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Transport of Proteins into Mitochondria

Posttranslational Transfer of ADP/ATP Carrier into Mitochondria *in vitro*

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The mitochondrial ADP/ATP carrier is an integral transmembrane protein of the inner membrane. It is synthesized on cytoplasmic ribosomes. Kinetic data suggested that this protein is transferred into mitochondria in a posttranslational manner. The following results provide further evidence for such a mechanism and provide information on its details.

1. In homologous and heterologous translation systems the newly synthesized ADP/ATP carrier protein is present in the postribosomal supernatant.

2. Analysis by density gradient centrifugation and gel filtration shows, that the ADP/ATP carrier molecules in the postribosomal fraction are present as soluble complexes with apparent molecular weights of about 120000 and 500000 or larger. The carrier binds detergents such as Triton X-100 and deoxycholate forming mixed micelles with molecular weights of about 200000–400000.

3. Incubation of a postribosomal supernatant of a reticulocyte lysate containing newly synthesized ADP/ATP carrier with mitochondria isolated from *Neurospora* spheroplasts results in efficient transfer of the carrier into mitochondria. About 20–30% of the transferred carrier are resistant to proteinase in whole mitochondria. The authentic mature protein is also largely resistant to proteinase in whole mitochondria and sensitive after lysis of mitochondria with detergent. Integrity of mitochondria is a prerequisite for translocation into proteinase resistant position.

4. The transfer *in vitro* into a proteinase-resistant form is inhibited by the uncoupler carbonylcyanide *m*-chlorophenylhydrazone but not the proteinase-sensitive binding.

These observations suggest that the posttranslational transfer of ADP/ATP carrier occurs via the cytosolic space through a soluble oligomeric precursor form. This precursor is taken up by intact mitochondria into an integral position in the membrane. These findings are considered to be of general importance for the intracellular transfer of insoluble membrane proteins. They support the view that such proteins can exist in a water-soluble form as precursors and upon integration into the membrane undergo a conformational change. Uptake into the membrane may involve the cleavage of an additional sequence in some proteins, but this appears not to be a prerequisite as demonstrated by the ADP/ATP carrier protein.

A large body of evidence has been presented that transfer of cytoplasmically synthesized proteins into mitochondria occurs by a posttranslational mechanism. The first support for such a mechanism came from kinetic experiments *in vivo* and in homologous cell-free systems [1–4]. More recently, mitochondrial proteins synthesized in heterologous cell-free systems were found to be translocated in reconstitution experiments employing isolated mitochondria [5–7]. Through these findings, attention is centered on the structure and properties of the extramitochondrial precursor forms. There appears to be no general rule for the structure of such precursors, since some are made as larger molecules with additional sequences, which are cleaved during or after transfer to the mitochondria [6–16], whereas others are not synthesized as larger molecules [4,5,17–20]. A route of the precursors through the cytosolic compartment is suggested by the experimental data in several cases [3–5,7]. Special attention should be given to proteins which are integral membrane components in their functional form. If

they pass through the cytosolic compartment the properties of the precursors can be anticipated to differ strongly from those of the membrane-bound form.

In this study, we have investigated the transfer of the ADP/ATP carrier, an integral transmembrane protein of the inner mitochondrial membrane [21−24]. We have already shown that ADP/ATP carrier synthesized in homologous or heterologous cell-free systems has the same apparent molecular weight as the functional protein and is made on free ribosomes [20]. We report here that the precursor form is present soluble in the cytosol fraction of *Neurospora* cell-free systems and of reticulocyte lysates programmed with *Neurospora* messenger RNA. It is not found as a monomer but as an oligomeric complex. The precursor binds detergents such a Triton X-100 and deoxycholate. It is transferred to isolated mitochondria in such a form that it becomes resistant to added proteinase. Proteinase resistance is a characteristic property of the mature functional protein in intact mitochondria. Integration into the membrane can be blocked by the uncoupler ClPhzC(CN)$_2$. The hypothesis is put forward, that the water-soluble precursor makes contact with the mitochondrial membrane through a specific binding site and in a second step is integrated into the membrane thereby undergoing a change in its conformation.

MATERIALS AND METHODS

Growth of Neurospora Cells and Cell Fractionation

Neurospora crassa (wild type, 74 A) was cultivated in Vogel's minimal medium [25]. Labelling of cells with [^{35}S]sulfate was performed as described [5]. Cell-free homogenates were prepared and amino acid incorporation was carried out according to published procedures [20]. *Neurospora* hyphae were converted to spheroplasts by suspending 10 g of hyphae (wet weight) in 50 ml of medium A containing 1 M sorbitol, 4.5 mM sucrose and Vogel's minimal medium as employed for cultivation of cells. Then 1 ml of a solution containing β-glucuronidase and arylsulfatase from *Helix pomatia* (5.2 and 4.5 U, respectively, Boehringer, Mannheim) were added. The mixture was kept at 25 °C for 30 min with gentle agitation. Then it was cooled to 0 °C and centrifuged for 10 min at 5000 × g. The pellet was washed once with medium A. The final pellet was transferred to a Dounce homogenizer together with 10 ml of a medium employed for protein synthesis in a homologous *Neurospora* cell-free system as described before [5,20]. Homogenisation was carried out by 10 strokes with a loosely fitting pestle. The homogenate was centrifuged twice for 5 min at 4000 × g, then mitochondria were sedimented by centrifugaton at 17300 × g for 12 min.

Protein Synthesis in Reticulocyte Lysates

Preparation of reticulocyte lysates, isolation of *Neurospora* poly(A)-containing RNA and incorporation of [^{35}S]methionine in reticulocyte lysates were carried out as described [20,26−28]. Postribosomal supernatants were prepared by centrifuging the cooled lysates for 1 h at 166000 × g in a Beckman rotor Ti 75.

Sucrose Gradient Centrifugation

Linear sucrose gradients were prepared by mixing 6 ml of 5 % sucrose with 6 ml 45 % sucrose (w/w). The solvent was 0.3 M KCl, 10 mM Tris-HCl pH 7.5 and when indicated contained 1 % Triton X-100. Samples to be centrifuged were first made 0.3 M KCl, or 0.3 M KCl and 1 % Triton. Samples of 0.5 ml were layered on the gradients. Centrifugation was carried out in a Beckman ultracentrifuge (rotor Ti 75) at 166000 × g and 1 °C for 15 h or 60 h. Gradient fractions were obtained by puncturing tubes at the bottom and collecting 1.3-ml fractions. Density of fractions was determined in a Zeiss refractometer. For electrophoretic analysis of total proteins, 0.1-ml aliquots were mixed with 0.1 ml acetone. Precipitated proteins were dissolved in dodecylsulfate-containing buffer as used for the solubilisation of immunoprecipitates (see below). The remaining 1.2 ml were subjected to immunoprecipitation.

Gel Filtration

Columns containing Sephadex G-100 (Pharmacia, Uppsala, Sweden) or Ultrogel AcA 34 (LKB, Bromma, Sweden) (55 × 0.9 cm) were equilibrated with 0.3 M KCl, 10 mM Tris-HCl pH 7.5 and, when indicated, 1 % Triton. For calibration, the following components were applied and eluted in the same buffer: dextran blue, thyroglobulin, ferritin, catalase, lactate dehydrogenase, serum albumin, cytochrome *c* and [^{3}H]-Triton X-100 (NEN Corp., Boston, MA). Supernatants of reticulocyte lysates (0.5 ml) were applied to the columns. The samples contained 0.3 M KCl and when indicated 1 % Triton. The flow rate was 18 ml/h with Sephadex G-100, and 6 ml/h with Ultrogel AcA 34. Fractions of 1.5 ml were collected. These were analysed by the same procedures as described for the sucrose density gradient fractions.

