SEPARATION OF MITOCHONDRIAL MEMBRANES OF *NEUROSPORA CRASSA*

I. Localization of L-Kynurenine-3-Hydroxylase

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INTRODUCTION

To date the various methods devised to determine the localization of mitochondrial enzymes have been applied primarily to mammalian mitochondria (1). The purpose of this study was to investigate methods of separating the mitochondrial membranes of the Ascomycete fungus *Neurospora crassa* and to identify, if possible, enzymes associated with the inner and outer membranes. The evidence presented below demonstrates that in *Neurospora* L-kynurenine-3-hydroxylase (EC 1.14.1.2) (KH) is found uniquely in the mitochondria and that it is localized in the outer mitochondrial membrane. A preliminary report of this investigation has appeared (2).

METHODS AND MATERIALS

Preparation of Mitochondria

All operations were carried out at 0°–4°C. Wild-type *Neurospora crassa*, strain KJT 1960A (3), was grown in shake culture for 18 hr at 30°C as previously described (4). Mycelium was harvested by filtering through cheesecloth, washed with cold 0.1 M Tris buffer at pH 8.0 containing 0.1 M sucrose, and collected by filtration on a Büchner funnel. Sand-ground preparations were made by homogenizing mycelium in a cold mortar with 1.5 times its wet weight of acid-washed sand and an equal amount of 0.25 M sucrose plus 0.15% bovine serum albumin (sucrose-BSA) (5). Homogenates were centrifuged two times for 10 min each at 1500 *g* in the SS-34 rotor of a Sorvall RC-2 centrifuge (Ivan Sorvall, Inc., Norwalk, Conn.). The final supernatant was centrifuged for 30 min at either 8000 *g* or 37,000 *g* to yield the crude mitochondrial preparation. Machine-ground preparations were made when larger quantities of mitochondria were needed. Mycelium, harvested and washed as indicated above, was suspended in cold sucrose-BSA, 7 ml/gram wet weight of mycelium, and processed as previously described (6). A similar machine method has been used to prepare *Neurospora* mitochondria capable of coupling phosphorylation to the oxidation of suitable substrates (7). Mitochondria were further purified by centrifuging the crude preparations in a density gradient (0.58–1.9 M sucrose) (8).

Osmotic Sonication Treatment of Mitochondria

In early experiments the osmotic sonication method of Sottocasa et al. (9) was used. However, this method was not optimum for *Neurospora* mitochondria and resulted in experiments of excessive duration. A modified procedure was used in later experiments, as described in the following. A sample of gradient-purified mitochondria (80–150 mg protein) was washed with 0.25 M sucrose and centrifuged 30 min at 105,000 *g* in a Spinco 30 rotor (Spinco Division of Beckman Instruments, Inc., Fullerton, Calif.). The supernatant, which contained no detectable KH activity and usually less than 0.1 mg of protein/ml, was discarded. Osmotic swelling was initiated by resuspending the remaining mitochondrial pellet in the centrifuge tube by hand with a Teflon pestle (Arthur H. Thomas Co., Philadelphia, Pa.) in the desired volume of 10 mM Tris-phosphate buffer, pH 8.0. The suspension was transferred to a glass vessel and resuspension was completed by vigorous hand manipulation of the pestle for 2–3 min. The suspension was subjected to additional vigorous homogenization during the last 2 min of the swelling period. After a 30 min osmotic swelling period one-third of the suspension volume of a freshly prepared solution of either 0.4 M sucrose or 1.8 M sucrose, both containing 2 mM adenosine triphosphate (ATP) and 2 mM $MgSO_4$, was added. The sucrose-ATP-$MgSO_4$ (SAM) was blended with the suspension by means of two or three passes of the pestle. As previously noted (9), the turbidity of the suspension increased when SAM was added. The resulting suspension usually had a protein concentration of 7–12 mg protein/ml and contained either 0.1 or 0.45 M sucrose. The 1.8 M SAM solution was used in initial experiments. It was later found that the lower density 0.4 M SAM resulted in better separations of kynurenine hydroxylase activity in the discontinuous gradients. 5 min after the addition of SAM to the suspension, 3.5-ml samples were transferred to 10-ml beakers in ice and mildly sonicated for either

15 or 30 sec at 3 amp (setting 3) or 15 sec at 4 amp (setting 4) with a Branson Sonifier, model LS75, equipped with a step horn (Branson Sonic Power, Co., Danbury, Conn.).

