

# SEPARATION OF MITOCHONDRIAL MEMBRANES OF *NEUROSPORA CRASSA*

## II. Submitochondrial Localization of the Isoleucine-Valine Biosynthetic Pathway

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### ABSTRACT

Separation of *Neurospora* mitochondrial outer membranes from the inner membrane/matrix fraction was effected by digitonin treatment and discontinuous density gradient centrifugation. The solubilization of four isoleucine-valine biosynthetic enzymes was studied as a function of digitonin concentration and time of incubation in the detergent. The kinetics of the appearance of valine biosynthetic function in fractions outside of the inner membrane/matrix fraction, coupled with enzyme solubilization patterns similar to that for the matrix marker, mitochondrial malate dehydrogenase, indicate that the four isoleucine-valine pathway enzymes are localized in the mitochondrial matrix.

### INTRODUCTION

The submitochondrial localization of enzymes involved in oxidative phosphorylation (1), carbohydrate metabolism (2), protein metabolism (3), lipid metabolism (4), heme synthesis (5), and nucleic acid metabolism (6) has been studied over the past few years by a number of workers. The major portion of this work has been done with mammalian mitochondria, employing different methods of mitochondrial disruption, e.g. osmotic shock, digitonin, diethylstilbestrol, and phospholipase. Submitochondrial localization of these enzymes has been dependent upon the development of enzymatic markers for outer membrane (1, 6), matrix (3), and inner membrane (7). Lately, in this laboratory, Cassady and Wagner (8) used an osmotic-sonic method to subfractionate the

mitochondria of *Neurospora crassa* and characterized the enzyme L-kynurenine-3-hydroxylase (KH) as an outer membrane marker, and succinate-cytochrome *c* reductase (SCCR) as an inner membrane marker. In this study, digitonin subfractionation has been used to uncover evidence that a group of four enzymes catalyzing the over-all biosynthesis of two branched chain amino acids, isoleucine and valine, from pyruvate-<sup>14</sup>C in isolated *Neurospora* mitochondria (9), behave as soluble enzymes loosely associated in the mitochondrial matrix. These enzymes are an acetohydroxy acid synthetase (AAS), an acetohydroxy acid reductoisomerase (RI), a dihydroxy acid dehydratase (DH), and a branch chained amino acid amino transferase (AT).

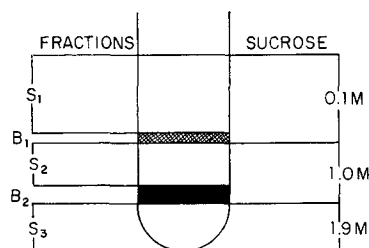


FIGURE 1 Submitochondrial fractionation of digitonin-treated mitochondria on discontinuous sucrose density gradient. Each gradient contained 40 mg of digitonin-treated mitochondrial protein in 0.1 M sucrose. Centrifugation at 39,000 *g* for 1 hr produced a transparent, pale orange layer, designated B<sub>1</sub>, and a heavy brownish-orange membranous band, designated B<sub>2</sub>.

## MATERIALS AND METHODS

### *Purification of Mitochondria*

Mitochondria from wild type *Neurospora crassa* strain LSDT(1969A) were prepared by the sand-ground method previously described (8). The crude mitochondrial fraction was washed once with 0.25 M sucrose, 0.15% bovine serum albumin (BSA), then centrifuged at 37,000 *g* in a Sorvall SS-34 rotor. Washed mitochondria were resuspended in 0.25 M sucrose, and 5 ml samples were placed on top of a discontinuous sucrose gradient composed of 4 ml of a 1.9 M sucrose cushion, followed by 2 ml of 1.0 M sucrose. A final 1.0 ml layer of 0.25 M sucrose was placed on top of the sample. The gradients were then centrifuged in a Spinco SW-41 rotor at 201,000 *g* for 60 min, and the mitochondrial band at the 1.0–1.9 M interface was collected. The mitochondria were diluted with 30 ml of 0.2 M sucrose solution and centrifuged for 12 min in a Spinco 50 rotor at 190,000 *g*. The final mitochondrial pellet was resuspended in 0.25 M sucrose, 0.15% BSA to give an adjusted protein of 40 mg/ml.

