Purification of argininosuccinase from *Neurospora* and comparison of some properties of the wild-type enzyme and an enzyme formed by inter-allelic complementation

By BRIAN B. COHEN* and JOHN O. BISHOP

Institute of Animal Genetics, West Mains Road, Edinburgh

1. INTRODUCTION

Argininosuccinase (ASAase) from rat liver catalyses the conversion of arginino-succinate (ASA) to arginine and fumarate (Ratner *et al.*, 1953) by a reversible reaction. Extracts of wild-type *Neurospora* mycelium possess catalytic properties similar to liver ASAase, and *arg-10* mutants, which lack this activity, accumulate ASA (Fincham & Boylen, 1957).

This paper describes a procedure for purifying ASAase from wild-type *Neurospora* and from a heterokaryon between two complementing *arg-10* mutants, and describes some properties of the enzymes.

2. MATERIALS AND METHODS

(i) *Stocks*

The wild-type strain of *Neurospora crassa* was ST*A*. The heterokaryon was formed using the *arg-10* mutant stocks B317-9-9a (Newmeyer, 1957) and 402-3*a* (Rice, 1963; derived by backcrossing K402*a* (D. G. Catcheside) three times to ST*A*).

(ii) *Culture of mycelium*

Mycelium was grown in 2-l. conical flasks containing 1·5 l. of 2% sucrose in 1% Vogel's medium (Vogel, 1955). The wall of each flask had three symmetrical indentations which caused turbulence and increased aeration. The flasks were shaken at 25°C. on a New Brunswick Gyratory Shaker at speed setting number 4. Flasks were inoculated with 5×10^6 wild-type conidia and harvested after 45 to 52 hours, or with 2×10^7 heterokaryon conidia and harvested after 36 to 42 hours. The mycelium was harvested in a basket centrifuge, frozen, lyophilized, powdered in a mortar and stored at -12°C. The yield of mycelium was 3 to 6 g. dry weight per flask.

* Present address: Department of Cell Biology, University of Glasgow.

(iii) *Extraction and purification of ASAase*

All operations were carried out at, or near, 0°C.

(a) *Wild-type*

The powdered mycelium was homogenized in 0·02 M sodium phosphate buffer, pH 7·9 (6·6 ml./g. dry weight), for 3 min., in a blendor (M.S.E. or Waring). The blendor was rinsed out with the same volume of buffer. This was added to the homogenate and the mixture centrifuged at $13,000 \times g$ for 30 min. to remove cell debris. The supernatant is referred to as *crude extract*.

The crude extract was treated with 1% (*w/v*) protamine sulphate, pH 7·5 (0·225 ml./ml. crude extract), and the precipitate removed by centrifugation. The protamine supernatant was stirred and slowly brought to 40% ammonium sulphate saturation with a saturated (0°C.) solution of ammonium sulphate, and after 30 min. centrifuged to remove the precipitate. The 40% supernatant was brought to 55% saturation, and centrifuged in the same way. The supernatant was discarded and the precipitate dissolved in 0·01 M *tris*-HCl (pH 7·5 at 20°C.) using a volume equal to $\frac{1}{10}$ of the volume of the 40% ammonium sulphate supernatant. The solution was then dialysed twice against 4 l. of the same buffer. The protein concentration was adjusted to 5 mg./ml. with 0·01 M *tris*-HCl and 0·15 vol. of calcium phosphate gel (30 mg./ml.) slowly added. After 30 min. the gel was removed by centrifugation and the supernatant (CP_1 super) retained. To this was added 0·444 vol. of gel, the mixture centrifuged and the supernatant discarded. The gel pellet was eluted with 0·006 M potassium phosphate, pH 6, using a volume of buffer equal to the second volume of gel added, the suspension was centrifuged and the supernatant discarded. The pellet was re-eluted with the same volume of 0·1 M buffer, the suspension centrifuged and the supernatant (E_2) retained. E_2 was adjusted to 10^{-3} M mercaptoethanol and dialysed overnight against 9 vol. of 10^{-3} M mercaptoethanol. The solution was adjusted to pH 6·5 and 200 to 500 ml. applied to a 2×33 cm. DEAE-cellulose column equilibrated with 0·01 M potassium phosphate, pH 6·5, containing 10^{-3} M EDTA and 10^{-3} M mercaptoethanol. The column was washed with the same buffer and the enzyme eluted with a 400 ml. linear potassium phosphate molarity gradient (0·05 to 0·11 M) at pH 6·5. The elution buffers were 10^{-3} M with respect to mercaptoethanol and EDTA. In some cases the enzyme was eluted without a gradient using 0·05 M buffer alone.

