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Purification of a Trifunctional Enzyme, Catalysing Three Steps of the Histidine Pathway, from *Neurospora crassa*

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1. A procedure is described for the purification of an enzyme from *Neurospora crassa* that has three catalytic functions. These are 1-*N*-(5′-phosphoribosyl)-ATP pyrophosphohydrolase, 1-*N*-(5′-phosphoribosyl)-AMP cyclohydrolase and histidinol dehydrogenase (L-histidinol–NAD oxidoreductase, EC 1.1.1.23), and are responsible for the catalysis of reactions 2, 3 and 10 in the histidine pathway. The ratio of these three catalytic activities remains approximately the same throughout the purification procedure. Evidence is presented that the purified preparations contain a single protein exhibiting association–dissociation equilibria.

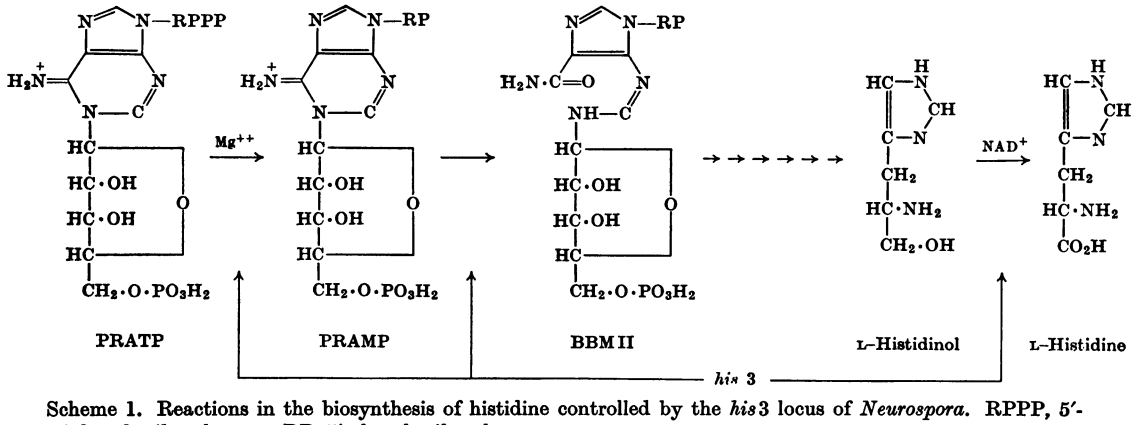
The biosynthetic pathway responsible for the formation of histidine has been extensively studied in *Salmonella typhimurium*, where nine structural genes are transcribed into a multicistronic messenger giving rise to ten enzymes (reviewed by Ames, Goldberger, Hartman, Martin & Roth, 1967).

In *Neurospora* most of the genes involved in histidine biosynthesis are widely separated and there is only one genetic region, *his* 3, which controls more than one enzymic step. These steps are the conversion of PRATP* into PRAMP carried out by PRATP pyrophosphohydrolase, the conversion of PRAMP into BBMII carried out by PRAMP 1,6-cyclohydrolase, and the oxidation of histidinol to histidine by histidinol dehydrogenase (L-histidinol–NAD oxidoreductase, EC 1.1.1.23); these are steps 2, 3 and 10 respectively in the histidine pathway (Ahmed, Case & Giles, 1964) (Scheme 1).

The question arose whether the *his* 3 region specifies three independent enzyme proteins or a single protein carrying three activities. Extensive genetic analysis has failed to resolve this question. Ahmed *et al.* (1964) considered the data best explained by a multi-enzyme hypothesis, whereas Catcheside (1965) considered that the data could be adequately explained if the *his* 3 region specified a multifunctional protein.

Creaser, Bennett & Drysdale (1965) found that

* Abbreviations: PRATP, 1-*N*-(5′-phosphoribosyl)-ATP; PRAMP, 1-*N*-(5′-phosphoribosyl)-AMP; BBMII, 5-(5′-phospho-D-ribosylaminoformimino)-1-(5″-phosphoribosyl)-imidazole-4-carboxamide.



Scheme 1. Reactions in the biosynthesis of histidine controlled by the *his* 3 locus of *Neurospora*. RPPP, 5′-triphosphoribosyl group; RP, 5′-phosphoribosyl group.

