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An immunological study of the uncoupling protein of brown adipose tissue mitochondria

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1. Ewes were injected with purified 32000-M_r uncoupling protein from mitochondria of brown adipose tissue of cold-adapted rats in order to raise antibodies. 2. The existence of antibodies in the plasma of ewes and the cross-reactivity of plasmas were demonstrated and studied by ^{125}I-labelled antigen–antibody reaction, double immunodiffusion, the inhibition of GDP binding to the 32000 M_r protein and by immunohistochemistry. 3. The antibodies raised against the homogeneous protein yielded a single immunoprecipitation band with detergent-solubilized mitochondrial membranes of brown adipose tissue from rat, hamster, guinea-pig, rabbit and with the purified uncoupling protein of these animals. No immunoprecipitation was obtained with the protein purified from brown adipose tissue of term lamb foetus. 4. The GDP-binding activity of the uncoupling protein (isolated or in solubilized membranes) was largely inhibited by the antiserum. 5. The anti-(rat uncoupling protein) could not cross-react with solubilized membranes from liver or muscle, nor with the purified beef heart or rat liver ADP/ATP translocator.

The physiological function of brown adipose tissue is heat production in newborn mammals, cold-adapted rodents and hibernating species. The biochemical mechanisms of this process have been recently elucidated (Nicholls, 1979; Locke & Nicholls, 1981). Energy arising from fatty acid oxidation is dissipated as heat through loose coupling of mitochondria. A specialized 32000 M_r protein of the mitochondrial inner membrane collapses the electrochemical proton gradient generated by respiration and limits ATP synthesis. The amount of this mitochondrial component, also named uncoupling protein, is increased when brown adipose tissue is in a thermogenic state (Ricquier & Kader, 1976; Heaton *et al.*, 1978; Desautels & Himms-Hagen, 1979; Ricquier *et al.*, 1979*a*); this protein can be characterized by its specific GDP-binding activity (Heaton *et al.*, 1978).

We have developed investigations on this membrane protein by using an immunological approach. In a first step we attempted to obtain antibodies against the pure 32000 M_r protein; in a second step we studied the immunological cross-reaction of 32000 M_r protein isolated from different species. We also tried to identify such a component in other tissues, since the GDP-binding assay and electrophoresis of mitochondrial proteins are not sensitive enough assays. Finally, antibodies were tested against the isolated ADP/ATP carrier of mitochondria, since this protein and the uncoupling protein do possess similar characteristics (roughly the same M_r, component of the inner mitochondrial membrane, affinity for purine di- or triphosphate nucleotides).

Abbreviations used: SDS, sodium dodecyl sulphate; Mops, 4-morpholinepropanesulphonic acid; IgG, immunoglobulin G.

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Experimental

Animals

Rats (Sprague–Dawley strain) were either kept at room temperature or were cold-adapted at 5°C for 3–5 weeks. Golden hamsters were exposed to 5°C for 3–5 weeks. Newborn guinea-pigs and rabbits were 0–1 day old; lamb foetuses were obtained 5 days before the calculated date of birth.

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Tissue sampling and isolation of mitochondria

Interscapular brown adipose tissue was collected from rats and new-born guinea-pigs, interscapular, axillary and cervical brown adipose tissue from hamsters and new-born rabbits and perirenal brown adipose tissue from lamb foetuses. Pieces of liver and muscle (thigh) were removed from room temperature and cold-adapted rats and hamsters. Brown adipose tissue mitochondria were isolated as described by Ricquier *et al.* (1982*a*); liver and muscle mitochondria were isolated as in Nedergaard & Cannon (1979).

Purification of the 32000 M_r uncoupling protein

Pure 32000 M_r protein was obtained from room temperature or cold-adapted rats, cold-adapted hamsters, new-born guinea-pigs, new-born rabbits and lamb foetuses by using hydroxyapatite filtration as previously described (Lin & Klingenberg, 1980; Ricquier *et al.*, 1982*b*). Evidence for the purity of the protein was obtained both from SDS/polyacrylamide-gel electrophoresis and from measurements of the GDP-binding capacity. Partially purified rat protein was also obtained by using affinity chromatography (Ricquier *et al.*, 1979*b*). The hydroxyapatite-purified proteins were characterized by the GDP-binding assay using equilibrium dialysis (Lin & Klingenberg, 1980; Ricquier *et al.*, 1982*b*) and by SDS/polyacrylamide-gel electrophoresis.