Those fractions containing ASAase were pooled and adjusted successively to 35, 45, 50 and 55% saturation with a saturated solution of ammonium sulphate. Each precipitate was removed by centrifugation and dissolved in 0·01 M potassium phosphate, pH 7·5, containing 10^{-3} M EDTA and 10^{-3} M mercaptoethanol (using 0·1 ml. buffer for every milligramme of protein in the pooled DEAE elutes). The enzyme solutions were then dialysed against 0·006 M potassium phosphate, pH 6·9, containing 10^{-3} M mercaptoethanol.

The active fractions were pooled and applied to a hydroxylapatite column (Tiselius *et al.*, 1956) previously equilibrated with 0·001 M potassium phosphate,

pH 6·9, and washed in with the 0·06 M buffer. A 2×15 cm. column was used to fractionate a solution containing 10 mg. protein. Inactive protein was eluted with 0·04 M buffer, and the enzyme with 0·06 M potassium phosphate, pH 6·9, containing 10^{-3} M mercaptoethanol. The 0·06 M active fractions were pooled and retained.

(b) Heterokaryon.

No procedure for purifying the heterokaryon enzyme completely has been developed. The following procedure gave the most satisfactory results.

Extraction and purification followed the wild type procedure up to the first 55% ammonium sulphate precipitation, except that the precipitation at 40% saturation was omitted.

For the preparation of CP_1, 0.1 volume of gel was used, and for adsorbing the enzyme from CP_1, 0.33 volume of gel. The gel was eluted with 0.006 M, 0.1 M and 0.15 M potassium phosphate, pH 6. The 0.1 M and 0.15 M eluates were pooled and fractionated on a DEAE column by a molarity gradient as described above. The active DEAE fractions were pooled and adjusted to 35, 45 and 55% ammonium sulphate saturation. The 45% precipitate was applied to a hydroxylapatite column and eluted with 0.04 and 0.08 M phosphate. The active fractions eluted with 0.08 M buffer were pooled and retained.

Estimation of ASAase

The standard assay measured the back reaction: arginine + fumarate→ASA. The assay mixture contained, in 0·2 ml., 12·5 μmoles L-arginine (guanido-C^{14}) monohydrochloride, specific activity 0·04 c/mole; 12·5 μmoles sodium fumarate, 5 μmoles sodium or potassium phosphate, pH 7·5, and enzyme. The mixture contained 0·025 ml. wild-type enzyme with an incubation time of 1 hour at 37°C. for preparations up to the calcium phosphate gel eluate and 30 min. for preparations from the DEAE step onwards, or 0·075 ml. heterokaryon enzyme with an incubation time of 2 hours.

The reaction was stopped by chilling the tubes in ice. 3·2 μl. aliquots were applied at 1 in. intervals to an 8×12 in. sheet of Whatman 3 MM chromatography paper alongside a standard solution and electrophoresed for 30 min. at 1200 V. using pyridine–acetic acid buffer (10% v/v pyridine, 0·4% v/v glacial acetic acid, pH 6·5). The apparatus was a smaller version of the tank described by Katz *et al.* (1959). After drying the paper, ASA was located by staining the reference strip with ninhydrin, and appropriate portions of the sample strips were cut out, placed in 10 ml. of counting fluid (0·5% w/v PPO, 0·03% w/v dimethyl POPOP in toluene) and the radioactivity measured in a Tricarb liquid scintillation counter.