Gradient Fractionation and Collection of Fractions

1-ml samples of the treated preparations were layered on either linear (0.58–1.9 M) or discontinuous sucrose density gradients and centrifuged 1 hr at 167,000 *g* in the SW-39 rotor of a Spinco Model L ultracentrifuge. The rotor and buckets were precooled to 4°C. The brake was not used. Discontinuous gradients were prepared in the cold no earlier than 30 min before they were to be used, by carefully layering 1.5 ml of 1.0 M sucrose on top of 1.5 ml of 1.9 M sucrose. Linear gradients were collected in equal fractions. Fractions from discontinuous gradients (Fig. 1) were carefully collected from the top of the tubes, by using 5-ml syringes with flat tipped 20-gauge hypodermic needles, and dispensed into 10-ml graduated test tubes. Protein was assayed immediately after the collection of fractions.

Enzyme Assays

Spectrophotometric assays were performed at 25°C in cuvettes with a 1 cm light path by the use of either a Cary model 14 recording spectrophotometer (Cary Instruments, Monrovia, Calif.) or a Gilford model 2000 recording spectrophotometer (Gilford Instrument Company, Oberlin, Ohio). Where necessary, fractions were diluted to 1 mg protein/ml with cold 0.25 M sucrose. Protein was estimated by the method of Lowry et al. (10). Succinate-cytochrome *c* reductase (SCCR) was assayed by the method of Tisdale (11). Acetohydroxy acid reductoisomerase (RI) was assayed by the method of Armstrong and Wagner (12) with α-aceto-α-hydroxybutyrate as substrate. Kynurenine hydroxylase (KH) was assayed by determining the production of 3-hydroxykynurenine with the method of Ghosh and Forrest (13) as modified: mitochondrial fractions were incubated in a reciprocating water bath for 1 hr at 30°C in 25-ml Erlenmeyer flasks; 10 mM potassium chloride was added to the 2.0 ml reaction mixture and 1–4 mg protein were added to start the reaction.

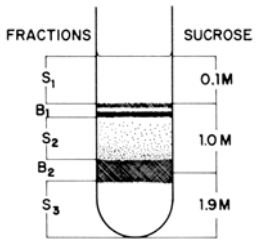

FIGURE 1 Appearance of three-layer discontinuous-gradients after centrifugation of mitochondria treated with the modified osmotic sonication method. S_1 is clear with a touch of pale orange color. B_1 consists of two, thin, sharply defined bands 0.5–1.0 mm apart: the upper band is a bright clear orange and the lower band is a slightly darker, light orange-brown. Both of the bands in B_1 contain KH activity. S_2 is a hazy, light orange color, darker than S_1. B_2 is brown with the top hazy in appearance and the lower edge well defined. S_3 is clear. Control gradients of untreated mitochondria in 0.1 M sucrose are clear with a well-defined B_2 band which contains all detectable KH activity. Occasionally one or two very faint bands are observed in the interface between 0.1 and 1.0 M sucrose.

Electron Microscopy

Freshly collected fractions were viewed following negative staining with 2% potassium phosphotungstic acid (PTA), pH 6.8. Fractions processed for plastic embedding were fixed for 1 hr in 2% buffered glutaraldehyde, pH 7.2, and centrifuged at either low speed (5000 rpm) or high speed (50,000 rpm) in a Spinco 50 rotor to yield the material analyzed. Samples were rinsed in three 10-min changes of buffer, fixed 1 hr in 1% osmium tetroxide, rinsed twice in distilled water, and stained in 0.5% uranyl acetate for 16–20 hr. Further processing was accomplished according to the procedure of Hawley and Wagner (14). Thin sections were cut on a Porter-Blum MT-1 ultramicrotome equipped with a diamond knife. Sections were either stained with lead citrate (15) or left unstained and viewed with a Siemens Elmiskop 1 or an RCA EMU-3G.