Preparation of antibodies

Purified mitochondrial 32000 M_r protein from brown adipose tissue of cold-adapted rats was injected intradermally into six ewes. The protein was in solution containing 20mM-Mops (pH 6.7), 20mM-Na_2SO_4 and 1–2% (w/v) Triton X-100. Before injection, the protein solution was emulsified with Freund's adjuvant (Difco Laboratories). The ewes were injected at 1 month intervals for 6 months with 0.4mg of protein and jugular blood was drawn every 2 weeks. For some experiments the IgG fraction was isolated from whole serum by $(NH_4)_2SO_4$ precipitation and DEAE-cellulose (Whatman DE-52) chromatography as described by Foxwell & Tanner (1981). The purity of the IgG fraction was tested using SDS/polyacrylamide-gel electrophoresis. Antibodies were stored at −80°C, or at −25°C in 50% (v/v) glycerol.

Ouchterlony double-immunodiffusion analysis

Immunoprecipitation reactions were carried out by double diffusion in 1% agarose/0.9% NaCl plates with or without 0.5% Triton X-100 for 48h at 5°C or at room temperature. After a 6 day washing with 0.9% NaCl the plates were stained with Coomassie Blue R 250 or Amido Black, destained and photographed.

Iodination of the 32000 M_r protein

For iodination the excess of Triton X-100 in the purified uncoupling protein was removed by sucrose-gradient centrifugation (Lin & Klingenberg, 1980; Ricquier *et al.*, 1982*b*); then the protein was freeze-dried. The labelling procedure was performed according to the chloramine-T method (Hunter & Greenwood, 1962); 2μg of protein was dissolved in 20μl of 0.1M-acetate buffer (pH 5.6) and 1mCi (10μl) of $Na^{125}I$ was added followed by 40μl of 0.1M-acetate buffer, pH 5.6. The reaction was initiated by the introduction of chloramine-T (100μg in 10μl of 0.1M-acetate buffer, pH 5.6) and stopped 45s later by the addition of sodium metabisulphite (240μg in 100μl of 0.1M-acetate buffer, pH 5.6). The labelled 32000 M_r protein was purified by gel filtration on a Sephadex G-100 column (0.9cm × 60cm) using 0.1M-barbitone buffer (pH 8.6) containing 0.3% bovine serum albumin and 0.02% merthiolate as eluant.

Binding studies with sheep plasmas

The labelled protein was incubated for 4 days at 4°C with the plasmas at multiple initial dilutions (1:10, 1:100 and 1:1000). The incubation volume was 0.4ml, comprising 0.1ml of labelled protein, 0.1ml of diluted plasma and 0.2ml of diluent. 'No-antibody' controls were included in each assay to quantify incubation damage. The peak fraction 22 of the elution profile corresponding to the iodinated 32000 M_r protein was diluted 1:300 to obtain about 5000–10000c.p.m./0.1ml. The diluent used was 0.025M-barbitone buffer (pH 8.6) containing peptidase inhibitors (Garel *et al.*, 1974). At the end of the incubation, bound and free labelled fractions were separated on talcum powder (Garel *et al.*, 1974). The ratio of bound (B) to free (F) 32000 M_r protein was calculated by using a correction for non-specific binding determined from the control tubes.

SDS/polyacrylamide-gel electrophoresis

SDS/polyacrylamide-gel electrophoresis was performed in rod or slab gels at homogeneous (10%) or gradient (10–20%) polyacrylamide concentrations mainly as described in Ricquier & Kader (1976). For counting of ^{125}I radioactivity in gels, non-stained rods were frozen on solid CO_2, cut in 1mm thick slices and counted in a γ-spectrometer (Kontron MR 480).

Purification of the mitochondrial ADP/ATP translocator

ADP/ATP carrier from beef heart mitochondria was a gift from Professor M. Klingenberg (Munich). Rat liver ADP/ATP carrier (carboxyatractylate-binding protein) was purified by using the method described in Klingenberg *et al.* (1979); its purity (apparent M_r 30000) was checked by SDS/polyacrylamide-gel electrophoresis.