The unit of ASAase activity is defined as the amount of enzyme catalysing the production of 1 μmole ASA per hour in the back reaction. Specific activity is expressed as units per milligramme soluble protein. Purification of one fraction over another is expressed as the ratio of specific activities of the fractions.

(iv) *Determination of Michaelis constants*

K_M (*ASA*)

The initial rate of formation of fumarate from ASA was measured by recording

S

the increase in optical density at 240 mμ. The potassium salt of ASA was used at concentrations between 0·1 and 0·7 mM. The reactions were run in 0·1 M phosphate buffer, pH 7·5.

K_M (*fumarate*)

The initial rate of disappearance of fumarate was measured in the same way. The reaction mixture contained 0·0625 M arginine and 0·07 M phosphate buffer, final pH 7·5. Fumarate concentrations were in the range 0·1 to 1 mM.

K_M (*arginine*)

A modification of the standard assay for the estimation of ASAase was used to measure the conversion of C^{14}-arginine to ASA. The reaction mixture contained 0·075 M phosphate buffer, pH 7·5, 0·0625 M fumarate and 0·25 to 2 mM arginine. After 5 min., 10 μl. of the reaction mixture was subjected to electrophoresis and radioactivity of the ASA determined as described above.

All the enzyme preparations used for K_M determinations were purified at least through the DEAE step, and checked for total absence of fumarase activity.

(v) *Sucrose density gradient centrifugation* (Martin & Ames, 1961)

0·2 ml. of fraction CP_1 was layered on a 4·8 ml. 5–20% sucrose gradient at 4°C. and centrifuged for 12 hours at an average speed of 38,400 r.p.m. in the Spinco No. SW39L rotor. Samples of seven drops were collected from the bottom of the tube and assayed for ASAase activity by the standard method.

(vi) *Starch gel electrophoresis*

The discontinuous buffer system of Poulik (1957) was used. Otherwise the procedure of Smithies (1955) was followed, with a voltage gradient of 2 to 3 V./cm. In some cases one of the horizontal gel slices was cut into segments (3 or 5 mm.) and each of these was assayed for ASAase activity by immersing the gel in reaction mixture for several hours at 37°C. Samples of the mixture were then removed and the conversion of C^{14}-arginine to ASA measured as described.

(vii) *Analytical centrifugation*

Samples were centrifuged in the standard rotor of the Spinco Model E ultracentrifuge using 12-mm. cells with Kel-F centre pieces. Homogeneity was examined using Schlieren optics and a protein concentration of about 4 mg./ml. Sedimentation coefficients were determined using ultra-violet optics and a protein concentration of 1 mg./ml. The molecular weight of wild-type ASAase was determined by the short-column equilibrium method of Yphantis (1960) using purified ASAase at a concentration of 4·5 mg./ml. The length of the column was 0·06 cm. and centrifugation was for 2·25 hours at 6550 r.p.m. and 20°C.