*his*3 mutants that were deficient in pyrophosphohydrolase or cyclohydrolase contained histidinol dehydrogenases that had altered kinetic properties when compared with the wild-type enzyme. This finding is inconsistent with a hypothesis that the enzymes specified by the *his*3 locus are separate entities. This evidence was extended when the histidinol dehydrogenases from two such mutants were shown to contain single amino acid replacements when compared with the wild-type enzyme (Bennett & Creaser, 1967).

Ahmed (1966, 1968) reported that PRATP pyrophosphohydrolase and histidinol dehydrogenase could not be separated by DEAE-cellulose chromatography or by Sephadex G-200 chromatography. All three enzyme activities associated with the *his*3 locus sedimented together during sucrose-density-gradient centrifugation. He interpreted these results to mean that the three enzymes specified by the *his*3 locus formed an enzyme aggregate.

Histidinol dehydrogenase has been purified from *Neurospora crassa* (Creaser, Bennett & Drysdale, 1967), and if this protein is specified by the entire *his*3 locus, as suggested above, then it should be able also to catalyse reactions 2 and 3 in the histidine pathway. Initially, the object of this work was to test this prediction.

MATERIALS AND METHODS

Organisms. *Neurospora crassa* strain Emerson *a* was used throughout this work. The organism was grown, harvested and stored by the methods of Creaser *et al.* (1967). *Salmonella typhimurium* histidine mutants EF-135, E-709 and I-648 were obtained from Dr P. Hartman. The organism was grown at 37° in the E medium of Vogel & Bonner (1956), containing 0·5% of D-glucose and trace elements as described by Ames, Garry & Herzenberg (1960). Histidinol (50 μM) was used as a source of histidine to de-repress the enzymes of the histidine pathway. Just before the stationary phase (E_{650} 0·9–1·1) the organisms were harvested by centrifuging at 5000*g* for 30 min., and washed twice with 250 ml. of 0·04 M-tris–HCl buffer, pH 7·5. Bacterial paste (5 ml.) was then subjected to treatment in a Hughes press at −15°, and the resulting material was allowed to thaw out and adjusted to a final volume of 20 ml. by addition of 0·05 M-tris–HCl buffer, pH 7·5. The nucleic acids were hydrolysed by treatment with 50 μg. of each of ribonuclease and deoxyribonuclease for 3 min. at 20°, and the cell debris was removed by centrifuging at 10000*g* for 20 min. The supernatant was dialysed for 2 hr. against two changes of 2 l. of the same tris–HCl buffer. The resulting extract was stored at −15°.

Chemicals. Chemicals used during the purification procedure were of reagent grade. All other chemicals were of A.R. grade unless otherwise stated. Sephadex G-100 and G-25 were obtained from Pharmacia, Uppsala, Sweden. DEAE- and triethylaminoethyl-cellulose (high capacity) and hydroxyapatite were obtained from Bio-Rad Laboratories, Richmond, Calif., U.S.A. L-Histidinol dihydrochloride was obtained from Haco A.-G., Berne, Switzerland. 5′-Phosphoribosyl 1-pyrophosphate was obtained from Mann Research Laboratories, New York, N.Y., U.S.A., and ATP and NAD^+ from Sigma Chemical Co., St. Louis, Mo., U.S.A. Deoxyribonuclease and ribonuclease were obtained from Worthington Biochemical Corp., Freehold, N.J., U.S.A.

PRATP was prepared by the method of Ames, Martin & Garry (1961) by using an extract of *S. typhimurium* EF-135, and purified by the method described by these authors, except that the salts were removed from the PRATP preparation by gel filtration on a column (30 cm. × 2 cm.) of Sephadex G-15. This method was found to remove ADP, which was not completely removed by DEAE-cellulose chromatography.

Enzyme assays. Histidinol dehydrogenase was assayed by the method described by Creaser *et al.* (1967). The enzyme was incubated with buffer, 2-mercaptoethanol and NAD^+ for 2 min. before the reaction was initiated by the addition of histidinol. One unit of enzyme activity is defined as the reduction of 1 μmole of NAD^+/min. under the conditions of assay.