Immunohistofluorescence procedure

For the immunofluorescence method small pieces of brown adipose tissue (4mm diameter) were fixed in alcoholic Bouin's fluid for 24h and then dehydrated before embedding in paraffin wax. Paraffin was removed from the sections (5μm thick) with toluene. The sections were then treated in the usual way (Dubois, 1977). The sections were incubated with ewe antiserum (1:50, 1:100, 1:200 and 1:400 dilutions) for 2h in a moist chamber at 5°C; after 3 x 3min washes with veronal-buffered saline, the sections were covered with rabbit anti-(ewe γ-globulin) conjugated with fluorescein isothiocyanate (Institut Pasteur, Paris, France) for 1h. The sections were then washed with the veronal-buffered saline, incubated in Evans Blue (0.1mg/litre) for 10min, washed with water, mounted in glycerol/veronal-buffered saline (4:1, v/v) and examined in a Leitz fluorescence microscope. The specificity of immunofluorescence was ascertained by using: 1, non-immunized-ewe plasma; 2, incubation without specific antibody; and 3, incubation of sections with antiserum saturated with antigen (10μg of antigen in 1ml of 1:200 diluted antiserum).

Results

The presence of antibodies against the 32000 M_r uncoupling protein and the cross-reactivity of plasmas were checked with the iodinated antigen–antibody reaction, double immunodiffusion, inhibition of GDP-binding activity and immunohistofluorescence.

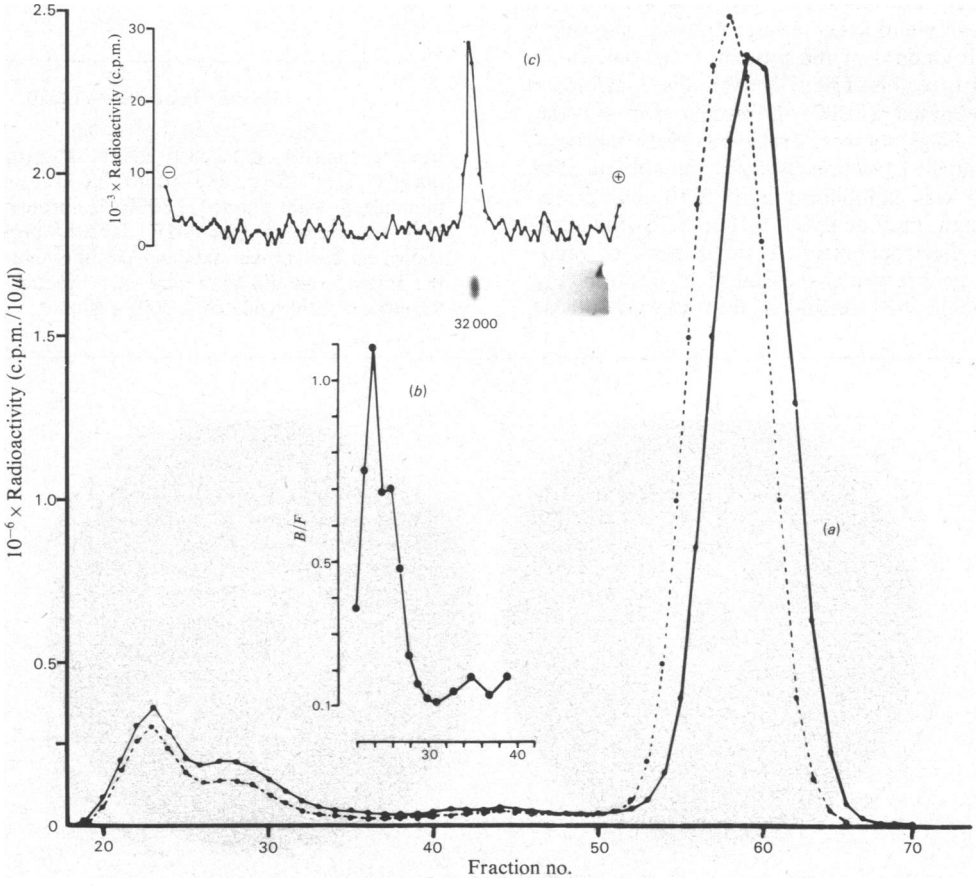

Fig. 1. *Iodination of the purified 32000 M_r protein*

(*a*) Elution profile on Sephadex G-100 of the reaction medium containing the 32000 M_r protein. The first peak (fractions 21–24) represents the pure labelled protein, the second peak (fractions 26–32) probably labelled Triton X-100, and the major peak the radioactive iodine. The solid and broken lines represent two separate experiments. (*b*) Immunoreactivity of the radioactive fractions 20–40. (*c*) A portion (10μl) of the radioactive fraction 22 was subjected to SDS/polyacrylamide-gel electrophoresis; the rod was sliced and radioactivity counted. The spot (Coomassie Blue) under the peak represents 5μg of pure 32000 M_r protein coelectrophoresed with the labelled protein.

