JOURNAL OF BACTERIOLOGY, Mar. 1972, p. 1001-1013

Vol. 109, No. 3
Printed in U.S.A.

Comparison of Cysteine and Tryptophan Content of Insoluble Proteins Derived from Wild-Type and mi-1 Strains of *Neurospora crassa*

WENDELL D. ZOLLINGER[1] AND DOW O. WOODWARD
Department of Biological Sciences, Stanford University, Stanford, California 94305

Received for publication 29 November 1971

The possibility of an amino acid substitution (cysteine for tryptophan) in a membrane protein of the [mi-1] strain of *Neurospora crassa* has been investigated in detail by using a double radioactive labeling procedure. Auxotrophic strains of *Neurospora* having wild-type [+] or [mi-1] cytoplasm have been grown under conditions which result in the specific labeling of protein tryptophan with ^{3}H and protein cysteine with ^{35}S. Although the least soluble 1 to 20% of the [mi-1] mitochondrial membrane protein was usually found to have a higher Cys/Trp ratio (ratio of cysteine plus half-cystine to tryptophan) than the corresponding [+] fraction, it has been shown that these differences were due mainly to the presence of differential amounts of a very insoluble, cysteine-rich (Cys-rich) material. The same Cys-rich material was found in variable amounts in both [+] and [mi-1] cultures, but the concentration was usually higher in the [mi-1] cultures. The Cys-rich material is clearly distinct from "structural protein" on the basis of amino acid composition and appears to have no direct relationship to the [mi-1] phenotype. In the absence of the Cys-rich material, no difference between the Cys/Trp ratios of corresponding [+] and [mi-1] membrane proteins could be detected. We conclude, therefore, that the previously postulated amino acid substitution of cysteine for tryptophan in [mi-1] membrane protein is incorrect.

The study of cytoplasmic mutants in eukaryotic microorganisms is a promising approach to the problem of understanding the complex interactions which take place between nuclear and cytoplasmic genes and gene products.

Poky [mi-1] is one of the best characterized strains of a class of respiratory-deficient, cytoplasmic mutants of *Neurospora crassa*. The [mi-1] strain, which was isolated by Mitchell and Mitchell (11), exhibits a pleiotropic mutant phenotype. Relative to wild-type strains, young cultures of [mi-1] have a 10- to 15-fold excess of cytochrome *c* and free fatty acids, little or no cytochrome *a* or cytochrome *b*, and 10 to 15% as much succinic oxidase and cytochrome oxidase (7, 24). As the cultures age, growth accelerates, cytochrome *c* drops to a level approaching that of wild type, and cytochromes *a* and *b* become detectable (7).

[1] Present address: Department of Bacterial Diseases, Walter Reed Army Institute of Research, Walter Reed Army Medical Center, Washington, D.C.

Woodward and Munkres (28) conducted a comparative study of a mitochondrial membrane protein (MSP) purified from wild-type and [mi-1] mitochondria. They found a number of interesting differences with respect to both composition and behavior. Amino acid composition data indicated that the [mi-1] MSP contained more half-cystine and less tryptophan than wild-type MSP (28). Binding studies produced evidence that the [mi-1] MSP associated with reduced nicotinamide adenine dinucleotide, adenosine triphosphate, and malate dehydrogenase (MDH) to a lesser extent than wild-type MSP. In addition, it was found that the presence of [mi-1] MSP resulted in a large increase in the Michaelis constant of MDH for malate, whereas wild-type MSP had no effect (28). Subsequent experiments (29) revealed that MSP which exhibited the same difference in half-cystine and tryptophan composition could also be isolated from other [mi-1] cell fractions (microsomes, nuclei,

and whole cell 100,000 × *g* supernatant fluid). On the basis of a number of criteria (27), these membrane protein preparations were judged to be relatively homogeneous. As a working hypothesis, these data were interpreted to mean that a mutant membrane protein was present in the [mi-1] strain and was responsible for its characteristic mutant phenotype. The mutation was postulated to involve an amino acid substitution (cysteine for tryptophan) resulting from an alteration in the protein's structural gene (presumably located in the mitochondrion).

Although this interpretation was considered the simplest, other interpretations of those data and the [mi-1] phenotype are also possible. For example, the [mi-1] phenotype could result from an alteration in the mitochondrial protein-synthesizing system or in mitochondrial ribonucleic acid (RNA) polymerase. Based on the data presented in this paper, the earlier interpretation of an amino acid replacement in "structural protein" is in error. These data also suggest that *Neurospora* "structural protein" preparations contained more than one protein, as is the case also in "MSP" isolated from mammalian systems (8).

In this report, we describe the use of a double radioactive labeling technique to investigate membrane proteins in more detail to determine what is responsible for the high half-cystine and low tryptophan found in [mi-1] MSP. The experimental approach is based on the finding of Woodward and Munkres (28) that the ratio of half-cystine to tryptophan in wild-type MSP was about 4:3, while the same ratio in [mi-1] MSP was near 5:2. These two ratios differ by a factor of 1.88. The specific labeling of protein half-cystine with sulfur-35 and protein tryptophan with tritium has made it possible to compare half-cystine to tryptophan ratios of many different wild-type and [mi-1] proteins and protein fractions simply by comparing $^{35}S/^{3}H$ ratios.

MATERIALS AND METHODS

Strains of N. crassa. Two sets of multiple auxotrophic mutant strains were used in this study: (i) *trp-1;cys-1,ylo-1;a* and *trp-1;cys-1,ylo-1;a[mi-1]*, which are referred to as TC102a[+] and TC203a [mi-1], and (ii) *trp-1;cys-1,ylo-1;me-7;a* and *trp-1; cys-1,ylo-1;me-7;a[mi-1]*, which are referred to as TCM23a[+] and TCM41a[mi-1]. The symbols [+] and [mi-1] denote the wild-type and poky cytoplasmic genotypes, respectively. The four strains listed above were derived by appropriate crosses from the following parent strains: *cys-1(84605),ylo-1(Y30539y);a*, which is blocked in the conversion of sulfite to thiosulfate; *trp-1(A10);A*, which is blocked in the conversion of shikimic acid to anthranilic acid; *me-7(4894);A*, which is blocked in the conversion of cysteine to cystathionine; and [mi-1-1.4], a vegetative isolate from a standard poky strain (FGSC no. 1575).

Double labeling of [+] and [mi-1] strains. Double label experiments I, II, and III were carried out with strains TC102a[+] and TC203a[mi-1]. The growth medium for these strains consisted of minimal salts (25) supplemented with 2% sucrose, 40 mg of anthranilic acid per liter, 20 to 40 mg of L-cysteine-hydrochloride per liter, 150 to 240 mg of L-methionine per liter, and radioactive label in the form of ^{35}S-cysteine and ^{3}H-anthranilic acid. The complete medium including the label was made up in a single vessel and then divided into separate flasks prior to autoclaving.

Experiments IV through VII were carried out with the improved strains TCM23a[+] and TCM41a [mi-1]. The composition of the growth medium used with these strains differed from that described above only in the levels of the various supplements added (per liter): 25 to 30 mg of anthranilic acid, 40 to 120 mg of L-cysteine, and 40 to 120 mg of L-methionine.

Cultures which had been inoculated with 3×10^5 to 6×10^5 conidia per ml of medium were grown at 30 C on a rotary shaker operating at 200 rev/min. The time of growth varied from 36 to 48 hr for the [+] strains and from 48 to 84 hr for the [mi-1] strains.

Cell fractionation. The mycelium was collected, rinsed with cold, distilled water, and pressed between paper towels to remove excess water. The mycelium was extracted in a mortar and pestle packed in ice by grinding with twice its weight of 0.2-mm acid-cleaned glass beads and twice its weight of cold 0.5 M STE [0.5 M sucrose, 0.05 M 0.001 M tris(hydroxymethyl)aminomethane(Tris)-hydrochloride, 0.001 M ethylenediaminetetraacetic acid (EDTA), pH 7.5]. After about 5 min of grinding, the extract was filtered through Whatman no. 1 filter paper in a Büchner funnel. Fresh buffer was added to the partially extracted mycelium, and it was ground for another 5 min. This procedure was repeated a total of three or four times, and the extracts were pooled.