### *Digitonin Treatment and Submitochondrial Fractionation*

Digitonin (Calbiochem, Los Angeles, Calif., 2 × recrystallized) at the desired concentration was dissolved by heating in an amount of 0.1 M sucrose which, when mixed 1:1 with the resuspended mitochondria, gave a protein concentration of approximately 20 mg/ml. The suspension was carefully mixed by five strokes of a Teflon pestle, transferred to a beaker, and magnetically stirred at 4°C for the times indicated. At the end of the treatment, the mixture was centrifuged at 37,000 *g* for 60 min, and

the supernatants were carefully removed and assayed for soluble enzymes. Controls (minus digitonin) were processed as above, and were assayed to give control levels of enzyme activity in the intact mitochondrial pellet.

Separation of the submitochondrial fractions after digitonin treatment was accomplished with a discontinuous sucrose gradient as previously described (8), with the modification that 1.0 ml of 1.9 M sucrose was used as a cushion; 1.5 ml of 1.0 M sucrose was layered above this, and a 2.0 ml sample of digitonin-treated mitochondria was layered on top. The gradient fractions observed, after centrifugation, are shown diagrammatically in Fig. 1.

### *Electron Microscopy*

The B<sub>1</sub> and B<sub>2</sub> fractions were fixed in 2% buffered glutaraldehyde, pH 7.2, postfixed in 1% osmium tetroxide for 1 hr, stained in 0.5% uranyl acetate, and prepared for electron microscopy with a Siemens Elmiskop 1 as previously described (8).

### *Enzyme Assays*

AAS was assayed by the method of Kuwana et al. (10). RI was assayed spectrophotometrically in a Cary model 14, using a 1 cm light path cuvette, by the method of Armstrong and Wagner (11). DH was assayed by the method of Altmiller and Wagner (12). Malate dehydrogenase (MDH) was assayed by the method of Ochoa (13). SCCR was assayed by the method of Tisdale (14), using a modification by Cassady and Wagner (8). KH was assayed by the method of Ghosh and Forrest (15). AT was assayed by the method of Coleman and Armstrong (16). Two systems were used in combination for over-all synthesis of valine from pyruvate: (a) the nonrespiring assay of Kiritani et al. (17), and (b) the respiring assay of Bergquist et al. (18). When used in combination, these systems are referred to as the "combined assay." Protein was estimated by the method of Lowry et al. (19). Total enzyme activities are expressed as percentage of total untreated control mitochondrial pellet levels except in the reductoisomerase assays, where a digitonin concentration of 0.025 mg per mg of mitochondrial protein was included in the assay of "control" pellets to remove a latency of the enzyme for its substrate without producing any solubilization of the activity.

## RESULTS

Preliminary studies employing digitonin concentrations in the range used by Schnaitman and Greenawalt (1) to subfractionate rat liver mitochondria into inner and outer membrane components (0.5–2.0 mg/10 mg mitochondrial

