Roodyn, D. B., and Wilkie, D. (1968), Biogenesis of Mitochondria, London, Methuen.
Schweyen, R., and Kaudewitz, F. (1970), *Biochem. Biophys. Res. Commun. 38*, 728.
Scott, W. A., Munkres, K. D., and Metzenberg, R. L. (1971), *Arch. Biochem. Biophys. 142*, 623.
Sebald, W., Birkmayer, G. D., Schwab, A. J., and Weis, H. (1971), *in* Proceedings of the International Symposium on Autonomy and Biogenesis of Mitochondria and Chloroplasts, Boardman, N. K., Linnane, A. W., and Smillie, R. M., Ed., Amsterdam, North Holland, p 339.
Sebald, W., Schwab, A. and Bücher, T. (1969), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 4*, 243.
Sebald, W., Schwab, A., Bücher, T., Olbrich, B., and Kaudewitz, F. (1968), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 1*, 235.
Senior, A. E., and Brooks, J. C. (1970), *Arch. Biochem. Biophys. 140*, 247.
Strauss, E. G., and Kaesberg, P. (1970), *Virology 42*, 437.
Swank, R. T., and Munkres, K. D. (1971), *Anal. Biochem. 39*, 462.
Swank, R. T., Sheir, G. I., and Munkres, K. D. (1971), *Biochemistry 10*, 3931.
Woodward, D. O., and Munkres, K. D. (1966), *Proc. Nat. Acad. Sci. U. S. 55*, 872.
Yang, S., and Criddle, R. S. (1970), *Biochemistry 9*, 3063.

Subcellular and Generic Distribution, Molecular Weights, and Proportions of Oligopeptides*

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ABSTRACT: *Neurospora crassa* mitochondria exhibited six proteins of molecular weight in the range of 2600 to 8800, together comprising 10–13% by weight and 35–45% by mole proportion of mitochondrial protein. A submitochondrial fraction, designated P_0, yielded the same molecular weights in two methods of molecular sieving. An insoluble membrane fraction and fraction P_0 were enriched in the concentration of these oligopeptides whose molecular weights were the same as those in whole mitochondria of *N. crassa*. In mitochondria of *Saccharomyces cerevisiae* and bovine heart, oligopeptides were similar to those of *N. crassa* in number of classes, molecular weights, and proportion. We observed the same classes and an enrichment of oligopeptides in both the electron transport particles and fraction P_0 of bovine mitochondria, but only a small quantity of one peptide of 3500 in bovine mitochondrial adenosine triphosphatase. Only small amounts and numbers of classes of oligopeptides appeared in the cytosol or microsomes of *N. crassa* or in purified rat liver nuclear membrane, and none appeared in synaptic complex of porcine brain. Nuclei of *N. crassa* and both neurofibrils and myelin of porcine brain, all of which were probably contaminated by mitochondria, yielded fewer classes and lower proportion of oligopeptides than did pure mitochondria. No experiment thus far has yielded any evidence indicating that the oligopeptides are products of proteolysis either *in vivo* or *in vitro;* rather, they appear to be synthesized directly by way of cytoribosomes.

Electrophoresis in polyacrylamide gels which contain the detergent sodium dodecyl sulfate (Shapiro and Maizel, 1969; Dunker and Rueckert, 1969; Weber and Osborn, 1969) (SPAGE[1]) permits analysis of monomeric molecular weights and proportions of polypeptides[2] in complex mixtures such as viruses (Dunker and Rueckert, 1969), ribosomes (Dzionara *et al.*, 1970), membranes (Schnaitman, 1969; Kiehn and Holland, 1970a), and even whole cells (Kiehn and Holland, 1970b). Studies of the molecular weights of mitochondrial proteins of *Neurospora crassa* and bovine heart by these SPAGE methods, and by the method of Hedrick and Smith (1968) reveal, however, that a substantial proportion of the total protein is less than 10,000 (Munkres *et al.*, 1971; Swank *et al.*, 1971), and that the methods are inadequate for high resolution of these small proteins. This paper describes the application of two improved methods of electrophoresis in studies of the subcellular and generic distribution, molecular weights, and proportions of oligopeptides in the range of 2000–10,000 in eucaryotic cells, with emphasis upon *N. crassa*.

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[1] Abbreviations used are: SDS, sodium dodecyl sulfate; BPB, bromophenol blue; ETP, electron transport particles; P_0, mitochondrial oligopeptide fraction; SPAGE, SDS–polyacrylamide gel electrophoresis; Temed, *N,N,N′,N′*-tetramethylethylenediamine.

[2] Here oligopeptides refers to polypeptides whose molecular weights are below 10,000, and polypeptides to all monomeric proteins.

Materials and Methods

Methods of stock maintenance and culture, and *in vivo* labeling with radioactive leucine were described by Swank *et al.* (1971).

Preparation and Source of Cell Fractions. Mitochondria and postmitochondrial supernatant (32,000*g* supernatant, cytosol) are prepared from *N. crassa* mycelial homogenates by differential centrifugation as described by Swank *et al.* (1971). The microsomal fraction is collected from the 32,000*g* supernatant by centrifugation in a Ti-60 rotor at 176,600*g* for 1 hr in an L2-65B Spinco centrifuge. Nuclei are prepared by the following method similar to that of Luck and Reich (1964). The mycelial homogenate is prepared as described by Swank *et al.* (1971), and centrifuged for 10 min at 4° and 3000*g*. The precipitate is suspended (in one-fifth of the original volume) in a solution containing 0.44 M sucrose, 1 mM EDTA, 50 mM Tris-Cl (pH 7.4, 25°), and 10 mM $CaCl_2$. The suspension is centrifuged four times at 500*g* for 5 min each, and the pellet is discarded each time. The supernatant is centrifuged for 10 min at 3000*g*. The nuclear pellet is suspended in the same solution and centrifuged for 10 min at 3000*g*.

Soluble and insoluble fractions of *N. crassa* mitochondria are prepared as follows. The mitochondrial pellet 2 (Swank *et al.*, 1971) is suspended (in $^1/_{20}$th of the volume of the mycelial homogenate) in 0.1 M sodium phosphate buffer (pH 7.4) at 4°. The suspension is probed with a Branson sonifier for four 15-sec periods at 4° and centrifuged 25 min at 32,000*g*. The soluble fraction is immediately heated in SDS solution. The pellet is washed twice with the original volume of buffer by centrifugation for 25 min at 32,000*g*, and the washes are discarded. The pellet, defined as the insoluble fraction, is immediately heated in SDS solution.