The various cell fractions were obtained from the filtered cell extract in the following manner. The extracts were centrifuged twice at 1,000 × *g* for 10 min, and the pellets which contained nuclei, cell debris, and fine glass fragments were discarded (unless otherwise specified). The resulting supernatant fluid was centrifuged at 16,000 × *g* for 25 min to pellet the mitochondria, and then at 100,000 × *g* for 1 hr to obtain a pelleted microsomal fraction and whole cell soluble protein (WCSP). The crude mitochondrial fraction (16,000 × *g* pellet) was further purified by twice subjecting the mitochondria to isopycnic centrifugation on discontinuous sucrose gradients. The gradients were composed of equal volumes of 1.6 M sucrose, 1.2 M sucrose, and 0.8 M sucrose, each made up in 0.05 M Tris-hydrochloride, 0.001 M EDTA, *p*H 7.5. The mitochondria were collected from the interface between 1.2 and 1.6 M sucrose, diluted 1:1 with TE buffer, and pelleted at 30,000 × *g* for 30 min. The material which sedi-

mented through the 1.6 M sucrose and pelleted is referred to as the gradient pellet (GP) fraction. All operations were carried out at 0 to 5 C.

Polyacrylamide gel electrophoresis. Electrophoresis at low *p*H was carried out by the method of Takayama et al. (23) with the following modifications. (i) Gels were subjected to pre-electrophoresis as suggested by Mitchell (12). (ii) After pre-electrophoresis, the gels were equilibrated overnight or longer with phenol-acetic acid-water (2:1:1, w/v/v) containing 5 M urea (3). (iii) Samples were dialyzed overnight against phenol-acetic acid-water containing 5 M urea. (iv) Gels were stained for 10 to 15 hr with 0.02% Coomassie brilliant blue in 10% trichloroacetic acid after fixation in 10% trichloroacetic acid for 4 to 6 hr. Destaining was also done with 10% trichloroacetic acid.

Except for sample preparation, electrophoresis in the presence of sodium dodecyl sulfate (SDS) was carried out by the procedures described by Weber and Osborne (26). Samples were dissolved in 2.5% SDS, 2.5% 2-mercaptoethanol, 0.01 M sodium phosphate buffer, *p*H 7.1. The dissolved samples were diluted 1:1 with 0.01 M sodium phosphate buffer containing 40% sucrose and 0.02% bromophenol blue.

Measurement of radioactivity. All protein samples to be counted were precipitated with 5% trichloroacetic acid and washed once with 5% trichloroacetic acid. Lipid was extracted from the acid precipitates by a series of three organic washes: 90% acetone, chloroform-methanol (2:1 v/v), 90% acetone. The extracted protein was washed once with distilled water and then dissolved in a small volume of 1% SDS in 0.1 M NaOH. One 0.05-ml portion of each sample was used for determination of protein, and a second 0.05-ml portion was added to 0.5 ml of NCS solubilizer (Nuclear-Chicago). After the samples were solubilized, 20 ml of toluene base scintillation fluid was added to each, and the samples were counted in a Packard Tri-Carb liquid scintillation spectrometer. Protein derived from cultures labeled with a single isotope were used to determine the counting efficiencies by the channels ratio method. The counting efficiency of neither tritium nor sulfur-35 was significantly affected by fivefold variation in the protein concentration or by the solubility properties of the labeled protein provided sufficient time was allowed for the NCS to completely solubilize the protein.

The radioactivity in sliced polyacrylamide gels was determined by a procedure essentially like that described by Basch (1). Analytical gels were frozen and sliced manually with a razor blade. The gels were kept frozen and held in position by a simple device which guided the blade and regulated the width of the slices. The standard deviation in weight of the gel slices cut in this manner was normally less than 5%. Each gel slice was placed in a scintillation vial, and sufficient distilled water was added to bring the total sample weight to 70 mg. A 0.5-ml amount of NCS solubilizer was added to each vial, after which the vials were capped and placed on a shaker at 30 C for 6 to 8 hr. At the end of this period, 20 ml of scintillation fluid was added to each vial, and the vials were allowed to stand with occasional shaking for 2 hr before counting.

Amino acid analysis. Protein samples were prepared for hydrolysis by washing with (i) 90% acetone, (ii) chloroform-methanol (2:1, v/v), (iii) 90% acetone, and (iv) distilled water. The delipidized protein was oxidized with performic acid as described by Moore (13) and hydrolyzed with 6 N HCl at 110 C for 36 hr in evacuated, sealed tubes. After hydrolysis, excess HCl was removed from the samples on a flash evaporator. Samples were analyzed by the method of Spackman et al. (22) on a Beckman model 120 amino acid analyzer.

Protein determination. Protein was assayed by the microbiuret method of Goa (6) with bovine serum albumin as a standard. The bovine serum albumin was standardized on the basis of its optical density at 280 nm ($e_{1\%}$ = 6.6).

Determination of specific activity of protein cysteine and protein methionine. Protein labeled with sulfur-35 was hydrolyzed after treatment with performic acid as described for amino acid analysis. One portion of the hydrolysate was run on the long column of the amino acid analyzer in the normal way, and the micromoles of cysteic acid and methionine sulfone were calculated. A second portion, identical to the first, was run on the long column, but the effluent was diverted directly to a fraction collector before being mixed with ninhydrin. One-minute fractions (1.2 ml) were collected. The tubes containing the cysteic acid and methionine sulfone peaks were diluted with 1.0 ml of distilled water, and each was transferred quantitatively to a scintillation vial. The vials were filled with 18 ml of dioxane base scintillator fluid and counted. For determination of the amount of ^{35}S applied to the column, a 0.05-ml portion of hydrolysate was diluted with 1.2 ml of long-column buffer and 1.0 ml of water and counted under the same conditions as the effluent fractions. Another 0.05-ml portion was counted in the standard NCS-toluene system to determine the relative counting efficiencies of the two systems. Results were expressed as equivalent counts per minute in the NCS-toluene system.

Determination of the specific activity of protein tryptophan. Protein, which was prepared as described above for amino acid analysis, was hydrolyzed in barium hydroxide by the procedure described by Noltman et al. (17). After removal of barium by precipitation as barium carbonate, the hydrolysate was evaporated to dryness on a flash evaporator, dissolved in a small volume of distilled water, and centrifuged. The tryptophan in the hydrolysate was converted to indole by incubating a portion of the hydrolysate with *Escherichia coli* tryptophanase, as described by Newton and Snell (16) for the assay of tryptophanase. The indole is taken up in the toluene phase as it is formed. After the reaction had proceeded for 30 to 40 min at 30 C, the toluene phase was carefully removed with a Pasteur pipette. One portion was counted in the standard NCS-toluene system, and a second portion was used to assay for indole with acid Ehrlich reagent, as outlined by Newton and Snell (16).

Tryptophanase was purified from *E. coli* strain B/1t7 by the procedure of Newton et al. (15) and was

stored as a precipitate in 65% saturated ammonium sulfate, 2 mM EDTA, and 5 mM 2-mercaptoethanol at 4 C.

Purification of cytochrome oxidase. Cytochrome oxidase (EC 1.9.3.1) was purified by a procedure developed by David Edwards (*personal communication*). Gradient-purified mitochondria were suspended in 0.5 M STE, frozen, and then thawed. The thawed mitochondria were pelleted and washed twice in 0.9% KCl. The washed mitochondria were suspended at a concentration of 10 mg/ml in 0.1 M Tris-hydrochloride, *p*H 7.4, and KCl was added to a concentration of 1.0 M. After the cytochromes were reduced by addition of a few crystals of dithionite, deoxycholate and cholate were added to a concentration of 2 mg/mg of protein and 1 mg/mg of protein, respectively. The insoluble material was spun down at 34,000 × *g* for 20 min, and the supernatant fluid was made 16% saturated in ammonium sulfate. The precipitate which formed immediately was spun down, and the resulting supernatant fluid was allowed to stand at room temperature overnight. The precipitate which formed was spun down, and the resulting supernatant fluid was made 28% saturated in ammonium sulfate. The precipitate which contained the cytochrome oxidase was spun down, dissolved in 1% cholate, 0.1 M Tris-hydrochloride, *p*H 8.0, and chromatographed on a Sephadex G-200 column equilibrated in the same 1% cholate buffer. Purified cytochrome oxidase was collected as the excluded peak. Cytochrome oxidase activity was measured by the spectrophotometric method of Smith (21).

