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A Rapid Immunological Procedure for the Isolation of Hormonally Sensitive Rat Fat-Cell Plasma Membrane

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1. A rapid method for the isolation of hormonally sensitive rat fat-cell plasma membranes was developed by using immunological techniques. 2. Rabbit anti-(rat erythrocyte) sera were raised and shown to cross-react with isolated rat fat-cells. 3. Isolated rat fat-cells were coated with rabbit anti-(rat erythrocyte) antibodies, homogenized and the homogenate made to react with an immunoadsorbent prepared by covalently coupling donkey anti-(rabbit globulin) antibodies to aminocellulose. Uptake of plasma membrane on to the immunoadsorbent was monitored by assaying the enzymes adenylate cyclase and 5'-nucleotidase and an immunological marker consisting of a ^{125}I-labelled anti-(immunoglobulin G)–anti-cell antibody complex bound to the cells before fractionation. Contamination of the plasma-membrane preparation by other subcellular fractions was also investigated. 4. By using this technique, a method was developed allowing 25–40% recovery of plasma membrane from fat-cell homogenates within 30min of homogenization. 5. Adenylate cyclase in the isolated plasma-membrane preparation was stimulated by 5μM-adrenaline.

A variety of lipolytic and anti-lipolytic hormones have been shown to exert their primary action at the fat-cell plasma membrane (Rodbell, 1970). Investigation of the mechanism of action of these hormones would be aided by the isolation of purified membrane fragments.

The ability to raise cross-reacting rabbit antisera against various isolated rat cells suggested the possibility of using the reaction of such antibodies with cell membranes as the basis for an immunological isolation of plasma-membrane fragments (Hales, 1972). In the present paper an immunological procedure is described for the rapid isolation of hormonally sensitive plasma-membrane fragments from rat fat-cell homogenates. A preliminary report of this work has been presented (Luzio *et al.*, 1974).

Experimental

Materials

Collagenase was obtained from Worthington Biochemical Corp., Freehold, N.J., U.S.A.; bovine serum albumin (fraction V) was from Armour Pharmaceutical Co., Eastbourne, Sussex, U.K.; adrenaline was from Hopkin and Williams Ltd., Chadwell Heath, Essex, U.K.; horse and rabbit IgG* (Cohn fraction II) were from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K.; electrophoretically pure DNAase 1 from bovine pancreas was supplied by Sigma Chemical Co., St. Louis, Mo., U.S.A.; pyruvate kinase was from Boehringer Corp. (London) Ltd., London W.5, U.K.; Swinny filter holder was from Millipore Corp., Bedford, Mass., U.S.A.; polytetrafluoroethylene/glass homogenizers were from Jencons Ltd., Hemel Hempstead, Herts., U.K.; [2-^{3}H]AMP (5000mCi/mmol) was from The Radiochemical Centre, Amersham, Bucks., U.K.; microgranular cellulose powder (CC41) was from Whatman Biochemicals Ltd., Springfield Mill, Maidstone, Kent, U.K.

Antisera

Anti-(rat erythrocyte) sera were raised in mixed-strain rabbits fed *ad libitum* and kept at the Department of Biochemistry, University of Cambridge. Rat erythrocytes were prepared using heparin as an anti-coagulant, and then washed with Krebs–Ringer bicarbonate buffer (1.3mM-Ca^{2+}, pH7.4) (Cohen, 1957). Rabbits were injected subcutaneously at monthly intervals, with 1ml of packed erythrocytes per injection per rabbit. For the first three monthly injections approx. 0.5mg of heat-killed mycobacteria (to act as adjuvant) was injected into the rabbits with the cells. Rabbits were bled from an ear vein 10 days after the third and subsequent injections. Antisera from several bleeds of different rabbits were pooled to provide the rabbit anti-(rat erythrocyte) serum used in

* Abbreviations: IgG, immunoglobulin G; DNAase, deoxyribonuclease; EGTA, ethanedioxybis(ethylamine)-tetra-acetic acid.

the experiments described below. Rabbit anti-(rat erythrocyte) serum was heated at 56°C for 30min before use to inactivate complement (Lachmann, 1968).

Donkey anti-(rabbit globulin)-precipitating serum was obtained from Wellcome Reagents Ltd., Wellcome Research Laboratories, Beckenham, Kent, U.K.

Immunoadsorbents

Immunoadsorbents were prepared by coupling IgG to a diazonium derivative of powdered cellulose (Whatman CC41) by the method of Gurvich *et al.* (1961) as described by Miles & Hales (1968). IgG from rabbit anti-(rat erythrocyte) serum and from donkey anti-(rabbit globulin) serum used to make immunoadsorbents was prepared by sodium sulphate precipitation (Deutsch, 1967). For the preparation of rabbit IgG immunoadsorbents, 100mg quantities of IgG were coupled to 250mg quantities of diazocellulose. For the preparation of donkey and horse IgG immunoadsorbents, 100mg quantities of IgG were coupled to 100mg quantities of diazocellulose. After coupling, the immunoadsorbents were washed to remove non-covalently bound protein, and stored at 4°C in 0.05M-sodium veronal buffer, pH8.0, containing 5g of bovine serum albumin/litre, 12g of NaCl/litre, 200mg of NaN_3/litre and 20mg of non-immune IgG (rabbit IgG for rabbit IgG immunoadsorbents, or horse IgG for donkey and horse IgG immunoadsorbents)/litre.

Protein uptake on to diazocellulose in the preparation of immunoadsorbents was: for rabbit anti-(rat erythrocyte) IgG immunoadsorbent, 162 $\pm 10\mu g$ per mg of cellulose base; for rabbit non-immune IgG immunoadsorbent, $193 \pm 15\mu g$ per mg of cellulose base; for donkey anti-(rabbit globulin) IgG immunoadsorbent, $388 \pm 15\mu g$ per mg of cellulose base; and for horse non-immune IgG immunoadsorbent, $390 \pm 10\mu g$ per mg of cellulose base.