Immunoprecipitation

For immunoprecipitation of ADP/ATP carrier from mitochondria, mitochondrial pellets were lysed in 1 ml 1 % Triton, 0.3 M KCl, 1 mM *p*-chloromercuribenzoate, 10 mM Tris-HCl pH 7.5, and freed from insoluble material by centrifugation for 15 min at 20000 × g. 100 μl of a solution containing 2 mg

immunoglobulin fraction, 0.3 M KCl, 10 mM Tris-HCl pH 7.5 was added to the supernatant per 0.25 mg mitochondrial protein and the mixture was kept at 4 °C for 4 h. The precipitate was collected by centrifugation, and washed twice with 0.3 M KCl, 10 mM Tris-HCl, 1% Triton pH 7.5 and three times with 10 mM Tris-HCl pH 7.5.

Immunoprecipitation of ADP/ATP carrier from reticulocyte lysates and from fractions of sucrose density gradients or gel filtration (1 − 1.4 ml) was performed after addition of KCl to a final concentration of 0.3 M and Triton to final concentration of 1% (if not already present). First, 10 µl of the immunoglobulin solution (see above) and after 5 min at 4 °C Sepharose-bound protein A (10 mg dry weight, preswollen in 100 µl 0.3 M KCl, 10 mM Tris-HCl pH 7.5, Pharmacia, Uppsala, Sweden) were added. The mixture was shaken for 5 min and then Sepharose beads were collected by centrifugation. They were washed as described above for direct immunoprecipitation.

Immunoprecipitates obtained by direct precipitation or with Sepharose-bound protein A were dissociated by heating for 2 min at 95 °C in 50 µl 2% sodium dodecylsulfate, 10 mM Tris-HCl pH 7.5, 2.5% (v/v) 2-mercaptoethanol.

Limited Proteolysis of Immunoprecipitates

Mitochondria (2 mg protein) were isolated from cells labelled by growth on [^{35}S]sulfate. After lysis with Triton, ADP/ATP carrier was immunoprecipitated. The washed immunoprecipitate was dissolved in 120 µl 2% sodium dodecylsulfate, 10 mM Tris-HCl pH 7.5, and 360 µl of 10 mM Tris-HCl pH 7.5 were added and the sample was kept at 95 °C for 2 min.

Mitochondria were isolated from unlabelled cells, immunoprecipitation was carried out and the precipitate dissolved in 60 µl 2% dodecylsulfate, 10 mM Tris-HCl pH 7.5. An immunoprecipitate was also obtained from the postribosomal supernatant of a reticulocyte lysate (1 ml) which was previously incubated with [^{35}S]methionine and Neurospora poly(A)-containing RNA for 60 min. This immunoprecipitate was dissolved in 60 µl 2% sodium dodecylsulfate, 10 mM Tris-HCl pH 7.5, and mixed with the dissociated precipitate from the unlabelled mitochondria. Then 360 µl 10 mM Tris-HCl pH 7.5 were added and the mixture kept at 95 °C for 2 min.

The two samples were each divided into two equal portions. To one of each portion were added 30 µl, to the other two 60 µl of a solution containing 1 mg/ml trypsin and 10 mM Tris-HCl pH 7.5. All four samples were kept at 23 °C for 15 min. Proteolysis was stopped by first adding 30 µl of 2-mercaptoethanol and 30 µl 20% sodium dodecylsulfate in 10 mM Tris-HCl pH 7.5, and then boiling for 2 min. 50 µl of each sample was then subjected to gel electrophoresis. Vertical gel

electrophoresis was performed according to Laemmli [29]. The concentrations of acrylamide/bisacrylamide were 17.5/0.12% (w/v).

Transfer in vitro of ADP/ATP Carrier

Protein synthesis in vitro was performed in a reticulocyte lysate in the presence of [^{35}S]methionine and Neurospora poly(A)-containing RNA for 1 h. Samples were cooled to 0 °C and centrifuged for 1 h at 166000 × g. Methionine and sucrose were added to the supernatant to final concentrations of 50 µM and 0.3 M, respectively. Mitochondria isolated from spheroplasts were gently resuspended in this mixture (0.5 − 1 mg protein/ml). The suspension was incubated for the times indicated in the individual experiments at 25 °C. Reaction was terminated by cooling to 0 °C, then mitochondria and supernatant were separated again by 12 min centrifugation at 17300 × g. The mitochondrial pellet was washed once with a solution containing 0.44 M sucrose, 2 mM EDTA, 100 mM Tris-HCl pH 7.8 (sucrose buffer). Then proteinase treatment was performed when indicated. For this purpose the mitochondrial pellets were resuspended in sucrose buffer and proteinase K (Boehringer, Mannheim) (100 µg/ml) was added and samples were incubated at 4 °C for 60 min. Proteolysis was stopped by the addition of 0.1 µmol/ml phenylmethylsulfonyl fluoride. Then to 1 ml of the mitochondrial suspensions 100 µl 3 M KCl, 100 mM Tris-HCl pH 7.5, 100 µl of 10 mM p-chloromercuribenzoate, and 60 µl of 20% Triton X-100 were added. After a clarifying spin, direct immunoprecipitation was performed.

RESULTS

Synthesis of ADP/ATP Carrier in Reticulocyte Lysates and Release into the Postribosomal Supernatant

Protein synthesis was carried out in rabbit reticulocyte lysates supplemented with Neurospora messenger RNA in the presence of [^{35}S]methionine. ADP/ATP carrier was immunoprecipitated from the postribosomal supernatant. A single radioactive band is seen after gel electrophoresis in the presence of dodecylsulfate (Fig. 1, lane 1). It is absent when messenger RNA was omitted, both in lysates pretreated with micrococcal nuclease and not pretreated (lanes 2 and 3). The apparent molecular weight is the same as that of the authentic protein (Fig. 1, lane 9) [17,20]. To make sure that the product in vitro actually represents ADP/ATP carrier, immunoprecipitates from reticulocyte supernatants and from Triton lysates of mitochondria from ^{35}S-labelled cells were subjected to limited proteolysis with trypsin. The patterns of labelled fragments from product in vitro and authentic protein are identical (Fig. 1, lanes 5 − 8). Essentially, two major bands are observed. This agrees with the
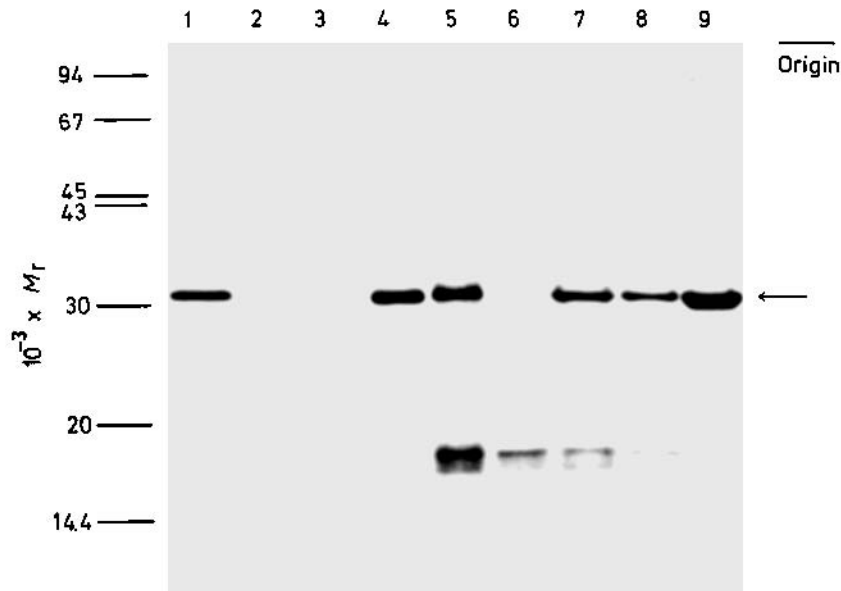
Fig. 1. *Synthesis of ADP/ATP carrier in a reticulocyte cell-free system.* A rabbit reticulocyte lysate was treated with micrococcal nuclease [28] and then incubated together with *Neurospora* poly(A)-containing RNA and [^{35}S]methionine in the presence of an energy regenerating system for 60 min. The postribosomal supernatant was prepared and immunoprecipitation with anti-(ADP/ATP carrier) immunoglobulin was performed. Immunoprecipitates were analysed by gel electrophoresis in the presence of dodecylsulfate and autoradiographs of the dried gels were obtained. (1, 4) Incubation in the presence of poly(A)-containing RNA; (2) incubation in the absence of poly(A)-containing RNA; (3) as in 2, but without nuclease treatment; (5, 6) ADP/ATP carrier immunoprecipitated from a reticulocyte lysate incubated as in 4; the immunoprecipitates were treated with 0.1 mg/ml and 0.2 mg/ml trypsin, respectively; (7, 8) ADP/ATP carrier immunoprecipitated from mitochondria as in 9; the immunoprecipitates were treated with 0.2 mg/ml and 0.1 mg/ml trypsin, respectively; (9) ADP/ATP carrier immunoprecipitated from mitochondria, which were isolated from cells grown in the presence of [^{35}S]sulfate. Arrow indicates position of ADP/ATP carrier stained with Coomassie blue

earlier observation that the cleavage products have a very high tendency to aggregate even in the presence of dodecylsulfate (M. Klingenberg, personal communication) [20, 30].