Radioactive compounds. ^{35}S-cysteine (25 to 30 mCi/mmole) was purchased from Amersham/Searle. Anthranilic acid was custom tritium-labeled by Amersham/Searle by using the catalytic exchange method. The crude ^{3}H-anthranilic acid was purified by paper chromatography in two solvent systems: isopropanol-ammonia-water (100:5:10, v/v/v) followed by methanol-butanol-benzene-water (2:1:1:1, v/v). The final product appeared to be greater than 95% pure as judged by paper chromatography in either of the solvent systems described above.

RESULTS

Specificity of labeling. The specificities of labeling achieved with two different pairs of strains under the conditions described above are compared in Table 1. Acid hydrolysis of performic acid-oxidized, whole cell soluble protein revealed that 85 to 95% of the protein-bound sulfur-35 was associated with cysteine and the remainder with methionine. Usually the specific activity of protein cysteine in the [mi-1] culture differed from that in the [+] culture by 10 to 20%. Appropriate corrections for this difference were made before computing the ^{35}S/^{3}H ratios and, subsequently, the double ratios (^{35}S/^{3}H ratio of a [mi-1] protein or protein fraction divided by the ^{35}S/^{3}H ratio of the corresponding [+] protein or protein fraction). The specific activity of protein tryptophan was consistently the same in the [+] and [mi-1] cultures and was equal to the specific activity of the ^{3}H-anthranilic acid in the initial medium. One-dimensional paper chromatography of alkaline hydrolysates of [+] or [mi-1] tritium-labeled protein from either pair of strains resulted in a single peak of tritium label which migrated the same distance as a sample of L-tryptophan. Thus, protein tryptophan is specifically and efficiently labeled with tritium by the procedure used.

Although specific labeling of protein cysteine with sulfur-35 was obtained with strains TC102a[+] and TC203a[mi-1], the labeling was not very efficient, and the specific activity of protein cysteine from both [+] and [mi-1] strains was found to decrease significantly with increasing culture age. In addition, it was found that, in both [+] and [mi-1] strains, the specific activity of protein cysteine derived from soluble protein was somewhat lower than

TABLE 1. *Labeling specificity obtained with two different pairs of auxotrophic strains*

Expt	Strain	^{35}S counts/min/ μmole of ½ Cys[a]	Percentage ^{35}S as ^{35}S-Met	^{3}H counts/min/ μmole of Trp
	Pair 1			
I	TC102a[+]	70,000	6	169,000[b]
	TC203a[mi-1]	59,000	9	168,000
II	TC102a[+]	60,000	6.5	193,000
	TC203a[mi-1]	50,000	11.6	188,000
	Pair 2			
V	TCM23a[1]	147,000	9	340,000
	TCM41a[mi-1]	175,000	11	340,000

[a] Abbreviations: ½ Cys, half-cysteine; Met, methionine; Tryp, tryptophan.
[b] The specific activity of ^{3}H-anthranilic acid in the initial medium was 172,000 counts per min per μmole.

the specific activity of protein cysteine derived from insoluble protein of the same culture. These disadvantages were overcome by the use of strains TCM23a[+] and TCM41a[mi-1], which were obtained from the former pair of strains by the introduction of the me-7 mutation which blocks conversion of cysteine to cystathionine.

Observation of high "double ratios." On the basis of the data obtained by Woodward and Munkres (28), it was predicted that a double ratio of about 1.9 would be obtained when comparing "structural protein" from the two strains. A comparison of any [+] and [mi-1] nonmutant protein should give a double ratio of 1.0. Table 2 lists the double ratios obtained for selected cell fractions in two experiments. Double ratios significantly greater than one were obtained for several cell fractions in both experiments. Purification of the crude mitochondrial fraction on discontinuous sucrose gradients routinely produced a small pellet of material which had sedimented through 1.6 M sucrose to the bottom of the tube. This pelleted material, which lacked spectrally detectable cytochromes, is referred to as the gradient pellet (GP) fraction. Analyses of the $^{35}S/^{3}H$ ratios of the GP fractions revealed that they often had an unusually high $^{35}S/^{3}H$ ratios relative to other cell fractions. Table 3 gives a comparison of the $^{35}S/^{3}H$ ratios and the double ratios of the gradient-purified mitochondria and the GP fractions obtained in eight separate experiments. The $^{35}S/^{3}H$ ratios for each experiment are normalized to the $^{35}S/^{3}H$ ratio of the [+] WCSP in the same experiment. The magnitude of the GP fraction $^{35}S/^{3}H$ ratio varied from experiment to experiment, but in those experiments in which the concentration of methionine in the growth medium was 100 mg/liter or greater, it was consistently high. In most experiments, the $^{35}S/^{3}H$ ratio of the [mi-1] GP fraction was higher than that of the [+] GP fraction, resulting in double ratios greater than one.

TABLE 2. *Double ratios obtained for selected cell fractions in two experiments*

Fractions	Double ratio[a]	
	Expt I	Expt II
Whole cell soluble protein	1.04	1.05
Microsomal fraction	0.98	1.95
Least soluble mitochondrial fraction	1.57 (1%)[b]	2.03 (8%)
1,000 × *g* pellet	1.58	ND[c]
Gradient pellet fraction	3.83	2.75

[a] The double ratio is the $^{35}S/^{3}H$ ratio of an [mi-1] fraction divided by the $^{35}S/^{3}H$ ratio of the corresponding [+] fraction.

[b] Values in parentheses refer to the percentage of total mitochondrial protein contained in that fraction.

[c] Not done.

Characterization of the GP. An investigation of material with a high $^{35}S/^{3}H$ ratio was undertaken to determine its relationship to "structural protein" and to the [mi-1] phenotype as well as to determine the basis for the variability in amount present in different experiments. The data in Table 3 suggest a correlation between the concentration of methionine in the growth medium and the $^{35}S/^{3}H$ ratio of the GP fractions. In separate experiments, the growth rate of strains TCM23a[+] and TCM41a[mi-1] was found to be directly proportional to the methionine concentration for the range between 40 and 120 mg/liter. Several experiments have indicated that the $^{35}S/^{3}H$ ratio of the GP fraction is also affected, though less strongly, by culture age. Data from experiment III, which are plotted in Fig. 1, show a gradual increase in the $^{35}S/^{3}H$ ratio with culture age. The GP fraction from even

TABLE 3. *Comparison of sulfur-35-to-tritium ratios of gradient-purified mitochondria and gradient pellet fractions in eight experiments*

Expt	Methionine concn in growth medium (mg/liter)	Gradient pellet fraction $^{35}S/^{3}H$			Gradient-purified mitochondria $^{35}S/^{3}H$		
		[+]	[mi-1]	Double ratio [mi-1]/[+]	[+]	[mi-1]	Double ratio ([mi-1]/[+])
I	250	2.84	10.8	3.83	1.28	1.16	0.91
III	240	2.2	4.4	2.0	0.99	0.96	0.97
II	150	4.36	2.29	0.55	1.60	1.20	0.75
IV	100	1.24	3.23	2.62	1.02	1.02	1.0
V	70	1.12	1.54	1.37	0.83	0.84	1.01
VI	70	0.92	0.92	1.0	0.73	0.80	1.09
VII	70	1.18	1.14	0.97	0.75	0.82	1.09
IV-A	40	0.94	0.98	1.05	0.68	0.76	1.12