Before quantities of immunoadsorbent were used in the preparation of plasma membrane from cell homogenates, the immunoadsorbent was always washed three times with 2ml of homogenizing buffer, with centrifugation at 300*g* for 2min after each wash.

Preparation of fat-cells

Male Wistar rats (120–140g), bred in the laboratory or obtained from Ralph Tuck Ltd., Rayleigh, Essex, U.K., and given free access to food and water, were used in all experiments.

Rats were killed by decapitation and the fat-cells prepared from the epididymal fat-pads by the method of Rodbell (1964), as described by Siddle & Hales (1974).

Antibody-coated fat-cells

In experiments where fat-cell homogenates were made to react with donkey anti-(rabbit globulin) IgG immunoadsorbent or horse non-immune IgG immunoadsorbent it was necessary to coat the cells with rabbit anti-(rat erythrocyte) antibodies before homogenization. As a routine this was done by incubating the fat-cells from four rats in 20ml of Krebs–Ringer bicarbonate buffer (1.3mM-Ca^{2+}, pH7.4), containing 40mg of bovine serum albumin/ml and 0.2ml of rabbit anti-(rat erythrocyte) serum for 30min at 37°C.

Homogenization of fat-cells

Fat-cells were homogenized in Krebs–Ringer bicarbonate buffer (1.3mM-Ca^{2+}, pH7.4) containing 1mg of bovine serum albumin/ml. Before cell disruption the isolated fat-cells were washed with 4×10ml of the homogenizing buffer. Cells were homogenized either by ten strokes at 1400rev./min in a Potter–Elvehjem glass homogenizer fitted with a polytetrafluoroethylene pestle (clearance 76.2–127μm), or by repeated aspiration (10–15 times) into a plastic syringe fitted with a Swinny filter holder containing a stainless-steel photo-etched support screen, without a filter (Avruch & Wallach, 1971). The homogenized mixture was centrifuged at 300*g* for 2min at 4°C, and the pellet and infranatant formed below the fat-cake were removed and called the homogenate. As a routine, isolated fat-cells from four rats were homogenized to give 2.5ml of homogenate, of which 0.5ml was kept for the assay of total amounts of subcellular markers.

Incubation of homogenate with immunoadsorbents

Unless otherwise stated, the following procedure was adopted in the experiments described. The fat-cell homogenate (2ml) was again centrifuged at 300*g* for 2min at 4°C to give a 300*g* pellet. The supernatant from this centrifugation was incubated with 2mg of washed immunoadsorbent for 15min at 4°C. During this period, the incubation vessel was inverted at 5min intervals to ensure mixing. The incubation mixture was then centrifuged at 300*g* for 2min at 4°C, and the pellet washed with 2ml of homogenizing buffer and again centrifuged at 300*g* for 2min at 4°C. The initial 300*g* pellet and final immunoadsorbent pellet were both resuspended in 1ml of homogenizing buffer before assays were performed. By using the above procedure, four fractions were obtained, the initial 300*g* pellet and then after the incubation, a supernatant, wash and immunoadsorbent pellet.

Assays

To measure the purity of plasma-membrane preparations, a number of subcellular markers

were assayed. DNA was assayed as a nuclear marker by the method of Kissane & Robins (1958). In experiments where the DNA in a fraction was to be decreased by incubation with DNAase, 100μl samples of the fraction were incubated for 30min at 37°C with 4μg of DNAase before assay of the DNA. Cytochrome oxidase (EC 1.9.3.1) was assayed as a mitochondrial marker by the method of Cooperstein & Lazarow (1951). NADH–cytochrome *c* reductase (EC 1.6.99.3) was assayed as an endoplasmic-reticulum marker (McKeel & Jarett, 1970), by the method of Dallner *et al.* (1966), and lactate dehydrogenase (EC 1.1.1.27) as a cytosol marker by the method of Keiding *et al.* (1974). Subcellular markers were always assayed at least in triplicate in each experiment.

To assess plasma-membrane recovery, the enzymes adenylate cyclase (EC 4.6.1.1) and 5′-nucleotidase (EC 3.1.3.5) were assayed. In addition, plasma-membrane recovery was determined by measuring the amount of ^{125}I label in subcellular fractions after coating the intact fat-cells with a trace label consisting of a rabbit anti-(rat erythrocyte) antibody–^{125}I-labelled anti-(rabbit IgG) complex before homogenization. ^{125}I-labelled anti-(rabbit IgG) was prepared as previously described (Beck & Hales, 1975), and had been used to investigate the cross-reaction of rabbit anti-(rat cell) antibodies with isolated rat cells (Hales, 1972). In the present study, ^{125}I-labelled anti-(rabbit IgG) was used to label only a small proportion of the rabbit anti-(rat erythrocyte) antibodies coating the fat-cell surface. In practice 0.2ml of rabbit anti-(rat erythrocyte) serum was incubated with a portion of ^{125}I-labelled anti-(rabbit IgG) (approx. 100000c.p.m.) for 30min at 37°C, before this mixture was incubated with the fat-cells from four rats. The cells were then washed, homogenized and fractionated in the usual way as described above. Whole fractions (after removal of samples for other marker assays) were subsequently counted for radioactivity for a minimum of 5min in a Wallac Decem GTL 300 automatic gamma counter (counting efficiency approx. 50%). The uptake of ^{125}I label on to fat-cells measured by this labelling technique was 0.7±0.1% (4) of the initial label added to the rabbit anti-(rat erythrocyte) serum.

