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THE CLEAVAGE AND SYNTHESIS OF CYSTATHIONINE IN WILD TYPE AND MUTANT STRAINS OF *NEUROSPORA CRASSA**

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Studies on the methionineless mutants of *Neurospora* have established that methionine synthesis goes over the pathway: cysteine + homoserine → cystathionine → homocysteine → methionine[1-3]. In this paper, experiments on the enzymic cleavage of cystathionine and on the reversibility of the above pathway are reported, and a paper chromatographic method for the determination of cystathionine is described.

MATERIALS AND METHODS

Cultures for enzyme preparations were grown under aeration in carboys containing 8–16 liters of the nutrient fluid described by HOROWITZ AND BEADLE[4]. Two methods were used to extract the enzymes (Method A and Method B). The preparations were kept cold throughout the operations.

Method A: The mycelium was extracted twice with 1 ml distilled H_2O per g wet weight of mycelium. After grinding in a mortar with sand, the material was centrifuged at 8,000 *g*. The supernatant was decanted and then brought to 50% of saturation with $(NH_4)_2SO_4$. After 20 minutes the precipitate was collected by centrifugation and dialyzed with stirring for 3–6 hours against 20 volumes of 4 m*M* tris(hydroxymethyl)aminomethane (Tris) buffer at pH 8.6. The buffer was renewed and dialysis repeated two times. The dialysate was next frozen, then thawed and the resulting precipitate removed by centrifugation. The supernatant was used as a source of the enzyme.

Method B: Mycelium to be extracted was frozen with dry ice and the cell walls broken in a blendor. This material was centrifuged at 19,000 *g* for 1 hour and the precipitate discarded. The supernatant was dialyzed for 2 hours against 20 volumes of 4 m*M* Tris buffer at pH 8.6. The buffer was renewed and the dialysis repeated two times. The dialysate was used as the source of the enzyme.

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Enzyme incubations were carried out under continuous stream of N_2. Each experimental incubation, containing enzyme, L-cystathionine, pyridoxal phosphate and Tris buffer was matched by a control incubation, except that cystathionine was omitted. Cystathionine was added to control incubations at the end of the run. Total disulfides were determined by the method of KASSEL AND BRAND[5]. Cystine was determined by the method of SULLIVAN AND HESS[6]. Since the enzyme solutions interfered with color formation, recovery experiments with cystine were necessary. Homocystine was determined by subtracting cystine from total disulfide values. Colorimetric results were confirmed by chromatography in critical experiments.

Homocystine, cystine and cystathionine were identified by paper chromatography before and after oxidation with H_2O_2. One developer, 6 *n*-propanol : 2 12 *N* HCl : 1 H_2O, permitted the separation of cysteine, homocysteine and homocystine from cystine and cystathionine, as seen in Table I. After oxidation with H_2O_2[7] cystine and cystathionine were found to separate with the propanol developer. Disulfide and sulfhydryl were detected by the method of TOENNIES[8]. After oxidation with H_2O_2 and chromatography, color was developed with ninhydrin. Homocystine was also characterized in a second developer, 2 *n*-butanol : 1 glacial acetic acid : 1 H_2O, which separates homocystine from cystine and cystathionine.

TABLE I

THE R_F'S OF SEVERAL AMINO ACIDS IN TWO DEVELOPING SOLVENTS BEFORE AND AFTER OXIDATION BY H_2O_2

Amino acid	*Developing solvent*	
	2 n-butanol 1 acetic acid 1 H_2O	*6 n-propanol 2 12 N HCl 1 H_2O*
Oxidized cystathionine*		0.13
Cystine	0.19	0.19
Cystathionine	0.21	0.22
Cysteic acid**		0.39
Homocystine	0.35	0.43
Methionine	0.69	0.47
Homocysteic acid***		0.52
Cystine		0.56
Homocysteine		0.62

* The product of oxidation of cystathionine by H_2O_2.
** The product of oxidation of cystine by H_2O_2.
*** The product of oxidation of homocystine by H_2O_2.

To study the biosynthesis of cystathionine, a paper chromatographic method for its determination was developed. Cultures were grown in quadruplicate by standard methods[4] for 72 hours. Solutions for chromatography were prepared by separately extracting 2 cultures with 1 ml hot H_2O per g wet weight. The two remaining cultures were separately dried and weighed. The solutions for chromatography were deproteinized with 5 % trichloracetic acid. The extracts and standard cystathionine solutions were applied to Whatman No. 1 paper in spots of constant diameter (3 mm). Cystathionine was oxidized with H_2O_2[7] and separated from other amino acids with the propanol developer (Table I). The dry chromatograms were dipped in a pyridine solution containing 0.4 % ninhydrin and the cystathionine content determined by visual comparison of the area and intensity of ninhydrin spots produced by known and unknown cystathionine solutions in the range of 0.2 to 0.5 mg per ml, with the results presented in Table II.