Properties of ADP/ATP Carrier in the Postribosomal Supernatant

In order to find out in which form the newly synthesized carrier released from the ribosomes is present in the postribosomal fraction, the following experiments were carried out.

From the postribosomal supernatant of a reticulocyte lysate the carrier was immunoprecipitated in the absence and presence of Triton X-100 (Fig. 2, lanes 4 and 5). Without addition of Triton there is unspecific coprecipitation of many other proteins. This unspecific reaction is also seen with non-immune serum in the absence of Triton but not in its presence (Fig. 2, lanes 6 and 7). Despite of this coprecipitation it is obvious that antibodies against the ADP/ATP carrier precipitate the same amount of carrier protein in the presence and absence of Triton (lanes 4 and 5). Furthermore, if the supernatant is treated with trypsin, the carrier is digested in the absence as well as in the presence of Triton X-100, so that immunoprecipitation

of intact protein or even fragments is not any longer observed (Fig. 2, lanes 1 – 3). The same experiments were performed with the supernatant of a homologous cell-free system [5, 20] and the results were the same (not shown).

It is concluded from these observations that the newly synthesized protein is accessible to immunoglobulins as well as to proteinase. Therefore it cannot be enclosed in vesicles or protected in a lipid phase, as is the functional protein (see below, Fig. 6).

The reticulocyte supernatant was subjected to sucrose density gradient centrifugation and ADP/ATP carrier was immunoprecipitated from gradient fractions. In a first experiment centrifugation was carried out for short time (15 h). Fig. 3 A shows total acetone-precipitable proteins in the gradient fractions analysed by gel electrophoresis and autoradiography. Hemoglobin, which is present in the reticulocyte supernatant in very high amounts, forms a band in the middle of the gradient. The immunoprecipitable ADP/ATP carrier is found as a single band in the gradient and sediments slightly faster than hemoglobin (Fig. 3 B). When centrifugation was performed for 60 h, ADP/ATP carrier was found in the two lowest fractions of the gradient (Fig. 3 C). Apparently, it has a density higher than 1.21 g/cm^3. The gradients
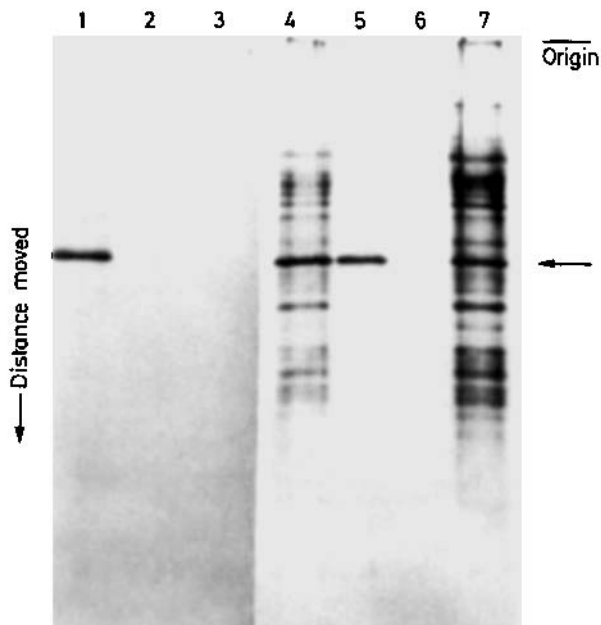
Fig. 2. *Accessibility to antibody and proteinase of ADP/ATP carrier synthesized* in vitro *in reticulocyte lysates*. Protein synthesis was carried out in a rabbit reticulocyte lysate in the presence of [^{35}S]-methionine and *Neurospora* poly(A)-containing RNA for 60 min. Then the postribosomal supernatant was prepared. Samples of the supernatant were treated as follows and then subjected to immunoprecipitation with immunoglobulins specific for ADP/ATP carrier or non-immune immunoglobulins and then with Sepharose-bound protein A (as indicated below). The immunoprecipitates were analysed by electrophoresis and autoradiography. (1, 5) Lysis with Triton; addition of specific immunoglobulins; (2) lysis with Triton; then treatment with trypsin (1 mg/ml) for 60 min at 23 °C followed by addition of trypsin soybean inhibitor (1 mg/ml) and cooling to 0 °C; addition of specific immunoglobulins; (3) treatment with trypsin (1 mg/ml) for 60 min at 23 °C, then addition of trypsin inhibitor as in 2; the sample was cooled and Triton was added; then specific immunoglobulins were added; (4) addition of specific immunoglobulins without lysis with detergent; (6) lysis with Triton and addition of non-immune immunoglobulins; (7) addition of non-immune immunoglobulins without lysis with detergent. Samples 1 – 3 and samples 4 – 7 were obtained from two different experiments. Arrow indicates position of ADP/ATP carrier

did not contain visible pellets and no immunoprecipitable radioactivity could be washed off from the bottom of the tubes (not shown).

Sedimentation was then carried out employing a gradient which contained Triton X-100 above the critical micellar concentration. The applied reticulocyte supernatant contained Triton. After 60 h centrifugation the ADP/ATP carrier formed a distinct band in the upper third of the gradient (Fig. 3 D). Apparently, the carrier protein binds detergent, which leads to a reduced specific density. This binding of detergent to the carrier synthesized *in vitro* is a specific property not shared by other proteins such as hemoglobin, as seen from its position in the detergent-containing gradient. It was also found that the sedimentation behaviour of *Neurospora* isocitrate lyase,

an enzyme of the glyoxysomal matrix, did not differ in detergent-free and detergent-containing gradients (not shown here).

For further analysis of the ADP/ATP carrier synthesized *in vitro*, the supernatant of reticulocyte lysates was subjected to gel filtration. When Sephadex G-100 was employed, the carrier was eluted with the void volume (Fig. 4). No indication of a monomer or of a dimer was found. In the electrophoretic patterns of the total proteins in the column fractions hemoglobin can be used as an internal marker. In contrast to the ADP/ATP carrier it enters the Sephadex gel.

In order to achieve resolution in the higher molecular weight region, chromatography was performed on Ultrogel AcA 34 (Fig. 5). The column was calibrated with marker proteins (Fig. 5C). The ADP/ATP carrier is detected in two bands in the elution diagram. The apparent molecular weights of the two bands were determined to be about 120000 and 500000 or higher. The second species was eluted close to the exclusion limit and therefore a precise value for its molecular weight cannot be given. In different experiments the distribution of ADP/ATP carrier between these two bands varied in the range of 2 : 1 or 1 : 2.