PRATP pyrophosphohydrolase and PRAMP cyclohydrolase were assayed independently by a complementation test *in vitro*. A wild-type *Salmonella* extract converts 5-phosphoribosyl 1-pyrophosphate into 5-amino-1-(5′-phosphoribosyl)imidazole-4-carboxamide, a by-product of the sixth reaction in the pathway, but extracts of mutants blocked in this part of the pathway fail to do so. If an unknown protein is mixed with a mutant extract the ability of the mixture to convert 5′-phosphoribosyl 1-pyrophosphate into 5-amino-1-(5′-phosphoribosyl)imidazole-4-carboxamide demonstrates the existence in the unknown protein of the enzyme activity absent from the mutant extract. Extracts of mutant I-648 (lacking cyclohydrolase) and mutant E-709 (lacking pyrophosphohydrolase) were used. The conditions of reaction and the assay of 5-amino-1-(5′-phosphoribosyl)imidazole-4-carboxamide were as described by Ahmed *et al.* (1964). This assay is sensitive (0·005 units of activity is detectable), but in our hands this method did not give quantitative results.

Alternatively pyrophosphohydrolase and cyclohydrolase can be measured together by measuring the overall reaction PRATP→PRAMP→BBM II. The hydrolysis of the 1–6 bond of the purine ring of PRAMP results in an increase in E_{290} (Smith & Ames, 1964, 1965), and this was used to measure the production of BBM II in a reaction mixture containing PRATP. One unit of activity is defined as the conversion of 1 μmole of PRATP into BBM II/min. under the conditions of assay, which were as follows. The assay solution in a 1 cm.-light-path cuvette, contained (in 1 ml.): 0·05 M-tris–HCl buffer, pH 8·6; 5 μmoles of 2-mercaptoethanol; 2 μmoles of $MgCl_2$; 0·15 μmole of PRATP; enzyme. Buffer, $MgCl_2$, 2-mercaptoethanol and enzyme were incubated together in the cuvette for 2 min., and the reaction was initiated by the addition of 0·05 ml. of the PRATP solution. The reaction temperature was 37°. Under these assay conditions the observed reaction velocity was proportional to enzyme concentration over the range 0·008–0·100 units.

The assay of the overall reaction described above was used as a routine to measure pyrophosphohydrolase and cyclohydrolase activities. Preparations that failed to catalyse the overall conversion were then tested by the

complementation technique *in vitro* for the presence of either activity alone.

Gel electrophoresis. Starch-gel electrophoresis was performed by the method of Smithies (1955) with a horizontal gel. Hydrolysed starch was obtained from Connaught Laboratories, Toronto, Ont., Canada, and gels were prepared by using starch concentrations 1% higher than those recommended by the manufacturer. Gels were prepared in 0·05M-tris–HCl buffer, pH 8·6, and the electrode vessels contained the same buffer at a concentration of 0·125M. Gels were run for 12hr. at 20v/cm. The ambient temperature was 4°.

Histidinol dehydrogenase activity was located on the gel by the method of Davidson *et al.* (1965), with L-histidinol as the proton donor.

Pyrophosphohydrolase and cyclohydrolase activities were located by cutting the gel into 4mm. strips and macerating each strip with 1ml. of the standard assay solution for the overall reaction. After 15min. at 37° the starch particles were removed by centrifuging, and the solutions were assayed colorimetrically for BBMII by the method of Ames *et al.* (1961).

Protein was located on the gel by staining with Amido Black.

Sucrose-density-gradient centrifugation. Experiments were carried out by the method of Martin & Ames (1961), with a linear 5–20% (w/v) sucrose gradient in 0·01M-sodium–potassium phosphate buffer, pH 6·8. Samples were sedimented in a Beckman L-2 preparative ultracentrifuge with an SW 39 head at 38000rev./min. for 18hr. The temperature was maintained at 4°. After centrifugation the tubes were pierced, and single-drop fractions were collected.

Amino acid analysis. Performic acid oxidation was carried out by the method of Hirs (1956). Oxidation of a standard amino acid mixture gave the following yields: cysteic acid, 84·5%; methionine sulphone, 89%; tyrosine, 89%. All other amino acids were recovered at 100% yields except tryptophan, which was completely destroyed by the oxidation procedure.

A known dry weight of protein was oxidized with performic acid and dissolved in a known volume of redistilled formic acid at 0°. This solution was dispensed into Pyrex tubes so that each tube contained approx. 0·3mg. of protein, and the formic acid was removed under a stream of N_2. Constant-boiling HCl (0·5ml.) was added to each tube and the solutions were frozen in liquid N_2. The solutions were then slowly thawed under a stream of N_2 and immediately sealed. The phials were incubated at 110° for 24, 48 or 72hr. The phials were then opened and the HCl was removed at 90° under a stream of N_2.

