

Kinetic Studies on the Transport of Cytoplasmically Synthesized Proteins into the Mitochondria in Intact Cells of *Neurospora crassa*

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The transport of cytoplasmically synthesized mitochondrial proteins was investigated in whole cells of *Neurospora crassa*, using dual labelling and immunological techniques.

In pulse and pulse-chase labelling experiments the mitochondrial proteins accumulate label. The appearance of label in mitochondrial protein shows a lag relative to total cellular protein, ribosomal, microsomal and cytosolic proteins.

The delayed appearance of label was also found in immunoprecipitated mitochondrial matrix proteins, mitochondrial ribosomal proteins, mitochondrial carboxyatractyloside-binding protein and cytochrome *c*. Individual mitochondrial proteins exhibit different labelling kinetics.

Cycloheximide inhibition of translation does not prevent import of proteins into the mitochondria.

Mitochondrial matrix proteins labelled in pulse and pulse-chase experiments can first be detected in the cytosol fraction and subsequently in the mitochondria. The cytosol matrix proteins and those in the mitochondria show a precursor-product type relationship.

The results suggest that newly synthesized mitochondrial proteins exist in an extra-mitochondrial pool from which they are imported into the mitochondria.

The majority of mitochondrial proteins are coded for by nuclear genes and are synthesized on cytoplasmic ribosomes. It has been estimated that some 85–95% of the total mitochondrial protein originates from cytoplasmic protein synthesis [1]. These proteins must subsequently be transported to their functional locations in the mitochondrion. A similar situation exists in the formation of chloroplasts in plant cells where the majority of the chloroplast proteins originate in the cytoplasm [2].

Despite the obvious importance of the transport process little is known about the selectivity which allows only mitochondrial proteins access to mitochondria and about the transport mechanism. In recent years a mechanism was proposed which integrates synthesis and transport into a simple unified mechanism [3–6]. According to this attractive hypothesis, mitochondrial proteins are translated by cytoplasmic ribosomes which are bound to the outer membrane of the mitochondria. Nascent polypeptides are discharged in a vectorial manner into the matrix, or membranes, in a manner analogous to the mechanism proposed for protein transport in exocrine cells [7]. The evidence for the general existence of such a mechanism is equivocal and many questions in relation

to how such a system could function remain unanswered.

We have studied the kinetics of synthesis of mitochondrial proteins and their subsequent appearance in the mitochondria in intact cells of *Neurospora crassa*. The results of our studies suggest that extra-mitochondrial pools of mitochondrial proteins exist. It is furthermore shown that newly synthesized mitochondrial proteins can be detected outside the mitochondrial fraction. Our data are not readily compatible with a mechanism in which synthesis and transport are functionally linked. The existence of cytoplasmic ribosomes bound to outer membranes of mitochondria could not be demonstrated in *Neurospora crassa*.

MATERIALS AND METHODS

Growth of Neurospora Cells

Hyphae of *Neurospora crassa* (wild type 74A) were grown in Vogel's minimal medium, supplemented with 2% sucrose, at 25 °C for 14 h as described [8]

with the following modification. The magnesium sulfate concentration was reduced to 0.08 mM. Sodium [³⁵S]sulfate (New England Nuclear Co., Boston, Mass.) (specific radioactivity 10–1000 Ci/mol) was added at the time of inoculation at a concentration of 250 μCi/l. The inoculum was 2×10^6 conida/ml. After 14 h growth, magnesium sulfate was added to the cultures at a concentration of 1 mM. After further growth for 1 h the cultures were cooled to 8 °C and maintained at this temperature for 2 h.

Pulse and Pulse-Chase Labelling of Cells

For pulse labelling, [³H]leucine (New England Nuclear Co., Boston, Mass.) (specific radioactivity 40–60 Ci/mol) was added at a concentration of 2 mCi/l. Samples of 200 ml were withdrawn at the intervals indicated in the different experiments and mixed with two volumes of iced water. The cells were immediately harvested by filtration using a specially constructed funnel to maintain the temperature at 0 °C. For a chase, unlabelled leucine was added to the cultures at a final concentration of 10 mM.

Fractionation of Cells

The filtered cells (0.5–1 g wet weight) were resuspended in 5 volumes of medium A (0.5 M sucrose, 5 mM NH₄Cl, 1 mM mercaptoethanol, 30 mM Tris-HCl, pH 7.4) with an Ultraturrax homogenizer for 6 s. The cells were broken in a specially designed small-scale grind-mill [9]. The time elapsed between filtration of the cells and collection of the ground suspension from the mill was kept as short as possible and usually did not exceed 2–3 min. Care was taken that the temperature during the whole procedure did not exceed 0 °C.

The ground suspension was centrifuged in a Sorvall RC5 refrigerated centrifuge twice for 5 min at $2000 \times g$ (max.). The pellet comprising cell walls and unbroken cells was discarded. The supernatant was taken as homogenate. It was centrifuged for 12 min at $17300 \times g$ (max.) to obtain the mitochondrial pellet. Mitochondria were washed by resuspending them in 1 ml medium B (0.44 M sucrose, 1 mM EDTA, 30 mM Tris-HCl, pH 7.6) and resedimenting then for 12 min at $17300 \times g$ (max.). This procedure was repeated once.

The postmitochondrial supernatant was centrifuged for 30 min at $48000 \times g$ (max.) and the resulting pellet was taken as the microsomal fraction. It was washed once with medium A by resuspension and resedimentation for 30 min at $48000 \times g$ (max.).

The postmicrosomal supernatant was centrifuged for 2 h at $165000 \times g$ (max.) in a Beckman Spinco centrifuge. The pellet contained the cytoplasmic ribo-

somes. The supernatant was taken as the cytosol fraction.

Isolation of Mitochondrial Proteins

Carboxyatractyloside-Binding Protein. This protein was isolated according to the procedure of Klingenberg *et al.* [10]. The purified protein displayed a single band after dodecylsulfate–polyacrylamide gel electrophoresis with an apparent molecular weight of 32000.