RESULTS

Presence of Kynurenine Hydroxylase in Neurospora Mitochondria

Kynurenine hydroxylase activity in sand-ground, crude mitochondrial preparations (CMP) was found in the 37,000 *g* pellet only and not in any of the soluble fractions. The activity remained in the mitochondrial band after centrifugation in a sucrose gradient. It is seen in Fig. 2 that the position of KH activity in the gradient coincides with that of the mitochondrial marker SCCR. Analysis of the pellet formed during the density gradient centrifugation revealed occasional trace amounts of KH activity. This activity was probably associated with mitochondria trapped in the more dense material and sedimented with it (7).

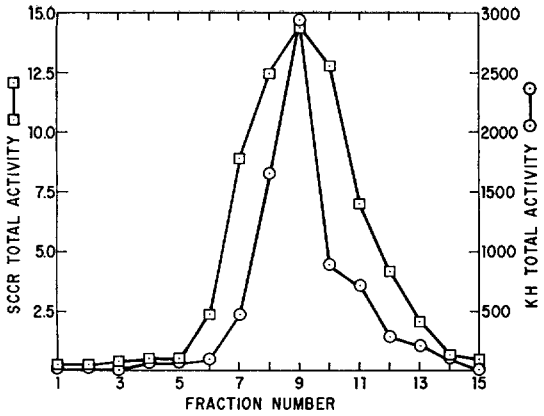

FIGURE 2 Distribution of total activity of succinate-cytochrome *c* reductase (SCCR, micromoles cytochrome *c* reduced per minute) and kynurenine hydroxylase (KH, millimicromoles 3-hydroxykynurenine produced per hour) in a linear sucrose density gradient (0.58–1.9 M sucrose) after centrifugation of a 37,000 *g* crude mitochondrial preparation for 1 hr at 198,000 *g* in the 50 rotor of a Spinco Model L ultracentrifuge. The brake was not used. Fractions are numbered from the bottom.

Mitochondrial Fractionation after Osmotic Sonication Treatment

Mitochondria were treated with the modified osmotic sonication method and subsequently centrifuged in linear sucrose gradients. It is clear, as seen in Fig. 3, that this treatment results in a substantial separation of KH and SCCR into fractions of different densities. A pale orange particulate band was observed near the top of the gradients at the interface between 0.45 and 0.58 M sucrose. This corresponds to a density range of approximately 1.06–1.07 g/ml. The major portion of the band was collected in the fraction with the highest KH activity.

Table I presents results obtained when a modified osmotic sonication preparation is centrifuged in the discontinuous gradient (Fig. 1). About 92% of the recovered KH activity is found in the light particulate B_1 fraction. The B_1 fraction, as seen in Fig. 4, consists of vesicles, some as small as 300–400 A, which are bounded by a single membrane. The vesicles appear to have no internal ultrastructure. They resemble the vesicles found in the outer membrane fraction of rat liver mitochondria (9, 16). No KH activity is detected in the heavy B_2 fraction where approximately 60% of the recovered SCCR is located. Vesicles seen in the B_2 fraction (Fig. 5) are large compared to those in the B_1 fraction and have an abundance of interior ultrastructure. It is noteworthy that the large vesicles are bounded by a single membrane and appear to retain some compartmental integrity. They are similar to vesicles observed in the inner membrane fraction of rat liver mitochondria (9). There appear to be two principal vesicle profiles present in the B_2 fraction: one is contracted and the other one is swollen (Fig. 5). Intact mitochondria have not been observed in the B_2 fraction.

Recovery of reductoisomerase (RI), an isoleucine-valine biosynthetic enzyme in *Neurospora* (17), was also followed in this experiment. Table I shows that approximately 51% of the recovered RI activity is present in the soluble S_1 fraction where little or no KH and SCCR activities are recovered. Significant RI activity, however, is also recovered in the heavy B_2 fraction, where no KH is recovered. It is apparent from Table I that the recovery pattern of RI is not like that of KH or SCCR.