Amino acid analysis was performed by the method of Spackman, Stein & Moore (1958) and Moore, Spackman & Stein (1958) with a Beckman 120B amino acid analyser. The instrument was fitted with a Honeywell-manufactured component that converted the 0–5mv range of the recorder into a 2·610–4·6358mv range. The colorimetric constant and the S.D. for each amino acid were obtained by performing seven analyses on samples containing 0·05μmole of each amino acid. Determinations of lysine gave an S.D. of 2·2% about the mean. Determinations of all other amino acids gave S.D. equal to or lower than this value.

Tryptophan was determined by reaction with *p*-dimethylaminobenzaldehyde by the method of Spies & Chambers (1949). Native protein (0·2–0·4mg. in 0·1ml. of water) was mixed with 0·9ml. of 10·7M-H_2SO_4 containing 3mg. of *p*-dimethylaminobenzaldehyde. After 15hr. at 25°, 0·01ml. of 0·04% $NaNO_2$ was added, and after incubation for a further 30min. the blue colour was measured at 590nm. A standard curve was prepared with pure tryptophan.

Sedimentation coefficients. Sedimentation coefficients were determined by using the Spinco model E ultracentrifuge by the method described by Schachman (1957). All experiments were performed in a 12mm. double-sector cell at a rotor speed of 50740rev./min. and at a temperature of 4°.

Approach-to-equilibrium experiments. The method employed has been described in detail by Klainer & Kegeles (1955). In this work only values of the observed refractive index gradient at the meniscus, $(dn/dx)_m$, were used to calculate the molecular weight. All experiments were performed with a 12mm. double-sector cell at a rotor speed of 13140rev./min. at a temperature between 20 and 21°. Schlieren optics were used throughout, and measurements were made from enlarged photographic prints. Five photographs were taken during the 2½hr. duration of the experiment, and values of $(dn/dx)_m/x_m c_m \omega^2$ (where x_m is the distance of the meniscus from the centre of rotation and c_m is the concentration of solute at the meniscus, expressed in appropriate units) were computed for each exposure. The values of this ratio were time-independent, and an average value was used to calculate the molecular weight. Evaluation of c_m from Eqn. 9 of Klainer & Kegeles (1955) requires the refractometric determination of the initial concentration, usually in a separate experiment in the synthetic-boundary cell. As the amount of protein was limited, an alternative procedure was used, in which the rotor was accelerated to 50740rev./min. at the end of the approach-to-equilibrium experiment. After a complete boundary had been formed further photographs were taken. The plateau concentration was found by graphical integration of the area under the Schlieren peak, and this was corrected for radial dilution to give the initial concentration.

Determination of protein. Total protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

RESULTS

Throughout this work no protein preparation has been obtained that contained pyrophosphohydrolase activity but not cyclohydrolase activity, nor vice versa. Thus any preparation unable to catalyse the overall reaction PRATP→BBMII also failed to catalyse either of its component reactions singly. These results are in agreement with those of Ahmed (1966). It seems therefore that, for practical purposes, the reaction sequence PRATP→PRAMP →BBMII may be treated as a single reaction in the wild-type organism.

It was found that purified histidinol dehydrogenase (Creaser *et al.* 1967) cannot catalyse the conversion of PRATP into BBMII. Investigation of the fractionation of pyrophosphohydrolase and cyclohydrolase during the purification of histidinol dehydrogenase showed that the three activities were not separated. Pyrophosphohydrolase and cyclohydrolase activities were found only in

Table 1. *Purification of PRAMP cyclohydrolase*

The starting material was approx. 200g. of dry mycelium.