Iodination of the protein and immunoreactivity test

The iodination efficiency (ratio between radioactivity of protein and total radioactivity eluted from the column) was about 15% using our procedure (Fig. 1*a*). As demonstrated by the immunoreactivity and the SDS/polyacrylamide-gel electrophoresis analysis (Figs. 1*b* and 1*c*) the purification on Sephadex G-100 was sufficient to obtain a good separation of the iodinated 32000 M_r protein. The immunoreactivity of the different fractions was determined by incubation during 72h at 4°C of plasma from the third puncture of ewe no. 363 (final dilution 1:40). Then immunoreactive fractions were localized in tubes nos. 21 to 24 (Fig. 1*b*); the highest immunoreactivity was found in fraction 22 which corresponded to the peak fraction of the elution profile. A poor immunoreactivity was observed in fractions 26–36 (Fig. 1*b*) corresponding to the second peak of the elution profile. These fractions on SDS/polyacrylamide-gel electrophoresis showed a very small amount of the protein. Fractions 26–36 contained probably Triton X-100. The localization of the iodinated 32000 M_r protein was substantiated by SDS/polyacrylamide-gel electrophoresis. The ^{125}I-labelled fraction 22 from the column (Fig. 1*a*; 10μl) was solubilized with SDS and β-mercaptoethanol and analysed using SDS/polyacrylamide-gel electrophoresis. A single peak of radioactivity was observed at the 32000 M_r position (Fig. 1*c*). It could be concluded that the 32000 M_r uncoupling protein was in the fractions 21–24 of the eluate of the column. The binding studies with sheep plasma using the iodinated 32000 M_r protein revealed the presence of antibodies in immunized ewes. The antibody titration curves obtained using the plasma of the third puncture from ewe no. 363 is given in Fig. 2 as an example. Antibodies were

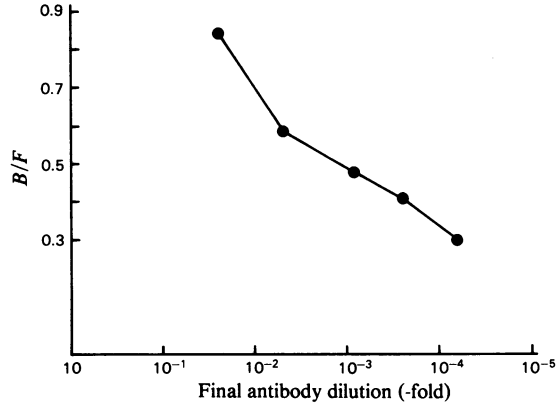

Fig. 2. *Antibody titration*
Binding capacity of labelled 32000 M_r protein to plasma from sheep 363 (third blood sampling) immunized with purified 32000 M_r protein. The ratio of bound to free (B/F) labelled protein is shown on the vertical axis and the final dilution of the antibody on the horizontal axis. Fraction 22 of the elution profile (diluted 1:300) was used.