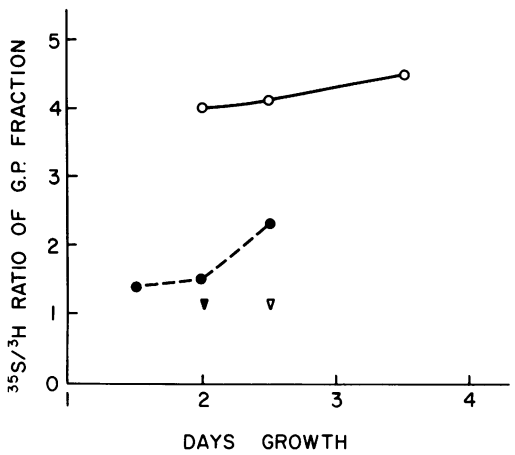

FIG. 1. *Effect of culture age on $^{35}S/^{3}H$ ratio of gradient pellet fraction from [+] and [mi-1] strains. Cultures of TC102a[+] (●) and TC203a[mi-1] (○) were grown for various periods of time in minimal medium supplemented (per liter) with 240 mg of L-methionine, 40 mg of L-cysteine, and 30 mg of anthranilic acid. In one [+] culture (▼) and one [mi-1] culture (▽), the level of methionine was reduced to 150 mg/liter and the level of cysteine to 20 mg/liter. Label was added as ^{35}S-cysteine and ^{3}H-anthranilic acid. Each $^{35}S/^{3}H$ ratio is relative to the $^{35}S/^{3}H$ ratio of the whole cell soluble protein of that same culture.*

the youngest [mi-1] culture has a markedly higher $^{35}S/^{3}H$ ratio than that of any of the [+] cultures. Both strains had a $^{35}S/^{3}H$ ratio near 1.0 for the GP fraction when the cultures were grown on medium containing a reduced level of methionine (150 mg/liter).

The material in the GP fractions which is responsible for the high $^{35}S/^{3}H$ ratios is largely associated with structures of relatively high density. Attempts to band these structures in a sucrose density gradient revealed that their density, though not sharply defined, was greater than or equal to 1.26 g/ml. *Neurospora* mitochondria normally band at a point in the gradient corresponding to a density of about 1.17 to 1.19 g/ml.

Attempts to solubilize the GP fraction with either 5 M urea in phenol-acetic acid-water (2:1:2, w/v/v) or with 2.5% SDS, 2.5% 2-mercaptoethanol, 0.05 M Tris-hydrochloride, *p*H 7.4, resulted in solubilization of 50 to 95% of the protein in different experiments. The insoluble residue remaining after treatment with either solvent was found to contain the bulk of the material with a high $^{35}S/^{3}H$ ratio (Table 4).

The unusual properties of the material responsible for the high $^{35}S/^{3}H$ ratio of the GP fractions raised several questions. (i) Is the sulfur-35 all associated with cysteine, or might it be associated with a sulfur-containing polysaccharide? (ii) Is the high $^{35}S/^{3}H$ ratio due to a high cysteine content or low tryptophan content or both? (iii) What is the relationship between this protein and mitochondrial "structural protein"? To obtain some information relative to these questions, the GP fractions from the [+] and [mi-1] cultures in experiment I were washed twice with 5 M urea in phenol-acetic acid-water (2:1:2, w/v/v), and the insoluble residues were oxidized with performic acid and hydrolyzed as described above. After removal of excess HCl, the hydrolysates were chromatographed on the long column of a model 120C Beckman amino acid analyzer. One-minute fractions were collected directly off the bottom of the column and assayed for radioactivity. Over 95% of the sulfur-35 in the hydrolysate applied to the column was recovered in the fractions corresponding to the elution time of cysteic acid and methionine sulfone. A second portion of each hydrolysate was analyzed in the normal way for amino acid composition. The results are shown in Table 5 and are compared to the amino acid composition of STA-4 MSP. The data in Table 5 indicate that the material from the GP fractions which has a high $^{35}S/^{3}H$ ratio is clearly different from MSP on the basis of its amino acid composition. The [+] and [mi-1] hydrolysates appear to have a very similar amino acid composition, and both have unusually high mole percentages of cysteic acid. The tryptophan content of this material was not determined directly, but determinations of the amount of tritium radioactivity per milligram of protein, as determined by the method

TABLE 4. *Comparison of $^{35}S/^{3}H$ ratios and double ratios of soluble and insoluble subfractions of gradient pellet fraction*

Fraction	$^{35}S/^{3}H$ ratio		Double ratio ([mi-1]/[+])
	[+]	[mi-1]	
Experiment I			
Gradient pellet, unfractionated	2.84	10.8	3.83
Soluble in phenol-acetic acid-urea	2.10	2.14	1.04
Insoluble in phenol-acetic acid-urea	5.5	18.4	3.30
Experiment IV			
Gradient pellet, unfractionated	1.24	3.23	2.62
Soluble in 2.5% SDS-2.5% 2-mercaptoethanol	1.18	1.14	0.97
Insoluble in 2.5% SDS-2.5% 2-mercaptoethanol	1.88	13.9	7.4

TABLE 5. *Comparison of amino acid composition of [+] and [mi-1] Cys-rich material with Neurospora mitochondrial structural protein*

Amino acid	STA-4 MSP[a]	[mi-1] Cys-rich material (more %)[b]	[+] Cys-rich material[c]
Lysine	6.43	5.6	ND[d]
Histidine	1.92	0.40	ND
Arginine	5.12	1.5	ND
½ Cystine	2.0	13.3	12.9
Methione	2.0	1.4	1.6
Threonine	5.46	5.8	5.8
Serine	5.9	9.3	8.7
Glutamic acid	9.6	3.7	4.4
Proline	4.5	6.0	5.9
Glycine	10.2	11.6	12.5
Alanine	10.8	9.0	9.1
Valine	7.5	7.9	8.1
Isoleucine	5.7	7.1	6.4
Leucine	9.5	8.2	8.3
Phenylalanine	4.3	1.8	2.4

[a] Mitchondrial membrane protein. Recalculated from Woodward and Munkres (28).

[b] Tyrosine and tryptophan were destroyed during hydrolysis of the cysteine (Cys)-rich material and are excluded from the calculations in each case.

[c] These values were calculated by assuming that the mole percentage of the three basic amino acids is the same as for the [mi-1] sample.

[d] Not done.

of Goa (6), indicated that the material was low in tryptophan as well as rich in cysteine plus half-cystine. Because of some uncertainty in the tryptophan content due to difficulty in obtaining accurate protein determinations on such highly insoluble material, the material will be referred to simply as Cys-rich material.

Isolation and fractionation of mitochondria. Purification of mitochondria on sucrose density gradients was not sufficient to remove completely all differences in the $^{35}S/^{3}H$ ratios of [+] and [mi-1] mitochondrial protein fractions. In several different experiments, the fraction representing the least soluble 1 to 20% of the mitochondrial protein had a double ratio significantly greater than one. A comparison of the $^{35}S/^{3}H$ ratios of [+] and [mi-1] protein fractions that were obtained by fractionating the mitochondrial proteins by two different procedures is given in Table 6. The only mitochondrial subfractions which exhibit a high double ratio such as that predicted for MSP are: (i) the 1% of the mitochondrial protein insoluble in 0.5% deoxycholate (experiment I) and (ii) 10% of the mitochondrial protein which remained insoluble after three successive washes with 0.1% SDS (experiment IV). Subsequent treatment of the latter fraction with 2.5% SDS, 2.5% 2-mercaptoethanol yielded an insoluble residue (about 3% of the mitochondrial protein) which had a still higher double ratio and a soluble fraction with a double ratio closer to one. The solubility properties and the $^{35}S/^{3}H$ ratios suggested that a small amount of the Cys-rich material found in larger amounts in the GP fractions was still present in the mitochondrial fractions. It was not possible, however, on the basis of these data to rule out the presence in the [mi-1] strain of a mitochondrial protein with an altered $^{35}S/^{3}H$ ratio.