Purification step	Activity (total units)		Ratio	Total protein (mg.)	Specific activity (units/mg.)	
	Histidinol dehydrogenase	PRATP→BBM II			Histidinol dehydrogenase	PRATP→BBM II
Protein extraction	700	550	1·27	60000	0·012	0·009
Precipitation of nucleic acids	600	550	1·09	35000	0·017	0·016
$(NH_4)_2SO_4$ fractionation	600	500	1·2	8200	0·073	0·061
Sephadex G-25 gel filtration	550	450	1·22	7800	0·070	0·058
DEAE-cellulose chromatography	310	230	1·35	2750	0·113	0·084
First hydroxyapatite chromatography	250	170	1·47	190	1·32	0·89
Sephadex G-100 gel filtration	82	64	1·28	12	6·83	5·33
Second hydroxyapatite chromatography	63	44	1·43	5·1	12·4	8·63

dehydrogenase-active fractions, but the two former activities became progressively decreased during the purification procedure. Loss of cyclohydrolase and pyrophosphohydrolase activities could be prevented by the inclusion of 0·2% (v/v) 2-mercaptoethanol in all solvents used during the purification. Under these conditions the three activities were found in the same fraction after each stage of purification, and the activities were in approximately the same ratio as was found in the crude cell extract. However, one of the effects of 2-mercaptoethanol was to alter the protein elution profile in several of the chromatographic procedures used, and as a result the purified preparation contained at least six protein components. A new purification procedure was therefore devised to obtain a pure protein able to catalyse all three reactions. This trifunctional enzyme is referred to as PRAMP cyclohydrolase to distinguish it from the unifunctional histidinol dehydrogenase prepared by Creaser *et al.* (1967).

Purification of PRAMP cyclohydrolase

The purification procedure devised involves eight steps, the results of which are summarized in Table 1. It was found to be unnecessary to include 2-mercaptoethanol in all buffers used during the purification. 2-Mercaptoethanol was only included where essential, because its use resulted in a small amount of denaturation during the course of the purification. Except where otherwise stated all procedures were performed at 4°.

Steps 1, 2 *and* 3. Procedures for extraction of the enzyme, precipitation of the nucleic acids and ammonium sulphate fractionation were identical with those used for the purification of histidinol dehydrogenase (Creaser *et al.* 1967), except that twice the volume of buffer was used for the extraction of protein from the dry mycelium.

Step 4: *gel filtration.* The ammonium sulphate precipitate obtained from step 3 was dissolved in 150ml. of 0·05M-tris–hydrochloric acid buffer, pH 7·5, and applied to a column (15cm. × 9cm.) of Sephadex G-25 previously equilibriated in 0·01M-sodium–potassium phosphate buffer, pH 6·8. The column was washed with the same phosphate buffer, and all the protein was collected. This procedure rapidly removes all the ammonium sulphate, and prepares the protein for the next stage of the purification (dialysis of the protein at this stage results in heavy losses of all activities). This step was carried out at room temperature.

Step 5: *batch ion-exchange adsorption.* The protein solution was mixed with 40g. of DEAE-cellulose, and the slurry was made up to 2l. with water. After adsorption of the protein (20min.) the exchanger was collected by filtration and washed with 2l. of 0·01M-sodium–potassium phosphate buffer, pH 6·8. The washing was repeated three times. The exchanger was then poured into a column (3cm. diam.), and the column eluted with 0·10M-sodium–potassium phosphate buffer, pH 6·8. Activity was eluted at the front together with a dark band. This dark band and the following 200ml. were collected.

Step 6: *hydroxyapatite chromatography.* The pro-

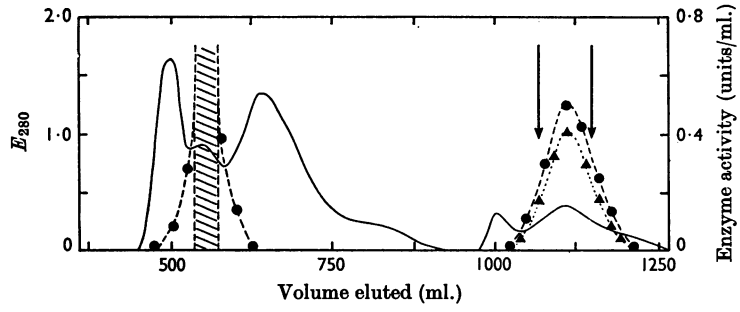


Fig. 1. Purification step 7: recycling gel chromatography. An 8ml. sample was chromatographed on a 1700ml. Sephadex G-100 column. The protein eluate was monitored (——), and either collected or recycled (shaded area). After two cycles the sample was completely eluted and the eluate was assayed for histidinol dehydrogenase activity (●), and for ability to convert PRATP into BBMII (▲). ↓, Fractions collected and pooled.

tein solution was diluted fourfold with water to give a phosphate concentration of 0·025M, and the solution was made 0·2% with respect to 2-mercaptoethanol. This solution was added to 40g. of hydroxyapatite, and the mixture was stirred for 10min. The mixture was then filtered, and the pad of hydroxyapatite was washed three times with 300ml. of 0·05M-sodium–potassium phosphate buffer, pH6·8. The hydroxyapatite was packed into a column, (3cm. diam.), and the column eluted with 0·15M-phosphate buffer, pH6·8, containing 0·2% 2-mercaptoethanol. Activity was eluted at the front by the buffer. A 100ml. volume of eluate was collected.