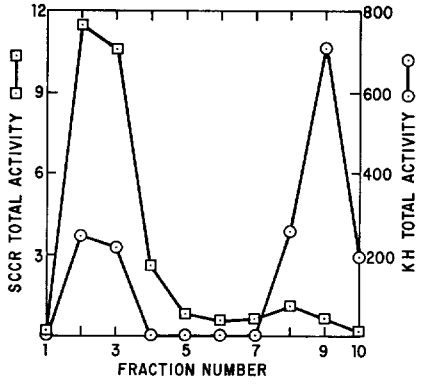

FIGURE 3 Distribution of total activity of succinate-cytochrome *c* reductase (SCCR, micromoles cytochrome *c* reduced per minute) and kynurenine hydroxylase (KH, millimicromoles 3-hydroxykynurenine produced per hour) in a linear sucrose density gradient (0.58–1.9 M sucrose) after treatment of mitochondria with the modified osmotic sonication method and centrifugation for 1 hr at 167,000 *g* in the SW-39 rotor of a Spinco Model L ultracentrifuge. The brake was not used. Fractions are numbered from the bottom. Sonication was in 0.45 M sucrose for 15 sec at 3 amp; the protein concentration of sonicated material was 14.6 mg/ml, and protein loaded onto gradients was 43.8 mg. Recovery from gradients was protein, 102%; SCCR, 62.3%, and KH, 66%.

TABLE I

Fractionation of Mitochondria By Modified Osmotic Sonication Method

Fraction	Protein	KH		SCCR		RI	
	mg/fraction	*Specific* activity*	*Units/fraction*	*Specific‡ activity*	*Units/fraction*	*Specific§ activity*	*Units/fraction*
S_1	6.84	19.4	133	0		0.809	5.53
B_1	14.0	156	2184	0.368	5.15	0.225	3.15
S_2	5.58	10.8	60	0.358	2.00	0.032	0.18
B_2	19.8	0		0.548	10.8	0.104	2.06
S_3	0.43	0		trace		0	
Recovery	46.6		2377		18.0		10.9
Control	46.6	89.3	4161	0.671	31.3	0.201	9.37
% Recovery	100	57.1		57.5		116.3	

* Millimicromoles of 3-hydroxykynurenine produced per hour per milligram.

‡ Micromoles cytochrome *c* reduced per minute per milligram.

§ Micromoles NADPH oxidized per minute per milligram.

Recovery of kynurenine hydroxylase (KH), succinate-cytochrome *c* reductase (SCCR), reductoisomerase (RI), and protein after treatment of mitochondria with the modified osmotic sonication method and subsequent centrifugation in a three-layer discontinuous sucrose gradient. Sonication was at 3 amp for 30 sec. The protein concentration of the sonicate was 7.76 mg/ml. Fractions were collected as shown in Fig. 1.

DISCUSSION

The results presented above establish the mitochondrial localization of kynurenine hydroxylase in *Neurospora crassa* and indicate that this enzyme is probably uniquely localized in the mitochondria. This finding extends the similar observation, made by Okamato et al. (18) in rat liver mitochondria, to a different organism. The biochemical evidence and electron microscope evidence indicate that the outer mitochondrial membrane is removed by the modified osmotic sonication treatment and is recovered in the light B_1 fraction on the discontinuous gradient, and that KH is localized in the outer membrane. Additionally, we find that the SCCR is localized in the inner mitochondrial membrane which is recovered in the heavy B_2 fraction. These localizations agree with those found previously in rat liver mitochondria for KH (18, 19, 16) and SCCR (9, 16), and have been further verified by treating *Neurospora* mitochondria with digitonin and sodium deoxycholate (20, 21).

In Table I, it is observed that reductoisomerase has a different recovery pattern than either the KH or the SCCR. The distinct recovery pattern of RI indicates that its localization and compartmentation within the mitochondria is different from that of either KH or SCCR. We suggest that RI may be localized in the mitochondrial matrix. This localization follows from the data which indicate that substantial RI activity is recovered both in the soluble S_1 fraction and in the heavy B_2 fraction. A similar intermediate recovery pattern and localization has been described for the malate dehydrogenase of rat liver mitochondria (22, 23). More recent data show that in *Neurospora* the malate dehydrogenase and dihydroxyacid dehydratase, another isoleucine-valine biosynthetic enzyme (24), are also localized in the mitochondrial matrix (20, 21).