3. RESULTS

(i) *Purification of wild-type ASAase*

Table 1 shows summarized data from a typical purification. In general there was no marked loss at any step of the procedure. The recovery of enzyme from the
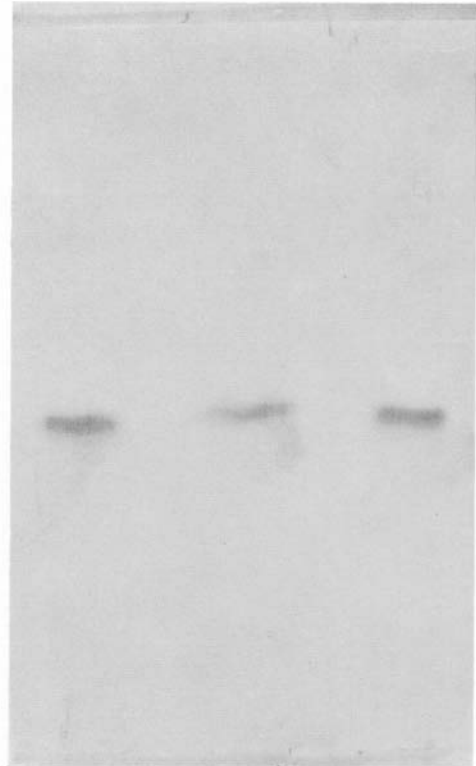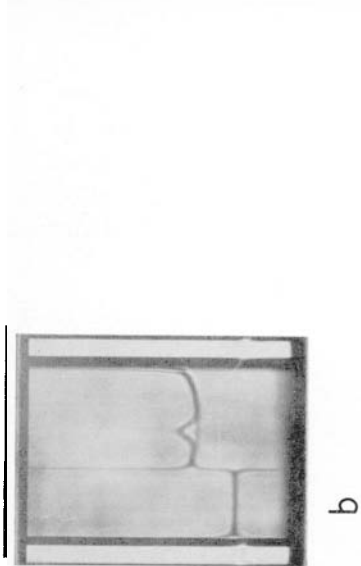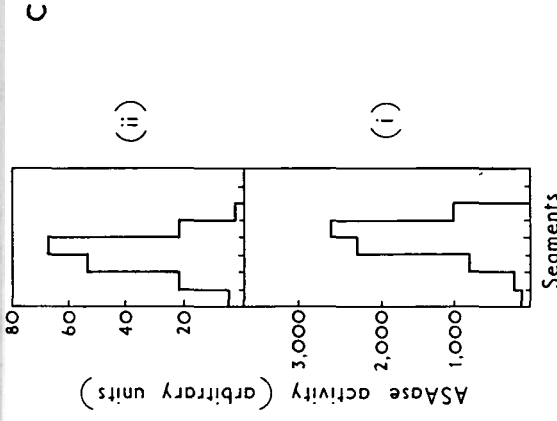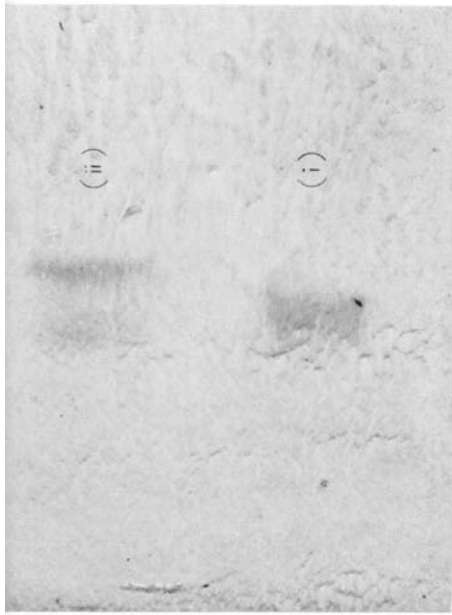
Plate Ia. Photograph of starch gel following electrophoresis of purified wild-type ASAase. The protein concentration was about 4 mg./ml. Migration from left to right.

Plate Ib. Sedimentation of purified wild-type ASAase. Sedimentation from left to right. Protein concentration 4 mg./ml. Photograph taken with bar angle of 50° 48 min. after reaching 42,040 r.p.m. at 20°C.