Step 7: *recycling gel filtration.* The protein solution was concentrated to about 8ml. by ultrafiltration, and applied to a column (100cm. × 5cm.) of Sephadex G-100. The column was equilibrated and eluted with 0·10M-sodium–potassium phosphate buffer containing 0·2% 2-mercaptoethanol. The column was maintained at 10° by a water jacket, and the elution rate was 70ml./hr. in the upward direction. Fig. 1 shows the protein elution pattern. Material from the shaded area was recycled into the bottom of the column, and when this material was eluted for the second time it was collected in fractions (5ml.). The fractions between the arrows in Fig. 1 were pooled.

Step 8: *second hydroxyapatite chromatography.* The material obtained from step 7 was diluted with 3vol. of water and made 0·1% with respect to 2-mercaptoethanol. The solution was then added to 6g. of hydroxyapatite and stirred for 10min., and the material was packed into a column (1·0cm. diam.). The column was eluted with 150ml. of 0·09M-sodium–potassium phosphate buffer, pH6·8, and then with 100ml. of the same buffer at 0·15M and containing 0·1% of 2-mercaptoethanol. All the activity was eluted in about 60ml. of the 0·15M buffer.

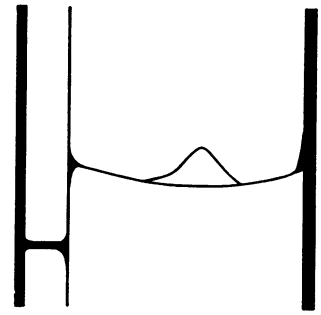


Fig. 2. Ultracentrifugation of the purified protein. A 0·27% solution in 0·10M-potassium phosphate buffer, pH6·8, was centrifuged at 50740rev./min. in a Spinco model E ultracentrifuge. The schlieren pattern shown was obtained after 70min. The temperature was 21°.

The overall yield of both activities was about 10% of the activity in the crude extract, and the yield of protein was 5mg. from 200g. of dry mycelium. Table 1 shows that the ratio of the two activities was approximately the same throughout the purification.

The purified protein was more stable than that at any stage during the purification, since the 2-mercaptoethanol could be removed by dialysis and the protein frozen and thawed several times without measurable decrease of enzyme activity.

Criteria of purity

Fig. 2 shows the sedimentation behaviour of a 0·27% solution of PRAMP cyclohydrolase in 0·10M-sodium–potassium phosphate buffer, pH6·8. The protein sediments as a single boundary with $S_{20,w}$ 7·5s. Visual examination shows that the schlieren peak is unsymmetrical, having a trailing edge on the solvent side of the boundary.

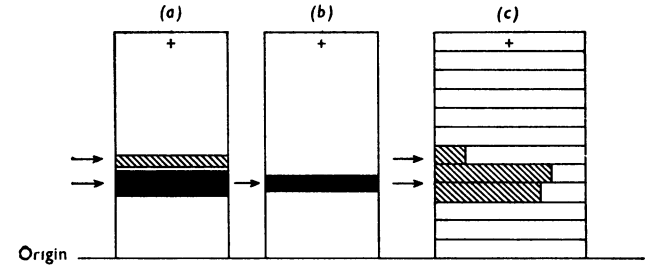


Fig. 3. Starch-gel electrophoresis of the purified protein. Conditions of the experiment were as described in the Materials and Methods section. (*a*) Gel stained for histidinol dehydrogenase activity; (*b*) gel stained for protein; (*c*) gel tested for ability to convert PRATP into BBMII. The gel was cut into twelve 4mm. strips. The width of the shaded area shown in (*c*) represents the amount of BBMII produced in 15min. at 37° by the corresponding strip.