SUMMARY

A procedure is described for the separation of the inner and outer membrane fractions of the mitochondria of *Neurospora crassa*. Kynurenine hydroxylase activity was found almost exclusively in the fraction believed, on the basis of electron microscope examination, to be the outer membrane. Results show that succinate-cytochrome *c* reductase is localized in the inner membrane. Acetohydroxy acid reductoisomerase, an enzyme in the isoleucine-valine biosynthetic pathway, may be localized in the mitochondrial matrix.

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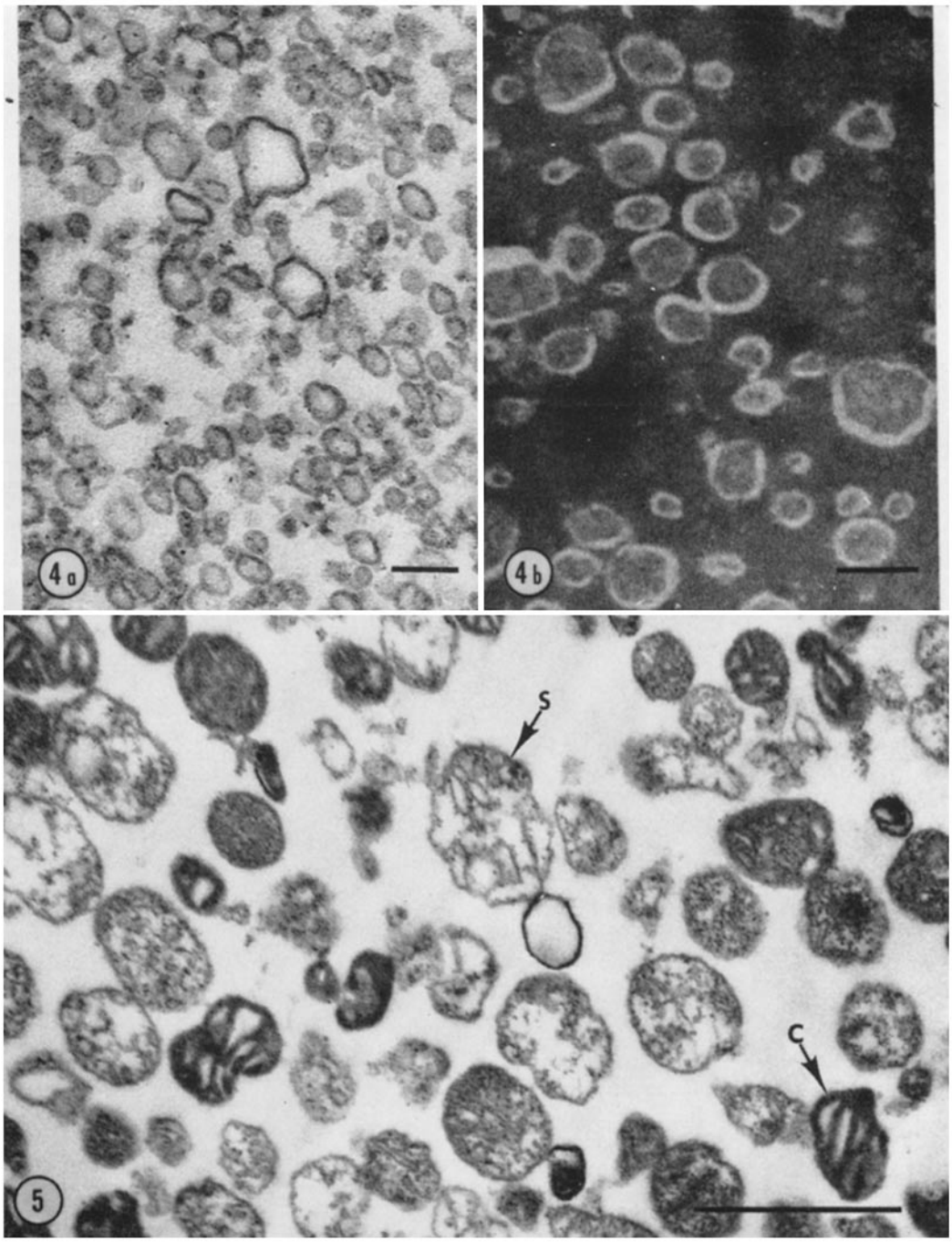

FIGURE 4 (*a*) Vesicles present in the B_1 fraction after a high-speed centrifugation, fixed with glutaraldehyde and osmium tetroxide, and stained with uranyl acetate. The bar represents 0.1 μ. × 96,000. (*b*) Vesicles present in the B_1 fraction are seen negatively stained with 2% phosphotungstic acid. The bar represents 0.1 μ. × 120,000.