The validity of comparing the $^{35}S/^{3}H$ ratios of heterogeneous protein fractions from the [+] and [mi-1] strains depends on the identity or equivalence of the two protein fractions

TABLE 6. *Comparison of the $^{35}S/^{3}H$ ratios in [+] and [mi-1] mitochondria and mitochondrial subfractions after fractionation by two different procedures*

Protein Fraction	$^{35}S/^{3}H$ ratio[a]		Double ratio ([mi-1]/[+])
	[+]	[mi-1]	
Exp I[b]			
Unfractionated mitochondria	1.28	1.16	0.91
Soluble at 0.1 mg of DOC/mg of protein	1.23	1.11	0.90
Soluble at 0.5 mg of DOC/mg of protein			
12% AS precipitate	1.01	0.96	0.95
12 to 35% AS precipitate	0.98	0.92	0.94
35% AS supernatant fluid	1.50	1.30	0.87
Insoluble at 0.5 mg of DOC/mg of protein	2.41	3.87	1.57
Exp IV[c]			
Unfractionated mitochondria	1.02	1.02	1.0
First supernatant fluid	1.09	1.06	0.97
Second supernatant fluid	1.09	0.86	0.79
Third supernatant fluid	0.70	0.71	1.02
Third pellet	0.58	1.17	2.03
Soluble in 2.5% SDS	0.52	0.71	1.4
Insoluble in 2.5% SDS	0.61	2.42	4.0

[a] All $^{35}S/^{3}H$ ratios are relative to the corresponding ratio for [+] whole cell soluble protein from the same experiment.

[b] Mitochondria were suspended in water at 10 mg/ml, and deoxycholate (DOC) was added to the concentrations indicated. Ammonium sulfate (AS) fractionation was carried out at *p*H 6.0 by addition of saturated ammonium sulfate solution to the percentage of saturation indicated.

[c] Mitochondria were washed three successive times at a protein concentration of about 10 mg/ml with a solution containing 1 mM EDTA, 1 mM 2-mercaptoethanol, 50 mM Tris-hydrochloride (*p*H 7.4), and 0.1% sodium dodecyl sulfate (SDS). The third pellet representing 10% of the mitochondrial protein was treated with 2.5% SDS in the same buffer.

with respect to the protein species present and the relative proportions of each. Since [+] and [mi-1] mitochondria are known to contain different relative proportions of several enzymes, and since the two kinds of mitochondria do not necessarily respond identically to a given fractionation procedure, it is possible that comparing "equivalent" heterogenous protein fractions might yield misleading results.

Because of these uncertainties, it was considered advisable to analyze the various fractions in more detail by using polyacrylamide gel electrophoresis to resolve the several proteins present in each fraction. Two approaches were taken. The first (experiment V) was to compare the total complement of mitochondrial proteins by first fractionating on the basis of solubility in SDS and then resolving the proteins of each fraction on polyacrylamide gels. The second approach (experiment VII) was to analyze on gels specific proteins which have been reported to be altered in the [mi-1] strain, namely, "structural protein" and cytochrome oxidase.

On the basis of several experiments, it became evident that, when cultures were grown in medium containing methionine at concentrations below about 70 mg/liter, very little of the Cys-rich material was observed in cell extracts. In experiments V and VII, this observation was utilized to minimize the amount of the Cys-rich material present. An [mi-1] protein with an altered Cys/Trp ratio should be more easily detected in the absence of the Cys-rich material. Strains TCM23a[+] and TCM41a[mi-1] were grown on minimal medium supplemented with 70 mg of methionine, 70 mg of cysteine-hydrochloride, and 25 mg of anthranilic acid per liter of medium. Radioactive label was added in the form of ^{35}S-cysteine and ^{3}H-anthranilic acid. An inoculum of 6×10^8 conidia per liter was used, and the cultures were grown for 36 hr in the case of the [+] cultures and 52 hr in the case of the [mi-1] cultures. The mycelium was harvested, and various cell fractions were obtained as described above.

In experiment V, gradient-purified mitochondria from each strain were washed three successive times at a concentration of about 10 mg/ml with a solution containing 0.1% SDS, 1 mM EDTA, 1 mM 2-mercaptoethanol, and 50 mM Tris-hydrochloride, *p*H 7.4. The insoluble material was pelleted at 80,000 $\times$ *g* for 60 min at 4 C and was resuspended with the aid of mild sonic treatment for 15 sec. The ^{35}S/^{3}H ratios of the three supernatant fractions and the final pellet were determined, and the values for equivalent [+] and [mi-1] fractions were compared. These results, along with similar data for the major cell fractions, are given in Table 7. The only two fractions which exhibit a double ratio significantly greater than one are the GP fraction and the P3 mitochondrial subfraction.

The equivalence of the [+] and [mi-1] mitochondrial subfractions was investigated by subjecting these fractions to polyacrylamide gel electrophoresis using a modification of the low *p*H system of Takayama as described above. The results are presented in Fig. 2. No major differences are evident between the [+] and [mi-1] gels in the case of the S1, S2, or S3 fractions, but clear differences are evident in the case of the P3 fraction. A comparison of the gels of the P3 fractions with a gel of purified cytochrome oxidase from the wild-type strain STA-4 reveals that several bands which are present in purified cytochrome oxidase and in the [+] P3 fraction appear to be missing or displaced in the gel of the [mi-1] P3 fraction.

The relationships between the observed difference in the ^{35}S/^{3}H ratio of the [+] and [mi-1] P3 fractions and the difference in the banding pattern on gels were investigated by slicing the gels and determining the ^{35}S/^{3}H ratio for each slice. The results of the counting are presented in Fig. 3. Each major band

TABLE 7. *Comparison of ^{35}S/^{3}H ratios for selected fractions from strains TCM23a[+] and TCM41a[mi-1]: experiment V*

Fraction	^{35}S/^{3}H ratio		Double ratio ([mi-1]/[+])
	[+]	[mi-1]	
Cell fractions			
Whole cell soluble protein	1.0	0.95	0.95
12% AS supernatant fluid[a]	0.95	0.95	1.0
12% AS precipitate	1.0	0.95	0.95
Microsomal fraction	0.84	0.91	1.08
Mitochondria	0.82	0.79	0.96
Gradient pellet	1.12	1.54	1.37
Mitochondrial subfractions[b]			
S1 (45)	1.05	0.88	0.84
S2 (22)	0.72	0.72	1.0
S3 (12)	0.65	0.70	1.08
P3 (20)	0.50	0.60	1.20

[a] Deoxycholate (2 mg/mg of protein) and cholate (1 mg/mg of protein) were added to the whole cell soluble protein. Enough saturated ammonium sulfate (AS) solution at neutral *p*H was then added to make the solution 12% saturated.

[b] Fractionation of mitochondria was carried out as in experiment IV (see footnote to Table 6). The numbers in parentheses are the percentages of total mitochondrial protein.