Cytochrome c. The procedure of Heller and Smith [11] was used with several modifications. 4 kg of frozen hyphae were homogenized with distilled water in a grind mill. Cell walls and unbroken cells were removed by low spin centrifugation. The homogenate was brought to pH 10 with conc. ammonia and stirred for 30 min at room temperature. The pH was adjusted to 8.5 with glacial acetic acid and the homogenate was centrifuged for 1 h at $14700 \times g$ (max.). The supernatant was diluted to 75 l with distilled water. 30 g of Biorex 70, 400 mesh (Biorad Laboratories, Richmond, Calif.) were added and the mixture was stirred overnight at 4 °C. Then the resin was collected by filtration and the extraction procedure was repeated once. The following steps were carried out according to Heller and Smith. 40–50 mg of cytochrome *c* were obtained with a ratio reduced/oxidised (A_{550}/A_{280}) of 1.25–1.40, indicating purity of the preparation. Dodecylsulfate gel electrophoresis displayed one single band with an apparent molecular weight of 12000.

Mitochondrial Matrix Proteins

Mitochondria were prepared from cells which were homogenized with medium B. The mitochondrial fraction was washed three times with the same medium. The final mitochondrial pellet was resuspended in 0.1 M sodium phosphate buffer, pH 7.0 at a protein concentration of 10 mg/ml and sonified 10 times 30 s with a Branson sonifier (model S 75) at step 5. The temperature was kept below 7 °C. Membranes were removed by centrifugation for 2 h at $165000 \times g$ (max.) in a Beckman Spinco centrifuge. The upper two-thirds of the supernatant were used as matrix protein fraction.

Ribosomal Proteins. Mitochondrial ribosomes were prepared according to procedures published previously [12]. The purified ribosomes were employed for raising antibodies against mitochondrial ribosomal proteins [13].

Preparation of Antibodies

Antigen preparations were emulsified with equal volumes of Freund's complete adjuvant (Difco, Detroit, Mi.). The following amounts of protein were

injected. Carboxyatractyloside-binding protein: 1–3 mg in 0.5 ml of 0.5% Triton X-100, 50 mM NaCl, 10 mM morpholinopropanesulfonic acid, pH 7.2. Cytochrome *c*: 2–3 mg in 0.5 ml distilled water. Matrix proteins: 5 mg in 1 ml 0.1 M sodium phosphate buffer, pH 7.2. Mitochondrial ribosomes: 2–3 mg protein in 0.5 ml 0.1 M NH_4Cl , 10 mM MgCl_2 , 30 mM Tris-HCl, pH 7.2.

These amounts of antigens were injected four times at intervals of 8–10 days into the neck region of rabbits. Eight to ten days after the last injection blood was drawn from the ear vein and the serum collected after coagulation of the blood.

Immunoglobulin fractions were prepared from the serum by ammonium sulfate precipitation. Titres of immunoglobulin preparations were determined by immunoprecipitation of antigens from ^{35}S -labelled mitochondria.

Conditions of Immunoprecipitation

Mitochondria (0.1–0.3 mg protein) were re-suspended in 1 ml 0.3 M KCl, 10 mM Tris-HCl, pH 7.5. Then 50 μl of 20% Triton X-100 (Sigma Chemical Co., St Louis, Mo.) in the same buffer were added. The microsomal fraction was treated in the same way with the exception that a suspension of unlabelled mitochondria in medium A (25 μl containing 0.1 mg protein) were added. To 0.5 ml of the cytosol fraction 0.5 ml of 0.6 M KCl, 10 mM Tris-HCl, pH 7.5 were added, then unlabelled mitochondria (0.2 mg protein in 50 μl medium A) and 50 μl of 20% Triton X-100 in 0.3 M KCl, 10 mM Tris-HCl, pH 7.5.

The lysates were subjected to a clarifying spin (15 min at 48000 $\times g$ max.) and appropriate amounts of immunoglobulin preparations were added. Samples were kept for 15 h at 4 °C and the precipitates were collected by centrifugation in a microcentrifuge. Pellets were washed twice with 1 ml 1% Triton X-100, 0.3 M KCl, 10 mM Tris-HCl, pH 7.2 and thereafter three times with 10 mM Tris-HCl, pH 7.2. The pellets were dissolved in 40 μl 1% sodium dodecylsulfate, 0.1 M Tris-HCl, 2.5% (v/v) mercaptoethanol for 15 h at 4 °C.

The percentages of total ^{35}S -radioactivity precipitated by the individual antibodies from isolated mitochondria were as follows: carboxyatractyloside-binding protein 5%; cytochrome *c* 0.7–1.0%; matrix proteins 20% and mitochondrial ribosomal proteins 3%.

Polyacrylamide Gel Electrophoresis

Separation of proteins by gel electrophoresis in the presence of dodecylsulfate was carried out on slab gels [14].

Determination of Radioactivity

For the determination of radioactivity in protein of cell fractions aliquots of 20–50 μl were applied to Whatman GF filters presoaked with trichloroacetic acid. The filters were passed three times through 5% trichloroacetic acid, once for 10 min through 5% trichloroacetic acid at 90 °C, twice through ethanol/ether (2/1, v/v) and once through ether. The dried filters were placed in scintillation tubes together with 0.5 ml 1% dodecylsulfate in 10 mM Tris-HCl, pH 7.5 and kept for 1 h at 60 °C. Then 12 ml of scintillation fluid [6 g PermaBlend III (Packard Instruments, Dreieichenhain, Germany), 600 ml toluene and 400 ml methoxyethanol] were added.

Radioactivity in immunoprecipitates was measured by directly adding 10–40 μl of the dodecylsulfate-solubilized samples to 12 ml scintillation mixture.

For determination of radioactivity in gel fractions, gels were sliced into 1-mm sections. The slices were placed in 0.2 ml 1% sodium dodecylsulfate, 10 mM Tris-HCl, pH 7.5 in 1.5 ml polyethylene microtubes (Eppendorf, Hamburg) and shaken for 4 h at 60 °C. Then 1 ml of scintillation mixture (6 g PermaBlend III, 700 ml toluene, 300 ml Triton X-100) was added.