RESULTS

Extracts of *Neurospora* were found to catalyze the cleavage of cystathionine to homocysteine and to cysteine as indicated in Table III. The production of homocysteine was of primary interest, since this is a step in the biosynthetic pathway of methionine[1]. This enzyme will be called cystathionase II since it is genetically separable from the cysteine-producing enzyme, cystathionase I. When extracts of a mutant (4894)

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TABLE II

THE QUANTITATIVE DETERMINATION OF CYSTATHIONINE AFTER OXIDATION WITH H_2O_2 AND PAPER CHROMATOGRAPHY IN 6 *n*-PROPANOL : 2 12 *N* HCl : 1 H_2O

Cystathionine concentration in mg/ml		
Actual	*Determined*	*Mean*
0.400	0.5, 0.4, 0.4, 0.4 0.36, 0.4, 0.5, 0.25	0.4 ± 0.080
0.333	0.4, 0.33, 0.28, 0.45 0.3, 0.33, 0.37, 0.25	0.34 ± 0.066
0.400	0.2, 0.45, 0.33, 0.36 0.4, 0.25, 0.25, 0.36	0.325 ± 0.085
0.333	0.3, 0.33, 0.25, 0.3 0.25, 0.33, 0.4, 0.3	0.307 ± 0.044
0.240	0.2, 0.3, 0.4, 0.3 0.37, 0.4, 0.33, 0.3	0.325 ± 0.066

TABLE III

CYSTATHIONINE CLEAVAGE IN WILD TYPE AND MUTANT STRAINS OF *Neurospora*

Columns 6 and 7 give an estimate of the amount of activity which could have been present and not detected. Vessels contained 4–7 γ pyridoxal phosphate (Ca^{++} salt) per ml, 0.12–0.18 mg L-cystathionine per ml. Activity is expressed as μmoles indicated product per 10 g wet wt. mycelium per h. No H_2S was detected. Reaction time, 2–4 hours.

Strain No.	*Prep. method*	*Activity*			*Minimum detectable activity*	
		Sulfhydryl	*Cysteine*	*Homocysteine*	*Cysteine*	*Homocysteine*
H98	B	0.247	0.245	0		0.016
4894	A	0.15	0	0.15	0.02	
H98-R1 *	A	0.07	0.08			0.03
4894-R1 *	B	0.402	0	0.402	0.01	
5256	A	0.23	0.06	0.17		

* Re-isolate after cross to wild type.

blocked between cystine and cystathionine were examined for capacity to cleave cystathionine, it was found that homocysteine, but not cysteine, was produced. Consequently, cystathionase II was characterized using extracts obtained from this mutant.

The activity of cystathionase II was increased by the addition of pyridoxal phosphate, as reported by BINKLEY AND HUDGKINS[9]. Optimal activity was found at pH 7.8. Activity was not increased by the addition of magnesium ion or reducing agents. Homocysteine was identified as a product by chromatography. Extracts of a strain (H98), blocked between cystathionine and homocysteine had no cystathionase II activity. The same preparation did produce cysteine from cystathionine (Table III). The colorimetric results were confirmed by chromatography. Similar results have been reported by WIJERSUNDERA AND WOODS[10] who found that a homocysteine-requiring strain of *E. coli* has no cystathionase II activity.

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To test the possibility that the absence of cystathionase II activity in mutant H98 might be due to an inhibitor in the extracts, they were mixed with an active cystathionase preparation. As seen in Table IV, no inhibition of cystathionase II was found. Continued dialysis failed to produce any cystathionase II activity in extracts of H98 nor did the addition of a known cofactor, pyridoxal phosphate.

A single gene suppressor of H98 was obtained through the courtesy of Doctor NORMAN H. GILES. The suppressor partly abolishes the methionine requirement of the mutant[11]. Suppressed H98 was found to have a low cystathionase II activity, as seen in Table V.

The same suppressor also removes the block in strain 4894 (blocked between cysteine and cystathionine). When extracts of suppressed 4894 were examined, cystathionase I activity was found.

TABLE IV

NON-INHIBITION OF CYSTATHIONASE II BY EXTRACTS OF MUTANT H98

Enzymes were prepared according to method B. Disulfide values alone were determined. Eight ml of enzyme solution was incubated with 1.5 mg L-cystathionine, 7 γ pyridoxal phosphate per ml at pH 8.05 for 2 hours. Activity is expressed as μmoles disulfide per 10 g fresh wt. mycelium per h.

Enzyme (ml)		*Activity*	
H98	*4894*	*Found*	*Expected*
8		0.25	
	8	0.40	
5	3	0.31	0.30
4	4	0.29	0.33
3	5	0.33	0.34

TABLE V

CYSTATHIONASE II ACTIVITY IN STRAINS CARRYING THE SUPPRESSOR

6 γ pyridoxal phosphate per ml, 0.16 mg L-cystathionine per ml. Activity is expressed as μmoles indicated product per 10 g wet wt. per h. Enzymes were obtained by method A. No H_2S was detected.