Detergent binding was also studied by gel filtration. The postribosomal supernatant of the reticulocyte lysates was made 1 % in Triton X-100 and chromatographed on Sephadex G-100 and on Ultrogel AcA 34, equilibrated with Triton-containing buffer. From Sephadex G-100 the carrier was eluted with the void volume as in the absence of Triton (Fig. 4). The analysis of acetone-precipitated column fractions shows that the elution behaviour of hemoglobin is not affected by the detergent. This shows that Triton does not effect dissociation into monomers. From Ultrogel the ADP/ATP carrier was eluted as a broad band corresponding to a molecular weight of 200000 – 400000. Labelled Triton X-100 was eluted from the column with an apparent molecular weight of 90000 in agreement with the reported size of Triton micelles [31]. Elution behaviour of marker proteins was not affected by the inclusion of Triton into the elution buffer. Apparently, the soluble protein complex containing the ADP/ATP carrier forms mixed micelles with Triton X-100. Gel filtration was also performed on Ultrogel AcA 34 in the presence of 1 % sodium deoxycholate, 0.3 M KCl, 10 mM Tris-HCl pH 8.0. As with Triton, the carrier was eluted corresponding to an apparent molecular weight of 200000 – 400000 (not shown).

Transfer of ADP/ATP Carrier from Postribosomal Supernatant of Reticulocyte Lysates into Isolated Mitochondria

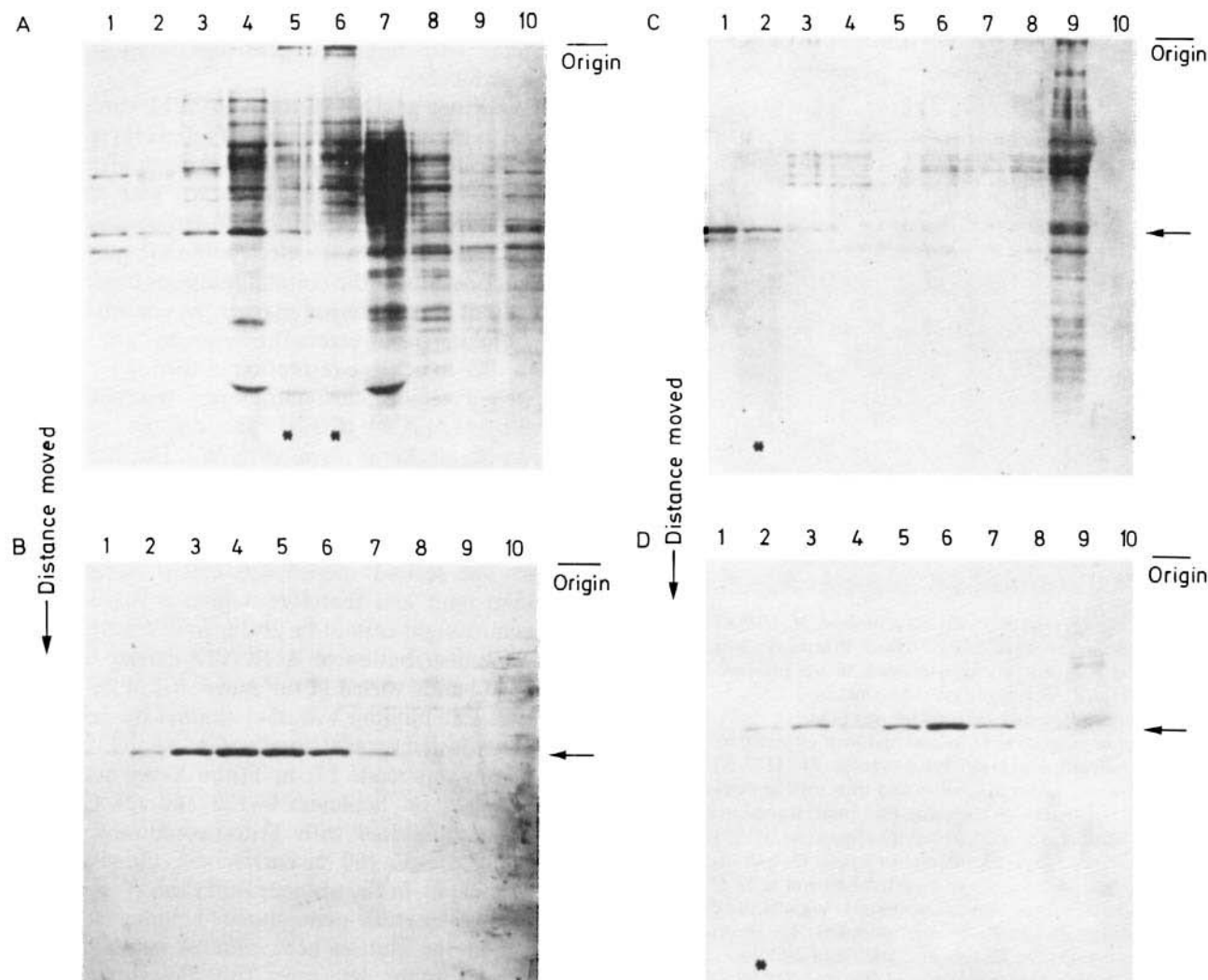Mitochondria were isolated from *Neurospora* spheroplasts and were resuspended in a postribosomal

Fig. 3. *Sucrose density gradient centrifugation of ADP/ATP carrier synthesized in a rabbit reticulocyte lysate.* A 166000 × g supernatant was obtained from a reticulocyte lysate after protein synthesis *in vitro* in the presence of [^{35}S]methionine. KCl was added to the postribosomal supernatant to a final concentration of 0.3 M. A first 0.5-ml aliquot was layered on a sucrose density gradient (5–45% sucrose) and centrifugation was performed for 15 h. Fractions of 1.3 ml were collected which were divided into 0.1-ml and 1.2-ml portions. The smaller portions were subjected to acetone precipitation to obtain total protein. The larger portions were subjected to immunoprecipitation after addition of Triton with antibodies against ADP/ATP carrier. A second 0.5-ml sample was centrifuged on the same gradient for 60 h. A third 0.5-ml aliquot was made 1 % in Triton and centrifuged on a gradient containing 1 % Triton for 60 h. Gradient fractions were subjected to immunoprecipitation as above. Acetone precipitates and immunoprecipitates were dissolved in buffer containing dodecylsulfate and subjected to gel electrophoresis and autoradiography. Asterisk indicates position of hemoglobin. Arrow indicates position of ADP/ATP carrier protein. Fractions are numbered from bottom to top. (A) 15 h centrifugation, acetone-precipitated proteins; (B) 15 h centrifugation; immunoprecipitation; (C) 60 h centrifugation, immunoprecipitation; (D) 60 h centrifugation in the presence of Triton; immunoprecipitation

supernatant from a reticulocyte lysate which was previously incubated with [^{35}S]methionine and *Neurospora* poly(A)-containing RNA. The mixture was kept for 60 min at 25 °C. Then mitochondria were re-isolated by centrifugation, lysed with Triton and the ADP/ATP carrier was immunoprecipitated. Fig. 6, lane 1 shows that carrier synthesized *in vitro* is present in the mitochondrial fraction.

In order to show that in this reconstituted system carrier becomes actually integrated into the mitochondria, the following experiments were carried out.