The samples were counted in a Packard Tricarb 2425 scintillation counter with efficiencies of 21% for ^3H and 40% for ^{35}S in samples labelled simultaneously with the two isotopes.

RESULTS

Pulse and Pulse-Chase Kinetics of [^3H]Leucine Incorporation into Mitochondria and Other Cell Fractions

A culture of *Neurospora* cells was grown in the presence of [^{35}S]sulfate to obtain homogeneous labelling of total cellular protein. A chase of unlabelled sulfate was given to ensure that proteins synthesized during the period of the consecutive pulse labelling do not incorporate radioactive sulfur. The culture was adjusted to 8 °C for 2 h. Then [^3H]leucine was added to the culture, which was maintained at 8 °C. Samples were withdrawn after 60, 90, 180, 360, 1440 and 2880 s and immediately brought to 0 °C. Cells were harvested and homogenized in a grind-mill. Cell walls and unbroken cells were removed by centrifugation. The homogenate was then separated into the mitochondrial, microsomal, ribosomal and cytosol fractions. The ^3H and ^{35}S radioactivities in the protein of these fractions were determined. The ^3H radioactivity represents the newly synthesized proteins, while the ^{35}S radioactivity represents the preexisting proteins.

In Fig. 1 the labelling kinetics of the four different fractions are plotted. Each fraction is compared to the total homogenate. Furthermore, uptake of [^3H]-

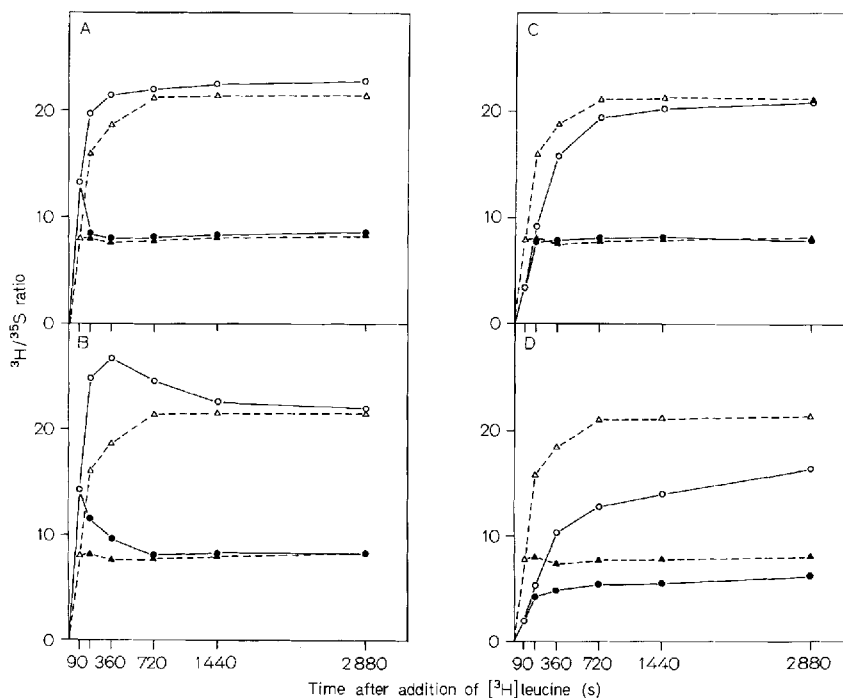


Fig. 1. Pulse and pulse-chase kinetics of incorporation of [^3H]leucine into various cell fractions of *Neurospora*. Cells grown in the presence of [^{35}S]sulfate were labelled with [^3H]leucine at 8 °C. One portion of the culture received a chase of unlabelled leucine at 90 s after addition of [^3H]leucine. At the times indicated, samples were withdrawn and immediately cooled to 0 °C and harvested. Cells were fractionated and ^3H and ^{35}S radioactivities in protein of the fractions were determined. (A) Cytoplasmic ribosomes; (B) microsomes; (C) cytosol; (D) mitochondria. For comparison the $^3\text{H}/^{35}\text{S}$ ratios of the total homogenate are included in each panel (broken lines). Open symbols: pulse; closed symbols: pulse and chase

leucine from the culture medium was measured and it was found that the uptake occurs exponentially with a $t_{1/2}$ of about 60 s.

Both the ribosomes and the microsomes became labelled faster than the homogenate. The microsomes are characterized by a rapid increase in the $^3\text{H}/^{35}\text{S}$ ratio which attains values in excess of the homogenate. The maximum value reached at 360 s is followed by a slow decrease resulting in a final $^3\text{H}/^{35}\text{S}$ ratio equal to that of the homogenate. This labelling pattern shown by the microsomes indicates that newly formed polypeptide chains first appear in this fraction and are then released giving rise to the decline in $^3\text{H}/^{35}\text{S}$.

The rapid increase in the $^3\text{H}/^{35}\text{S}$ ratio shown by both the ribosomes and microsomes can be explained by the fact that the nascent chains on the ribosomes are the primary sites of incorporation. RNA analysis showed that the microsomal fraction contained 0.12 mg RNA/mg protein; approximately 3–5% of the total cellular RNA was found in this fraction. The RNA content of the ribosomes was 0.5–0.6 mg/mg protein representing 88% of the total cellular RNA.

The appearance of label in the cytosol fraction is characterized by a lag period, and shows a distinctly slower rate of increase than the homogenate (Fig. 1C). The mitochondrial fraction shows not only a marked lag relative to the homogenate, but also shows a lag

with respect to the cytosol (Fig. 1D). The first three fractions reach the same $^3\text{H}/^{35}\text{S}$ ratio as the homogenate, the mitochondrial fraction has not attained this ratio even after 2880 s.