Plate Ic. Comparison of electrophoretic mobility of wild-type (i) and heterokaryon (ii) ASAase. Migration from left to right. The very

BRIAN B. COHEN and JOHN O. BISHOP

(*Facing p.* 246)

Table 1. *Purification of ASAase from wild-type mycelium*

	ASAase activity (units)	Total protein (mg.)	Specific activity (units/mg.)	Purification	Recovery (%)
Crude extract	77,722	52,890	1·47		
55% (NH₄)₂SO₄	63,302	5,015	12·6	8·6	82
Calcium phosphate gel step 1 super (CP₁)	57,330	1,886	30·41	21	74
Calcium phosphate gel eluate (E₂)	39,250	634	62	42	50
Total DEAE fractions	12,790	91	141	96	16
Pooled (NH₄)₂SO₄ ppts.	9,041	23·3	388	264	12
Total hydroxylapatite fractions	5,770	5·7	1,013	690	7

180 g. of powder was extracted and ASAase purified through the hydroxylapatite step. Recoveries and purifications are with respect to crude extract.
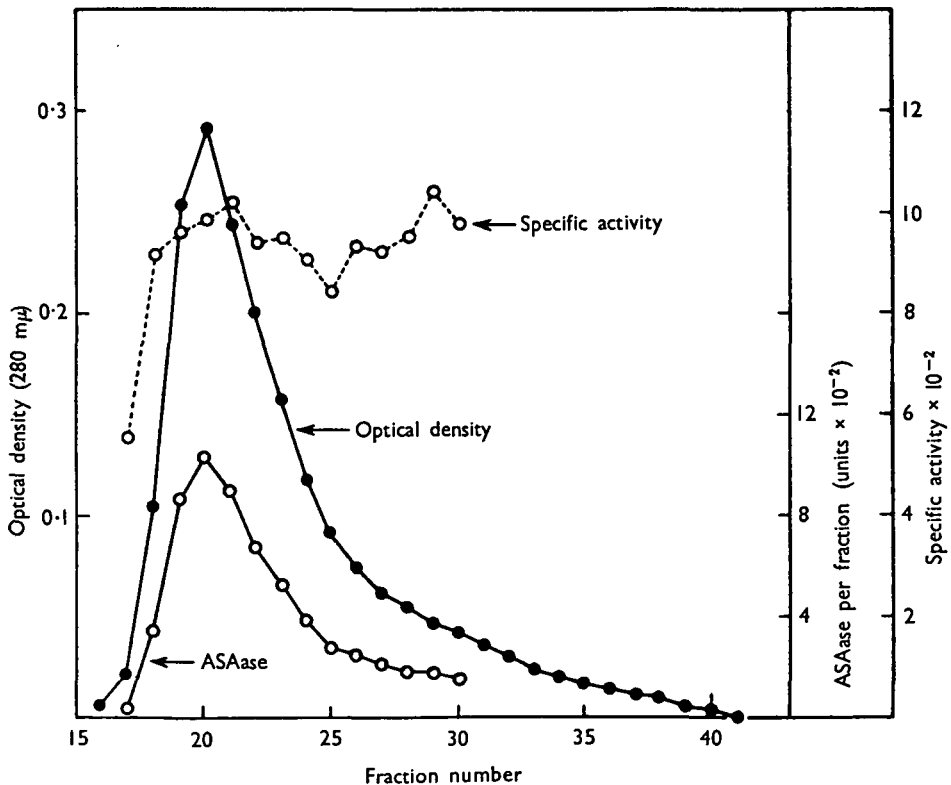

Fig. 1. Chromatography of wild-type ASAase on hydroxylapatite. The profile shown was obtained by elution with 0·06 M phosphate buffer.

DEAE–cellulose column in this particular preparation was unusually low. An indication of the homogeneity of the purified enzyme was the uniform specific activity of the successive fractions eluted from hydroxylapatite with 0·06 M buffer (Fig. 1). The active hydroxylapatite fractions were pooled and concentrated to 5 mg. protein per millilitre and the homogeneity of the preparation further tested by starch-gel electrophoresis (Plate I*a*) and by analytical ultracentrifugation (Plate I*b*). By both methods the preparation appeared homogeneous.