The following preparations were gifts: bovine heart mitochondria, electron transport particles, and F_1 (Senior, Brooks, and Green); mitochondria of derepressed *Saccharomyces cerevisiae* (Morimoto and Halvorson, 1971); lipid-extracted rat liver membrane (Kasper); porcine brain synaptic complex, neurofibrils, and myelin (Kornguth). Dr. H. Pitot provided normal rats and those with Morris hepatoma.

Preparation of Mitochondrial Oligopeptide Fraction. A protein fraction, designated P_0, is isolated from either mitochondria or from the acetone powder of disintegrated cells. Whichever the source, there is little difference in the proportions and the molecular weights of the oligopeptides, or in their isoelectric points (G. I. Sheir and R. T. Swank, unpublished data). But the powder is easier to handle and produces greater yields of P_0, and hence is generally used. It is prepared after physical disruption of cells in liquid nitrogen (Munkres and Richards, 1965).

The process of isolation is as follows: mycelia powder or mitochondria or acetone powders of rat liver and rat hepatoma are extracted at 4° with 20 volumes of a solution containing 0.5% sodium dodecylsarcosine, 1 mM ATP, 0.05% 2-mercaptoethanol, and 10 mM Tris-Cl (pH 7.4) at 25°. For large preparations, homogenization is performed in a Waring blender at high speed; for small, in a Potter-Elvehjem homogenizer with motor-driven pestle. The suspension is centrifuged at 20,000*g* for 15 min. The supernatant is slowly adjusted to pH 6.0 with HCl at 4°; solid ammonium sulfate is slowly added to the solution to 48% saturation. The solution is stirred for 15 min and stored at 4° for 1 hr. The precipitate is collected from it by centrifugation at 20,000*g* for 15 min, suspended in a small volume of glass-distilled water at pH 6.0, and dialyzed in acetylated dialysis tubing against three changes of glass-distilled water at 4°.

SDS-Polyacrylamide Gel Electrophoresis (SPAGE). Proteins are dissolved in 2% SDS at 100° and the solution is adjusted to 8 M urea and 1% mercaptoethanol as described by Swank *et al.* (1971). A SPAGE system with 12.5% acrylamide, 1.25% bisacrylamide, 8 M urea, and 0.1% SDS is used (Swank and Munkres, 1971). Sperm-whale myoglobin (3–5 μg) is added to each sample as an internal standard for molecular weight determinations. Proteins are stained with coomassie blue as described by Swank *et al.* (1971).

Alkaline-Urea Polyacrylamide Gel Electrophoresis. Acrylamide gel contain: 50 mM glycine–NaOH buffer, 0.15% Temed (v/v), 0.02% ammonium persulfate, 11–20% (w/v) acrylamide in 1% increments per gel, bisacrylamide at $^1/_{15}$th of the acrylamide concentration, and 8 M urea at a final pH of 10.4. Solutions with urea (enzyme grade) are prepared just before use to avoid excess cyanate formation. Gels are cast in Pyrex tubing (6 × 70 mm) and overlaid with water. Electrode reservoir solutions contain 50 mM glycine–NaOH buffer (pH 10.4). A Büchler Polyanalyst apparatus is used with 12 tubes, the lower reservoir being maintained at a constant temperature of 15°. The gels are subjected to a current of 50 mA for 2 hr to remove persulfate. Samples of lyophilized fraction P_0, or standard proteins, are dissolved at 1–3 mg/ml in a freshly prepared solution containing 8 M urea and 6 mM dithiothreitol, and adjusted to pH 11.0 with NaOH. Sample solutions (0.005–0.05 ml) are layered on top of the gels beneath the buffer in the electrode reservoir. A solution of 0.003 ml of 0.02% BPB in 50% sucrose is added to each sample. Samples are subjected to electrophoresis toward the anode at 4 mA/gel for 4–5 hr at 15°. Gels are removed from the tubes with a syringe plunger, stained 2–4 hr (depending upon the gel concentration) in a solution containing 0.05% (w/v) coomassie blue, 5% trichloroacetic acid, and 5% sulfosalicylic acid, and destained with the same acid solution. The distance from the origin traversed by each protein is recorded to the nearest 0.5 mm. The gels are scanned at 550 nm with a Gilford recording attachment, and the positions of the proteins were recorded to the nearest 0.1 mm. A standard curve of slope *vs.* molecular weight is constructed by the method of Hedrick and Smith (1968) on the basis of the results obtained from duplicate gels of six acrylamide concentrations. The slopes of linear plots of the logarithm of the distance (in millimeters) traversed by proteins *vs.* the gel concentration (Ferguson plots) are estimated by least-squares regression analysis.

Densitometry and Radioactivity Measurements. Absorbancies of stained proteins in gels are analyzed with a Gilford Model 2000 recording spectrophotometer at 550 nm, set at full-scale absorbancy of 0–1.0. A Gilford gel scan attachment with slit dimensions (0.05 × 2.36 mm) is driven at 1–2 cm/min, and the recorder is driven at 2 in./min.

Radioactivities of proteins in gels are analyzed by using a Gilson automatic gel crusher as described by Swank *et al.* (1971). Myoglobin, methylated with [^{3}H]dimethyl sulfate, was used in electrophoresis as a reference.

Determination of the Relative Proportions of Polypeptides in Molecular Weight Class Intervals. The abscissi of graphs of the distribution of radioactivity (or absorbancy of stained proteins) in SPAGE as a function of distance are transformed to a molecular weight scale with previously established calibrations (Swank and Munkres, 1971) in relation to the position of internal labeled or unlabeled marker proteins (Swank *et al.*, 1971). Tracings of graphs are cut from paper. Relative areas under the curve in fixed molecular weight class intervals are determined by cutting and weighing the paper to the nearest 0.01 mg. The total weight of the paper under the graphic tracing is set to equal 100% of the protein weight; a close approximation since 98% of the total radioactivity