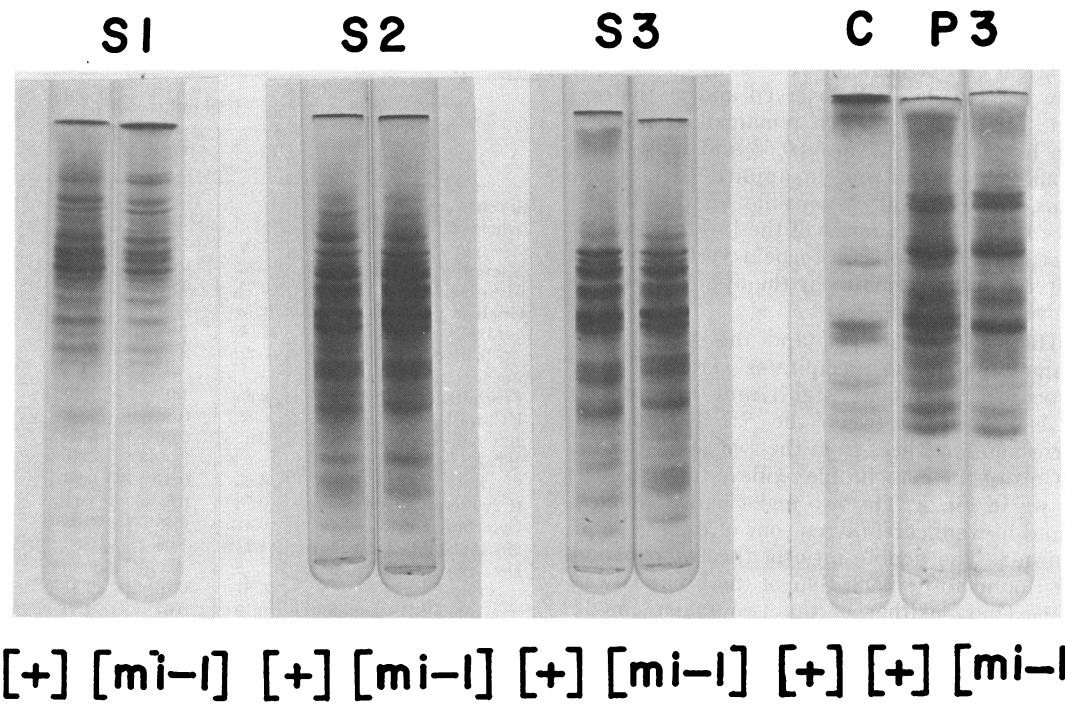

FIG. 2. *Comparison of [+] and [mi-1] mitochondrial proteins on low pH polyacrylamide gels. Mitochondrial proteins were separated into fractions S1, S2, S3, and P3 by washing the mitochondria three times in succession with a solution containing 0.1% sodium dodecyl sulfate, 1 mM EDTA, 1 mM 2-mercaptoethanol, and 50 mM Tris-hydrochloride (pH 7.4). The protein fractions were dialyzed against 5 M urea in phenol-acetic acid-water (2:1:1 w/v/v) and resolved on low pH polyacrylamide gels as described in the text. The gel labeled "C" is of purified cytochrome oxidase from wild-type mitochondria.*

which is common to both gels has the same $^{35}S/^{3}H$ ratio in both gels. Some differences in the $^{35}S/^{3}H$ ratios are apparent in the areas where a band appears to be missing in one of the gels. These differences, however, probably contribute little to the overall difference between the P3 fractions, since they tend to cancel each other out. The material principally responsible for the overall difference in the $^{35}S/^{3}H$ ratio of the P3 fractions appears to be at the origin (about 50% of the protein applied) and smeared through the first 10 to 12 mm of the gels.

The [+] and [mi-1] gradient-purified mitochondria obtained in experiment VII were fractionated in exactly the same way by a procedure developed by David Edwards (*personal communication*) for the purification of *Neurospora* cytochrome oxidase. The first portion of this procedure, which is described above, is essentially the same as the procedure used by Yang and Criddle (30) for the preparation of yeast "structural protein" and by Richardson et al. (18) for the isolation of beef MSP.

The $^{35}S/^{3}H$ ratios for equivalent cell fractions and subfractions from the two strains are compared in Table 8. The GP fraction and the detergent-insoluble mitochondrial subfraction have relatively low $^{35}S/^{3}H$ ratios and have double ratios less than one. This suggests that only a small amount of the Cys-rich material is present in these cultures and that slightly more is present in the [+] culture than in the [mi-1] culture. The cytochrome oxidase fraction and the 16% ammonium sulfate fraction have double ratios close to one.

The detergent-insoluble fraction and the 16% ammonium sulfate precipitate were analyzed in more detail by resolving the proteins on low *p*H polyacrylamide gels, slicing the gels, and comparing the $^{35}S/^{3}H$ ratios of corresponding slices from the [+] and [mi-1] gels. The results for the detergent-insoluble fraction are given in Fig. 4 and 5. The detergent-insoluble fractions were colorless in appearance and represented approximately 15% of the mitochondrial protein.

The gels of the detergent-insoluble fractions (Fig. 4) are essentially identical and show two major bands and several minor bands superim-

posed on a background smear. The $^{35}S/^{3}H$ ratios of the [+] and [mi-1] gels are essentially identical except near the end of the gels where very few counts were observed and at the origin. The material which remained at the origin has a double ratio of 0.58 and represents about 25% of the protein applied to the gel. Thus, the material responsible for the difference in the $^{35}S/^{3}H$ ratios of the [+] and [mi-1] fractions again appears to be a very insoluble material which remains at the origin or enters the gel as a smear.

The gels of the [+] and the [mi-1] 16% ammonium sulfate precipitates were similar in appearance, and no significant differences were observed between the $^{35}S/^{3}H$ ratios of corresponding slices from the two gels.

Gels of the cytochrome oxidase fractions are shown in Fig. 4. The two major bands, labeled a and b, were carefully cut out of each gel and counted. The double ratio of the two a bands was found to be 0.98; that of the b bands was 0.91. Thus, neither of the two major [mi-1] cytochrome oxidase fractions differs from the corresponding [+] fractions in its Cys/Trp ratio. Fewer minor bands are present in more highly purified preparations of cytochrome

TABLE 8. *Comparison of $^{35}S/^{3}H$ ratios for selected subcellular fractions from strains TCM23a[+] and TCM41a[mi-1]: experiment VII*

Fraction	$^{35}S/^{3}H$ ratio		Double ratio ([mi-1]/[+])
	[+]	[mi-1]	
Cell fractions			
Whole cell soluble protein	1.0	1.0	1.0
Microsomal fraction	0.82	0.95	1.16
Mitochondria	0.75	0.82	1.09
Gradient pellet	1.18	1.14	0.97
Alkaline SDS-soluble[a]	1.06	1.08	1.02
Alkaline SDS-insoluble[a]	5.9	2.5	0.42
Mitochondrial subfractions			
Freeze-thaw wash	0.85	1.0	1.20
KCl washes	0.88	0.88	1.0
16% AS[b] precipitate	0.67	0.66	0.98
16% AS overnight precipitate	0.65	0.625	0.96
G-200 retarded fraction	0.78	0.87	1.11
Cytochrome oxidase	0.455	0.48	1.05
28% AS supernatant fluid	1.17	1.09	0.93
Detergent-insoluble fraction	0.87	0.70	0.81
Alkaline SDS-soluble[a]	0.79	0.67	0.85
Alkaline SDS-insoluble[a]	6.9	3.1	0.45

[a] Material was suspended in 2% sodium dodecyl sulfate (SDS)-0.1 N NaOH for 30 min at room temperature. Only 1 to 2% of the material in these fractions was insoluble under these conditions.

[b] Ammonium sulfate.

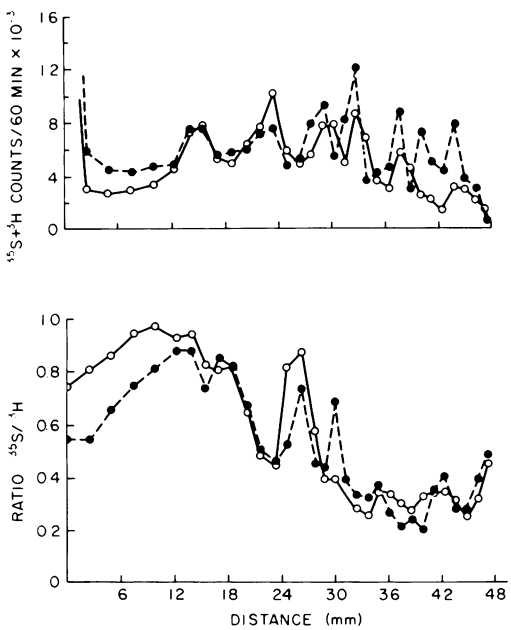

FIG. 3. *Comparative analysis of the P3 fraction proteins from TCM23a[+] mitochondria (●) and from TCM41a[mi-1] mitochondria (○) after resolution on low pH gels (Fig. 2). The gels were sliced into 1.5-mm slices and counted. The total ^{35}S plus ^{3}H counts (upper plot) and the $^{35}S/^{3}H$ ratio (lower plot) were determined for each slice.*