An extension of this experiment was the administration of a chase of unlabelled leucine after 90 s labelling with [^3H]leucine. The immediate check it imposes on the incorporation into the homogenate demonstrates the effectiveness of the chase. Moreover, in separate experiments it was also found that incorporation of [^3H]leucine into nascent polypeptide chains on mitochondrial ribosomes was blocked without a detectable lag. In the ribosomal and microsomal fractions, the $^3\text{H}/^{35}\text{S}$ ratios decrease, obviously because of replacement of labelled nascent chains by unlabelled ones. The kinetics of the decrease in the microsomal fraction is slower than in ribosomes which may indicate a complex slow movement of labelled proteins out of this heterogeneous fraction. In the cytosol the chase effects a rapid increase of ^3H radioactivity which reflects the chasing of nascent chains from the ribosomes. Mitochondrial proteins show a similar slow increase of the $^3\text{H}/^{35}\text{S}$ ratio as in the pulse kinetics. In both cases a rapid initial rise is observed, which is followed by the onset of a slow increase after about 720 s. As in the pulse experiment, mitochondrial proteins do not reach the $^3\text{H}/^{35}\text{S}$ ratio of the homo-

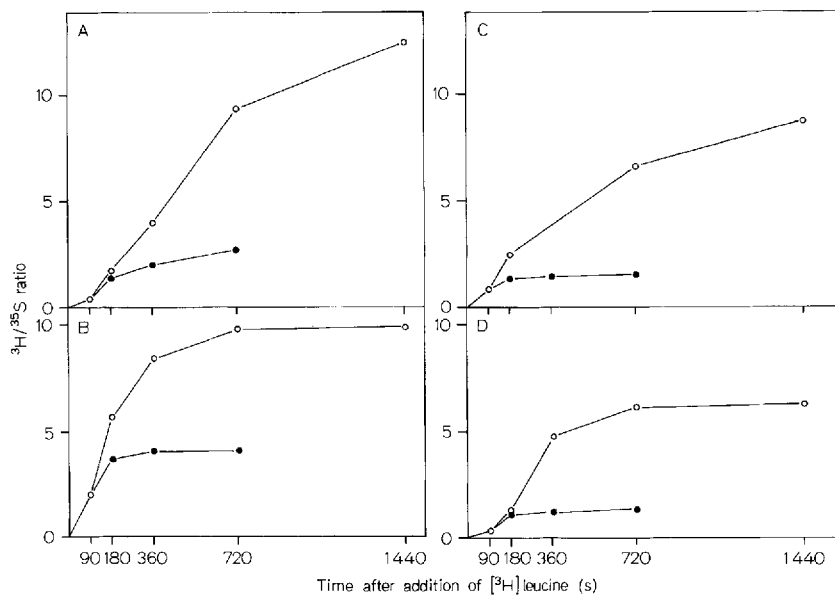


Fig. 2. Pulse and pulse-chase kinetics of incorporation of [^3H]leucine into immunoprecipitable proteins of mitochondria. Cells were labelled as outlined in the legend to Fig. 1. Mitochondria were isolated and solubilized with Triton X-100. The following proteins were precipitated with specific antibodies: (A) mitochondrial matrix proteins; (B) carboxyatractyloside-binding protein; (C) mitochondrial ribosomal proteins; (D) cytochrome *c*. The $^3\text{H}/^{35}\text{S}$ ratios of the immunoprecipitates are plotted versus time of incorporation. Open symbols: pulse; closed symbols: pulse and chase

genate even after 2880 s. It should be noted, however, that in labelling experiments performed at 25 °C, in which the labelling kinetics of the various fractions could not be resolved, the mitochondria reached the same $^3\text{H}/^{35}\text{S}$ ratio both after pulse and chase as the other cell fractions.

These data suggest that the mitochondrial proteins subsequent to their synthesis on cytoplasmic ribosomes are located outside the mitochondria before eventual transport into the mitochondria.

Pulse and Pulse-Chase Kinetics of [^3H]Leucine Incorporation into Individual Mitochondrial Proteins

Cells were labelled as outlined above and mitochondria were isolated. Four different protein fractions were immunoprecipitated from the detergent-solubilized mitochondria with specific antibodies: mitochondrial matrix proteins; the carboxyatractyloside-binding protein; proteins of mitochondrial ribosomes; cytochrome *c*.

The kinetics of [^3H]leucine incorporation can be seen in Fig. 2, where the $^3\text{H}/^{35}\text{S}$ ratios are plotted. The individual proteins show distinct labelling kinetics, however all four proteins show a distinct lag. There is also a distinct post-chase increase in the $^3\text{H}/^{35}\text{S}$ ratio in all four proteins, clearly indicating that migration of protein continues when incorporation of further label into nascent chains is not detectable.

When the immunoprecipitates were analysed by dodecylsulfate gel electrophoresis the matrix proteins

were resolved into a number of fractions (Fig. 3). The $^3\text{H}/^{35}\text{S}$ ratios of these different fractions increased in an individual manner. The different behaviour of the various fractions is shown more clearly in Fig. 4, where the $^3\text{H}/^{35}\text{S}$ ratios of several gel fractions are plotted against time.

Gel electrophoretic analysis of the carboxyatractyloside-binding protein is presented in Fig. 5. A single peak of radioactivity is seen in these profiles. The $^3\text{H}/^{35}\text{S}$ ratios of the total immunoprecipitates do not differ significantly from those of the peak fractions.

In Fig. 6 gel electrophoretic analysis of immunoprecipitated cytochrome *c* is shown. One single band with an apparent molecular weight of 12–13000 was resolved. It should be noted that the antibody was found to cross-react with chemically prepared apocytochrome *c* [15] and that apocytochrome *c* is not resolved from cytochrome *c* by the applied electrophoresis.

The data obtained with antibodies against the various mitochondrial proteins would support the view that extramitochondrial pools of mitochondrial proteins exist and suggest that the pool may vary in size depending on the protein in question.