(ii) *Purification of heterokaryon ASAase*

Table 2 shows a summary of the purification procedure. Complete purification was never achieved. The data suggest that better purification and recovery would be achieved by omitting the CP_2 step. The 45% ammonium sulphate precipitate was fractionated on a hydroxylapatite column and yielded an active peak which was shown by both analytical ultracentrifugation and starch-gel electrophoresis to contain two components.

Table 2. *Purification of ASAase from heterokaryon mycelium*

	ASAase activity (units)	Total protein (mg.)	Specific activity (units/mg.)	Purification	Recovery (%)
Crude extract	2,021	41,743	0·048		
55% $(NH_4)_2SO_4$ ppt.	1,933	5,360	0·36	7·5	95·6
Calcium phosphate gel step 1 super (CP_1)	1,477	2,418	0·61	12·6	73·1
Calcium phosphate gel eluate, 0·1 M (CP_2)	382	707	0·54	11·2	18·9
Calcium phosphate gel eluate, 0·15 M (CP_2)	504	551	0·92	19	24·9
Total DEAE fractions	285	60	4·7	97	14·1
45% $(NH_4)SO_4$ ppt.	78·5	13·4	5·9	121	3·9
55% $(NH_4)_2SO_4$ ppt.	81·3	21·6	3·8	78	4·0

242 g. was extracted and ASAase purified to the final ammonium sulphate step. Recovery and purification is with respect to crude extract.

(iii) *Kinetic properties*

The pH optimum for the back reaction was close to 7 for both enzymes. This is consistent with the value found by Fincham & Boylen (1957) for the wild-type enzyme.

K_M values are shown in Table 3. The K_M for arginine was not determined for the heterokaryon enzyme. The other two K_M values appear to be the same for both enzymes.

Table 3. *Michaelis constants of ASAase extracted from wild-type and heterokaryon mycelia. In several cases, the results of two separate determinations are shown*

	$K_M \times 10^4$	
Substrate	Wild-type	Heterokaryon
ASA	1·78	2·64
	2·49	
Fumarate	4·84	4·10
	5·22	2·47
Arginine	8·07	—

(iv) *Physical properties*

A purified preparation of the wild-type enzyme, examined in the ultracentrifuge at a concentration of 1 mg./ml., gave $S_{20,w} = 8·7 \times 10^{-13}$. Fraction CP_1 of the wild-type and heterokaryon enzymes, and a mixture of the two, were analysed by sucrose gradient centrifugation (Fig. 2). In each case a single peak of enzyme activity was located at the same position in the gradient showing that the sedimentation coefficients of the two enzymes are identical. Calculated by the method of Martin & Ames (1961) the sedimentation coefficient was again found to be 8·7 *s*.

By starch gel electrophoresis at pH 8·7 the mobilities of the two enzymes were found to be very similar, and probably identical (Plate I*c*).

The molecular weight of the wild-type enzymes, determined by the short-column equilibrium method (Yphantis, 1960), was found to be 176,000 (assuming a partial specific volume of 0·74). The frictional ratio, estimated from the molecular weight and the sedimentation coefficient, was 1·23, indicating a nearly spherical molecule.

In the presence of 0·5% sodium dodecyl sulphate, the wild-type enzyme showed a single component in the analytical ultracentrifuge with a sedimentation coefficient of 2·3 *s*.

4. DISCUSSION

The procedure for purifying ASAase from wild-type mycelium is reliable and quite efficient. 10% of the ASAase in the crude extract can be recovered with a 700-fold purification. The purified enzyme is homogeneous by the criteria of sedimentation and starch-gel electrophoresis.

Our failure to isolate ASAase from the heterokaryon by the same procedure may reflect a structural difference between the two enzymes. The existence of such a difference is supported by the chromatographic properties of the two enzymes on hydroxylapatite columns, the wild-type enzyme being eluted with 0·06 M phosphate buffer, the heterokaryon enzyme largely with 0·08 M buffer. At the same time, there is an indication in the lower recovery after ammonium sulphate precipitation that the heterokaryon enzyme is more labile.