Genotype of strain	*Time of incubation (hours)*	*Disulfide*	*Activity*	
			Cysteine	*Homocysteine*
S-1 *	2	0.05	0.03	0.02
S-1 me-2 **	6	0.103	0.018	0.085
S-1 me-2 **	9	0.072	0.033	0.039
S-1 me-3.1 ***	6	0.034	0.012	0.022

* Strain carrying the suppressor alone.
** Strain carrying the suppressor and the gene for homocysteine requirement.
*** Strain carrying the suppressor and the gene for cystathionine requirement.

To determine whether cystathionine can be synthesized from methionine over a pathway that does not include cysteine a paper chromatographic method for its determination was used. The occurrence of L-cystathionine in partially purified extracts of wild type *Neurospora* was also determined by a cystathionine-requiring mutant of *Neurospora* (36104) and found to agree with levels determined by chro-

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matography. The preparation did not support the growth of the homocysteine-requiring mutant (H98), indicating the absence of homocysteine and methionine. The results of study of the biosynthesis of cystathionine are presented in Table VI. The production of cystathionine in mutant 4894 growing in the presence of methionine is not dependent upon the presence of sulfate in the medium. Wild type *Neurospora* (5256) shows similar behavior, cystathionine production being independent of sulfate. Extracts of wild type *Neurospora* which can cleave cystathionine to cysteine and to homocysteine appear to convert homocysteine to cysteine in the presence of serine and pyridoxal phosphate. In these experiments, cysteine was detected by the method of SULLIVAN AND HESS[6]. These findings indicate that transsulfuration in *Neurospora* is reversible.

TABLE VI

CYSTATHIONINE ACCUMULATION BY DIFFERENT STRAINS OF *Neurospora* IN 72 HOUR CULTURES

Growth conditions		*Strain number and genotype*					
		me-2 (H98-R)		*Wild type (5256)*		*me-3.1 (4894-R)*	
mg Na_2SO_4 per culture	*mg Methionine per culture*	*Cystathionine content*		*Cystathionine content*		*Cystathionine content*	
		mg dry wt. of culture	*Per cent*	*mg dry wt. of culture*	*Per cent*	*mg dry wt. of culture*	*Per cent*
0	1.2	77.0	0.13	65.0	0.13	64.5	0.15
5.6	1.2	71.25	1.5	68.25	0.13	59.0	0.26
5.6	0.6	58.25	1.8	64.25	0.078	53.75	0.14
5.6	0.3	46.0	2.2	63.25	0.051	38.75	0.19
5.6	0.15	33.0	2.4	58.75	0.021		

The cystathionine content of growing cultures of H98 increases as the methionine content of the growth medium is decreased. The increased accumulation by biochemical mutants of metabolites when the nutritional requirement is limited has been reported[12]. It is possible that methionine regulates its own synthesis by an inhibition of cystathionine synthesis, analogous to the feed-back system proposed by UMBARGER[13] and others. However, this effect is complicated by differences in growth rate of the cultures in various levels of methionine, and by the fact that cystathionine slowly disappears from the mycelium of aging cultures.

DISCUSSION

The hypothesis that each gene has a primary product which controls a chemical process of the cell has been supported by the observation that single gene nutritional mutants commonly have but a single nutritional requirement[14]. In addition it has been found in a number of cases that the enzyme that synthesizes the required metabolite is missing in the mutant[15]. The present data add two examples of this effect.

The return of cystathionase II activity in suppressed strains indicates that the S-1 gene operates by reopening the original pathway of methionine synthesis, and not some alternative pathway. The results concerning cystathionase II are similar to those of YANOFSKY[16] who found that a suppressed tryptophanless mutant contained tryptophan desmolase activity.

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The production of cystathionine found with 4894 mutant growing in the presence of methionine indicates that cystathionine can be synthesized from methionine over a pathway that does not include cysteine. The apparent conversion of homocysteine to cysteine by enzyme extracts and the absence of cystathionase I activity in a me-3 mutant with its return in the suppressed strain suggests that transsulfuration in *Neurospora* is a reversible process.

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SUMMARY

Wild type *Neurospora* was found to cleave cystathionine to cysteine and to homocysteine. A mutant which requires homocysteine for growth was found to have no enzyme for the production of homocysteine from cystathionine. A cystathionine-requiring mutant was found to be deficient in the cysteine-producing enzyme. It was found that a suppressor, obtained by GILES, which causes these mutants to grow on minimal medium, returns enzyme activity to the mutants.

A paper chromatographic method for the determination of cystathionine is described.

Evidence is presented that transsulfuration in *Neurospora* is a reversible process.

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