First, as a control, mitochondria were isolated from spheroplasts which were prepared from cells grown on [^{35}S]sulfate. The ADP/ATP carrier which was immunoprecipitated from these mitochondria after lysis, forms a single band upon gel electrophoresis (Fig. 6, lane 6). When these mitochondria, before lysis, were incubated with proteinase K at 4 °C for 1 h, the carrier was largely resistant to proteolysis (lane 7). Apparently, this membrane protein is not easily accessible to the proteinase in intact mitochondria. When mitochondria were first lysed with Triton and
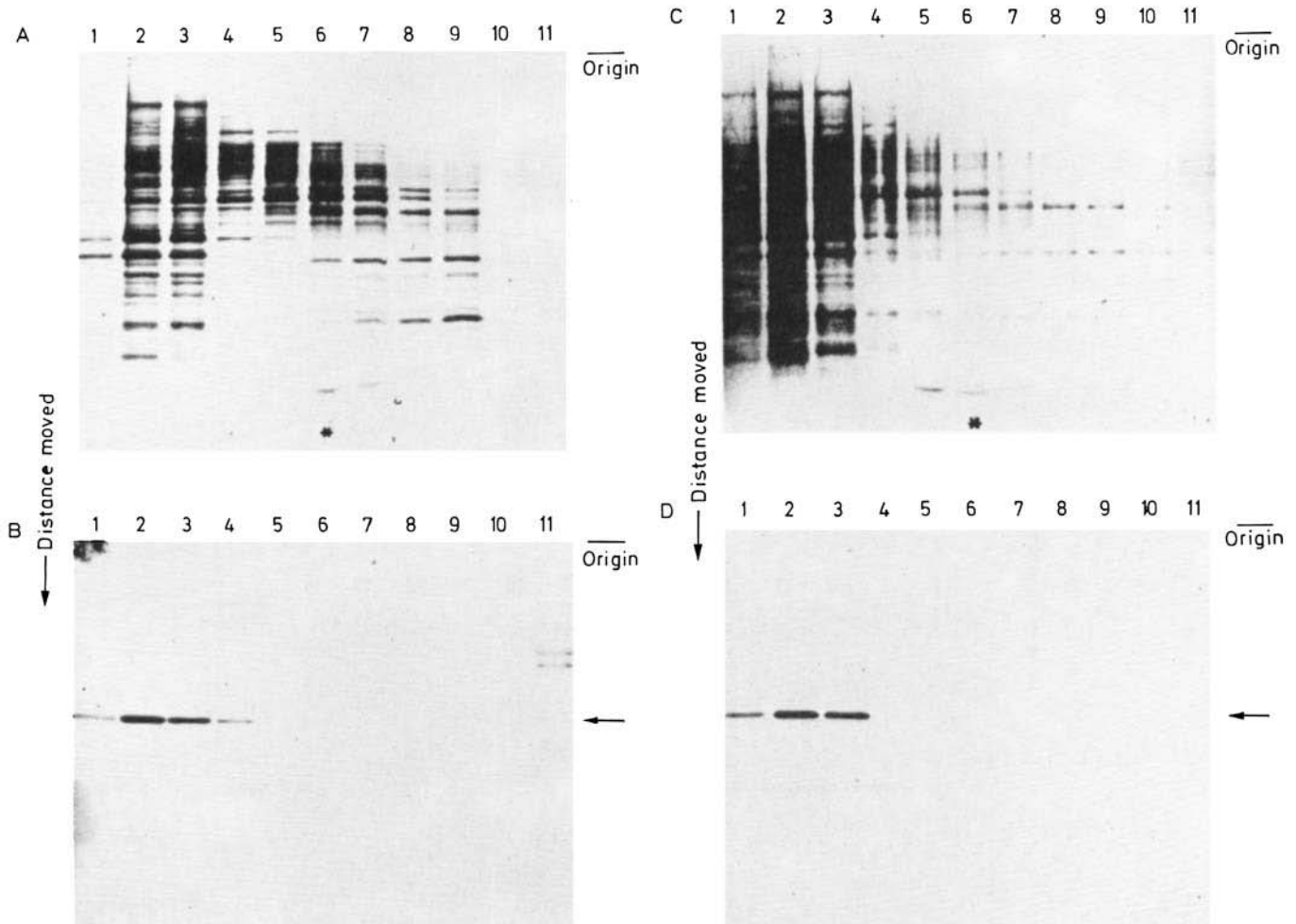
Fig.4. *Gel filtration of ADP/ATP carrier synthesized in a rabbit reticulocyte lysate on Sephadex G-100.* A 166000×g supernatant was obtained from a reticulocyte lysate after protein synthesis *in vitro* in the presence of [^{35}S]methionine. KCl was added to the supernatant to a final concentration of 0.3 M. Gel filtration on Sephadex G-100 of 0.5-ml aliquots was performed as described below; fractions of 1.5 ml were collected. Aliquots of 0.1 ml were subjected to acetone precipitation. From the rest, immunoprecipitates were obtained with antibodies against ADP/ATP carrier and subjected to gel electrophoresis and autoradiography. Fractions are numbered according to their appearance from the column. (A, B) Supernatant chromatographed without further treatment: (A) acetone precipitates; (B) immunoprecipitates. (C, D) Supernatant was supplemented with 1% Triton and chromatographed on a column equilibrated with buffer containing 1% Triton: (C) acetone precipitates; (D) immunoprecipitates. Arrow indicates position of ADP/ATP carrier. Asterisk indicates position of hemoglobin

then treated with proteinase K, the carrier was completely digested into pieces which no longer can be immunoprecipitated (lane 8).

When mitochondria, into which protein synthesized *in vitro* had been transferred, were treated with proteinase K, part of the immunoprecipitable carrier was not digested by proteinase K (Fig.6, lane 2). However, when these mitochondria were first lysed and then treated with proteinase, the carrier transferred *in vitro* was digested (lane 3). The amount of carrier which becomes proteinase-resistant may be underestimated, since resuspension and incubation of mitochondria in the reticulocyte lysate leads to a higher sensitivity of the preexistent carrier protein to proteinase (Fig.6, lanes 9 and 10). Temperature dependence of the transfer *in vitro* was studied in a

parallel experiment. When incubation of mitochondria with the postribosomal supernatant was carried out at 0 °C, the amount of ADP/ATP carrier bound to the mitochondria was about 30−40% as compared to the incubation at 25 °C (Fig.6, lane 4). The uptake into proteinase-resistant position was however completely blocked (lane 5).