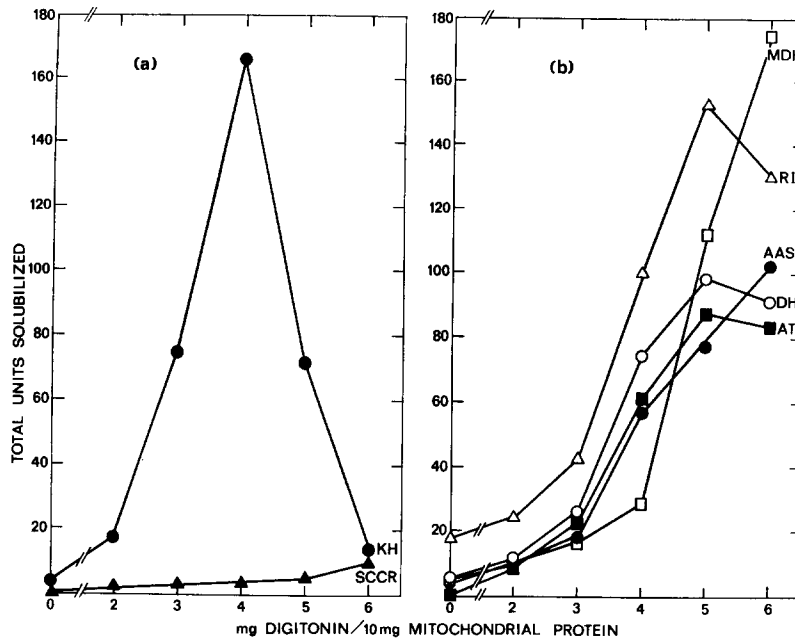


FIGURE 2 Mitochondrial enzymes present in 37,000 *g* supernatant as a function of digitonin concentration. A mitochondrial suspension at 40 mg per ml was treated with the indicated levels of digitonin for 20 min, and the suspension was centrifuged at 37,000 *g* for 60 min. Total enzyme activities were determined in the pellet only on the "0" digitonin control, unless otherwise stated in the text. The levels of activity present in the 37,000 *g* supernatant are expressed as percentages of the control pellet activity. Units of enzyme activity are as follows: Fig. 2 a, succinate cytochrome *c* reductase (SCCR),  $\mu$ moles cytochrome *c* reduced per minute; kynurenine hydroxylase (KH),  $\mu$ moles 3-OH kynurenine produced per hour. Fig. 2 b, acetoxy acid synthetase (AAS),  $\mu$ moles  $\alpha$ -acetolactate produced per hour; reductoisomerase (RI)  $\mu$ moles NADPH oxidized per hour; dihydroxy acid dehydratase (DH),  $\mu$ moles ketoisovalerate produced per hour; branch chain amino acid aminotransferase (AT),  $\mu$ moles ketoisoleucine produced per 10 min; malic dehydrogenase (MDH),  $\mu$ moles NADH oxidized per min.

protein) showed these levels ineffective for sub-fractionation of *Neurospora* mitochondrial membranes. The optimum concentration of digitonin for solubilization of *Neurospora* mitochondria was determined over a range of 2–6 mg/10 mg mitochondrial protein by sedimenting purified mitochondria, previously incubated in digitonin for 20 min, at 37,000 *g* for 60 min, and assaying the supernatant for solubilized enzyme activities. These activities included KH, the outer membrane marker (8), SCCR, the inner membrane marker, MDH, the matrix marker (20), and the four individual enzymes (AAS, RI, DH, and AT) that are necessary for the synthesis of valine from pyruvate. Fig. 2 a reveals that peak solubilization of KH occurred at a concentration of 4 mg digitonin/10 mg mitochondrial protein, while at higher or lower concentrations fewer units of enzyme were solubilized. In contrast, very little SCCR was

solubilized from the inner membrane at the optimal digitonin concentration for KH release, while at higher concentrations significantly more SCCR was released, suggesting partial disruption of the inner membrane/matrix component by the detergent (Fig. 2 a). It can be seen in Fig. 2 b that, as contrasted to the peak release of KH and the relative insolubility of the SCCR, MDH activity was continuously released over a concentration range of 2–4 mg digitonin/10 mg protein, and between 4 and 6 mg/10 mg protein a sharp increase in the levels solubilized was observed. This further suggests that concentrations higher than 4 mg digitonin/10 mg protein solubilize matrix enzymes to an even greater extent than enzymes bound to the inner membrane. The decrease in KH activity observed at high digitonin concentrations (Fig. 2 a) may be due to disruptive interaction of digitonin with components of the outer mitochondrial mem-