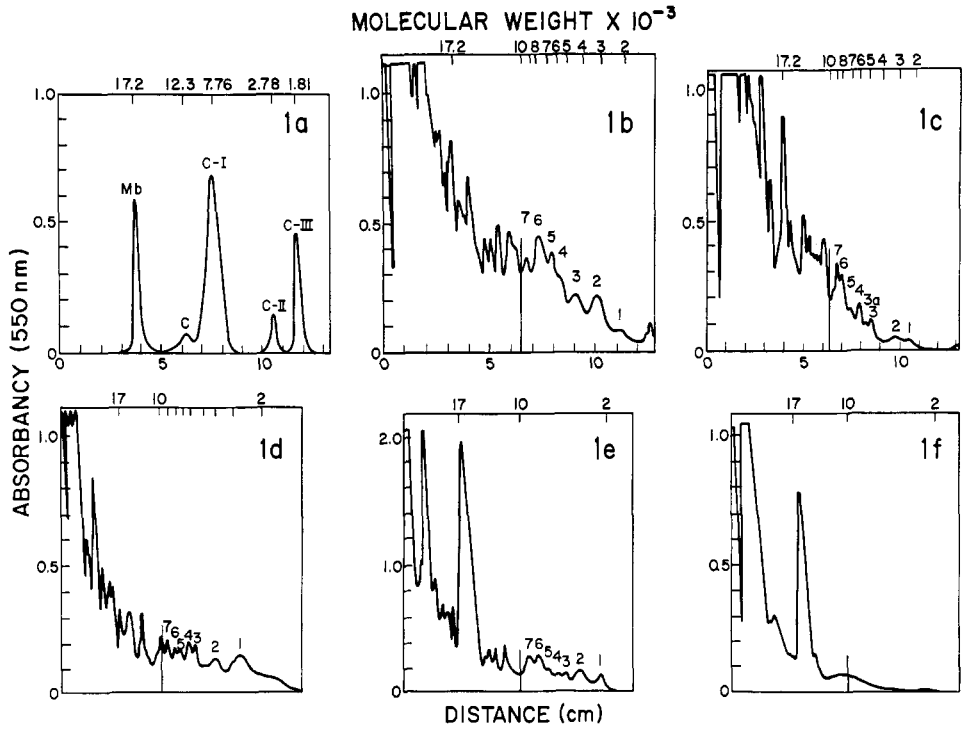

FIGURE 1: SPAGE analysis of mitochondrial and submitochondrial proteins. Proteins were dissolved in a solution of SDS–urea and mercaptoethanol, electrophoresed toward the anode (toward the right in the figures) at 7–8 V/cm for 17 hr in a high-resolution SPAGE system, stained, and recorded as described in Methods. Sperm-whale myoglobin (mol wt 17,200) was included as an internal marker. The vertical line from the abscissa within the figures is the demarcation of mol wt 10,000. (a) Cyanogen bromide fragments (30 μg) of horse heart cytochrome *c* (containing a trace of uncleaved cytochrome *c*) and 5 μg of sperm-whale myoglobin; (b) *N. crassa* mitochondria (107 μg, expt 278-6); (c) *S. cerevisiae* mitochondria (100 μg, expt 282-1); (d) heavy bovine heart mitochondria (46 μg, expt 274-1); (e) *N. crassa* mitochondrial-insoluble fraction (35 μg, expt 284-1); (f) *N. crassa* mitochondrial-soluble fraction (33 μg, expt 284-6).

applied to the gel is recovered after the gel is crushed into segments and analyzed. The relative mole proportions of the polypeptides are calculated by dividing the relative weight proportion in each interval by the midpoint value of the molecular weight class interval. The relative mole proportion in each interval is normalized to "mole per cent" by division of the mole proportion by the sum of the proportions in all intervals.

Because of the low amount of radioactivity that was incorporated into mitochondrial proteins, relative to their absorbancy after staining them with coomassie blue, and because the limit of resolution of the densitometer was 0.05 mm but that of the gel crusher was 1 mm, densitometry was used to measure the position of proteins in the gel for molecular weight estimates; it may, however, underestimate the proportions of oligopeptides because in the process of fixing and staining them, they are leached from the gel (Swank and Munkres, 1971). The suggestion has been made (de St. Groth *et al.*, 1963) that coomassie blue does not stain peptides as well as it does larger proteins, but quantitative estimates of the concentration of the larger ones with this dye is possible within a certain limit of that concentration, the error of estimate of relative concentration being about 3%. In order to determine that range of protein concentration in which absorbancy was proportional to the concentration, we analyzed a series of concentrations of a given preparation.

Proteinase Assays. The principle of the assays of proteinases depends upon a colorimetric measurement of the colored product formed with the diazotizable amine of *p*-nitroaniline that is released by the hydrolysis of *N*-α-benzoyl-arginine-*p*-nitroanilide or *N*-succinyl-L-phenylalanine-*p*-nitroanilide by trypsin- or chymotrypsin-like enzymes, respectively (Willig and Körber, 1967). The assays are calibrated with crystalline trypsin and chymotrypsin. Under our conditions, trypsin (2.3 μg) yielded an absorbancy of 0.1 in a 15-min incubation. Chymotrypsin (7 μg) yielded an absorbancy of 0.1 in a 60-min incubation. Linear proportionality of enzyme concentration and absorbancy was found when the latter was below 0.5.

Chemicals. The following chemicals were employed: L-[^{14}C]-leucine, sp act. 216 mCi/mmole (Calbiochem); coomassie brilliant blue (CoLab Laboratories); *N'*,*N*-tetramethylethylenediamine and 2-mercaptoethanol (Eastman Organic Chemical Co.); sperm-whale myoglobin, porcine adrenocorticotrophic hormone, bovine trypsin inhibitor, bovine insulin, sucrose, urea (enzyme grade), phenylmethylsulfonyl fluoride, benzethonium chloride, sodium dodecylsarcosine, diisopropyl fluorophosphate, *N*-α-benzoyl-DL-arginine-*p*-nitroanilide, *N*-succinyl-L-phenylalanine-*p*-nitroanilide, *N*-1-naphthylethylenediamine, and ammonium sulfamate (Mann Research Labs.); acrylamide and *N'*,*N*-methylenebisacrylamide (Matheson Coleman and Bell); L-[4,5-^{3}H]-leucine, sp act. 53.9 Ci/mmole (Schwarz BioResearch Co.); bacitracin, chloramphenicol, spinach ferridoxin, glucagon, bovine ribonuclease S protein, dithiothreitol, adenosine triphosphate, sodium dodecyl sulfate, crystalline trypsin (Type XI, 8200 *N*-Bz-L-ArgOEt units/mg, dicyclohexylcarbodiimide treated), crystalline chymotrypsin (Type II, 42 Bz-L-TyrOEt units/mg); Triton X-100 (Sigma Chem. Co.); and cycloheximide (Upjohn Co.).

Cyanogen bromide peptides of sperm-whale myoglobin I

TABLE I: Survey of Occurrence of Membrane Oligopeptides.