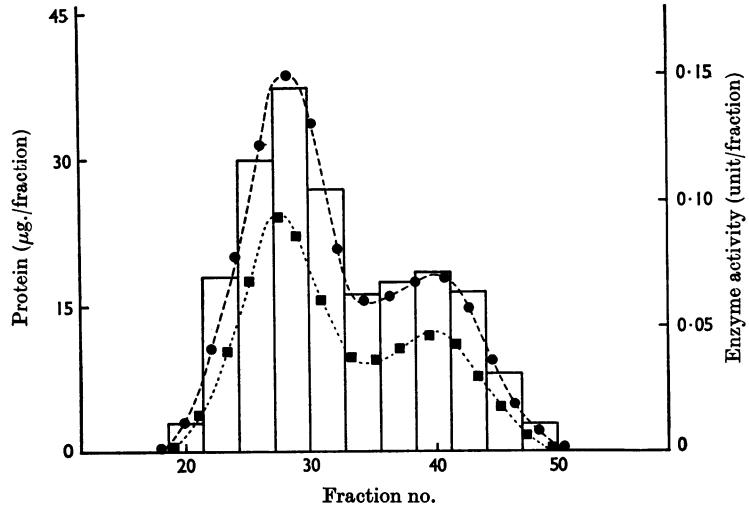


Fig. 4. Sucrose density-gradient centrifugation of the purified protein. The distribution is shown of a 200μg. (0·1ml.) sample after centrifugation for 18hr. at 38000rev./min. Distributions of histidinol dehydrogenase activity (●) and ability to convert PRATP into BBMII (■) were obtained by assaying alternate one-drop fractions from the same gradient. The protein histogram was obtained by assaying three-drop fractions from a gradient centrifuged in parallel. Sedimentation was from right to left. The gradient contained 75 one-drop fractions. Those fractions not shown in the figure contained no detectable protein or enzyme activity.

Fig. 3 shows the result of starch-gel electrophoresis of the purified protein. There is a single band of protein coincident with a heavy band of dehydrogenase activity. A minor, slightly more mobile, band of dehydrogenase activity exists, but no protein can be detected at this point. The method for locating cyclohydrolase and pyrophosphohydrolase activity in the gel has a low resolution, but ability to convert PRATP into BBMII is clearly found in the same part of the gel as histidinol dehydrogenase activity.

The protein sediments as a complex zone during sucrose-density-gradient centrifugation, as shown in Fig. 4. The sum of the contributions to the protein histogram is 196μg., which represents a recovery of 98% of the protein applied to the gradient. The protein distribution shown in Fig. 4 has been obtained consistently in sucrose-density-gradient centrifugation experiments, and it demonstrates the physical heterogeneity of the preparation. However, since the zones of protein and enzyme activity are coincident, the protein at all points in the distribution has the same specific activity and must therefore be chemically identical.

Table 2. *Amino acid composition of PRAMP cyclohydrolase*

The values given are for 100g. of protein.

Amino acid	$10^{-2}\times$ Residues recovered (moles)	Weight contribution (g.)
Lys	6·27	8·19
His	2·04	3·07
Arg	3·11	4·93
CyS*	1·24‡	1·31
Asp	7·45	8·72
Thr	5·20§	5·31
Ser	5·47§	4·93
Glu	10·00‖	13·07
Pro	4·77	4·72
Gly	6·24	3·68
Ala	9·70	7·01
Val	7·27‖	7·33
Met†	2·04‡	2·71
Ile	5·09‖	5·85
Leu	7·73‖	8·99
Tyr	0·76‡	1·25
Phe	3·28	4·89
Trp	0·68¶	1·28
Total		97·24

* Determined as cysteic acid.

† Determined as methionine sulphone.

‡ Corrected for incomplete recovery after performic acid oxidation.

§ Extrapolated to zero time of hydrolysis.

‖ Recoveries increase with time of hydrolysis. Value given is for 72hr. hydrolysate.

¶ Determined by the method of Spies and Chambers (1949).

Amino acid composition and molecular weight

The results of amino acid analyses of a preparation of PRAMP cyclohydrolase oxidized with performic acid are given in Table 2. The protein was weighed before performic acid oxidation and the values are expressed as moles/100g. The amino acid composition was used to calculate the partial specific volume of the protein by the method of Cohn & Edsall (1943), which gave a value of 0·74ml./g.