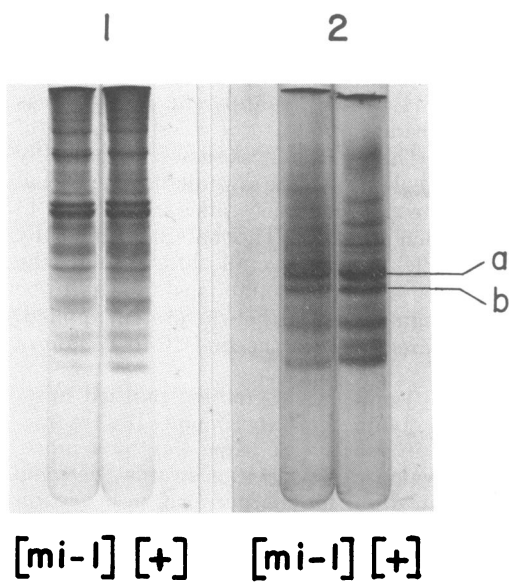

FIG. 4. *Low pH polyacrylamide gels of the detergent-insoluble fraction (1) and the cytochrome oxidase fraction (2) from [+] and [mi-1] mitochondria (experiment VII).*

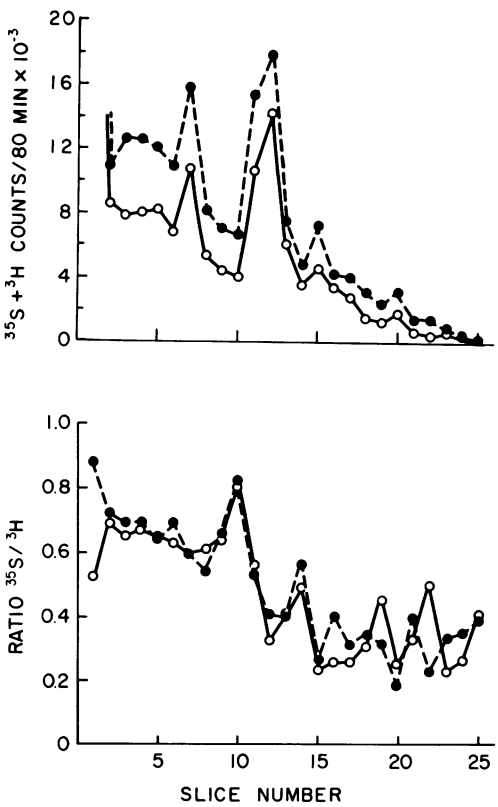

FIG. 5. *Comparative analysis of low pH gels of the detergent-insoluble fraction obtained from TCM23a[+] mitochondria (●) and from TCM41a [mi-1] mitochondria (○) in experiment VII. The gels were sliced into 1.8 mm-slices and counted. The total ^{35}S plus ^{3}H counts (upper plot) and the $^{35}S/^{3}H$ ratio (lower plot) were determined for each slice.*

oxidase.

When grown on nonlimiting levels of methionine, [mi-1] cultures were consistently found to have higher levels of Cys-rich material than [+] cultures. Synthesis of the Cys-rich material was inhibited in this experiment by growth on a reduced level of methionine.

In spite of the low concentration of Cys-rich material in these cultures and the fact that in this experiment the [+] culture contained more than the [mi-1] culture, the characteristic [+] and [mi-1] phenotypes were not detectably altered. Thus, mitochondria extracted from the [mi-1] culture had 5-fold more cytochrome *c* and over 10-fold less cytochrome *a* than [+] mitochondria; moreover, the [mi-1] culture grew up more slowly than the [+] culture. This suggests that no direct or causal relationship exists between the level of Cys-rich material and the [mi-1] phenotype.

DISCUSSION

Based on the postulated amino acid difference between "structural protein" from [mi-1] and wild type, it was predicted that the half-cystine-to-tryptophan ratio of [mi-1] "structural protein" would be 1.9 times higher than the same ratio for [+] "structural protein."

In most double label experiments, the least soluble 5 to 20% of the mitochondrial protein, the GP fraction, and often the microsomal fraction, had higher $^{35}S/^{3}H$ ratios in the [mi-1] strain than in the [+] strain. However, it appears that these differences were due predominantly to the presence of differential amounts of a Cys-rich (high in cysteine plus half-cystine) material in the two strains. This view is supported by several observations. (i) No major differences between the $^{35}S/^{3}H$ ratios of [+] and [mi-1] insoluble mitochondrial proteins or GP fraction protein were found that persisted in the absence of the Cys-rich material or that were not reversed by a reversal of the relative concentrations of the Cys-rich material in the [+] and [mi-1] cell extracts. (ii) Each time a double ratio was found to deviate from one by more than 15 to 20%, it was possible to isolate from the [mi-1] fraction or the [+] fraction, or both, a relatively minor subfraction which had the properties of the Cys-rich material. The remainder of the original fraction was then found to have a double ratio close to one. (iii) Although more of the Cys-rich material was normally found in the [mi-1] cell extracts than in [+] cell extracts, it is not unique to [mi-1] strains since material which has the same density, solubility properties, and amino acid composition has been isolated from both [+] and [mi-1] strains.

After correcting for any differences found between the specific activities of [+] and [mi-1] protein half-cystine and protein tryptophan, the double ratio of WCSP was found in four separate experiments to be 1.01 ± 0.05, where the limits indicate the range of the values. A double ratio close to one was also obtained for the quasi-crystals (9, 20) isolated from WCSP of [+] and [mi-1] strains.

The in vivo function and location of the Cys-rich material has not been determined. Amino acid composition data on fractions which were greatly enriched in the Cys-rich material indicate that the protein present is very different than "structural protein." In addition, it is much less abundant in the cell and for the most part considerably less soluble in detergents and phenol-acetic acid-urea solvent than "structural protein."

The Cys-rich material does not appear to bear a causal relationship to the [mi-1] phenotype, but rather might be related in much the same way as cytochrome *c* or other enzymes (5) whose concentration or regulation is altered in the [mi-1] strain. Large variations in the concentrations of the Cys-rich material in cell extracts of both the [+] and [mi-1] strains had no detectable effect on either phenotype.

The results of this study suggest the need for a reinterpretation of the earlier data of Woodward and Munkres (28, 29). The presence of differential amount of Cys-rich material in those [+] and [mi-1] "structural protein" preparations may have been partially responsible for the data reported. Since the early method used to prepare "structural protein" involved a continual selection for the least soluble protein, and since two rounds of purification on sucrose gradients does not result in complete removal of the Cys-rich material from the mitochondria, it is quite possible that those preparations contained some of the Cys-rich material.

Reported alterations in cytochrome oxidase (4), succinic oxidase (7), and the ultrastructure of mitochondrial inner membranes (2) all suggest a defect in [mi-1] strains which affects the function and structure of membranes and membrane proteins. Several lines of evidence indicate that [mi-1] strains might have an altered protein-synthesizing system. Such an alteration could result in either a quantitative change in the synthesis of normal proteins or qualitative changes resulting from infidelity of transcription or translation. Growth of [+] strains in the presence of 2 mg of chloramphenicol per ml, which selectively inhibits the mitochondrial protein-synthetic system, results in the production of mitochondria which closely resemble [mi-1] mitochondria with respect to cytochrome spectrum and disorganization of the inner mitochondrial membrane (2). In addition, we have found that cytochrome oxidase activity and the levels of heme *a* and *b* are the same in the [+] mitochondria synthesized in the presence of chloramphenicol as in [mi-1] mitochondria (W. D. Zollinger, Ph.D. thesis, Stanford University, Stanford, Calif., 1970). The finding of Sebald et al. (19) that isolated [mi-1] mitochondria synthesize protein at a rate only about 10% as high as [+] mitochondria and our finding that the auxotrophic [mi-1] strains employed in this study contain a low level of spectrally and electrophoretically normal cytochrome oxidase suggest that the [mi-1] mitochondrial protein-synthetic system is functional but operates at a much reduced rate. However, the only components known to be specified by mitochondrial deoxyribonucleic acid are mitochondrial ribosomal RNA species and transfer RNA (tRNA) species. Brambl (*unpublished data*) has recently demonstrated that at least four mitochondrial tRNA species of [mi-1] differ qualitatively from the corresponding tRNA species of wild-type mitochondria. This result suggests that the defective protein-synthesizing system could be due to transcriptional abnormalities in [mi-1] mitochondria. Thus, an abnormal RNA polymerase could theoretically be responsible for the altered membranes, membrane proteins, and protein-synthesizing system, and for the altered tRNA species.