Source	Oligopeptides Present	No. of Mol Wt Classes[a]	% of Total Protein (w/w) Densitometry[f]	Radioactivity[f]
N. crassa				
Whole mitochondria	Yes	6	10 ± 1	12 ± 0.2
Mitochondrial insoluble	Yes	7	20 ± 5	
Mitochondrial soluble	Yes	1	3 ± 2	
Mitochondrial fraction P_0	Yes	9	60	
Microsomal	Trace	2	4 ± 1	3
Whole nuclei	Yes[b]	2–4	4 ± 2	
Cytosol	Trace	1–2	2	2.3 ± 1.1
Bovine heart				
Whole mitochondria	Yes	7	17	
Mitochondrial fraction P_0	Yes	6	Nd[c]	
ETP[d]	Yes	7	Nd	
F_1 (ATPase)	Trace	1	3	
Rat liver				
Nuclear membrane	Trace	1	1	
Fraction P_0	Yes[e]	6	Nd	
Porcine brain				
Synaptic complex	No	0	0	
Neurofibrils	Yes[b]	4	5	
Myelin	Yes[b]	2	3	
S. cerevisiae				
Whole mitochondria	Yes	8	8	

[a] Molecular weights and proportions were determined from densitometric or radioactivity measurements after electrophoresis in high-resolution SPAGE as described in Methods. [b] Contaminated with mitochondria. [c] Not determined but see Figure 2. [d] Electron transport particles. [e] Determined in alkaline–urea polyacrylamide electrophoresis system. [f] Average and standard deviation of three experiments with *N. crassa* fractions. The quantity of protein applied to the gel column, ranging from 25 to 150 μg, did not influence these estimates. Note however that in a few instances such as *N. crassa* mitochondrial soluble, cytosol, and rat liver nuclear membrane, where the proportion of oligopeptides was low, only a broad diffuse peak with indeterminate molecular weight was observed, perhaps representing the leading edge of a peak at or above 10,000 (Figures 1 and 2).

(mol wt 8270) and II (mol wt 6420) were prepared as described by Swank and Munkres (1971).

Results

Occurrence, Molecular Weights, and Proportions of Oligopeptides. The number of molecular weight classes and the weight proportions of oligopeptides in subcellular fractions of various genera are in Tables I and II and Figures 1 and 2. Mitochondria of *N. crassa* exhibit six classes of molecular weights below 10,000. These six also occur in a mitochondrial membrane fraction, but lower concentrations and fewer classes are in the soluble fraction of these mitochondria. Furthermore, the observed twofold enrichment of the concentration of oligopeptides in the mitochondrial membrane fraction, which comprises 45–55% of the total mitochondrial protein, indicates that the oligopeptides are associated with these membranes. A similar inference can be drawn from similar results with bovine mitochondria and their membraneous electron transport particles. In contrast to mitochondria, cytosol and microsomes of *N. crassa* yield only one or two classes below 10,000, and these peptides comprise 2–4% of the total protein in each of these fractions.

Fewer classes in lower proportion are in *N. crassa* nuclei than in mitochondria alone; however, it is likely that these preparations are contaminated with mitochondria. No significant quantity of oligopeptides is in purified rat liver nuclear membrane (Table I), but they are in fraction P_0 of rat liver and hepatoma (Figure 3 and Table III).

The proportion and number of oligopeptide classes are about the same in bovine heart and *N. crassa* mitochondria. Both electron transport particles and mitochondria of beef heart exhibit the same number of oligopeptide classes, but only one oligopeptide of 3500 is in F_1 of these mitochondria.

Synaptic complex, neurofibrils, and myelin of porcine brain probably do not have significant proportions of oligopeptides; the ones in neurofibrils and myelin are probably derived from brain mitochondria which contaminate these preparations (S. Kornguth, personal communication).

The molecular weights of mitochondrial oligopeptides of *N. crassa*, bovine heart, and yeast are in Table II. Within the error of determination, the weights of most of them in these genera are homologous. The same ones are greater in concentration in both the membrane and the P_0 fraction than in mitochondria of *N. crassa* (with the exception of the 9750 one in P_0). Similarly, their concentration is greater in both the electron transport particles and P_0 of bovine mitochondria.

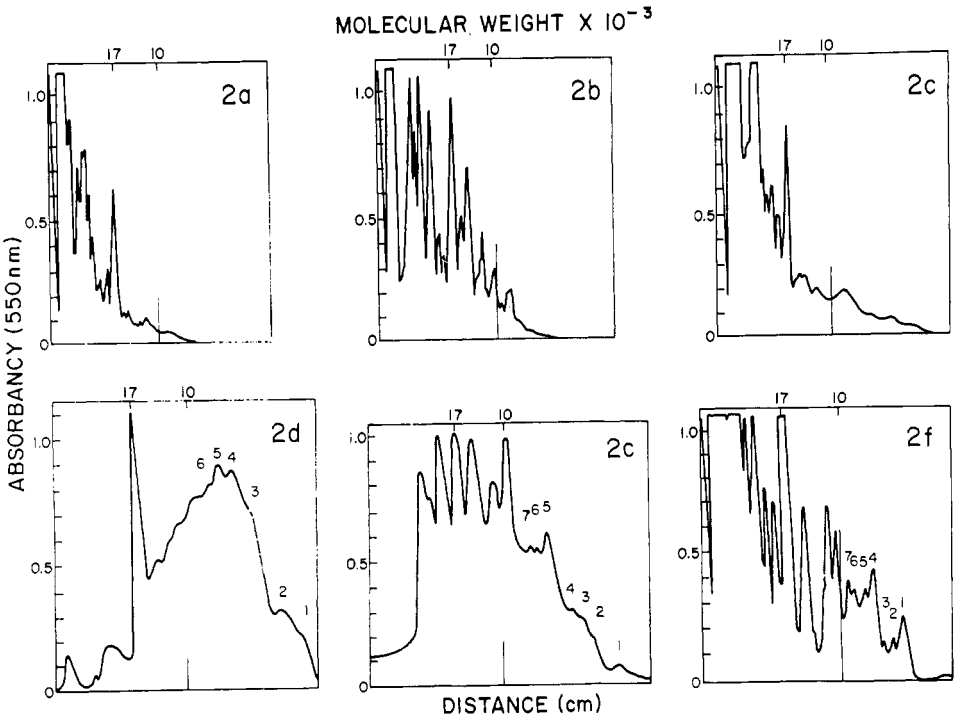

FIGURE 2: SPAGE analysis of extramitochondrial and submitochondrial proteins. Conditions as in Figure 1. (a) *N. crassa* postmitochondrial supernatant (cytosol) (32,000*g*) (48 μg, expt 275-7); (b) *N. crassa* microsomes (69 μg, expt 286-9); (c) *N. crassa* nuclei (126 μg, expt 286-8); (d) *N. crassa* mitochondrial fraction P_0 (100 μg, expt M-12); (e) heavy bovine heart mitochondrial fraction P_0 (100 μg, expt M-12); (f) heavy bovine heart mitochondrial electron transport particles (58 μg, expt 283-1).