Approach-to-equilibrium experiments were performed with a preparation of concentration 0·27% in 0·1M-sodium–potassium phosphate buffer, pH6·8. Ten measurements gave a mean $\pm$ S.D. value of 126000 $\pm$ 4000 for the molecular weight.

DISCUSSION

These results show that in *Neurospora* reactions 2, 3 and 10 of the histidine pathway are catalysed by a single protein species. The reasons for the physical heterogeneity of PRAMP cyclohydrolase preparations are unknown. The results of starch-gel electrophoresis and sucrose-density-gradient centrifugation might suggest that the preparations contain two protein species, each with the same catalytic activities. Thus Fig. 4 could represent two independently sedimenting protein zones, incompletely separated. However, the protein sediments as a single boundary in the analytical ultracentrifuge. The simplest explanation of these results is that protein–protein interactions are occurring in the preparations, and that these are responsible for the complex distributions obtained after zonal centrifugation in sucrose gradients. The single unsymmetrical boundary that is obtained after analytical centrifugation is consistent with the proposal that the protein exhibits association–dissociation equilibria (Schwert, 1949; Gilbert, 1955; Rao & Kegeles, 1958). Ahmed (1966, 1968) suggested that this trifunctional protein is an aggregate, composed of different subunits, but at present there is no direct evidence to support this hypothesis.

The phenomenon whereby several reactions are catalysed by a single protein has been shown to be widespread (Reed & Cox, 1966). In many cases multifunctional proteins catalyse sequential reactions in a particular pathway. In these cases it can be argued that the multifunctional protein is more efficient than a comparable series of unifunctional proteins in that the intermediates in the reaction sequence are held in close proximity to the catalytic sites that act on them. No such rationale can be used to explain the existence of a protein that catalyses reactions 2, 3 and 10 of a biosynthetic sequence, although the possibility cannot be ignored that *in vivo* all ten reactions in the histidine pathway are catalysed by a large enzyme aggregate. This seems unlikely because, among histidine mutants of *Neurospora*, only *his*3 mutants have multiple enzymic defects. We have considered the possibility that this multifunctional protein might be concerned with the control of histidine biosynthesis, but this appears to be ruled out by the finding that the second and third reactions in the pathway are not sensitive to histidine or histidinol.

Genetic segments analogous to the *his*3 region of *Neurospora* have been found in *Saccharomyces cerevisiae* (Fink, 1964, 1966) and *Aspergillus nidulans* (Berlyn, 1967), and it it therefore likely that in these organisms also reactions 2, 3 and 10 of the histidine pathway are catalysed by a single protein. A different situation exists in *Salmonella typhimurium*, where it has been shown that histidinol dehydrogenase is readily separable from PRATP pyrophosphohydrolase and PRAMP cyclohydrolase (Whitfield, Smith & Martin, 1964). However, only a small difference was reported in the sedimentation coefficients of the latter two enzymes (Whitfield *et al.* 1964), and the question of

the separate identity of PRATP pyrophosphohydrolase and PRAMP cyclohydrolase in *Salmonella* needs further examination.

A major point of interest is the relationship between PRAMP cyclohydrolase preparations and the histidinol dehydrogenase prepared by Creaser *et al.* (1967). The two proteins differ in several respects. Histidinol dehydrogenase catalyses only one reaction, whereas PRAMP cyclohydrolase catalyses all three reactions controlled by the *his* 3 locus. Further, PRAMP cyclohydrolase oxidizes histidinol to histidine at a higher rate than the histidinol dehydrogenase prepared by Creaser *et al.* (1967). It appears therefore that by using techniques that prevent losses of pyrophosphohydrolase and cyclohydrolase activities, the protein has been maintained in a form that has a higher specific dehydrogenase activity. We are interested to know whether the differences in the properties of the proteins prepared by the two methods are due to chemical differences or solely to physical differences. If Ahmed (1968) is correct in thinking that the trifunctional protein is an aggregate composed of dissimilar subunits, then the loss of pyrophosphohydrolase and cyclohydrolase activities that occurs when the purification procedure of Creaser *et al.* (1967) is used might be due to the removal of one of the components of the aggregate during the purification. Preliminary observations suggest that this is not the case. Preparations of histidinol dehydrogenase and PRAMP cyclohydrolase have very similar amino acid compositions, suggesting that they are chemically identical. This could be examined more rigorously by comparing the compositions of tryptic digests of the two proteins.

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