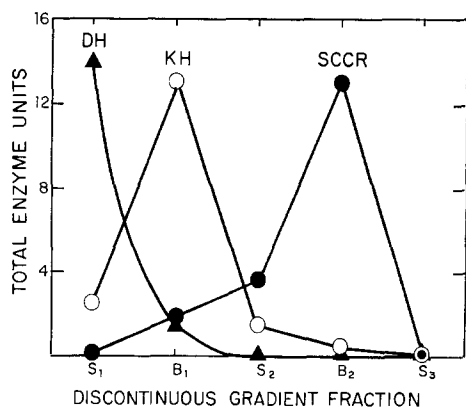


FIGURE 3 Distribution of enzymes after 20 min of incubation in 4 mg digitonin per 10 mg mitochondrial protein. Treated mitochondria were centrifuged at 167,000 *g* for 1 hr in discontinuous sucrose gradients. Total enzyme units in the gradient fractions are expressed as follows: dihydroxy acid dehydratase (DH)  $\mu$ moles ketoisovalerate  $\times 10^1$  produced per hour; kynurenine hydroxylase (KH),  $\mu$ moles 3-OH kynurenine produced per hour; succinate cytochrome *c* reductase (SCCR),  $\mu$ moles cytochrome *c* reduced per hour.

brane, perhaps lipid essential for enzyme activity. Cassady and Wagner (8) have observed that *Neurospora* KH is rather tightly bound to the outer mitochondrial membrane after disruption with sonication. Mayer and Staudinger (23) have shown that KH of rat liver mitochondria has a lipid dependency for activity.

The release of the four mitochondrial enzymes necessary for isoleucine-valine biosynthesis was also studied as a function of digitonin concentration, and as shown in Fig. 2 b, the pattern of release of these enzymes (AAS, RI, DH, and AT) closely resembles that of soluble matrix enzyme MDH, and not that produced by either of the membrane-bound marker enzymes KH or SCCR. The soluble nature of one of the isoleucine-valine (iv) pathway enzymes, the DH, is further illustrated in Fig. 3. Mitochondria were incubated for 20 min in 4 mg digitonin/10 mg protein; the inner membrane marker SCCR had an activity peak sharply concentrated in the heavy membranous B<sub>2</sub> fraction, while the peak activity of the outer membrane marker KH was sharply concentrated in the light membranous B<sub>1</sub> fraction. DH, while distributed throughout the gradient, was predominantly found in the light sucrose S<sub>1</sub> fraction. Using osmotic-sonic disruption, Cassady and Wagner

(8) found a similar gradient distribution for RI. Cassady (20) has also observed a similar gradient distribution for DH and AAS after disruption of mitochondria with deoxycholate. The presence of isoleucine-valine enzymes in the S<sub>1</sub> fraction suggests their release by digitonin from a matrix pool of soluble enzymes, both before and during discontinuous gradient centrifugation. Not only was this release of soluble iv enzymes a function of digitonin concentration, but also, as shown in Table I, of incubation time in the detergent.

Electron microscope examination of the B<sub>1</sub> and B<sub>2</sub> fractions revealed that the pale, transparent orange B<sub>1</sub> layer was comprised predominantly of empty vesicles bounded by single membranes (Fig. 4 a). Similar vesicles were seen in digitonin-treated rat liver mitochondrial preparations by Schnaitman and Greenawalt (1) and interpreted to be outer mitochondrial membrane. The dark brown-orange pigmented B<sub>2</sub> fraction was composed of larger vesicles bounded by a single smooth membrane and frequently containing an electron-opaque matrix (Fig. 4 b). The B<sub>1</sub> and B<sub>2</sub> fractions obtained by digitonin fractionation are ultrastructurally similar to the same fractions obtained by

TABLE I  
Valine Synthesis\* in Discontinuous Gradient Fractions as a Function of Time of Digitonin Treatment

Incubation time in digitonin	Total nmoles valine / 2 hr per fraction		
	S <sub>1</sub>	B <sub>1</sub>	B <sub>2</sub>
<i>min</i>			
5	535	199	1548
10	1073	239	1377
15	2383	353	1809

\* "Combined assay" for valine was prepared in a standard assay mixture containing 0.25 M sucrose, 0.15% bovine serum albumin, 20 mM L-phenylalanine, and 3 mM MgCl<sub>2</sub> in 0.1 M Tris at pH 7.8. Pyruvate concentration was 5  $\mu$ moles/ml assay medium. "Cofactor assay" components added were 0.25 mM nicotinamide adenine dinucleotide phosphate (NADP), 0.3 mM TPP, 0.4 mM pyridoxal-5-phosphate, and 25 mM glucose-6-phosphate. "Respiring assay" components added were 1 mM ADP, 2 mM inorganic phosphate, and 5 mM succinate. Assays were run 2 hr at 37°C. Digitonin treatment was performed as described in Materials and Methods, using 4.8 mg digitonin/10 mg mitochondrial protein.