TABLE II: Molecular Weights of Oligopeptides in Mitochondria and Submitochondrial Fractions.

Component	Molecular Weight[b] and Source							
	Whole Mitochondria				Submitochondrial Fraction			
					N. crassa		Bovine Heart	
No.[a]	*S. cerevisiae*	Bovine Heart	*N. crassa*	Av[c]	Insoluble	P_0	ETP	P_0
7	9100	8900 (100)	*d, e*	9000	*e*	9750 (920)	8800	9200
6	8300	8200 (100)	8800 (55)	8700	8600 (460)	8100 (500)	8100	
5	7400	6800 (50)	8600 (400)	7500	7880 (380)	7200 (270)	6800	7700
4	6300	6000 (0)	6640 (270)	6500	6750 (380)	6600 (320)	6000	6200
3[a]	5600					5750 (100)		
3	5000	5250 (150)	5310 (300)	5300	5700 (370)	5000 (250)	5100	5100
2[a]					4320 (300)	4000 (280)	4600	4200
2	3200	3950 (150)	4120 (350)	4000	3380 (390)	3600 (160)	3800	3700
1	2600	2500 (100)	2600 (400)	2600	2280 (240)	2200 (240)	*e*	*e*
No. of expt	1	2	10		4	3	1	1

[a] Numbered in ascending order from the anode as in Figures 1 and 2. [b] Determined after densitometry of proteins stained in a high-resolution SDS–polyacrylamide gel electrophoresis system as described in Methods. Standard deviations are in parentheses. [c] Weighted average of the three genera. [d] Next higher component mol wt 10,800 ± 290. [e] Not observed.

An Alternate Estimate of Molecular Weights. The intrinsic charge of proteins of molecular weights less than 10,000 (relative to those greater than) is a more important determinant of their electrophoretic mobilities in SPAGE (Swank and Munkres, 1971). Thus some of the oligopeptides might be charge isomers of the same weight. In order to test this hypothesis, we determined their weights in fraction P_0 by another electrophoretic system with polyacrylamide gel, according to the principle reported by Hedrick and Smith (1968). Such electropherograms are in Figure 3. The system is calibrated in Figure 4. The molecular weights are in Table III. The weight of the major protein in Figure 3a is

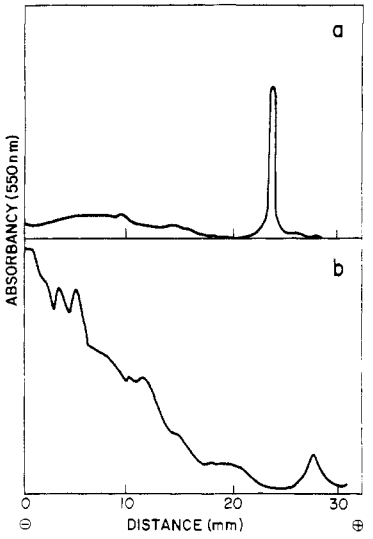

FIGURE 3: Alkaline-urea polyacrylamide electropherograms of *N. crassa* mitochondrial protein fraction P_0. Fraction P_0 (a, 9 μg; b, 20 μg) was electrophoresed through a 15% (w/w) gel at pH 10.4 in 8 M urea at 4 mA for 4 hr at 15°. Parts a and b illustrate patterns obtained with freshly isolated and "aged" preparations, respectively.

3600. (Proteins homologous in mobility to those of *N. crassa* P_0 are also observed in that fraction of rat liver and hepatoma.) A protein faster in mobility than that of the 3600 one is also occasionally observed in the *N. crassa* preparation; it may correspond to the 2200 protein observed in the SPAGE system. But estimate of its weight was not possible with the gel concentrations that we used (Figure 4). The proteins whose mobilities are slower than that of the major one (the smaller peaks in Figure 3a and the larger ones in Figure 3b) were greater in concentration than the 3600 one with greater total protein

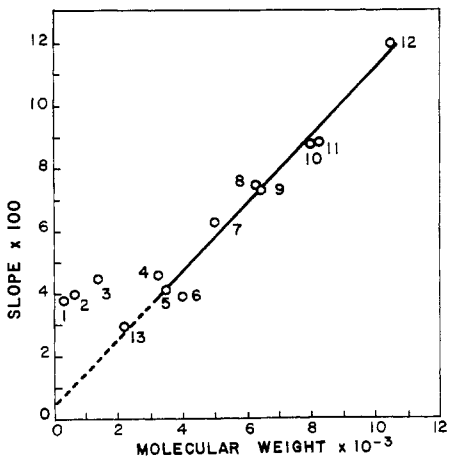

FIGURE 4: Calibration of alkaline-urea polyacrylamide gel electrophoresis system. Slopes were obtained from plots of log mobility of the following compounds as a function of acrylamide concentration. 1, phenol red; 2, bromophenol blue; 3, bacitracin; 4, insulin (B chain); 5, glucagon; 6, adrenocorticotrophic hormone; 7, spinach ferridoxin; 8, bovine trypsin inhibitor; 9, cyanogen bromide peptide (II) of myoglobin; 10, adrenocorticotrophic hormone (dimer); 11, cyanogen bromide peptide (I) of myoglobin; 12, bovine ribonuclease S protein; 13, insulin (A chain). The molecular weights employed and the preparation of cyanogen bromide peptides were described by Swank and Munkres (1971).

TABLE III: Molecular Weights of Oligopeptides in *Neurospora* Mitochondrial Subfraction, P_0.[a]

Component No.[b]	Mol Wt[c] $\bar{x}$	s
7	8950	1000
6	8600	900
5	7000	800
4	5900	700
2	3600	400
1	[d]	

[a] Determined by method of Hedrick and Smith after electrophoresis in polyacrylamide gels at pH 10.4 containing 8 M urea (see Methods). [b] The numbering system employed in the SPAGE analysis (Table II) is employed here. Component 4 here may be unresolved components 3 and 4 in SPAGE. [c] Average ($\bar{x}$) and standard deviation (s) of three experiments. [d] Not determined.

concentration and in "aged" preparations; their molecular weights are also in Table III.

Since the molecular weights determined by the two methods agree and since the alkaline electrophoresis separates proteins on the basis of both their charge and weight, the hypothesis that the oligopeptides are simply charge isomers is excluded. But since the heavier ones increase in proportion to the lower with greater protein concentration as well as after long storage of the preparations, the proteins greater than 3600 may be aggregates that are not completely dissociated in the SDS system. The propensity of the oligopeptides to aggregate in an oligomeric series and the weight of 3500 were previously demonstrated by molecular sieving in electrophoresis with a solvent containing phenol, acid, and urea (Munkres *et al.*, 1971).