Adenylate cyclase was assayed by incubating 15μl samples of homogenate or subcellular fraction for 20min at 30°C with 15μl of assay medium containing 180mM-Tris/180mM-sodium β-glycerophosphate (adjusted to pH7.4 with HCl), 5mM-$MgSO_4$, 6mM-potassium phosphoenolpyruvate, 20mM-theophylline, 1mM-ATP, 4mM-EGTA and 1.5units of pyruvate kinase. Adrenaline (dissolved in 0.1M-HCl at 10mg/ml and rapidly diluted with water) or 300mM-NaF was added to incubations as 1μl portions when required. The incubation was terminated by boiling for 5min, and, after the addition of 30μl of 4.4mM-$CaCl_2$ per incubation tube, 10μl samples from each incubation tube were assayed for cyclic AMP content by radioimmunoassay (Siddle *et al.*, 1973).

5′-Nucleotidase was assayed by the radioassay of Avruch & Wallach (1971), modified as described previously (Newby *et al.*, 1975). Samples (50μl) of homogenate or subcellular fraction were incubated for 1h at 37°C with 500μl of assay mixture containing 50mM-Tris (adjusted to pH8.0 with HCl), 2mM-$MgCl_2$, 0.2mg of adenosine, 200μM-AMP and tracer [^{3}H]AMP (20000–30000c.p.m.). The incubation was terminated by the addition of 100μl of 0.15M-$ZnSO_4$. Unhydrolysed AMP was precipitated by the addition of 100μl of 0.15M-$Ba(OH)_2$. Then 500μl of supernatant was added to 5ml of scintillant and radioactivity determined in a scintillation counter, with internal standards. The scintillant was prepared by adding 8g of PPO (2,5-diphenyloxazole), 200mg of POPOP [1,4-bis-(5-phenyloxazol-2-yl)benzene] and 1 litre of Triton X-100 to 2 litres of toluene.

Protein was measured by the method of Lowry *et al.* (1951). To measure IgG coupled during the preparation of immunoadsorbents, the latter were washed with protein-free buffer before assay, and horse or rabbit IgG was used as standard. Protein contents of homogenates and subcellular fractions were assayed with bovine serum albumin as standard.

Membrane-protein uptake on to donkey anti-(rabbit globulin) IgG immunoadsorbent was measured after elution from the immunoadsorbent with 0.01M-HCl. This procedure breaks the rabbit IgG–donkey anti-(rabbit globulin) IgG bond (Beck & Hales, 1975). Direct measurement of plasma-membrane protein on the immunoadsorbent cannot be done, since the small amount of membrane protein is masked by the large amount of IgG covalently linked to the aminocellulose.

Samples (50μl) of the immunoadsorbent membrane complex prepared in homogenizing buffer as described were first washed to remove bovine serum albumin. Each 50μl sample was washed with 5×200–300μl of Krebs–Ringer bicarbonate buffer (1.3mM-Ca^{2+}, pH7.4), with centrifugation at 15000*g* for 1min between washes. The sample was then incubated overnight at 4°C with 120μl of 0.01M-HCl followed by centrifugation at 300*g* for 2min to remove immunoadsorbent. A 90μl portion of the supernatant was then added to 10μl of 10M-$HClO_4$. This mixture was then centrifuged at 15000*g* for 1min and the protein pellet formed was assayed. Samples of donkey anti-(rabbit globulin) IgG immunoadsorbent that had not been made to react with fat-cell homogenates were always run in the above assay to determine the background acid elution of protein from the immunoadsorbent. No measurable amounts of protein could be eluted

from samples of horse non-immune IgG immunoadsorbent that had been made to react with fat-cell homogenates.

Electron microscopy

For morphological investigation of immunoadsorbent membrane pellets, portions of the pellet were fixed for 15min at room temperature (20°C) in 0.1M-cacodylate buffer, pH7.4, containing 2.5% glutaraldehyde. Subsequent washing, post-fixation with OsO_4, dehydration, embedding and electron microscopic examination were performed as previously described (Hales *et al.*, 1974).

Results

In the initial experiments, antisera raised against rat erythrocytes were used as a source of anti-cell antibodies for preparation of immunoadsorbent, since it was thought that such antisera were unlikely to contain antibodies to cell membranes other than the plasma membrane, owing to the absence of intracellular organelles in erythrocytes. The use of rabbit anti-(rat erythrocyte) IgG immunoadsorbent in the preparation of plasma-membrane fragments from isolated rat fat-cells is shown schematically in Fig. 1.

No conditions were found under which plasma-membrane uptake on to rabbit anti-(rat erythrocyte) IgG immunoadsorbent was greater than on to rabbit non-immune IgG immunoadsorbent. Neither increasing the time of incubation with immunoadsorbent nor incubating with a larger amount of immunoadsorbent resulted in a greater specific uptake of plasma membrane on to rabbit anti-(rat erythrocyte) IgG immunoadsorbent. However, nonspecific uptake of plasma membrane on to immunoadsorbent (or on to cellulose or aminocellulose) could be increased by long incubations with large amounts of immunoadsorbent. More than 20% of fluoride-stimulated adenylate cyclase was recovered in the aminocellulose pellet after the incubation of 4ml of fat-cell homogenate with 20mg of aminocellulose for 6h at 4°C. This same pellet contained over 20% of homogenate cytochrome oxidase, so that non-specific uptake of membrane on to aminocellulose could not be used as a preparative method for plasma membranes (Luzio, 1972).