Further studies on [mi-1] mitochondrial RNA polymerase and the components and products of the [mi-1] mitochondrial protein synthetic system are in progress. It seems significant to the final interpretation, however, that only a few proteins are produced by the mitochondrial protein-synthesizing system (R. A. Lansman, D. O. Woodward, and D. M. Kaplan, Fed. Proc. **30:** 1225) and that these appear to be minor components as opposed to major membrane proteins or quasi-crystalline protein (9, 20). By this criterion, these proteins produced in quantity must be specified by nuclear gene(s) and synthesized on cytoplasmic ribosomes.

ACKNOWLEDGMENTS

This investigation was supported by National Science Foundation grant GB8716 and Public Health Service grant GM10067 from the National Institute of General Medical Sciences to D.O.W. and by Public Health Service predoctoral fellowship T-5T01-GM-15814 from the National Institute of General Medical Sciences to W.D.Z. Photography for figures was a courtesy of the U.S. Army, Walter Reed Army Institute of Research, Washington, D.C.

LITERATURE CITED

1. Basch, R. S. 1968. An improved method for counting tritium and carbon-14 in acrylamide gels. Analyt. Biochem. **26:**184-188.
2. Birkmayer, G. D., and T. Bücher. 1969. Ferrochelatase in wild-type and in cytoplasmic mutants of *Neurospora crassa*. Fed. Eur. Biochem. Soc. Let. **5:**28-30.
3. Cotman, C. W., H. R. Mahler, and T. E. Hugli. 1967. Resolution of insoluble proteins in rat brain subcellular fractions. Arch. Biochem. Biophys. **120:**384-396.
4. Edwards, D. L., and D. O. Woodward. 1969. An altered cytochrome oxidase in a cytoplasmic mutant of *Neurospora*. Fed. Eur. Biochem. Soc. Let. **4:**193-196.
5. Flavell, R. B., and D. O. Woodward. 1970. The regulation of synthesis of Krebs cycle enzymes in *Neurospora* by catabolite and end product repression. Eur. J. Biochem. **13:**548-553.
6. Goa, J. 1953. A microbiuret method for protein determination. Determination of total protein in cerebrospinal fluid. Scand. J. Clin. Lab. Invest. **5:**218-222.

7. Haskins, G. A., A. Tissieres, H. K. Mitchell, and M. B. Mitchell. 1953. Cytochromes and the succinic acid oxidase systems of poky strains of *Neurospora*. J. Biol. Chem. **200**:819-826.
8. Kaplan, D. M., and R. S. Criddle. 1971. Membrane structural proteins. Physiol. Rev. **51**:249-272.
9. Kuehn, G. D., B. A. McFadden, R. A. Johnson, J. M. Hill, and L. K. Shumway. 1969. The facile isolation of a structural phospholipoprotein from *Hydrogeneomonas facilis* and *Neurospora crassa*. Proc. Nat. Acad. Sci. U.S.A. **62**:407-414.
10. Lenaz, G., N. F. Haard, A. Lauwers, D. W. Allmann, and D. E. Green. 1968. Mitochondrial structural protein. I. Methods of preparation and purification: characterization by gel electrophoresis. Arch. Biochem. Biophys. **126**:746-752.
11. Mitchell, M. B., and H. K. Mitchell. 1952. A case of "maternal" inheritance in *Neurospora crassa*. Proc. Nat. Acad. Sci. U.S.A. **38**:442-449.
12. Mitchell, W. M. 1967. A potential source of electrophoretic artifacts in polyacrylamide gels. Biochim. Biophys. Acta **147**:171-174.
13. Moore, S. 1963. On the determination of cystine as cysteic acid. J. Biol. Chem. **238**:235-237.
14. Munkres, K. D., and D. O. Woodward. 1966. On the genetics of enzyme locational specificity. Proc. Nat. Acad. Sci. U.S.A. **55**:1217-1224.
15. Newton, A. W., Y. Morino, and E. E. Snell. 1965. Properties of cystalline tryptophanase. J. Biol. Chem. **240**: 1211-1218.
16. Newton, A. W., and E. E. Snell. 1962. An inducible tryptophan synthetase in tryptophan auxotrophs of *Escherichia coli*. Proc. Nat. Acad. Sci. U.S.A. **48**:1431-1439.
17. Noltman, E. A., T. A. Mahowald, and S. A. Kuby. 1962. Studies on adenosine triphosphate transphosphorylases. II. Amino acid composition of adenosine triphosphate-creatine transphosphorylase. J. Biol. Chem. **237**:1146-1154.
18. Richardson, H. H., H. O. Hultin, and S. Fleisher. 1964. Interactions of mitochondrial structural protein with phospholipids. Arch. Biochem. Biophys. **105**:254-260.
19. Sebald, W., T. Bücher, B. Olbrich, and F. Kaudequitz. 1968. Electrophoretic pattern of an amino acid incorporation *in vitro* into the insoluble mitochondrial protein of *Neurospora crassa* wild-type and mi-1 mutant. Fed. Eur. Biochem. Soc. Let. **1**:235-240.
20. Shannon, C. F., and J. M. Hill. 1971. A cytoplasmic protein from *Neurospora crassa* resembling membrane proteins. Biochemistry **10**:3021-3029.
21. Smith, L. 1955. Spectrophotometric assay of cytochrome *c* oxidase, p. 427. *In* D. Glick (ed.), Methods of biochemical analysis, vol. 2. Interscience Publishers Inc., New York.
22. Spackman, D. H., W. H. Stein, and S. Moore. 1958. Automatic recording apparatus for use in the chromatography of amino acids. Anal. Chem. **30**:1190-1206.
23. Takayama, K., D. H. MacLennan, A. Tzagoloff, and C. D. Stoner. 1964. Studies on the electron transfer system. LXVII. Polyacrylamide gel electrophoresis of the mitochondrial electron transfer complexes. Arch. Biochem. Biophys. **114**:223-230.
24. Tissieres, A., H. K. Mitchell, and F. A. Haskins. 1953. Studies on the respiratory system of the poky strain of *Neurospora*. J. Biol. Chem. **205**:423-433.
25. Vogel, H. J. 1964. Distribution of lysine pathways among fungi: evolutionary implications. Amer. Natur. **98**:435-446.
26. Weber, K., and M. Osborne. 1969. The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. J. Biol. Chem. **244**:4406-4412.
27. Woodward, D. O. 1968. Functional and organizational properties of *Neurospora* mitochondrial structural protein. Fed. Proc. **27**:1167-1173.
28. Woodward, D. O., and K. D. Munkres. 1966. Alterations of a maternally inherited mitochondrial structural protein in respiratory-deficient strains of *Neurospora*. Proc. Nat. Acad. Sci. U.S.A. **55**:872-880.
29. Woodward, D. O., and K. D. Munkres. 1967. Genetic control, function, and assembly of a structural protein in *Neurospora*, p. 489-502. *In* H. J. Vogel, J. D. Lampen, and V. Bryson (ed.), Organizational biosynthesis. Academic Press Inc., New York.
30. Yang, S., and R. S. Criddle. 1969. Identification of a major membrane fraction as a product of synthesis by isolated yeast mitochondria. Biochem. Biophys. Res. Commun. **35**:429-436.