Effect of Cycloheximide on Pulse-Chase Kinetics of [^3H]Leucine Incorporation into Mitochondrial Proteins

Neurospora cells were pulse labelled with [^3H]leucine as described above. After a 180-s pulse of [^3H]leucine, a chase of unlabelled leucine was added

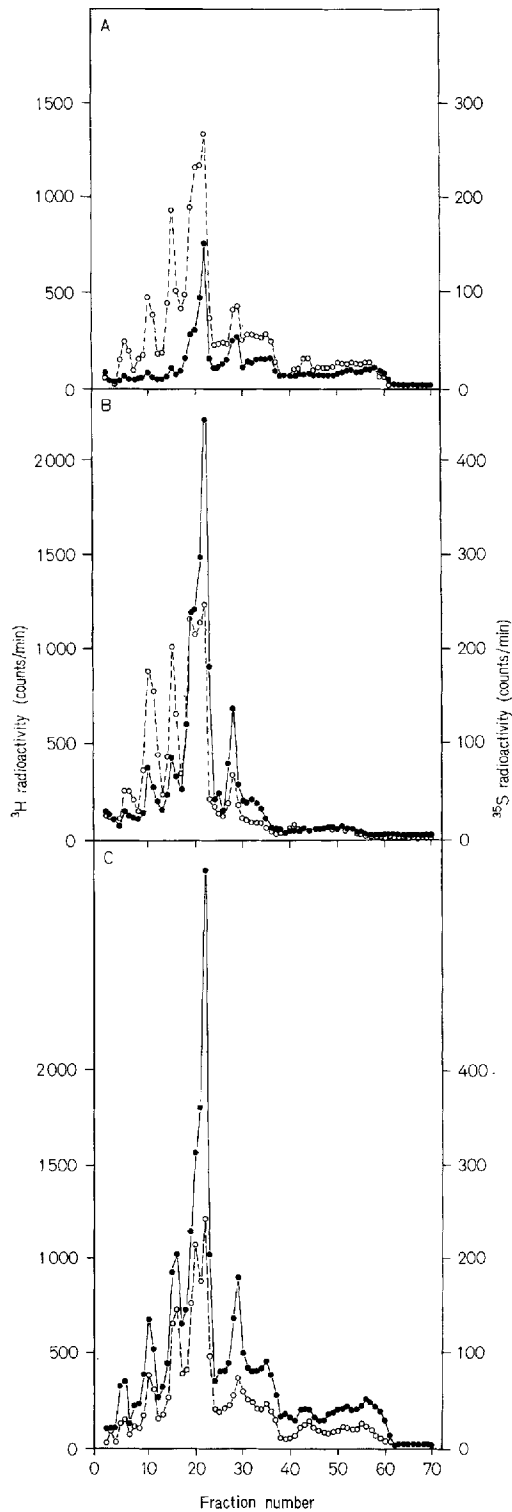


Fig. 3. Dodecylsulfate gel electrophoresis of mitochondrial matrix proteins immunoprecipitated from mitochondria after pulse labelling of cells with [^3H]leucine. Immunoprecipitates were dissolved with dodecylsulfate and subjected to gel electrophoretic separation in the presence of dodecylsulfate. Gels were sliced and the $^3\text{H}/^{35}\text{S}$ ratios determined in the fractions. (A) 180-s pulse; (B) 720-s pulse; (C) 2880-s pulse. (●—●) ^3H radioactivity; (○---○) ^{35}S radioactivity

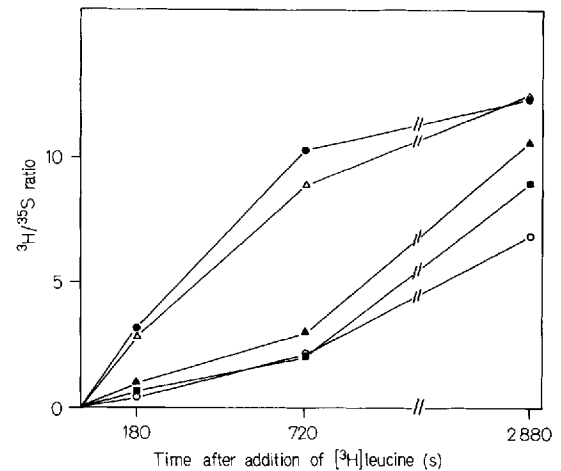


Fig. 4. $^3\text{H}/^{35}\text{S}$ ratios of various gel electrophoretic fractions of immunoprecipitated matrix proteins at different times of pulse labelling. (▲—▲) Fraction 5, (■—■) fraction 9/10, (○—○) fraction 15/16, (Δ—Δ) fraction 21/22, (●—●) fraction 28/29

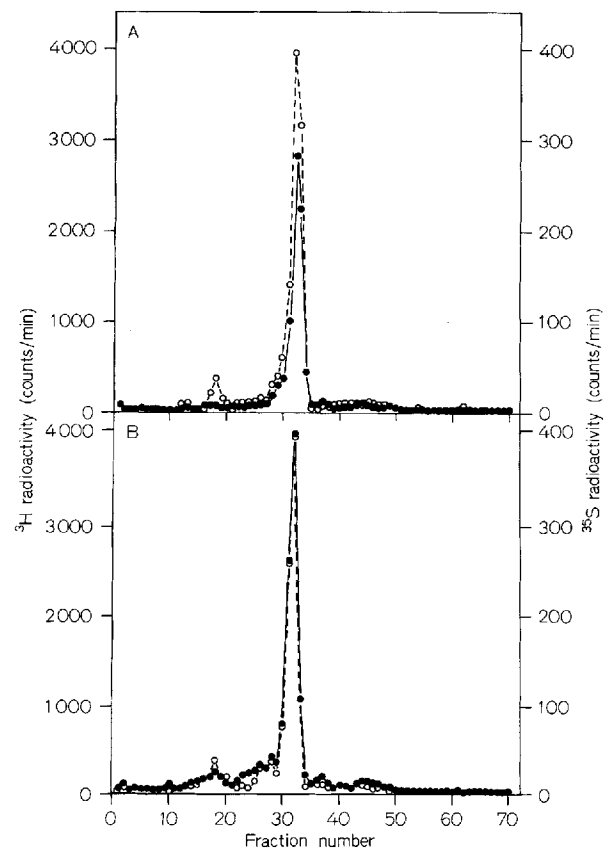


Fig. 5. Dodecylsulfate gel electrophoretic analysis of immunoprecipitated carboxyatractyloside-binding protein from mitochondria. Carboxyatractyloside-binding protein was immunoprecipitated from mitochondria isolated from cells (A) pulse-labelled for 180 s, and (B) pulse-labelled for 180 s and chased for 600 s. After electrophoretic separation, $^3\text{H}/^{35}\text{S}$ ratios were determined in the gel slices. (●—●) ^3H ; (○---○) ^{35}S