An alternative approach for the immunological preparation of fat-cell plasma membranes was to coat the cells with rabbit anti-(rat erythrocyte) antibody before homogenization, and then to make

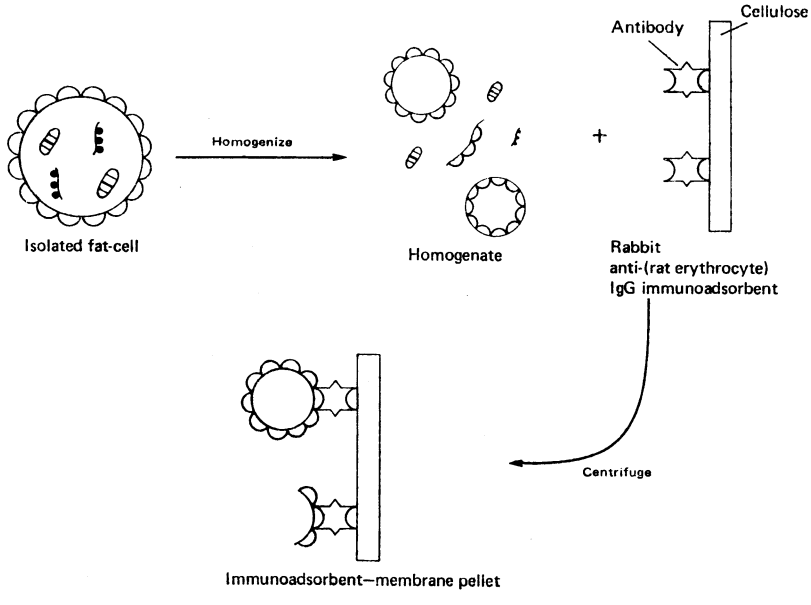


Fig. 1. *Diagrammatic representation of the use of rabbit anti-(rat erythrocyte) IgG immunoadsorbent in the preparation of plasma-membrane fragments from isolated rat fat-cells*

Isolated rat fat-cells were homogenized and centrifuged briefly at low speed to remove the fat-cake and large cell debris. The resulting homogenate which contained open and resealed plasma-membrane fragments was made to react with rabbit anti-(rat erythrocyte) IgG immunoadsorbent, which was then isolated by brief centrifugation at low speed.

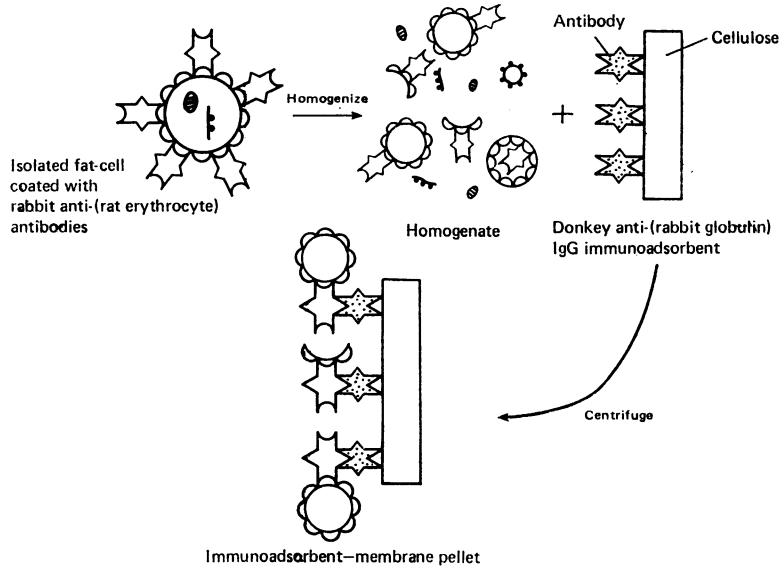


Fig. 2. *Diagrammatic representation of the use of donkey anti-(rabbit globulin) IgG immunoadsorbent in the preparation of plasma-membrane fragments from isolated rat fat-cells coated with rabbit anti-(rat erythrocyte) antibodies*

Isolated rat fat-cells were coated with rabbit anti-(rat erythrocyte) antibodies, washed, homogenized and centrifuged briefly at low speed to remove the fat-cake and large cell debris. The resulting homogenate which contained open and resealed plasma-membrane fragments was made to react with donkey anti-(rabbit globulin) IgG immunoadsorbent, which bound antibody-coated plasma-membrane fragments and was then isolated by brief centrifugation at low speed.

the homogenate containing antibody-coated plasma-membrane fragments react with donkey anti-(rabbit globulin) IgG immunoadsorbent. This is shown schematically in Fig. 2, and by using this method, greater uptake of plasma membrane on to donkey anti-(rabbit globulin) IgG immunoadsorbent relative to horse non-immune IgG immunoadsorbent was shown.

The time-course of uptake on to donkey anti-(rabbit globulin) IgG immunoadsorbent of plasma-membrane fragments from homogenates of rabbit anti-(rat erythrocyte) antibody-coated fat-cells was investigated, and it was found that uptake was maximal at 30min and 80% of maximal at 15min (Fig. 3). Alteration of the quantity of immunoadsorbent used in the incubation also affected recovery of plasma membrane (Fig. 4). Since it was desirable to develop a rapid isolation of plasma membrane by using small, economical quantities of immunoadsorbent, a standard technique was developed whereby 2ml of homogenate prepared from the antibody-coated cells of four rats was made to react with 2mg of immunoadsorbent for 15min at 4°C. A flow diagram illustrating the overall fractionation scheme developed is shown in Scheme 1.

By using the fractionation scheme shown in Scheme 1, the recovery of plasma-membrane markers in the donkey anti-(rabbit globulin) IgG immunoadsorbent pellet was compared with the recovery in a horse non-immune IgG immunoadsorbent pellet, and contamination of this fraction with other subcellular markers was also examined. The results of these experiments are summarized in Tables 1 and 2. The recovery of the plasma membrane markers 5′-nucleotidase, basal adenylate cyclase, fluoride-stimulated adenylate cyclase and membrane-bound ^{125}I-labelled anti-(rabbit IgG) in each fraction showed good agreement, although for fluoride-stimulated adenylate cyclase the total recovery of initial homogenate activity was low. The specific activities of the plasma-membrane enzymes 5′-nucleotidase, basal and fluoride-stimulated adenylate cyclase were increased 7.6±1.4-, 8.0±2.7- and 5.8±1.3-fold respectively in the donkey anti-(rabbit globulin) IgG immunoadsorbent plasma membrane pellet relative to the homogenate values. Allowing for complete recovery of the initial homogenate enzyme activity these values imply a purification of plasma membrane from homogenate of 7.8-, 7.7- and 8.1-fold respectively. Contamination of this immunoadsorbent