An approximation of the isoelectric point of a protein is obtained in the method of Hedrick and Smith (1968) by extrapolation of the plot of log mobility against gel concentration to the ordinate and comparison of that intercept with the intercepts of proteins of known isoelectric points. Such analysis reveals that the majority of the oligopeptides are acidic with isoelectric points in the range of 4–6, a conclusion confirmed by the method of isoelectric focusing (G. I. Sheir and M. Minssen, unpublished data). Thus, although unaged fraction P_0 yields simple electrophoretic patterns at alkaline pH in urea (Figure 3a), as was observed by others with similar preparations (reviewed by Munkres *et al.*, 1971), this electrophoretic system is not likely to be useful for analyzing charge differences of these oligopeptides from different genera or mutants because of the large total net charge of these proteins at alkaline pH. Electrophoresis near the isoelectric point of these proteins, in the absence of detergent, is also not useful for such comparisons since they form numerous aggregates in an oligomeric series (Munkres *et al.*, 1971); a not unexpected observation, since proteins tend to aggregate at their isoelectric point.

Analyses of many preparations of fraction P_0 of *N. crassa* by electrophoresis in the alkaline and SPAGE systems and by isoelectric focusing reveal that long storage of lyophilized samples at −20° apparently leads to an irreversible aggregation of the oligopeptides, a phenomenon which we are cur-

rently investigating. The results given in Tables I–III and Figure 2 are from experiments with relatively "unaged" preparations.

Biogenesis of Mitochondrial Oligopeptides. When 5 mM mercuric chloride, an inhibitor of yeast proteinases (Maddox and Hough, 1970), was included in the isolation medium, change in the proportion or the number of classes of mitochondrial oligopeptides was not observed.

When the time between the preparation of the mycelial homogenate and the isolation of the mitochondria was either 30 or 90 min, difference in the proportion or number of classes of oligopeptides was not observed.

The tryptic- and chymotryptic-like proteolytic activities of subcellular fractions of *N. crassa* and bovine heart mitochondria are in Table IV. Purified mitochondria of both *N. crassa* and bovine heart essentially lack these activities. Cytosol and microsomes of *N. crassa* exhibit much greater specific activities than do mitochondria. Moreover, on a cellular basis, the quantity of total cytosol protein is about four- to fivefold greater than that of mitochondria; hence, less than 1% of the total cellular activity is obtained in the purified mitochondrial fraction. This observation alone renders unlikely the possibility that mitochondrial oligopeptides are formed by enzymic proteolysis during isolation of the mitochondria, particularly since cytosol and microsomes, although relatively high in these activities, exhibit only very small amounts of oligopeptides. Furthermore, the quantity of proteinase in the most active cell fraction the cytosol (in trypsin and chymotrypsin equivalents) seems trivial, about 0.05% of the protein by weight. It is highly unlikely that this quantity could hydrolyze 10–13% of mitochondrial polypeptides to oligopeptides at 0° during the hour between the preparation of the homogenate and the recovery of the mitochondria.

Some mitochondrial enzymes may exhibit latency in assay because mitochondria are impermeable to the substrate. Mitochondria are assayed for proteinase activity after they are disrupted with 0.1% Triton X-100, a treatment that overcomes the latency of mitochondrial malate dehydrogenase (K. D. Munkres, unpublished), and that does not inhibit the cytosol proteinases. Thus, neither latency nor inhibition appears to contribute to the absence of mitochondrial proteinase activity.

Once having obtained mitochondria *in vitro* free of the potentially proteolytic *milieu*, they are immediately heated at 100° for 3 min in neutral 2% SDS and the solution is adjusted to 8 M urea and 1% mercaptoethanol, a treatment that undoubtedly inactivates the trace of proteolytic activity remaining. Incubation of the *Neurospora* proteinases with 1% SDS at room temperature for 30 min inactivates about 96% of the chymotryptic-like, and 50% of the tryptic-like activity.

TABLE IV: Proteolytic Activities of Cell Fractions of *Neurospora* and Bovine Heart Mitochondria.

Cell Fraction[a]	Proteinase Equiv[b] T	C
N. crassa		
Mitochondria (crude)	30	100
Mitochondria (purified)	4	7
Cytosol	100	500
Microsomes	14	1100
Bovine heart mitochondria	0.6	7

[a] The cell fractions were prepared from exponentially growing mycelia of wild-type 74A as previously described (Swank *et al.*, 1971) and in Methods. "Crude" mitochondria refers to the first 16,000*g* pellet after differential centrifugation. Purified mitochondria were subjected to two additional washes with extraction buffer and isopycnic sucrose gradient centrifugation. Mitochondria were assayed in the presence of 0.1% Triton-X 100. [b] Micrograms of trypsin (T) or chymotrypsin (C) equivalents per gram of protein as described in Methods.

Discussion

Molecular Weights. The existence in mitochondria of little proteins is demonstrated by two methods of molecular sieving utilizing electrophoresis in polyacrylamide gels. Molecular sieving by electrophoresis in an acid–urea system also reveals them (Munkres *et al.*, 1971). Laico *et al.* (1970) observed oligopeptides in bovine heart mitochondria with a weight of about 5000. These authors discussed several reasons why these little proteins may have escaped notice in other laboratories. In addition to these reasons, the present method of analysis allows the resolution of oligopeptides in contrast to previously described methods (Swank and Munkres, 1971). Thus, oligopeptides are unresolved by SPAGE with 8% acrylamide and low cross-linkage, and appear as a broad peak with apparent weight between 5000 and 10,000 (Swank *et al.*, 1971; Swank and Munkres, 1971). Furthermore, because the oligopeptides collectively constitute only 10–13% by weight of total mitochondrial protein, some investigators may have observed them but considered them to be trivial. But in mole proportion they constitute 35–45% of total mitochondrial protein, and therefore may be important.

Biogenesis of Mitochondrial Oligopeptides. Our observation that the synthesis of mitochondrial oligopeptides *in vivo* in *N. crassa* is completely inhibited by cycloheximide indicates that their synthesis is directly by cytoribosomes as is 90% of mitochondrial protein (Swank *et al.*, 1971). But because of the possibility that they might be synthesized by a mechanism involving both cytoribosomes and posttranslational cleavage, and because they might arise by proteolysis during their isolation, additional evidence for proteolysis was sought, but not found.