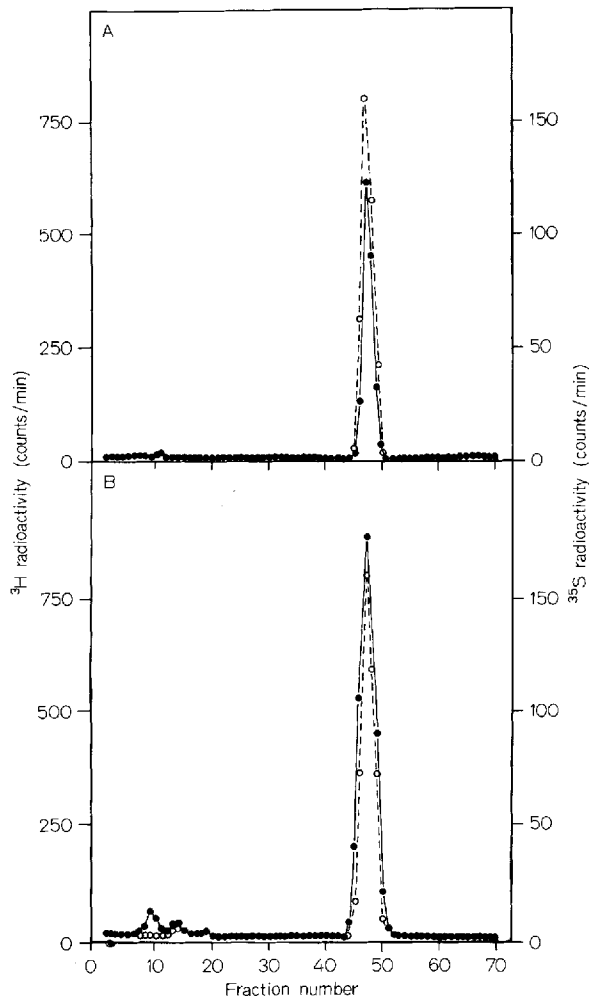


Fig. 6. Dodecylsulfate gel electrophoretic analysis of immunoprecipitated cytochrome *c* from mitochondria. Cytochrome *c* was immunoprecipitated from mitochondria isolated from cells (A) pulse labelled for 180 s, and (B) pulse labelled for 180 s and chased for 600 s. After gel electrophoretic separation, $^3\text{H}/^{35}\text{S}$ ratios were determined in the gel slices. (●—●) ^3H ; (○---○) ^{35}S

together with cycloheximide (0.1 mg/ml). Samples were withdrawn immediately and after further incubation intervals as indicated. The cells were homogenized and fractionated as described above. Fig. 7 demonstrates that incorporation of [^3H]leucine into the homogenate is stopped immediately after the chase plus cycloheximide.

In a separate experiment it was shown that cycloheximide completely blocks cytoplasmic translation within 5–10 s after addition to *Neurospora* cells. The chase was added together with cycloheximide to ensure that mitochondrial ribosomes would not continue to synthesize labelled polypeptides. Chloramphenicol was not found to be effective for blocking mitochondrial translation in intact cells since only partial inhibition could be obtained. The effectiveness of

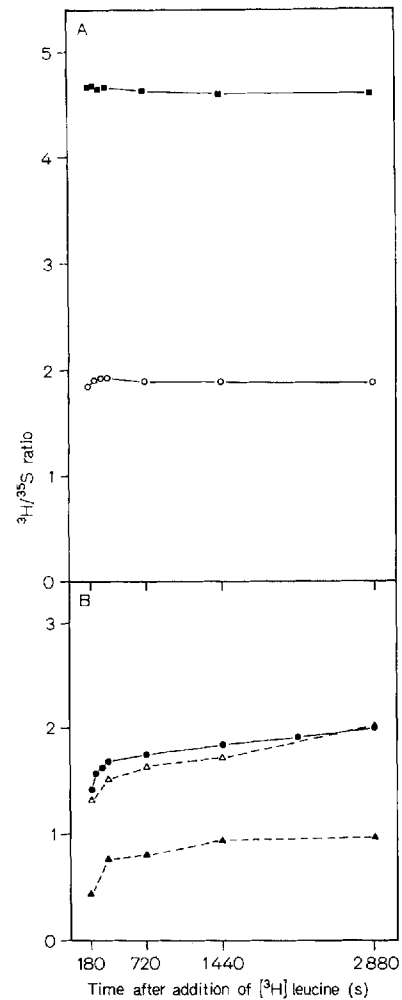


Fig. 7. Kinetics of labelling of various cellular fractions with [^3H]leucine after cycloheximide poisoning of cells. Cells grown in the presence of [^{35}S]sulfate were labelled with [^3H]leucine for 180 s. Then 0.1 mg/ml cycloheximide and a chase of unlabelled leucine were added. Samples were withdrawn at the times indicated and the $^3\text{H}/^{35}\text{S}$ ratios were determined in the total homogenate, in the cytosol and mitochondrial fraction, and in matrix protein and carboxyatractyloside-binding protein immunoprecipitated from the mitochondria. (A) (■—■) Total homogenate; (○—○) cytosol. (B) (●—●) Mitochondria; (△---△) carboxyatractyloside-binding protein; (▲---▲) matrix proteins

cycloheximide can also be seen by the immediate arrest of the labelling of the cytosol fraction (Fig. 7).

It is clearly shown that labelling of mitochondrial protein continues after application of cycloheximide when cytoplasmic translation is shut off.