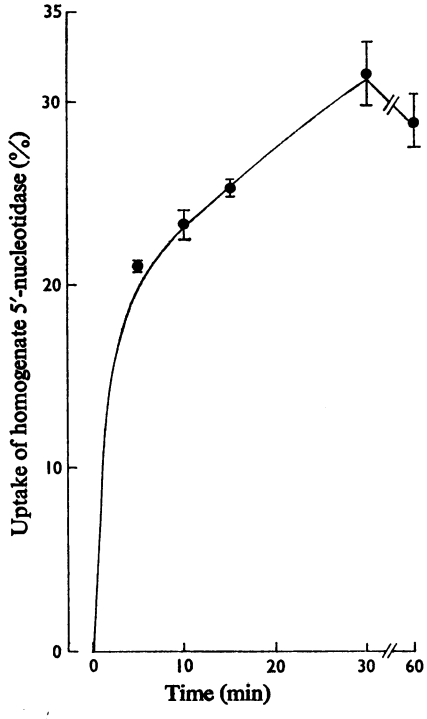


Fig. 3. *Time-course of uptake of plasma-membrane fragments from homogenates of rabbit anti-(rat erythrocyte) antibody-coated rat fat-cells, on to donkey anti-(rabbit globulin) IgG immunoadsorbent*

Isolated fat-cells from four rats were coated with rabbit anti-(rat erythrocyte) serum, washed, homogenized and incubated with 2mg of donkey anti-(rabbit globulin) IgG immunoadsorbent as described in the text. The time of incubation of homogenate with immunoadsorbent varied in different experiments. Each point on the graph represents the mean±S.E.M. of three determinations of the percentage uptake of homogenate 5'-nucleotidase on to immunoadsorbent.

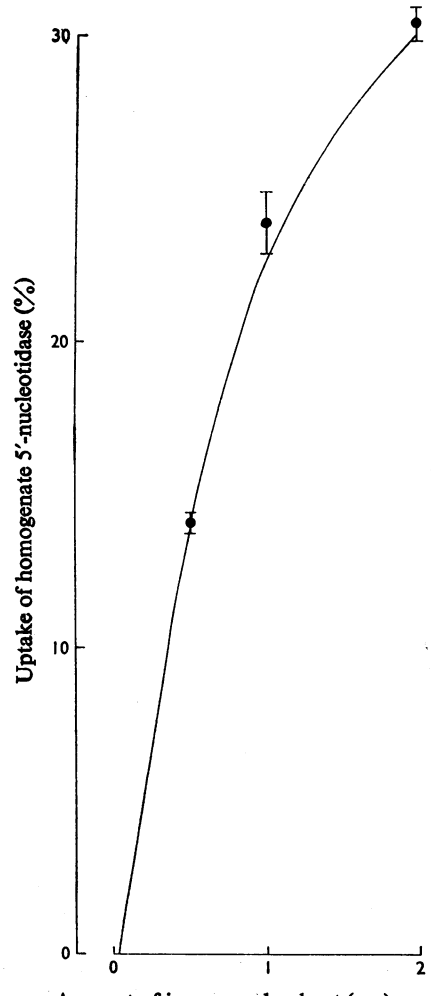


Fig. 4. *Uptake of plasma-membrane fragments from homogenates of rabbit anti-(rat erythrocyte) antibody-coated rat fat-cells on to donkey anti-(rabbit globulin) immunoadsorbent*

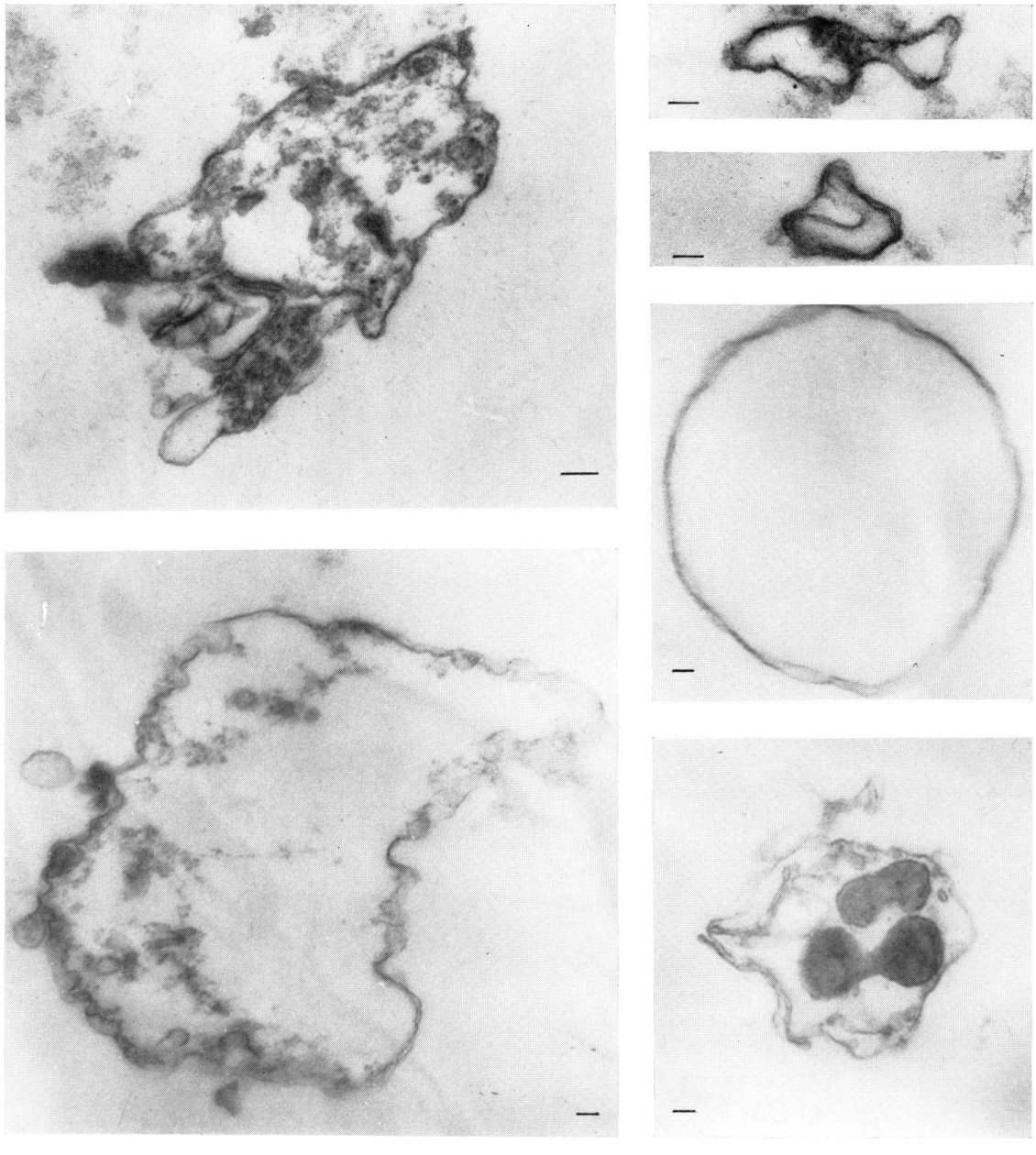
Isolated fat-cells from four rats were coated with rabbit anti-(rat erythrocyte) serum, washed, homogenized and incubated with different amounts of donkey anti-(rabbit globulin) IgG immunoadsorbent as described in the text. Each point on the graph represents the mean±S.E.M. of three determinations of the percentage uptake of homogenate 5'-nucleotidase on to immunoadsorbent.