FIGURE 5 Large vesicles observed in the B_2 fraction after a low-speed centrifugation. The vesicles appear to be present in two principal conformations: swollen (*s*) and contracted (*c*). Note the absence of an outer membrane around the vesicles. Fixed with glutaraldehyde and osmium tetroxide, and stained with uranyl acetate and lead citrate. The bar represents 1 μ. × 30,000.

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BIBLIOGRAPHY

1. ASHWELL, M., and T. S. WORK. 1970. *Annu. Rev. Biochem.* **39**:267.
2. CASSADY, W. E., and R. P. WAGNER. 1968. *Genetics.* **60**:168.
3. WAGNER, R. P., and A. BERGQUIST. 1963. *Proc. Nat. Acad. Sci. U. S. A.* **49**:892.
4. KIRITANI, K., S. NARISE, A. BERGQUIST, and R. P. WAGNER. 1965. *Biochim. Biophys. Acta.* **100**:432.
5. HALL, D. O., and J. W. GREENAWALT. 1964. *Biochem. Biophys. Res. Commun.* **17**:565.
6. BERGQUIST, A., D. A. LABRIE, and R. P. WAGNER. 1969. *Arch. Biochem. Biophys.* **134**:401.
7. HALL, D. O., and J. W. GREENAWALT. 1967. *J. Gen. Microbiol.* **48**:419.
8. GREENAWALT, J. W., D. O. HALL, and O. C. WALLIS. 1967. *Methods Enzymol.* **10**:142.
9. SOTTOCASA, G. L., B. KUYLENSTIERNA, L. ERNSTER, and A. BERGSTRAND. 1967. *J. Cell Biol.* **32**:415.
10. LOWRY, O. H., J. H. ROSEBROUGH, A. L. FARR, and R. J. RANDALL. 1951. *J. Biol. Chem.* **193**: 265.
11. TISDALE, H. D. 1967. *Methods Enzymol.* **10**:213.
12. ARMSTRONG, F. B., and R. P. WAGNER. 1961. *J. Biol. Chem.* **236**:2027.
13. GHOSH, D., and H. S. FORREST. 1967. *Genetics.* **55**:423.
14. HAWLEY, E. S., and R. P. WAGNER. 1967. *J. Cell Biol.* **35**:489.
15. REYNOLDS, E. S. 1963. *J. Cell Biol.* **17**:208.
16. SCHNAITMAN, C., and J. W. GREENAWALT. 1968. *J. Cell Biol.* **38**:158.
17. KIRITANI, K., S. NARISE, and R. P. WAGNER. 1966. *J. Biol. Chem.* **241**:2047.
18. OKAMATO, H., S. YAMAMOTO, M. NOZAKI, and O. HAYAISHI. 1967. *Biochem. Biophys. Res. Commun.* **26**:309.
19. BEATTIE, D. S. 1968. *Biochem. Biophys. Res. Commun.* **31**:901.
20. CASSADY, W. E. 1969. Ph.D. Dissertation. The University of Texas at Austin.
21. CASSADY, W. E., and R. P. WAGNER. 1969. *J. Cell Biol.* **43**(2, Pt. 2):18 *a.* (Abstr.)
22. SCHNAITMAN, C., V. G. ERWIN, and J. W. GREENAWALT. 1967. *J. Cell Biol.* **32**:719.
23. BRDICZKA, D., D. PETTE, G. BRUNNER, and F. MILLER. 1968. *Eur. J. Biochem.* **5**: 294.
24. ALTMILLER, D. H., and R. P. WAGNER. 1970. *Arch. Biochem. Biophys.* **13**:160.