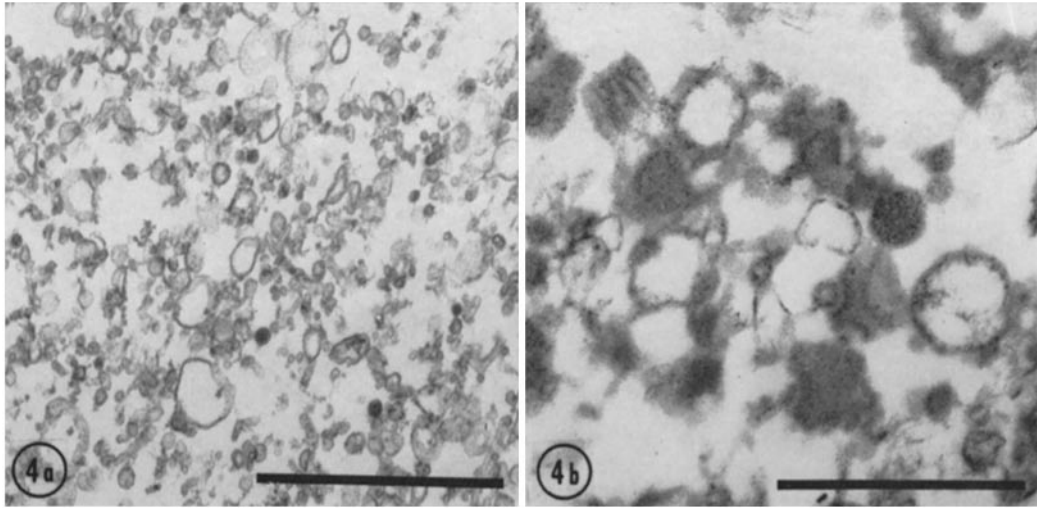


FIGURE 4 Appearance of  $B_1$  fraction (Fig. 4 a) and  $B_2$  fraction (Fig. 4 b) after 5 min digitonin treatment (4.8 mg digitonin/10 mg mitochondrial protein). The  $B_2$  fraction contains large, single membrane-bounded vesicles, but no intact mitochondria. Electron-opaque material is seen inside the membranes. The  $B_1$  vesicles are at least three to four times smaller than the  $B_2$  vesicles and exhibit no interior ultrastructure. The length of the bar represents  $1 \mu. \times 33,000$ .

the osmotic-sonic treatment reported previously (8).

The difficulty in assigning a specific submitochondrial localization for the iv enzymes was that these enzymes, as well as valine biosynthetic activity, were never found discretely localized in any one fraction, but were spread through the discontinuous gradients between the  $S_1$  and  $B_2$  regions, making it difficult to eliminate the possibility of a ubiquitous distribution. However, it was possible to rule out this consideration since it could be demonstrated not only that the digitonin-mediated solubilization of iv enzymes was a function of detergent concentration, but also that the appearance of valine biosynthetic activity into fractions above the  $B_2$  (including the four enzymes whose activities are required for valine synthesis) was a function of the time of incubation in digitonin before centrifugation. As shown in Table I, incubation in digitonin for 5, 10, and 15 min before centrifugation produced a marked increase over time of valine synthetic activity in the  $B_1$  fraction, and more particularly the  $S_1$  fraction, where a greater than 300% increase over the 5-min valine level was observed at 15 min. The levels of valine synthesis in the  $B_2$  fraction, on the contrary, either remained relatively stable, as seen in Table I, or, in some experiments, dropped as much

as 43% in 15 min. In this experiment (Table I), it is observed that the total specific activity of valine synthesis increases with increasing incubation time in digitonin. This is probably due to the increasing degradation of inner membrane/matrix compartmentation by digitonin, resulting both in the release of latent activity which is not assayable when compartmentation is intact, and in the elimination of permeability or other factors which might normally limit the amount of pyruvate or thiamine pyrophosphate (TPP) cofactor accessible to the AAS.