plasma-membrane pellet by other subcellular markers was low except for DNA.

Preliminary electron-microscopic investigation of the immunoadsorbent membrane preparation showed that it consisted largely of closed vesicles up to 2.0μm in diameter, often with smaller vesicles inside them (Plate 1). Very few mitochondria or nuclei were seen in the preparation. This morphological appearance is very similar to that reported for fat-cell plasma membrane prepared on density gradients (McKeel & Jarett, 1970; Jarett, 1974).

The adrenaline sensitivity of the adenylate cyclase in fat-cell plasma membrane prepared immunologically as described in Scheme 1, was also investigated. The adenylate cyclase in the membrane preparation retained fluoride sensitivity and was also activated by adrenaline at concentrations of 5μM and greater (Table 3).

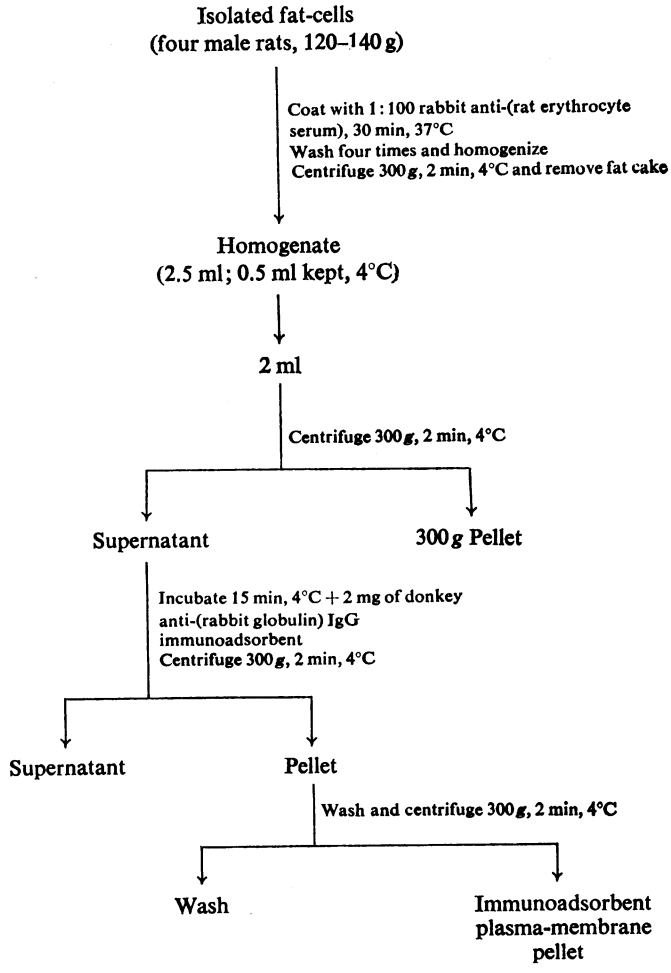
The high recovery of homogenate DNA in the donkey anti-(rabbit globulin) IgG immunoadsorbent membrane pellet appeared to be due to non-specific



EXPLANATION OF PLATE 1

Electron micrographs of the immunoadsorbent membrane preparation

Several types of profile are observed, usually being large vesicles, which often contain smaller vesicles and occasionally intracellular organelles. The bars represent 0.1 μm.