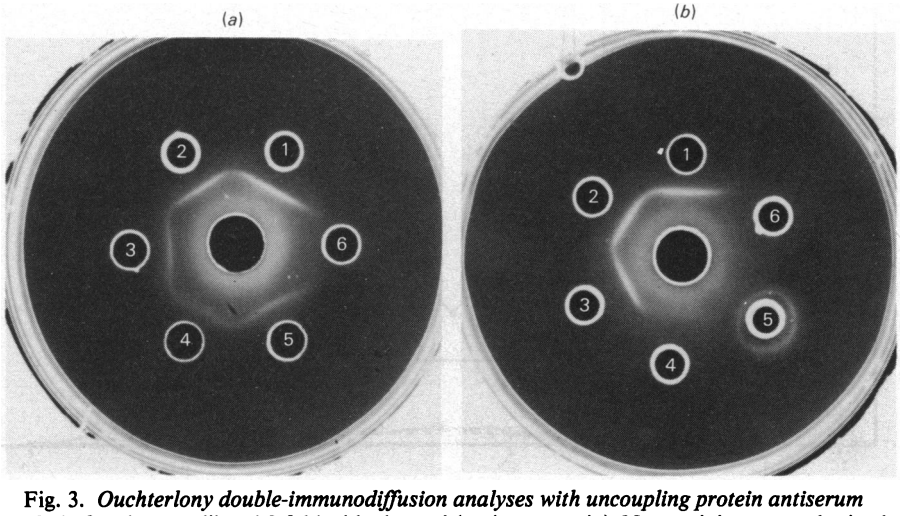

Fig. 3. *Ouchterlony double-immunodiffusion analyses with uncoupling protein antiserum*
Centre wells: 50μl of antiserum diluted 2-fold with glycerol (anti-rat protein). No precipitate was obtained with serum from non-immunized ewe. The same results were observed with IgG fractions. (*a*) Surrounding wells 1–4 contained respectively 20μl of pure and 2-, 4- or 8-fold diluted cold-adapted rat 32000 M_r protein solution (0.6μg/μl) purified using hydroxyapatite filtration. Well 5 contained 20μl of partially purified rat protein (1μg/μl) using affinity chromatography and well 6 contained 20μl of 2% Triton X-100 solution. (*b*) Outer wells contained purified protein from brown adipose tissue of respectively cold-adapted hamster (well 1, 9μg), new-born guinea-pig (well 2, 8μg), new-born rabbit (well 3, 6μg), lamb foetus (well 4, 22μg). Well 5 contained ADP/ATP carrier from beef heart mitochondria (20μg) and well 6 contained ADP/ATP carrier from rat liver mitochondria (16μg).

present at a low titre during the course of the immunization procedure and reached a plateau on the third blood sampling 6 weeks after the first immunization.

Ouchterlony double diffusion

Ewe antiserum raised against the purified uncoupling protein from brown adipose tissue mitochondria from cold-adapted rats showed a single immunoprecipitation line with the purified protein and with the partially purified protein obtained from affinity chromatography experiments (Fig. 3*a*).

Inhibition of the GDP-binding activity

The presence of antibodies was also substantiated by the inhibition of the GDP-binding activity of detergent-solubilized mitochondrial membranes, or of purified protein, when they were preincubated with antiserum for 2h before the assay (Table 1).

Cross-reaction studies

Cross-reactions of antiserum against the uncoupling proteins of cold-adapted rat brown adipose tissue were analysed by using double immunodiffusion and immunohistofluorescence. For the purified 32000 M_r uncoupling protein a single precipitin line was observed with uncoupling protein from rat, hamster, new-born guinea-pig and new-born rabbit; no marked precipitation was observed with the protein from lamb foetus (Fig. 3*b* and Table 2). The same observation was made with detergent-solubilized mitochondrial membranes of brown adipose tissue from different animals; the precipitation line was thinner for room temperature rats than for cold-adapted rats (Table 2). No pre-

Table 1. *Inhibition of the GDP-binding activity of the uncoupling protein by antiserum*

The assays were carried out as described in Lin & Klingenberg (1980) and Ricquier *et al.* (1982).

Uncoupling protein fractions	Sheep antiserum added	GDP binding (nmol/mg of protein)
—	50 μl	0.01
Triton X-100-solubilized mitochondrial membranes		
Cold-adapted rat	Non-immunized (50 μl)	2.93
	Immune (50 μl)	0.03
Rabbit	Non-immunized (50 μl)	2.05
	Immune (50 μl)	0.62
Isolated 32000 M_r protein*		
Cold-adapted rat	Non-immunized (100 μl)	4.1
	Immune (100 μl)	0.25
Cold-adapted hamster	Non-immunized (100 μl)	5.0
	Immune (100 μl)	0.24

* Hydroxyapatite pass-through fraction.

Table 2. *Cross-reaction studies with anti-(rat uncoupling protein)*

Data were obtained from double immunodiffusions.