The possibility that the oligopeptides were either synthetically incomplete peptides or the intermediates of rapid turnover was not indicated by the results of an *in vivo* pulse-chase experiment (Swank *et al.*, 1971).

In other experiments with the proteinase inhibitors $HgCl_2$ and phenylmethylsulfonyl fluoride, with variation in the time in isolation of mitochondria, and with consideration of the intracellular location of proteinases, the results indicated that the oligopeptides were not formed by enzymic proteolysis.

Occurrence of Oligopeptides in Other Membranes. Although our limited survey of eucaryotic cellular membranes indicates that oligopeptides may be primarily mitochondrial, the question remains: are the smaller quantities observed in microsomal and nuclear preparations either intrinsic to these organelles, or do they represent simply a slight contamination of these preparations by mitochondria?

In gram-negative bacteria, a major component of the membrane–wall complex consists of a peptide of mol wt 7000 (Braun *et al.*, 1970). In addition, in *Pseudomonas aeruginosa*, cytochrome *c* (Horio *et al.*, 1958) and cytochrome c_{551} (Dayhoff, 1970) are 7600–8100 and 9000, respectively. Similarly, the

weight of bovine microsomal cytochrome b_5 is 9400 (Dayhoff, 1970). Also, other bacterial redox proteins such as ferridoxin (Malkin and Rabinowitz, 1967) and rubredoxin (Newman and Postgate, 1968) are about 6000. In rat liver, a plasma membrane phospholipoglycoprotein's weight is 10,000 (Emmelot *et al.*, 1964) and several little proteins are in rat liver microsomes (Got *et al.*, 1967). Laico *et al.* (1970) reported that a major protein of the membranes, not only of bovine heart mitochondria but also of bovine outer retinal rods and human red blood cell, exhibits a weight of 5000. Similarly, sarcotubule membranes are largely composed of a peptide of 6500 (Byung and Masaro, 1970).

Functions of Oligopeptides in Mitochondria. At present, the functions of mitochondrial oligopeptides are unknown. It is unlikely that they are enzymic *per se*, although the existence of examples as bacterial ferridoxin, rubredoxin, cytochrome *c*, and bovine cytochrome b_5 may be relevant analogies. Possibly the oligopeptides may serve as catalytic or regulatory subunits of mitochondrial enzymes. The small peptide observed by Senior and Brooks (1970) in association with the membrane-bound mitochondrial adenosine triphosphatase, and confirmed here, may be an example. Mitochondrial oligopeptides may function in the selective transport of ions in a manner similar to some hormones or antibiotics. The highly insoluble mitochondrial peptide mitochondrocuprein of neonatal rat liver, whose weight is about 5000, binds copper and may function in the synthesis of cytochrome oxidase (Porter, 1971). Finally, the observation that the oligopeptides collectively constitute between 35 and 45% of the mole proportion of total mitochondrial protein may indicate either a structural or organizational function.

Small peptides such as the ionophorous antibiotics (Lardy and Ferguson, 1969), parathyroid hormone (Rasmussen *et al.*, 1967), and a mitochondrial peptidolipid (Rossi *et al.*, 1967) or the R factor (Lehninger and Remmert, 1959), which affect mitochondrial functions, may be considered as functional or structural analogs of naturally occurring mitochondrial oligopeptides. In fact, a peptide ionophore was recently isolated from bovine heart mitochondria (Blondin *et al.*, 1971).

Submitochondrial Fraction P_0 and Oligopeptides. We isolate a portion of mitochondrial membrane protein, designated P_0, with the aid of the detergent sodium dodecyl sarcosine, rather than sodium dodecyl sulfate, since the former is apparently less of a denaturant than the latter, at least for microtubule protein (Stephens, 1968). The observation that the preparation consists largely of oligopeptides suggests that our method does not select denatured proteins, in general. Moreover, their small size suggests that views about denaturation, derived from studies of large enzyme proteins, may not be appropriate here. Perhaps more suitable analogies may be derived from the peptide hormones, which are notoriously difficult to denature and which in some cases become more helical in detergent solutions (Bornet and Edelhoch, 1971). Furthermore, the insolubility of such membrane proteins may be directly related to their small size and not necessarily because they are denatured or unusually high in hydrophobic amino acid composition. Fisher (1964) presents a thermodynamic model that predicts why little proteins tend to aggregate and be water insoluble.

The physicochemical basis underlying the method of isolation of fraction P_0 is not clear, although models involving mixed micelles of the detergent sodium dodecylsarcosine with peptides could be considered. The purification procedure preferentially selects oligopeptides at two steps. At the ammonium sulfate precipitation and during subsequent washing and dialysis of the precipitate, the larger proteins are relatively more soluble (R. T. Swank, unpublished data).

Preliminary work indicates that the weights of the isolated oligopeptides are not distorted by association with phospholipid, carbohydrate, or nucleotides (G. I. Sheir and M. Minssen, unpublished data). The failure of the oligopeptides, before their isolation, to react with a periodic–Schiff stain after SPAGE, and their incorporation of radioactive leucine, indicates that they are not simply glycolipids such as observed in SPAGE of other membranes (Lenard, 1970).

Other laboratories observe that a subfraction of mitochondria, called structural protein (prepared in a manner similar but not identical to the preparation of fraction P_0 described here) contains products synthesized by mitoribosomes (Roodyn and Wilkie, 1968). Since these products are primarily 17,500, 27,700, and 33,500 (Swank *et al.*, 1971), but they constitute only 10% by weight of total mitochondrial protein, they perhaps may be in fraction P_0, although the bulk of this fraction consists of oligopeptides that are apparently synthesized by cytoribosomes.

References

Blondin, G. A., De Castro, A. F., and Senior, A. E. (1971), *Biochem. Biophys. Res. Commun. 43*, 28.

Bornet, H., and Edelhoch, H. (1971), *J. Biol. Chem. 246*, 1785.

Braun, V., Rehn, K., and Wolff, H. (1970), *Biochemistry 9*, 5041.

Byung, P. T., and Masaro, E. J. (1970), *Biochemistry 9*, 2909.

Dayhoff, M. O. (1970), Atlas of Protein Sequence and Structure, Vol. 5, Silver Springs, Md., National Biomedical Research Foundation.

de St. Groth, S. F., Webster, R. G., and Datyner, A. (1963), *Biochim. Biophys. Acta 71*, 377.

Dunker, A. K., and Rueckert, R. R. (1969), *J. Biol. Chem. 244*, 5074.

Dzionara, M., Kaltschmidt, E., and Wittman, E. (1970), *Proc. Nat. Acad. Sci. U. S. 67*, 1909.