Scheme 1. *Schematic representation of the fractionation scheme developed for the immunological preparation of rat fat-cell plasma membrane*

uptake on to the aminocellulose, as it was also obtained if horse non-immune IgG immunoadsorbent or aminocellulose were incubated with the homogenate. To lessen DNA contamination of the immunoadsorbent membrane pellet, the fractionation scheme used was modified to remove a greater proportion of the nuclei (with minimal loss of plasma membrane) before incubation of the homogenate with immunoadsorbent. A series of fractionations were carried out in which, after removal of the fat-cake, the homogenate was centrifuged at 1000*g* for 2min at 4°C (replacing the 300*g* centrifugation step), and the supernatant formed then incubated with donkey anti-(rabbit globulin) IgG immunoadsorbent in the usual manner. The 1000*g* pellet contained a similar percentage of homogenate plasma membrane to the 300*g* pellet, but was 2–3-fold enriched in DNA. Recovery of plasma membrane in the immunoadsorbent pellet in these experiments was 25.0±1.1% (6), with recovery of homogenate DNA in this pellet decreased to 8.3±1.6% (6). This latter value was higher than that in an equivalent non-immune horse IgG immunoadsorbent pellet [2.4±0.8% (3)] but could be lowered to 2.2±0.5% (3) by incubation with DNAase. Recovery of other subcellular markers and of membrane protein showed that, with the exception of DNA, the purity of this membrane preparation was not significantly different from that described above.

A number of modifications of the basic fractionation scheme shown in Scheme 1 were investigated to examine the parameters affecting recovery of

Table 1. *Recovery of plasma-membrane markers in fat-cell fractions after immunological preparation of plasma membrane*

Isolated rat fat-cells were coated with rabbit anti-(rat erythrocyte) antibodies, washed, homogenized and fractionated as described in the text. In each experiment the cells from eight rats were fractionated, the homogenate being divided after the 300*g* centrifugation step into two equal portions, one incubated with donkey anti-(rabbit globulin) IgG immunoadsorbent (*a*) and the other with horse non-immune IgG immunoadsorbent (*b*). The values presented are means±S.E.M. of the pooled data from the number of experiments stated and are percentages of total recovered activity in each half of the eight rat experiments. The recovery of ^{125}I-labelled anti-(rabbit IgG) is the recovery of this label used as a plasma-membrane marker as described in the text. The recoveries of other subcellular markers in these experiments are shown in Table 2.

	Recovered activity (% of total)							
	5′-Nucleotidase		^{125}I-labelled anti-(rabbit IgG)		Adenylate cyclase (basal)		Adenylate cyclase (+10mM-NaF)	
Fraction								
300*g* Pellet	10.8±4.1		6.8±1.2		3.5±3.8		7.9±1.6	
	(*a*)	(*b*)	(*a*)	(*b*)	(*a*)	(*b*)	(*a*)	(*b*)
Supernatant	47.7±2.1	78.5±3.0	52.7±3.6	73.6±1.7	61.6±10.8	95.4±4.9	54.4±3.7	87.5±2.6
Wash	6.2±1.0	6.5±0.9	7.0±0.7	9.6±0.8	0.1±0.1	0.7±0.7	1.3±0.7	3.0±1.9
Immunoadsorbent pellet	35.3±2.4	4.2±0.6	33.5±3.8	10.0±1.1	34.8±7.8	0.4±0.4	36.4±4.0	1.6±0.9
No. of expts.	6		4		3		5	
Total homogenate activity recovered (%)	96.9±2.0		88.0±3.4		104.0±6.0		72.0±4.1	

plasma membrane on the immunoadsorbent. Recovery of plasma membrane from the homogenate could be increased by incubating the supernatant remaining after a first incubation with immunoadsorbent with a fresh portion of the immunoadsorbent. Although incubating the homogenate of the fat-cells from four rats with 4mg of immunoadsorbent produced no greater recovery of plasma membrane than incubating with 2mg of immunoadsorbent, recovery could be increased by a factor of 50% if the supernatant from the first incubation with 2mg of immunoadsorbent was itself incubated with a fresh portion of 2mg of immunoadsorbent. Uptake of the antibody-coated plasma-membrane fragments on to the immunoadsorbent could be prevented by incubating in the presence of an excess of rabbit non-immune IgG. A further parameter affecting recovery of plasma membrane on the immunoadsorbent was the amount of rabbit anti-(rat erythrocyte) serum used to coat the fat cells before homogenization (Table 4).

Discussion

Fragments of hormonally sensitive rat fat-cell plasma membrane could be rapidly isolated on to donkey anti-(rabbit globulin) IgG immunoadsorbent from homogenates of antibody-coated cells, although the direct use of rabbit anti-(rat erythrocyte) IgG immunoadsorbent proved unsuccessful. The reasons for the lack of success with the direct method are not clear. It is possible that in the indirect method the existence of an IgG molecule between the immunoadsorbent and the plasma membrane acts as a 'spacer arm' analogous to the hydrocarbon arm often required when Sepharose affinity adsorbents are used to isolate large molecules or subcellular structures (Krug *et al.*, 1971). Alternatively, it is possible that a far lower proportion of covalently bound IgG molecules on the rabbit anti-(rat erythrocyte) IgG immunoadsorbent had antibody activity than on the donkey anti-(rabbit globulin) IgG immunoadsorbent.

The recovery of plasma-membrane markers from fat-cell homogenates was comparable with the recoveries previously obtained by other workers using density-gradient-centrifugation techniques (Jarett *et al.*, 1971; Avruch & Wallach, 1971; Jarett, 1974). Three markers were used to measure plasma-membrane recovery, namely two enzyme markers, adenylate cyclase and 5′-nucleotidase, and a ^{125}I-labelled antibody. There was extremely good correlation between their recovery in the different fractions (Table 1). This close correlation contrasts with a report of Chang *et al.* (1975) that, during fractionation of isolated rat fat-cells, recovery of 5′-nucleotidase did not correlate with the recovery of cell-surface-membrane receptors labelled with ^{125}I-labelled insulin, ^{125}I-labelled cholera toxin and ^{125}I-labelled plant lectins. This seems more remarkable in view of the characterization of 5′-nucleotidase as an 'ectoenzyme' in several mammalian cell types, including fat-cells (Trams & Lauter, 1974; de Pierre & Karnovsky, 1974; Newby *et al.*, 1975), and the failure with fat-cells to demonstrate any intracellular component (Newby *et al.*, 1975).