Antigen added (20 μl)	Cross-reaction with anti-(uncoupling protein) from rat brown adipose tissue mitochondria (50 μl)
Triton X-100 solubilized mitochondrial membranes (1 mg)*	
Brown adipose tissue from cold-adapted rat	++
Brown adipose tissue from room temperature rat	+
Brown adipose tissue from cold-adapted hamster	++
Brown adipose tissue from new-born rabbit	++
Brown adipose tissue from lamb foetus	—
Liver of cold-adapted or room temperature rat	—
Liver of cold-adapted hamster	—
Muscle of cold-adapted or room temperature rat	—
Muscle of cold-adapted hamster	—
Purified brown adipose tissue 32000 M_r protein (4–12 μg)	
Cold-adapted rat	++
Room temperature rat	++
Cold-adapted hamster	++
New-born guinea-pig	++
New-born rabbit	++
Lamb foetus	—
Hydroxyapatite pass-through of solubilized liver or muscle mitochondrial membranes (10–15 μg)*	
Cold-adapted rat	—
Cold-adapted hamster	—
Purified ADP/ATP carrier (15–20 μg)	
Beef heart	—
Rat liver	—

* Detergent-solubilized membranes and hydroxyapatite pass-through fractions of solubilized liver and muscle mitochondrial membranes were prepared as for brown adipose tissue mitochondria.