Emmelot, P., Box, C. J., Bendetti, E. L., and Rumke, P. (1964), *Biochim. Biophys. Acta 90*, 126.

Fisher, H. F. (1964), *Proc. Nat. Acad. Sci. U. S. 51*, 1285.

Got, K., Polya, G. M., Polya, J. B., and Cockerill, L. M. (1967), *Biochim. Biophys. Acta 135*, 225.

Hedrick, J. L., and Smith, A. J. (1968), *Arch. Biochem. Biophys. 126*, 155.

Horio, T., Higashi, T., Nakai, M., Kusai, K., and Okunuki, K. (1958), *Nature (London) 182*, 1307.

Kiehn, E. D., and Holland, J. J. (1970a), *Biochemistry 9*, 1716.

Kiehn, E. D., and Holland, J. J. (1970b), *Nature (London) 226*, 544.

Laico, M. T., Ruoslahti, E. I., Papermaster, D. S., and Dreyer, W. J. (1970), *Proc. Nat. Acad. Sci. U. S. 67*, 120.

Lardy, H. A., and Ferguson, S. M. (1969), *Annu. Rev. Biochem. 38*, 991.

Lehninger, A. L., and Remmert, L. F. (1959), *J. Biol. Chem. 234*, 2459.

Lenard, J. (1970), *Biochemistry 9*, 1129.

Luck, D. J. L., and Reich, E. (1964), *Proc. Nat. Acad. Sci. U. S. 52*, 931.

Maddox, I. S., and Hough, J. S. (1970), *Biochem. J. (London) 117*, 843.

Malkin, R., and Rabinowitz, J. C. (1967), *Annu. Rev. Biochem. 36*, 113.

Morimoto, H., and Halvorson, H. O. (1971), *Proc. Nat. Acad. Sci. U. S. 68*, 324.

Munkres, K. D., and Richards, F. M. (1965), *Arch. Biochem. Biophys. 109*, 465.
Munkres, K. D., Swank, R. T., and Sheir, G. I. (1971), *in* Proceedings of the International Symposium on Autonomy and Biogenesis of Mitochondria and Chloroplasts, Boardman, N. K., Linnane, A. W., and Smillie, R. M., Ed., Amsterdam, North Holland Publishing, p 152.
Newman, D. J., and Postgate, J. R. (1968), *Eur. J. Biochem. 7*, 45.
Porter, H. (1971), *Biochim. Biophys. Acta 229*, 143.
Rasmussen, H., Shirasu, H., Ogata, E., and Hawker, C. (1967), *J. Biol. Chem. 242*, 4669.
Roodyn, D. B., and Wilkie, D. (1968), The Biogenesis of Mitochondria, London, Methuen and Co.
Rossi, C. R., Sartorelli, L., Tato, L., Baretta, L., and Siliprandi, N. (1967), *Arch. Biochem. Biophys. 118*, 210.
Schnaitman, C. A. (1969), *Proc. Nat. Acad. Sci. U. S. 63*, 412.
Senior, A., and Brooks, J. (1970), *Arch. Biochem. Biophys. 140*, 247.
Shapiro, A. L., and Maizel, J. V., Jr. (1969), *Anal. Biochem. 29*, 505.
Stephens, R. E. (1968), *J. Mol. Biol. 33*, 517.
Swank, R. T., and Munkres, K. D. (1971), *Anal. Biochem. 39*, 462.
Swank, R. T., Sheir, G. I., and Munkres, K. D. (1971), *Biochemistry 10*, 3924.
Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244*, 4406.
Willig, F., and Körber, W. (1967), *Z. Gastroent. 5*, 23.

Yeast Diphosphopyridine Nucleotide Specific Isocitrate Dehydrogenase. Purification and Some Properties*

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ABSTRACT: Yeast diphosphopyridine nucleotide specific isocitrate dehydrogenase (EC 1.1.1.41) has been purified to homogeneity by the criteria of disc gel electrophoresis, sedimentation velocity, and sedimentation equilibrium. Purification of the enzyme is facilitated by preferentially eluting the enzyme from ion-exchange columns with citrate, a modifier of the enzyme. Citrate also stabilizes the enzyme during isolation and preserves the kinetic cooperativity with respect to isocitrate. The enzyme has an apparent molecular weight of 3×10^5 as determined by sedimentation equilibrium. A subunit molecular weight of 3.9×10^4 was estimated by polyacrylamide gel electrophoresis in 0.1% sodium dodecyl sulfate. Thus the enzyme probably contains eight subunits. The kinetic order of the reaction catalyzed by the purified enzyme, as determined by the slope of a Hill plot, is 3.5 with respect to isocitrate in the absence of AMP. Addition of AMP decreases this order, to a value as low as 2.6 in some preparations. The order of reaction with respect to DPN^+ is 1 at both high and low isocitrate concentrations, in contrast to the order of 2 observed for the partially purified enzyme.

The DPN-specific isocitrate dehydrogenase from yeast (*threo*-D_s-isocitrate + $DPN^+ \rightarrow$ α-ketoglutarate + CO_2 + DPNH, EC 1.1.1.41) is activated by AMP, and citrate may either activate or inhibit, depending upon the isocitrate and citrate concentrations (Hathaway and Atkinson, 1963). Kinetic studies on relatively crude preparations of the enzyme demonstrated a reaction order of 4 for isocitrate and of 2 for DPN^+, AMP, and Mg^{2+} (Atkinson *et al.*, 1965). Because of the linearity of Hill plots over a wide concentration range, it was suggested that their slopes might be an indication of the actual number of binding sites for each ligand.

The enzyme has been purified to apparent homogeneity as a prerequisite to a study of its physical properties and ligand-binding behavior. The purification and some physical properties are reported here, and the results of binding studies in the following paper (Kuehn *et al.*, 1971).

Materials and Methods

Materials. Sodium dodecyl sulfate from Fisher was further purified by recrystallization from ethanol according to Burgess (1969). Glyceraldehyde 3-phosphate dehydrogenase (rabbit muscle), hexokinase (yeast), and ribonuclease (beef pancreas) were from Boehringer Mannheim, fumarase (pig heart) from Calbiochem, glutamate dehydrogenase (beef liver) from Sigma, and bovine serum albumin from Pentex. DEAE-cellulose (Whatman DE-52 microgranular) was purchased from H. Reeve Angel Inc., phosphocellulose (standard grade) from Schleicher & Schuell, and Bio-Gel A-1.5m (100–200 mesh) from Bio-Rad Laboratories. Fresh, compressed baker's yeast (*Saccharomyces cerevisiae*) was purchased from Standard Brands, Inc. A Manton-Gaulin single-stage homogenizer was

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