The two enzymes were indistinguishable by sedimentation analysis and starch-gel electrophoresis. Thus, while they may differ in minor ways, they are probably very similar molecules. Previous comparisons of heterokaryon and wild-type

enzymes by sedimentation analysis (Partridge & Giles, 1963; Gross & Webster, 1963) and starch-gel electrophoresis (Fincham & Coddington, 1963) also showed the two to be indistinguishable by these means. The catalytic properties of the two enzymes were also much the same, as estimated by pH optima and K_M values. This
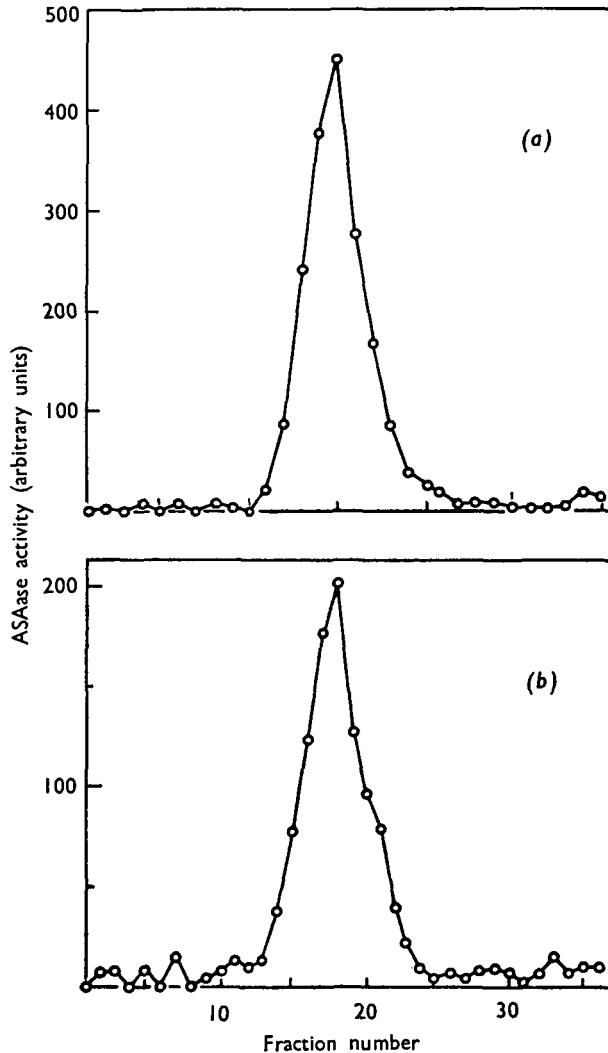

Fig. 2. Sedimentation of wild-type and heterokaryon ASAase through sucrose gradients. Direction of sedimentation from right to left. (a) Wild type. (b) Heterokaryon.

result is less to be expected since earlier studies have shown wild-type and heterokaryon enzymes generally to have different K_M values (Fincham, 1959; Suyama & Bonner, 1964).

Our findings are readily explained in terms of the current theory of intracistronic complementation (Brenner, 1959; Fincham, 1959). Of particular interest is the

great reduction in sedimentation coefficient observed when the purified wild-type enzyme was treated with S.D.S., suggesting strongly that the active enzyme molecule is made up of dissociable sub-units, like the other complementation enzymes which have been studied in this way, glutamic dehydrogenase (Fincham & Coddington, 1963) and tryptophan synthetase (Carsiotis *et al.*, 1963).

SUMMARY

Argininosuccinase has been purified from wild-type *Neurospora crassa*, strain ST*A*. The purified enzyme, which is homogeneous by the criteria of analytical centrifugation and starch-gel electrophoresis, has a molecular weight of about 175,000. The enzyme has also been partially purified from a heterokaryon between the *arg*-10 mutant stocks B317-9-9*a* and 402-3*a*.