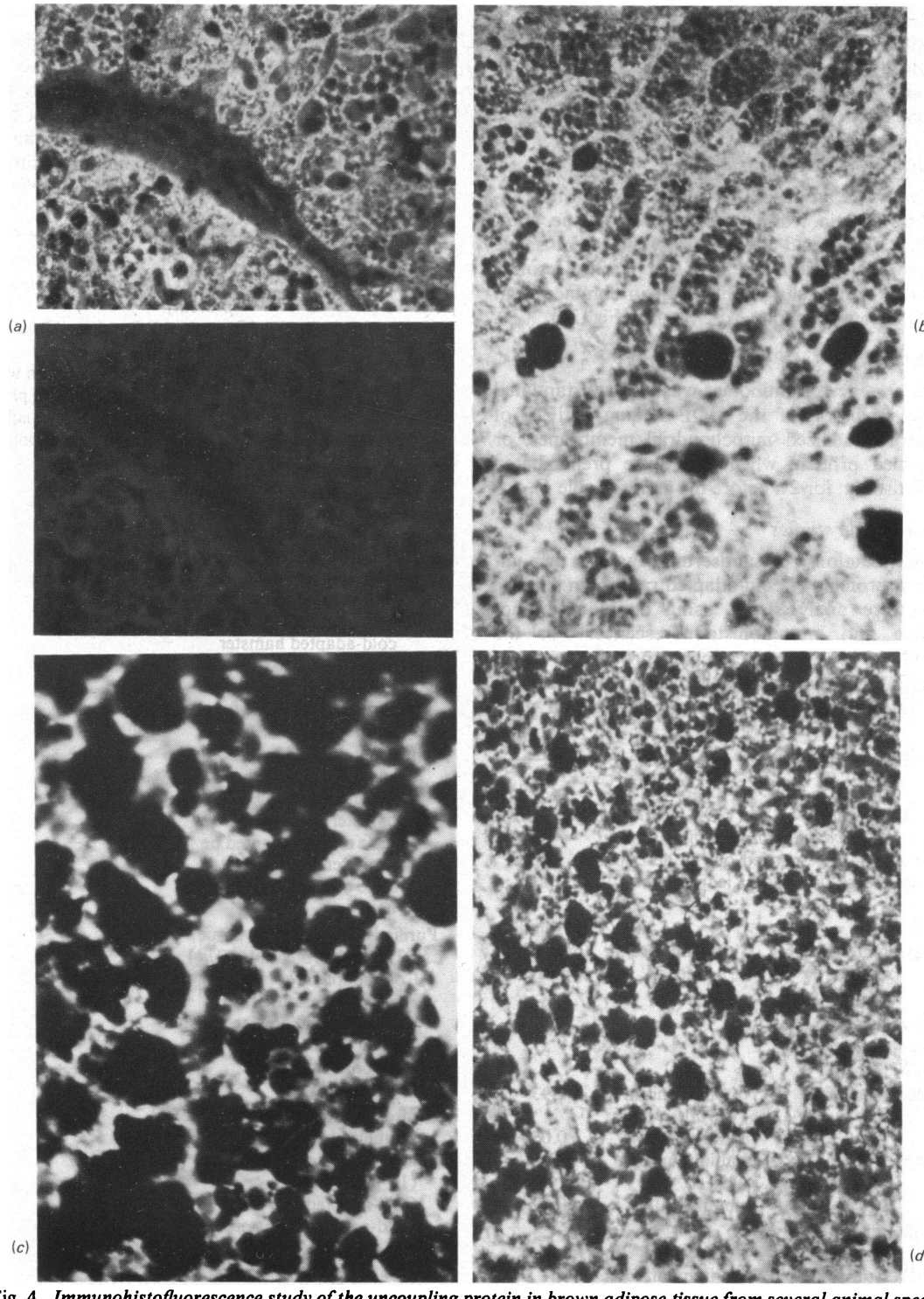

Fig. 4. *Immunohistofluorescence study of the uncoupling protein in brown adipose tissue from several animal species* Antibodies against rat protein diluted 1:200 were used. (*a*) Brown adipose tissue from cold-adapted rat (upper part, immunofluorescence; lower part, inhibition in presence of added 32000 M_r protein); (*b*) brown adipose tissue from cold-adapted hamster; (*c*) brown adipose tissue from new-born guinea pig; (*d*) brown adipose tissue from new-born rabbit. Inside the brown adipocytes the immunofluorescence can be visualized in the cytoplasm, around the black lipid droplets. The inhibition of the fluorescence when 32000 M_r protein was added to the incubation medium of slices was obtained for each animal species; added ADP/ATP carrier could not extinguish the fluorescence. Magnification 400×.

cipitation occurred when the ADP/ATP carrier from beef heart or rat liver mitochondria was used as an antigen, even at high concentrations (Fig. 3*b* and Table 2). Moreover, no precipitin line was observed with solubilized mitochondrial membranes from liver and muscle and with the corresponding hydroxyapatite pass-through fractions prepared by the same purification procedure as brown adipose tissue uncoupling protein.

Immunofluorescence staining was observed in brown adipose tissue sections from cold-adapted rat, cold-adapted hamster, new-born guinea-pig and new-born rabbit. The fluorescence was localized in the cytoplasm between fat droplets; it was intense with rat and hamster tissue (Fig. 4).

Discussion

Several groups have prepared antibodies against mitochondrial membrane proteins, mainly with yeast cytochrome *c* oxidase and ATPase (see Cabral & Schatz, 1979; Sebald & Wild, 1979) and for bovine heart ADP/ATP translocator (Eiermann *et al.*, 1977). Nevertheless, immunological studies of proteins of membrane organelles are not so far developed as those of soluble proteins. This is the first report of antibody production against the uncoupling protein of brown adipose tissue mitochondria.

The presence of antibodies was established by using ^{125}I labelling of the antigen followed by immunoreactivity titration, double immunodiffusion and immunohistofluorescence. The data were confirmed by the inhibition of the GDP-binding activity of mitochondrial brown adipose tissue membranes and of purified 32000 M_r protein. Immunotitration data show that antibody titre is not so high as with hormone antibodies, which is in agreement with the low antigenicity of some membrane proteins.

Antibodies were prepared against the uncoupling protein isolated from brown adipose tissue of cold-adapted rats, and it was observed that there was no species-specificity between rat, hamster, guinea-pig and rabbit; thus the 32000 M_r proteins isolated from different species possess similar antigenic determinants. A similar conclusion was drawn by Eiermann *et al.* (1977) for the ADP/ATP translocator of one particular organ, but they also concluded an organ specificity of the immunological properties of the carrier. We did not observe any cross-reaction of anti-(uncoupling protein) plasma with the ADP/ATP carrier isolated either from rat liver mitochondria or from beef heart mitochondria, which indicates that uncoupling protein and ADP/ATP translocator, although they are somewhat similar proteins, do not have common antigenic determinants that are recognized by the antisera used. It was obviously more interesting to look at a possible cross-reaction with the ADP/ATP carrier isolated from rat brown adipose tissue mitochondria (and not from liver) but we failed to obtain it pure and it was always contaminated by uncoupling protein to an extent of 50%. Contrary to the rodents and lagomorph studied, no immunoprecipitation could be observed with the 32000 M_r protein isolated from the mitochondria of lamb brown adipose tissue; it may result from a different composition or structure of the protein in this species. Cross reactions of antibodies with mitochondrial protein fractions isolated from liver or muscle of cold-adapted or room temperature animals were negative. These data show that the 32000 M_r uncoupling protein of brown adipose tissue is specific for this organ and that other tissues do not synthesize such a component in significant amount. This conclusion makes exciting the idea to look at the synthesis and processing system of this specialized and unique membrane protein. This is now possible since we have the antibodies available. However, the data obtained do not prove that the antibodies exhibit no cross-reactivity with other proteins which might have been present as contaminants during immunization.

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