To confirm that this increase is due to protein import into the mitochondria and not to an incompletely blocked mitochondrial incorporation of [^3H]leucine, matrix proteins and carboxyatractyloside-binding protein were immunoprecipitated from the mitochondria. Both matrix proteins and carboxyatractyloside-binding protein showed an increase in their $^3\text{H}/^{35}\text{S}$ ratio

after cycloheximide poisoning of the cells. The increase is only some 25–35% of that after chase alone. This is explained by the fact that after 180-s pulse labelling a large part of the total [^3H]leucine incorporated is still present in nascent chains on the ribosomes. An elongation rate of the nascent chains at 8 °C of about one amino acid per second can be calculated from the growth rate and the ribosome content. A rough estimate of the distribution of [^3H]leucine among nascent and completed chains suggests a percentage of some 50–60% in completed chains after 180-s pulse. This value would agree with the observation in Fig. 7, that the $^3\text{H}/^{35}\text{S}$ ratio of the cytosol is only some 40% of that of the total homogenate.

The data obtained in these experiments with cycloheximide show that transport of proteins into mitochondria is not dependent on their synthesis. Completed chains in the extramitochondrial pools can still be transported into the mitochondria. The pools appear to be exhausted rapidly when protein synthesis is blocked by cycloheximide. It cannot however be stated that the exhaustion of the extramitochondrial pools is complete.

Estimation of Newly Synthesized Mitochondrial Matrix Proteins in Extramitochondrial Fractions

Neurospora cells were pulse and pulse-chase labelled as outlined for Fig. 1. The mitochondrial, microsomal and cytosolic fractions were prepared at times indicated in Fig. 8 and subjected to immunoprecipitation with antibodies against mitochondrial matrix proteins. ^3H and ^{35}S radioactivities in the immunoprecipitates of each fraction were determined and also the total ^3H radioactivities related to 0.1 g of cells (wet weight). From the ^{35}S radioactivities it was calculated that about 30% of total matrix proteins were recovered in the cytosolic fraction and some 5% in the microsomal fraction. The high amount of matrix proteins leaked out from the mitochondria into the cytosol fraction was not surprising in view of the well-known lability of fungal mitochondria and in view of the rather rough procedures which must be applied to break the cells. For the determination of newly formed matrix proteins outside the mitochondria it was assumed that the matrix proteins which leaked out from the mitochondria had the same $^3\text{H}/^{35}\text{S}$ ratios as those remaining in the mitochondria. As will be discussed later this assumption is not quite true; however it appears justified as a working approximation. The total ^3H radioactivities in the three fractions were corrected for this leak-out and are plotted in Fig. 8.

The mitochondria showed kinetics similar to those already displayed by the $^3\text{H}/^{35}\text{S}$ ratios in Fig. 2. Only a small part of the total ^3H -labelled matrix proteins was found in the microsome fraction. In

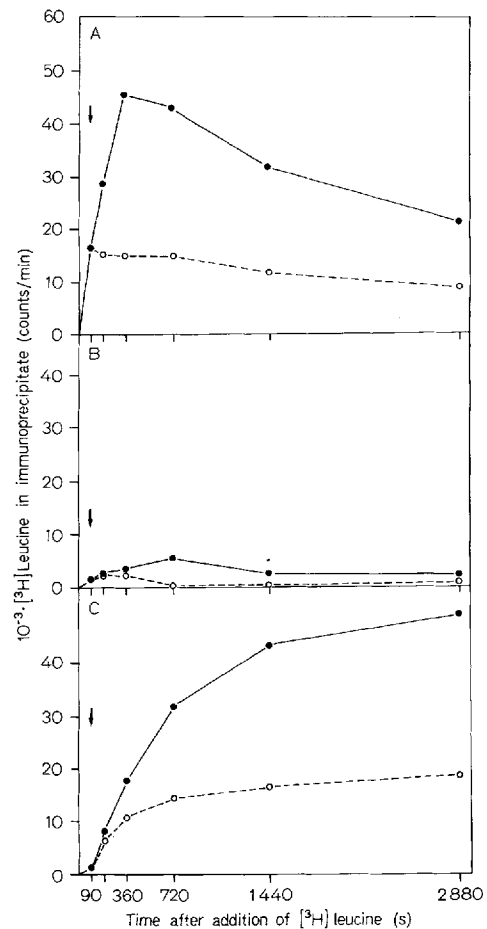


Fig. 8. Incorporation of [^3H]leucine into matrix proteins immunoprecipitated from the cytosol, microsomes and mitochondria after pulse and pulse-chase labelling of *Neurospora* cells. Cells were labelled as outlined in the legend to Fig. 1. Samples were withdrawn at the times indicated and matrix proteins were immunoprecipitated after fractionation of cells. Radioactivities are related to 0.1 g of *Neurospora* cells (wet weight). Cytosol and microsome fractions are corrected for contamination by mitochondrial matrix proteins as outlined in the text. (A) Cytosol; (B) microsome fraction; (C) mitochondria. The chased samples are drawn with broken lines and open symbols

contrast, a high proportion was detected in the cytosol fraction. The time course of appearance of ^3H -labelled matrix proteins in the cytosol fraction represents typical precursor-product type kinetics. Matrix proteins in the cytosol show a high specific ^3H -label at early times, while matrix proteins in the mitochondria are only slightly labelled. The label in the cytosol reaches a maximum at 360 s and then declines, whereas the label in the mitochondria continuously increases. Similar kinetics are observed after the chase.

Matrix proteins immunoprecipitated from the cytosol were analysed by dodecylsulfate gel electrophoresis (see Fig. 9). Two conclusions may be drawn from the radioactivity profiles. Firstly, proteins corresponding to the various gel electrophoretic fractions

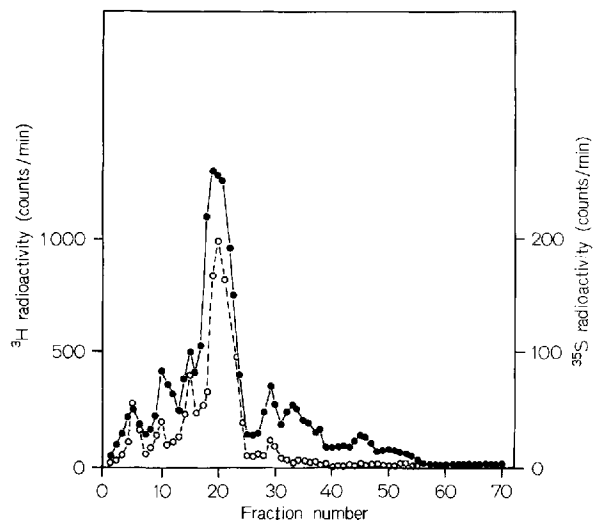


Fig. 9. Dodecylsulfate gel electrophoretic analysis of matrix proteins immunoprecipitated from the cytosol fraction. Cytosol was prepared from cells labelled with [^3H]leucine for 720 s and matrix proteins were immunoprecipitated. After electrophoresis gels were sliced and the $^3\text{H}/^{35}\text{S}$ ratios determined in the fractions. (●—●) ^3H ; (○—○) ^{35}S .