The reaction kinetics of the two enzymes were compared in several respects, and they were found to be indistinguishable. The enzymes were also indistinguishable by starch-gel electrophoresis, and sedimented at the same rate through a sucrose gradient. It seems likely, however, that the enzymes do differ physically since they showed different affinities for both calcium phosphate gel and hydroxylapatite during purification.

REFERENCES

BRENNER, S. (1959). The mechanism of gene action. *Ciba Foundation Symposium*, pp. 304–316. London: Churchill.

CARSIOTIS, M., APPELLA, E. & SUSKIND, S. R. (1963). Purification and properties of tryptophan synthetase from *Neurospora crassa*. *Genetics Today* (S. J. Gearts, ed.), vol. 1, p. 52.

FINCHAM, J. R. S. (1959). On the nature of the glutamic dehydrogenase formed by inter-allele complementation at the *am* locus of *Neurospora crassa*. *J. gen. Microbiol.* **21**, 600–611.

FINCHAM, J. R. S. & BOYLEN, J. (1957). *Neurospora crassa* mutants lacking argininosuccinase. *J. gen. Microbiol.* **16**, 438–448.

FINCHAM, J. R. S. & CODDINGTON, A. (1963). Complementation at the *am* locus of *Neurospora crassa*: a reaction between different mutant forms of glutamate dehydrogenase. *J. molec. Biol.* **6**, 361, 373.

GROSS, S. R. & WEBSTER, R. E. (1963). Some aspects of interallelic complementation involving leucine biosynthetic enzymes of *Neurospora*. *Cold Spring Harb. Symp. quant. Biol.* **28**, 543–547.

KATZ, A. M., DREYER, W. J. & ANFINSEN, C. B. (1959). Peptide separation by two-dimensional chromatography and electrophoresis. *J. biol. Chem.* **234**, 2897–2900.

MARTIN, R. G. & AMES, B. N. (1961). A method for determining the sedimentation behaviour of enzymes. *J. biol. Chem.* **236**, 1372–1379.

NEWMEYER, D. (1957). Arginine synthesis in *Neurospora crassa*; Genetic studies. *J. gen. Microbiol.* **16**, 449–462.

PARTRIDGE, D. W. H. & GILES, N. H. (1963). Sedimentation behaviour of adenylsuccinase formed by interallelic complementation in *Neurospora crassa*. *Nature, Lond.*, **199**, 304–305.

POULIK, M. D. (1957). Starch gel electrophoresis in a discontinuous system of buffers. *Nature, Lond.* **180**, 1477–1479.

RATNER, S., ANSLOW, W. P. & PETRACK, B. (1953). Biosynthesis of urea. VI. Enzymatic cleavage of argininosuccinic acid to arginine and fumaric acid. *J. biol. Chem.* **204**, 115.

RICE, JANET. (1963). Complementation at the *arg-10* locus of *Neurospora crassa*, Ph.D. Thesis, University of Leicester.

SINGER, T. P. & KEARNEY, E. B. (1950). The L-amino acid oxidases of snake venom. *Archs Biochem.* **29**, 190–209.

SMITHIES, O. (1955). Zone electrophoresis in starch gels: group variations in the serum proteins of normal human adults. *Biochem. J.* **61**, 629–641.

SUYAMA, Y. & BONNER, D. (1964). Complementation between tryptophan synthetase mutants of *Neurospora crassa*. *Biochim. biophys. Acta*, **81**, 565–575.

TISELIUS, A., HJERTEN, S. & LEVIN, Ö. (1956). Protein chromatography on calcium phosphate columns. *Archs Biochem. Biophys.* **65**, 132–155.

VOGEL, H. J. (1955). *Microb. Genet. Bull.* **5**, 13.

YPHANTIS, D. (1960). Rapid determination of molecular weights of peptides and proteins. *Ann. N.Y. Acad. Sci.* **88**, 586–601.