It is not surprising that the levels of valine synthesis in the  $B_2$  fraction relative to the other fractions remained high over time, even as considerable units of the enzymes were leached out of the matrix and into the upper fractions of the gradients, since one of the two components of the "combined assay" for valine synthesis, the "respiring assay," contains adenosine diphosphate (ADP), inorganic phosphorus, and succinate. These respiratory cofactors have been observed to stimulate valine synthesis from pyruvate at high specific activity only in intact *Neurospora* mitochondria capable of respiration coupled with oxidative phosphorylation (9) and the inner membrane/matrix mitochondrial subfraction containing the Krebs cycle and electron-transport

chain enzymes (unpublished observation). Presumably, actively respiring, coupled mitochondria are capable of generating endogenous pools of reduced cofactors, such as NADPH, which are supplied exogenously in the "cofactor assay" component of the "combined assay." With increased incubation time in digitonin, more inner membrane/matrix surface would become exposed, thus permitting greater availability of the respiratory substrates and, consequently, greater efficiency of valine synthesis, despite the loss of enzyme units into the gradients. Accordingly, under these experimental conditions, the B<sub>2</sub> fraction, consisting of inner membrane/matrix, exhibited continuously high levels of valine production since only this fraction is capable of utilizing both component assay systems of the "combined assay" (20).

#### DISCUSSION

It has been previously speculated (21) that the four enzymes in the biosynthetic pathway from pyruvate may reside inside the mitochondrion as an organized multienzyme complex. While our data neither completely confirm nor negate this speculation, the demonstration *in vitro* that the individual activities of each of the enzymes, when solubilized into the S<sub>1</sub>, or light sucrose fraction of a discontinuous gradient, retain the ability to catalyze valine synthesis from pyruvate, emphasizes that a loose association of the four enzymes is sufficient for valine synthesis. The similar pattern of release exhibited by each of the four enzymes (Fig. 4) indicates that no one enzyme was any more tightly bound to a membrane component than any of the others, and, further, the presence of high levels of enzyme units at the top of the gradient (0.1 M sucrose) would argue against a tendency of the enzymes to associate into a tight aggregate such as the one reported by Burgoyne et al. (22) formed by five aromatic amino acid biosynthetic enzymes in *Neurospora*.

The successful removal of the outer membrane of the mitochondrion with a minimum of contamination with other components is a prerequisite for enzyme localization studies. While several methods are available to achieve this, digitonin is a particularly useful tool in that, in addition to being able to effectively separate the outer from the inner membrane by varying the concentration and time of exposure of the detergent, one can determine the kinetics of soluble enzyme loss from the mitochondrial matrix. A clear optimum of digitonin

concentration for maximum removal of outer membrane exists, below which little matrix enzyme activity is released, and above which partial solubilization of the inner membrane and its associated membrane-bound enzymes occurs, with concomitant loss of matrix material. Schnaitman and Greenawalt (1) have reported similar findings. Because of the stringent limitations imposed by the factors of concentration and time on the digitonin technique, it is impossible to state with certainty that a given enzyme is matrix-localized, but on the basis of the kinetics of appearance of pathway function of the isoleucine-valine biosynthetic enzymes outside the inner membrane/matrix fraction, coupled with enzyme solubility properties similar to mitochondrial malate dehydrogenase, we can state with some confidence that all four enzymes are localized in the matrix.

We wish to thank Mrs. Esther Eakin for her excellent technical assistance, and Mr. Robert Riess for taking the electron micrographs. Dr. Marvin Collins kindly performed the transaminase assays.

This work was supported by grants GM 12323 and 2T01 GM 00337 from the National Institutes of Health United States Public Health Service, and a grant from the Robert A. Welch Foundation, Houston, Texas.

Received for publication 13 May 1971, and in revised form 15 December 1971.

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