Mitochondria were isolated from spheroplasts by gentle homogenisation of cells because it was found that mitochondria isolated from hyphae had a lower capacity to take up ADP/ATP carrier from the reticulocyte lysate. When mitochondria were isolated by grinding of spheroplasts with sand [5], their ability to integrate ADP/ATP carrier into a proteinase-resistant form was almost completely lost. Also, the ability to bind it into a proteinase-sensitive form was
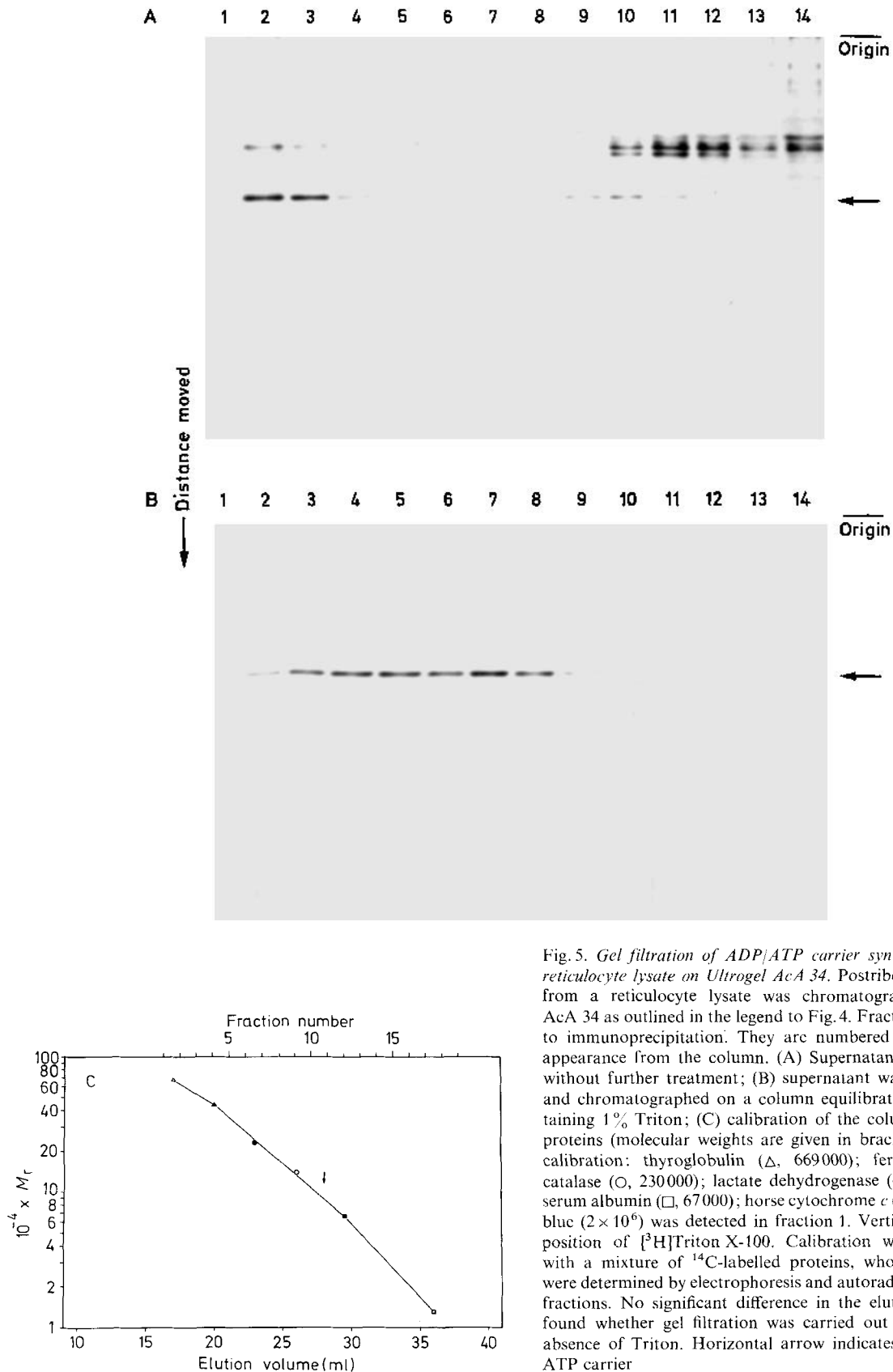
Fig. 5. *Gel filtration of ADP/ATP carrier synthesized in a rabbit reticulocyte lysate on Ultrogel AcA 34.* Postribosomal supernatant from a reticulocyte lysate was chromatographed on Ultrogel AcA 34 as outlined in the legend to Fig. 4. Fractions were subjected to immunoprecipitation. They are numbered according to their appearance from the column. (A) Supernatant chromatographed without further treatment; (B) supernatant was made 1% Triton and chromatographed on a column equilibrated with buffer containing 1% Triton; (C) calibration of the column. The following proteins (molecular weights are given in brackets) were used for calibration: thyroglobulin ($\triangle$, 669000); ferritin ($\blacktriangle$, 440000); catalase (O, 230000); lactate dehydrogenase ($\bullet$, 140000); bovine serum albumin ($\square$, 67000); horse cytochrome c ($\blacksquare$, 13000). Dextran blue (2×10^6) was detected in fraction 1. Vertical arrow indicates position of [^{3}H]Triton X-100. Calibration was also performed with a mixture of ^{14}C-labelled proteins, whose elution volumes were determined by electrophoresis and autoradiography of column fractions. No significant difference in the elution behaviour was found whether gel filtration was carried out in the presence or absence of Triton. Horizontal arrow indicates position of ADP/ATP carrier
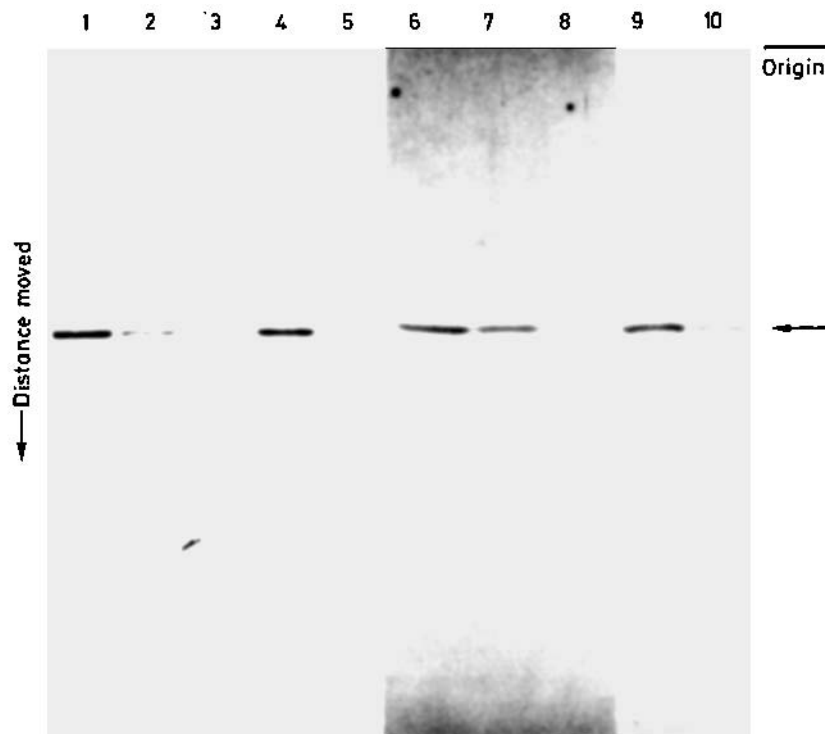
Fig. 6. *Transfer into isolated mitochondria of ADP/ATP carrier synthesized in a reticulocyte lysate.* Protein synthesis was carried out in a reticulocyte lysate programmed with *Neurospora* poly(A)-containing RNA in the presence of [^{35}S]methionine. The postribosomal supernatant was prepared and incubated with mitochondria isolated from *Neurospora* spheroplasts for 60 min. Mitochondria were reisolated and treated in the following way. (1 − 3) Mitochondria were incubated with supernatant at 25 °C. (1) Lysis with Triton and immunoprecipitation; (2) mitochondria were treated with 100 µg/ml proteinase K for 60 min at 4 °C, then 0.1 mM phenylmethylsulfonyl fluoride was added; mitochondria were lysed with Triton and immunoprecipitation was carried out; (3) mitochondria were lysed with Triton, then treated with proteinase K as in 2 and subsequently subjected to immunoprecipitation. (4, 5) Mitochondria were incubated with supernatant at 0 °C. (4) Lysis with Triton and immunoprecipitation; (5) treatment with proteinase K, then lysis with Triton and immunoprecipitation as in 2. (6 − 10) Mitochondria were isolated from cells grown in the presence of [^{35}S]sulfate and treated as follows. (6) Lysis with Triton and immunoprecipitation; (7) mitochondria were treated with proteinase K as in 2, then immunoprecipitation was performed; (8) mitochondria were lysed with Triton, treated with proteinase K as in 7, then immunoprecipitation was carried out; (9) mitochondria were resuspended in a supernatant from a reticulocyte lysate (not incubated for protein synthesis *in vitro*) for 60 min at 25 °C, then reisolated by centrifugation, lysed with Triton and subjected to immunoprecipitation; (10) mitochondria were incubated with reticulocyte lysate supernatant and reisolated as in 9, then treated with proteinase K for 60 min at 4 °C, then phenylmethylsulfonyl fluoride and Triton were added and immunoprecipitation was carried out. Immunoprecipitates were dissolved in dodecylsulfate-containing buffer and analysed by gel electrophoresis and autoradiography. Arrow indicates position of ADP/ATP carrier stained with Coomassie blue

reduced. The integrity of these mitochondria was strongly affected by the grinding with sand. This was indicated by the observation that adenylate kinase activity was only 20% as compared to mitochondria isolated by homogenisation of cells. For mitochondria from other sources it has been shown that adenylate kinase is located in the intermembrane space and is released from mitochondria after rupture of the outer membrane [32].

It is concluded from these experiments that in a reconstituted system, the ADP/ATP carrier can be translocated into mitochondria. Transfer is dependent on the integrity of the mitochondria. Furthermore, the soluble form of the carrier in the postribosomal fraction can be considered as a precursor form. The mode of transfer is clearly posttranslational.

The kinetics of the transfer *in vitro* are shown in Fig. 7 and 8. The appearance of the carrier in the mito-

chondria is accompanied by a commensurate decrease of precursor in the supernatant (Fig. 7, lanes 1 − 4). The carrier associated with mitochondria before proteinase treatment shows a rapid increase during the first 15 min, whereas the proteinase-resistant portion shows a continuous increase during the 60-min incubation. Apparently, there is a rapid binding of the precursor to the mitochondria, but a slow integration into the mitochondria.