leak out from the mitochondria to different degrees; e.g. fractions 8–16 are found in the cytosol in lower amounts relative to fractions 21/22 (cf. Fig. 3); secondly, the $^3\text{H}/^{35}\text{S}$ ratios of the various fractions from the cytosol are different from those of the mitochondria at early times, e.g. at 720 s in mitochondria the ratio of fraction 9/10 is 2 and of fraction 21/22 is 8–9; in the cytosol fraction 9/10 has a ratio of 13, fraction 21/22 a ratio of 8. This observation is in agreement with that detailed in Fig. 4 that the newly formed proteins corresponding to the electrophoretic fractions 21/22 are more rapidly transported into the mitochondria than those corresponding to fraction 9/10.

These data confirm the existence of extramitochondrial pools of mitochondrial proteins. They furthermore suggest that these pools are different in size for different mitochondrial proteins. Finally, they indicate that the extramitochondrial pools are located in the cytosol, at least in the case of the matrix proteins. This finding however must be interpreted with care, since the fractionation of cells may result in redistribution of proteins.

DISCUSSION

The mechanism of protein transport into mitochondria may turn out to be quite complex. In discussing protein transport three categories of protein must be recognized, *viz.* proteins of the mitochondrial matrix which are translocated across two membranes (inner and outer); proteins of the inner membrane which have to pass the outer membrane and eventually become integrated into the membrane as tightly bound

membrane proteins or as loosely attached proteins; and proteins of the outer membrane which need not be translocated across membranes.

It is not immediately apparent whether a single mechanism is involved or whether each of these different types of protein have different modes of transport. Conscious of the lack of information on the nature of the underlying transport mechanism or mechanisms we here attempt to evaluate possible transport mechanisms.

In recent years Butow and his colleagues [3–6] have proposed that the synthesis and transport of the mitochondrial proteins are functionally coupled. Their proposal is that mitochondrial proteins are synthesized on polysomes which are bound to the mitochondria. The mitochondrial proteins are inserted *in situ* in a manner analogous to the vectorial transport of proteins into the lumen of the endoplasmic reticulum. An alternative mechanism which we have proposed is that mitochondrial proteins exist in the cytoplasm prior to their import into the mitochondria [16,17].

In trying to assess the validity of these suggested mechanisms the following criteria may be employed.

According to the proposal of Butow *et al.* mitochondrial proteins should be labelled with similar kinetics to cytosolic proteins. According to our proposal the separation in time between synthesis and transport would impose a lag in the labelling of mitochondrial proteins in the mitochondria relative to the labelling of proteins in the cytosol.

In the direct insertion hypothesis all mitochondrial proteins would have very similar kinetics of appearance in the mitochondria, whereas our alternative would allow differences in the labelling kinetics of individual mitochondrial proteins depending on the size of the extramitochondrial pool.

As cycloheximide inhibition of protein synthesis is almost immediate a corresponding inhibition of transport would be expected, when synthesis and transport are functionally linked. An immediate inhibition of protein synthesis need not necessarily be accompanied by an immediate halt in the import of proteins into the mitochondria, when completed mitochondrial proteins exist in extramitochondrial pools. Labelling of proteins in mitochondria could continue until the pools of mitochondrial proteins were depleted.

In the case of coupled synthesis and transport newly synthesized mitochondrial proteins would not be expected to be preferentially located outside the mitochondria. In the case of the existence of extramitochondrial pools newly synthesized proteins passing through a precursor type pool would be initially most abundant in extramitochondrial fractions.

In applying these criteria to the experimental data a note of caution however must be sounded, as certain

experimental difficulties may well obscure the real picture.

It cannot be excluded *a priori* that newly synthesized mitochondrial proteins pass through a special mitochondrial pool from which they could leak out more readily to preexisting proteins in the course of fractionation. There is, however, no evidence in favour of such a pool. Data obtained in studies *in vitro* would argue against the existence of such a pool [18, 19].

The possibility exists that newly imported proteins in the mitochondria have a different conformation to that of the functional protein such that antibodies against the isolated proteins do not recognize them. Experience with the detection of nascent polypeptide chains and of precursors of secretory proteins by immunological techniques would not support such a possibility [7, 20].

The mechanism as suggested by Butow and that which we propose could conceivably both operate for different groups of proteins. On the other hand it would seem extravagant to have highly diversified mechanisms for the transport of proteins which are similar in structure and functional location within the mitochondrion.

Despite these reservations the results we have presented strongly support the existence of extramitochondrial pools of proteins and do not support the existence of a secretory model as proposed by Butow. Furthermore isolation of mitochondria in the presence of 5 mM magnesium ions and cycloheximide failed to reveal attachment of ribosomes to the outer mitochondrial membranes although contamination by vesicles studded with cytoplasmic ribosomes was observed.

The location of the pools of newly synthesized mitochondrial proteins in the cell remains to be defined concisely. On the basis of the fractionation studies newly synthesized matrix proteins would appear to be preferentially located in the cytosol. However, redistribution artifacts cannot be ruled out and it is conceivable that the cytosol location of the matrix proteins originated by leakage from compartments such as endoplasmic reticulum vesicles or the space between the inner and outer mitochondrial membranes.

On the other hand, the results of a study on synthesis and transport of mitochondrial proteins in *Neurospora crassa in vitro* clearly support the view that the cytosol may represent the site of an extramitochondrial precursor pool [18, 19].

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