂SO₄ fractionation] is due to the removal of the other two isoenzymes, and the true yield and purification are almost 5-fold higher than those quoted in Table 1. The enzyme can be stored for at least 6 months at –20°C in 50 mM-Bistrispropane/HCl buffer, pH 7.4, containing 0.4 mM-dithiothreitol, 1 mM-benzamidine, 0.1 mM-phosphoenolpyruvate and 50% (v/v) glycerol. When the enzyme became less active, full activity was restored by adding fresh dithiothreitol.

Partial purification of the tyrosine-sensitive and phenylalanine-sensitive 3-deoxy-D-arabino-heptulosonate 7-phosphate synthases

It has been reported that a mixture of these two isoenzymes was eluted from a column of agarose later in the presence of tyrosine and phenylalanine

Fig. 2. Purification of tryptophan-sensitive 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase on phosphocellulose

The column was prepared and run as described in the Materials and methods section. Fractions 1–61 were 14 ml each and fractions 62–73 were 7 ml each. At the arrow, 1 mM-phosphoenolpyruvate was added to the elution buffer. The fractions were monitored for A_{280} (●) and 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase activity in the absence (○) and in the presence (△) of 1 mM-tryptophan, and the specific activity in the absence of tryptophan was calculated (▲). Fractions 66–69 were pooled.

than in their absence (Halsall & Doy, 1969; Halsall *et al.*, 1971). Successive gel filtrations in the absence and in the presence of inhibitors were used to obtain a partial purification of these isoenzymes, although the two enzymes were not separated by the gel-filtration steps (Hoffmann *et al.*, 1972). It was found that the phenylalanine-sensitive isoenzyme obtained from the DEAE-cellulose column shown in Fig. 1 [58–68%-satn.-(NH_4) $_2\text{SO}_4$ fraction] had a lower apparent molecular weight, as determined by gel filtration in Ultrogel AcA-34, in the presence of phenylalanine than in its absence, and the isoenzyme was unaffected by tyrosine (results not shown). Similarly, the tyrosine-sensitive isoenzyme obtained from the same DEAE-cellulose column was unaffected by phenylalanine but had a lower apparent molecular weight in the presence of tyrosine than in its absence. The two enzymes were separated from each other by gel filtration on Ultrogel AcA-34 even in the absence of amino acids. Consequently successive gel filtrations in the presence and in the absence of the appropriate inhibitory amino acids, as described in the Materials and methods section, purified these two isoenzymes considerably and separated them from each other and from a small amount of 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase activity that was not inhibited by any of the aromatic amino

Fig. 3. Electrophoresis of 3-deoxy-D-arabino-heptulosonate 7-phosphate synthases in 7% polyacrylamide gels. The gels were run as described in the Materials and methods section and stained for protein (a) or 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase activity (b). (1) 20 μg of purified tryptophan-sensitive isoenzyme; (2) 40 μg of partially purified phenylalanine-sensitive isoenzyme; (3) 40 μg of partially purified tyrosine-sensitive isoenzyme.

acids. The partial purifications of these two enzymes are summarized in Table 1. Both isoenzymes were unstable and obtained in low yield, and neither was pure, although each was free from other 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase activities (Fig. 3).

Electrophoresis in 7% polyacrylamide gels

When purified tryptophan-sensitive 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase was electrophoresed in 7% polyacrylamide gels, one band of protein was observed, and this coincided with the band obtained when gels were stained for activity (Fig. 3). The enzyme appeared to be homogeneous, in contrast with the preparation obtained by Ip & Doy (1979), which gave two bands of protein after polyacrylamide-gel electrophoresis, only one of which was active. The band of tryptophan-sensitive isoenzyme activity clearly had a different mobility from the active bands obtained with the partially purified preparations of the phenylalanine-sensitive and tyrosine-sensitive isoenzymes (Fig. 3).

Gel filtration on Ultrogel AcA-34

Tryptophan-sensitive 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase was eluted from Ultrogel AcA-34 in a volume corresponding to a molecular weight of 200 000, as estimated by comparison with standard markers, and it was unaffected by the presence of 0.1 mM-tryptophan. Previous gel-filtration studies of this enzyme indicated that it was heterogeneous, with apparent molecular weights ranging from 79 000 to 165 000 (Doy, 1968). Possibly these smaller species were generated by proteolysis, whereas proteinase inhibitors were included in all the buffers used in our experiments. The apparent molecular weight of the phenylalanine-sensitive isoenzyme was lowered from 180 000 to 110 000 in the presence of 0.1 mM-phenylalanine, and that of the tyrosine-sensitive isoenzyme was lowered from 210 000 to 130 000 in the presence of 0.1 mM-tyrosine. Although there is some error in the absolute values of these molecular weights ($\pm 10 000$), the relative differences in the sizes of the three isoenzymes were clearly seen when the isoenzymes were chromatographed together and assayed in the presence and in the absence of the appropriate inhibitory amino acids. In earlier studies of the behaviour of the phenylalanine-sensitive and tyrosine-sensitive isoenzymes on gel-filtration columns, much lower values were obtained for the apparent molecular weights of the two isoenzymes in the presence and in the absence of inhibitors (Doy, 1968; Halsall & Doy, 1969). The apparent molecular weight of the insensitive isoenzyme was 210 000, and this was unaffected by aromatic amino acids.

Polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate

When purified tryptophan-sensitive 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase was subjected to electrophoresis in 5% polyacrylamide gels in the presence of sodium dodecyl sulphate only one band of protein was detected (Fig. 4), suggesting that the enzyme was homogeneous and contains a single type of subunit. The subunit molecular weight was estimated to be $52 000 \pm 3000$.

Fig. 4. Electrophoresis of purified tryptophan-sensitive 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase in 5% polyacrylamide gels in the presence of sodium dodecyl sulphate

The cross-linking was performed and the gels were run as described in the Materials and methods section. (a) 10 μ g of tryptophan-sensitive isoenzyme; (b) 25 μ g of tryptophan-sensitive isoenzyme cross-linked with dimethyl suberimidate.

Fig. 5. Sedimentation-equilibrium centrifugation of purified tryptophan-sensitive 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase

The plot of $\log(\text{concentration})$ ($\ln c$) against the square of the distance from the centre of rotation (r^2) was obtained from absorbance measurements recorded after 16 h centrifugation at 8000 rev./min in a Beckman model E ultracentrifuge.

by comparison with standard markers. When the enzyme was treated with the cross-linking reagent dimethyl suberimidate before electrophoresis on 3% (results not shown) and 5% polyacrylamide gels in the presence of sodium dodecyl sulphate, four bands of protein were observed, corresponding in decreasing order of intensity to monomers, dimers (mol.wt. $104\,000 \pm 2000$), trimers (mol.wt. $156\,000 \pm 2000$) and tetramers (mol.wt. $210\,000 \pm 10\,000$) (Fig. 4).

Ultracentrifugation studies of tryptophan-sensitive 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase

It has been shown that the behaviour of the tryptophan-sensitive isoenzyme on gel-filtration columns is unaffected by the presence of tryptophan (Halsall *et al.*, 1971). In agreement with this result, it was found that the sedimentation coefficient of the enzyme, $8.74 \pm 0.38S$, measured by sedimentation-velocity centrifugation, was not altered by the presence of phosphoenolpyruvate, D-erythrose 4-phosphate, 3-deoxy-D-arabino-heptulosonate 7-phosphate, phosphate, tryptophan or combinations of these.

The molecular weight of the native enzyme was found to be $200\,000 \pm 4000$ by sedimentation-

equilibrium centrifugation, and there was no evidence of inhomogeneity (Fig. 5). This agrees well with the value obtained from gel-filtration studies, suggesting that the enzyme is a globular protein. These results and those obtained from polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate suggest that tryptophan-sensitive 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase is a tetramer of like subunits, and the results of the cross-linking studies and sedimentation-velocity centrifugation are consistent with this conclusion.

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References

- Andrews, P. (1965) *Biochem. J.* **96**, 595–606
- Carpenter, F. H. & Harrington, K. T. (1972) *J. Biol. Chem.* **247**, 5580–5586
- Castellino, F. J. & Barker, R. (1968) *Biochemistry* **7**, 2207–2217
- Cohen, P., Duewer, T. & Fischer, E. H. (1971) *Biochemistry* **10**, 2683–2694
- Davies, G. E. & Stark, G. R. (1970) *Proc. Natl. Acad. Sci. U.S.A.* **66**, 651–656
- Davis, B. J. (1964) *Ann. N.Y. Acad. Sci.* **121**, 404–427
- Dixon, M. (1953) *Biochem. J.* **54**, 457–458
- Doy, C. H. (1968) *Rev. Pure Appl. Chem.* **18**, 41–78
- Doy, C. H. & Brown, K. D. (1965) *Biochim. Biophys. Acta* **104**, 377–389
- Fischer, E. H. & Krebs, E. G. (1958) *J. Biol. Chem.* **231**, 65–71
- Gaertner, F. H. & De Moss, J. A. (1970) *Methods Enzymol.* **17**, 387–401
- Halsall, D. M. & Doy, C. H. (1969) *Biochim. Biophys. Acta* **185**, 432–446
- Halsall, D. M., Catcheside, D. E. A. & Doy, C. H. (1971) *Biochim. Biophys. Acta* **227**, 464–472
- Herrmann, K. M. & Poling, M. D. (1975) *J. Biol. Chem.* **250**, 6817–6821
- Hoffmann, P. J., Doy, C. H. & Catcheside, D. E. A. (1972) *Biochim. Biophys. Acta* **268**, 550–561
- Ip, K. & Doy, C. H. (1979) *Eur. J. Biochem.* **98**, 431–440
- Jensen, R. A. & Nasser, D. S. (1968) *J. Bacteriol.* **95**, 188–196
- Jensen, R. A., Nasser, D. S. & Nester, E. W. (1967) *J. Bacteriol.* **94**, 1582–1593
- Kawahara, K. & Tanford, C. (1966) *Biochemistry* **5**, 1578–1584
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Lumsden, J. & Coggins, J. R. (1977) *Biochem. J.* **161**, 599–607

- Schoner, R. & Herrmann, K. M. (1976) *J. Biol. Chem.* **251**, 5440–5447
- Shapiro, A. L. & Maizel, J. V. (1969) *Anal. Biochem.* **29**, 505–514
- Steinmetz, M. A. & Deal, W. C. (1966) *Biochemistry* **5**, 1399–1405
- Tanford, C., Kawahara, K. & Lapanje, S. (1967) *J. Am. Chem. Soc.* **89**, 729–736
- Ullmann, A., Goldberg, M. E., Perrin, D. & Monod, J. (1968) *Biochemistry* **7**, 261–265
- Weber, K. & Osborn, M. A. (1969) *J. Biol. Chem.* **244**, 4406–4412