A quantitative evaluation of the transfer *in vitro* is shown in Fig. 8. The percentage of carrier protein transferred to mitochondria, was 70 − 80% of total input. The percentage of carrier protein integrated into a proteinase-resistant position was usually about 30% of the total carrier protein associated with mitochondria. From all the proteins synthesized *in vitro* in the supernatant, only about 20 − 25% became associated with mitochondria after 60-min incubation
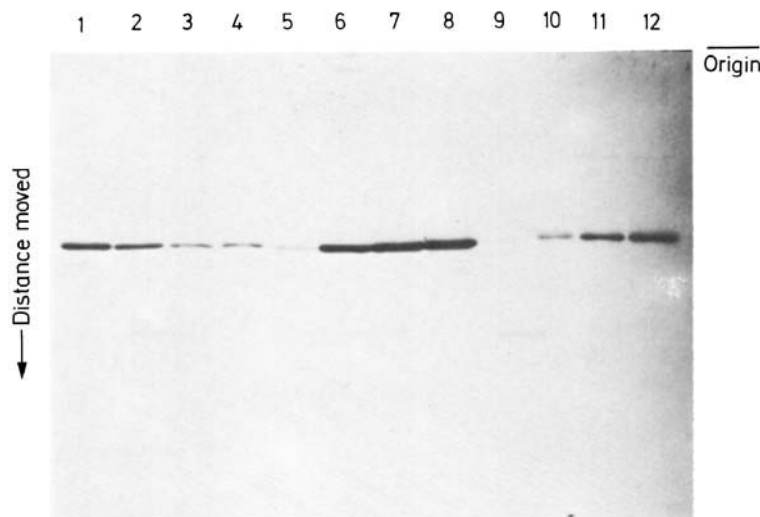
Fig. 7. *Transfer* in vitro *of ADP/ATP carrier into mitochondria*. Protein synthesis *in vitro* in a reticulocyte lysate was performed in the presence of *Neurospora* poly(A)-containing RNA and [^{35}S]methionine for 60 min. The postribosomal supernatant was prepared. To 3 ml of the supernatant was added 1 ml 1.2 M sucrose and 0.2 mM methionine. Then isolated mitochondria (2 mg) were resuspended. Immediately after resuspension a 1-ml aliquot was withdrawn, and further 1-ml aliquots after 15 min, 30 min and 60 min of incubation at 25 °C. After withdrawal, the samples were centrifuged for 12 min at $17300 \times g$ to obtain the mitochondria and the postmitochondrial supernatant. Triton X-100 was added to the postmitochondrial supernatants and immunoprecipitation performed with Sepharose-bound protein A. The mitochondrial pellets were resuspended in 2 ml 0.44 M sucrose, 2 mM EDTA, 10 mM Tris-HCl pH 7.5. To one half of each suspension Triton was added in a final concentration of 1% and direct immunoprecipitation was carried out. The other half of each suspension was treated with proteinase K (100 µg/ml) for 60 min at 4 °C, then phenylmethylsulfonyl fluoride was added and direct immunoprecipitation performed after addition of Triton. (1−4) Supernatant (0, 15, 30 and 60 min); (5−8) mitochondria (0, 15, 30 and 60 min); (9−12) mitochondria treated with proteinase K (0, 15, 30 and 60 min)

(Fig. 8). This is explained by the fact that mitochondrial proteins constitute only a fraction of the proteins synthesized in the cell-free system.

Specificity of binding of the ADP/ATP carrier to the mitochondria is demonstrated by the following experiment. Poly(A)-containing RNA was isolated from cells grown on acetate. In the presence of acetate *Neurospora* cells form glyoxysomes and the glyoxysomal isocitrate lyase is induced [33]. Protein synthesis in a reticulocyte lysate was promoted with this RNA and a postribosomal supernatant was incubated with mitochondria from non-induced cells. After incubation for 60 min, isocitrate lyase was not detectable in the mitochondrial fraction, even not before treatment with proteinase K (Fig. 8).

Inhibition of Transfer
by Carbonylcyanide m-*Chlorophenylhydrazone*
[ClPhzC(CN)$_2$]

Studies *in vivo* with whole cells and studies *in vitro* with cell-free homogenates had shown that transfer of ADP/ATP carrier into mitochondria is inhibited by ClPhzC(CN)$_2$ [1, 34]. In the reconstituted system described here, ClPhzC(CN)$_2$ was added after resuspending the mitochondria in reticulocyte supernatant. Then incubation was carried out exactly as described for Fig. 7. ADP/ATP carrier was immunoprecipitated from re-isolated supernatant, and from

mitochondria before and after proteinase treatment. It is apparent from Fig. 9 that ClPhzC(CN)$_2$ does not inhibit the binding of the carrier protein to the mitochondria, since decrease in the supernatant and increase in the mitochondria is comparable to the ClPhzC(CN)$_2$-free incubation (Fig. 9, lanes 1−4, 5−9). However, the appearance of a proteinase-resistant form is strongly reduced. This suggests that ClPhzC(CN)$_2$ specifically inhibits membrane integration of the ADP/ATP carrier, but not its binding. It remains to be clarified whether it is the breakdown of the proton gradient or some other effect on the membrane which leads to this decrease of integration into the mitochondria.

DISCUSSION

We have shown in earlier publications that a number of mitochondrial proteins synthesized on cytoplasmic ribosomes are translocated into mitochondria by a posttranslational mechanism [1−5, 12, 17, 18, 34]. In particular for the ADP/ATP carrier this was suggested from experiments *in vivo*. Kinetic data lent strong support to the view that transfer occurs via an extramitochondrial precursor form. In whole cells, transfer of the newly synthesized carrier protein was found to continue, when polypeptide chain elongation was blocked by cycloheximide. Furthermore, evidence was presented that free ribosomes and not
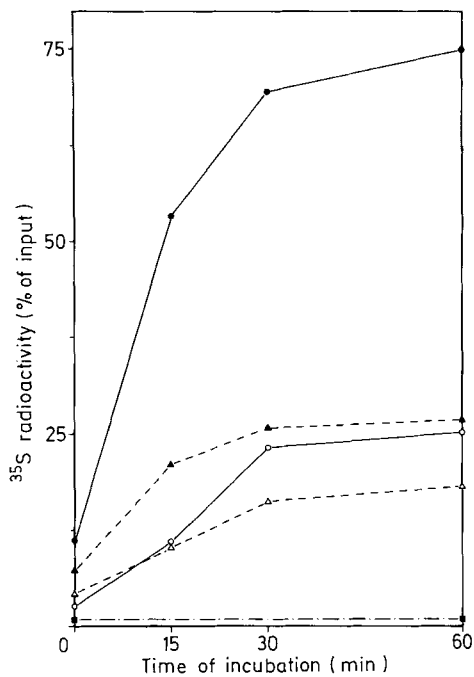
Fig. 8. *Kinetics of transfer of ADP/ATP carrier into mito-chondria.* Transfer *in vitro* of carrier synthesized in reticulocyte lysate to mitochondria was performed as described for Fig. 7. The amount of ^{35}S-labelled carrier transferred into proteinase-sensitive and proteinase-resistant positions was determined. For this purpose, immunoprecipitates were obtained from mitochondria after reisolation from the incubation mixture by direct immunoprecipitation. To the supernatant fractions the same amounts of unlabelled mitochondria as present during incubation were added and also direct immunoprecipitation was carried out. Immunoprecipitates were subjected to horizontal slab gel electrophoresis in the presence of dodecylsulfate [5] and the radioactivity in the 32000-M_r peaks measured. The radioactivity of ADP/ATP carrier in the mitochondria was related to the sum of radioactivities of ADP/ATP carrier in postmitochondrial supernatant and in mitochondria before proteinase treatment. Also, total ^{35}S radioactivity in tri-chloroacetic-acid-precipitable protein of the whole incubation mixture and in reisolated mitochondria was determined and related to each other. In a parallel experiment, a reticulocyte lysate was programmed with poly(A)-containing RNA from cells grown on acetate and the postribosomal supernatant incubated with mitochondria from cells grown on sucrose. Isocitrate lyase was immunoprecipitated from reisolated supernatant and mitochondria. Radioactivity in the 67000-M_r peaks was determined after gel electrophoretic analysis. (▲——▲) Cl₃AcOH-precipitable radioactivity in mitochondria before proteinase treatment; (△---△) Cl₃AcOH-precipitable radioactivity in mitochondria after proteinase treatment; (●——●) ADP/ATP carrier immunoprecipitated from mitochondria before proteinase treatment (○——○) ADP/ATP carrier immunoprecipitated from mitochondria after proteinase treatment; (■· ·—·■) isocitrate lyase immunoprecipitated from mitochondria before proteinase treatment

membrane-bound ribosomes synthesize the carrier protein. The newly synthesized carrier was found to have the same apparent molecular weight as the functional protein in the membrane [20]. Particularly, an amino terminal extension of the type of 'signal sequences' described for secretory proteins [35—38] could be excluded. In both homologous and hetero-

logous cell-free systems the newly formed carrier was found in the postribosomal supernatant.

Experiments reported here show that this product formed *in vitro* is present in soluble form. It is not enclosed in vesicles or membranes, since antibody and proteases have access. Also, upon sedimentation analysis the product formed *in vitro* behaved like a soluble protein. Employing gel filtration two forms of the product in the postribosomal supernatant were detected with molecular weights of about 120000 and 500000 or larger. Obviously, the newly synthesized carrier is not present as a monomer or dimer. Rather, it exists as a homooligomer or as a complex with other protein.

A distinctive property of the carrier is its ability to bind detergent. Both sedimentation analysis and gel filtration suggest that protein and detergent form large mixed micelles. This ability to interact with detergent is consistent with the idea that the carrier can exist either in a water-soluble form or in an integral membrane form. This is in notable similarity to melittin, the main component of bee venom [39,40]. This highly hydrophobic protein can exist in water-soluble form as a tetramer [41]. Upon contact with membranes it is converted to an integral membrane protein [41 — 43]. This protein forms mixed micelles with detergents such as Triton X-100 or Brij 58 [41].

We describe in this report the transfer of ADP/ATP carrier synthesized *in vitro* into isolated mitochondria, so that it becomes resistant to added proteinase. Protection against proteinase action is taken as a criterion for a movement of the extramitochondrial form across the outer mitochondrial membrane. In preliminary experiments we have found that the extramitochondrial form of the ADP/ATP carrier in the presence of Triton is bound to hydroxyapatite in contrast to the carrier isolated from the membrane; the proteinase-resistant carrier transferred into isolated mitochondria was not bound to hydroxyapatite (unpublished results). We therefore suggest that transfer into a proteinase-resistant location represents integration into the inner membrane. ClPhzC(CN)₂, an uncoupler of oxidative phosphorylation inhibits transfer into a proteinase-resistant location. Significantly, the binding to the mitochondria is not inhibited. We have already reported in an earlier publication that the protonophor ClPhzC(CH)₂ inhibits protein transfer *in vivo* and in a homologous cell-free system [1,34]. The data presented here confirm and extend these findings. The mechanism of this inhibition remains to be elucidated.

The data taken together clearly support the view that soluble ADP/ATP carrier in the postribosomal supernatant is a precursor form of the membrane-integrated form which is transported to and integrated into the mitochondrial inner membrane by a post-translational mechanism. On the basis of the experi-
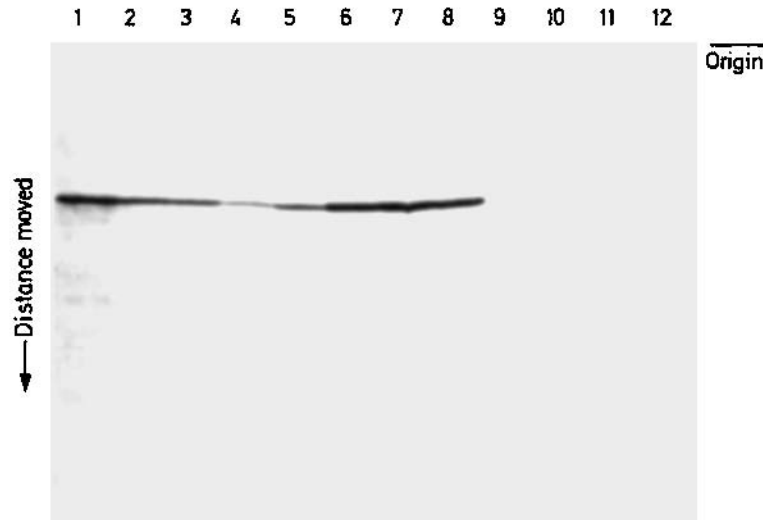
Fig. 9. *Inhibition of transfer* in vitro *of ADP/ATP carrier by carbonylcyanide* m-*chlorophenylhydrazone [ClPhzC(CN)₂]*. Incubation of isolated mitochondria with postribosomal supernatant was performed as described for Fig. 7 with the exception that ClPhzC(CN)₂ (final concentration 12.5 μM) was added immediately after resuspension of mitochondria. All other steps were carried out as in Fig. 7. (1−4) Supernatant (0, 15, 30 and 60 min); (5−8) mitochondria (0, 15, 30 and 60 min); (9−12) mitochondria treated with proteinase K (0, 15, 30 and 60 min)

ments reported here and in earlier publications we propose the following mechanism of intracellular translocation of ADP/ATP carrier. The protein is synthesized on free ribosomes and released into the supernatant in a soluble form and occurs there as an oligomeric complex. This precursor is rapidly transferred to the mitochondria, viz. its pool size is relatively small as compared to those of other extramitochondrial precursors, e.g. of matrix proteins [1−3]. The pool of nascent ADP/ATP carrier polypeptide on the ribosomes may be larger than the pool of the precursor in the extramitochondrial space [2]. Uptake of the precursor into the membrane may probably be dissected into several steps. The data presented suggest that in a first step the precursor is bound to the outer membrane; this step does not require energetisation of the membrane. It is speculated that a receptor protein exists, which determines the specific uptake into mitochondria. In a following reaction the precursor would then become integrated into the inner membrane. We suspect that the inner membrane and the outer membrane must exert specific interaction which is only possible in intact and energized mitochondria. A change in conformation then will lead to the formation of an integral transmembrane protein.

This hypothesis for the intracellular transfer of an insoluble membrane protein may also apply for other mitochondrial proteins. In some cases cleavage of an additional sequence or of a polyprotein precursor appears to be involved [9,11,44,45]. However, as shown here, this is not a prerequisite for such a post-translational transfer. For all mitochondrial proteins which have to cross completely the inner membrane, such as subunits of F₁-ATPase, citrate synthase,

carbamoylphosphate synthase, and ornithine trans-carbamoylase larger precursors were reported [6−8, 12−14,16]. In analogy, the small subunit of ribulose-bisphosphate carboxylase which is synthesized on cytoplasmic ribosomes in plant cells and which is transported into the stroma space of chloroplasts is synthesized as a larger precursor [15,46,47]. However, it was also reported that another mitochondrial matrix protein, aspartate aminotransferase, in its mature form, has the ability to associate specifically with isolated mitochondria [48]. In the case of F₁-ATPase subunits, ClPhzC(CN)₂ was described to inhibit processing in whole cells. This was attributed to a reduced intramitochondrial ATP level [49]. If ATP is necessary for processing it is not excluded, however, that ClPhzC(CN)₂ has an additional effect on the membrane. Interestingly, Wickner and coworker have recently reported a posttranslational membrane insertion of M 13 coliphage coat protein [50−53]. According to this work, coat protein which is synthesized as a larger precursor (procoat) [54], is present as a soluble oligomeric protein in the cytoplasm of the bacterial cell, before it is cleaved and integrated into the membrane. ClPhzC(CN)₂ was reported to inhibit *in vivo* processing of procoat protein but not its binding to the membrane [55]. It should however be noted that a quite different mechanism for the assembly of this protein has been suggested, namely a cotranslational insertion [56].

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