Table 2. *Recovery of subcellular markers in fat-cell fractions after immunological preparation of plasma membrane*

Isolated rat fat-cells were coated with rabbit anti-(rat erythrocyte) antibodies, washed, homogenized and fractionated as described in the text. In each experiment the cells from eight rats were fractionated, the homogenate being divided after the 300*g* centrifugation step into two equal portions, one incubated with donkey anti-(rabbit globulin) IgG immunoadsorbent (*a*) and the other with horse non-immune IgG immunoadsorbent (*b*). The values presented are means±S.E.M. of the pooled data from the number of experiments stated and are percentages of total recovered activity in each half of the eight rat experiments. The recovery of plasma-membrane markers in these experiments is shown in Table 1.

Fraction	Recovered activity or content (% of total)									
	Cytochrome oxidase		NADH–cytochrome *c* reductase		Lactate dehydrogenase		DNA		Protein	
300*g* Pellet	2.6±0.3		2.6±0.3		7.0±0.1		26.5±6.6		7.4±2.1	
	(*a*)	(*b*)	(*a*)	(*b*)	(*a*)	(*b*)	(*a*)	(*b*)	(*a*)	(*b*)
Supernatant	80.7±5.8	84.5±4.1	85.8±2.7	91.9±1.8	83.2±0.9	86.8±0.7	49.3±5.5	54.4±6.6	81.8±5.3	82.0±7.6
Wash	10.9±3.1	10.2±1.5	4.6±0.8	4.0±0.8	5.6±0.1	4.5±0.1	10.6±3.9	9.0±3.5	6.3±1.8	14.3±3.3
Immunoadsorbent pellet	5.8±0.7	2.7±0.5	7.0±1.1	1.5±1.0	4.2±0.1	1.7±0.1	13.6±2.8	10.9±2.2	4.5±0.5	0
No. of expts.	5		5		3		3		6	
Total homogenate activity or content recovered (%)	107.6±5.5		97.0±5.3		100.0±1.1		103.0±5.5		100.9±8.1	

Table 3. *Adrenaline sensitivity of rat fat-cell plasma membrane isolated on donkey anti-(rabbit globulin) IgG immunoadsorbent*

Rat fat-cell plasma membranes were isolated on donkey anti-(rabbit globulin) IgG immunoadsorbent as described in the text. Plasma membrane was isolated from eight rats, and adenylate cyclase was assayed in the initial homogenate and in the immunoadsorbent–plasma-membrane fraction as described in the text. The values presented are means±S.E.M. of four determinations. ND, not determined.

	Adenylate cyclase activity (pmol of cyclic AMP formed/10min per mg of protein, at 30°C)	
	Homogenate	Immunoadsorbent–plasma-membrane pellet
Basal	342±26	2539±223
+NaF, 10mM	1375±79	7560±246
+Adrenaline, 5μM	ND	3505±276
+Adrenaline, 100μM	ND	7650±230

Table 4. *Recovery of plasma membrane on immunoadsorbent after preincubating the intact fat-cells with different concentrations of rabbit anti-(rat erythrocyte) serum*

In each experiment isolated fat-cells were prepared from four rats, washed and incubated with different amounts of rabbit anti-(rat erythrocyte) serum, in 20ml of Krebs–Ringer bicarbonate buffer (1.3mM-Ca^{2+}, pH7.4) containing 40mg of bovine serum albumin/ml, for 30min at 37°C. The cells were then washed and homogenized, and plasma membrane was isolated on 2mg of donkey anti-(rabbit globulin) IgG immunoadsorbent, as described in the text. The values presented are means±S.E.M. of three determinations of the percentage uptake of homogenate 5′-nucleotidase on to immunoadsorbent.

Amount of rabbit anti-(rat erythrocyte) serum incubated with fat-cells from four rats (μl)	Uptake of homogenate 5′-nucleotidase on to immunoadsorbent (%)
20	12.5±0.9
200	26.3±0.3
400	34.5±1.4
2000	41.5±1.3

The use of immunological markers to follow plasma membrane during cell fractionation has previously been reported, but mainly by indirect and only partially quantitative means (Kamat & Wallach, 1965; Gahmberg & Simons, 1970; Allan & Crumpton, 1970; Wilson & Boyle, 1972), although

Boone *et al.* (1969) followed ^{125}I-labelled horse antibody previously bound to intact cells, during preparation of plasma-membrane fragments from HeLa cells. The present work shows that the use of ^{125}I-labelled anti-IgG in following the distribution of cell-surface-bound antibodies during cell fractionation may well prove convenient.

Isolation of plasma-membrane fragments on solid-phase immunoadsorbents has advantages over classical density-gradient-centrifugation techniques. Indeed one may question the theoretical soundness of using differential centrifugation in a density gradient to isolate a randomly fragmented and resealed large subcellular structure. Whereas structures such as nuclei, mitochondria and lysosomes are reasonably homogeneous in size and shape, the same is not true of the sheets and vesicles of plasma-membrane fragments. The present method is rapid and uses mild preparative conditions (physiological buffer, low centrifugal force). The damage which may result from the subjection of fat-cell plasma membrane to the classical density-gradient technique includes loss of coupling between hormone receptors and adenylate cyclase (Jarett *et al.*, 1971, 1974). The immunological method is also applicable to small quantities of tissue and is theoretically capable of selecting vesicles of membrane which are not inside-out. The latter potential derives from the fact that intact cells are initially made to react with antibody. Therefore antibody on vesicles which are sealed and inside-out would not be available for reaction with the immunoadsorbent. Since a major interest in the isolation of membrane vesicles is the study of transport mechanisms, correct orientation of the vesicles is essential.

The capacity of the immunological technique for the preparation of plasma membrane should be adequate for structural studies. The isolated fat-cells of 40 rats (120–140 g body wt.) would require only 2 ml of the rabbit anti-(rat erythrocyte) serum and 20 mg of the donkey anti-(rabbit globulin) immunoadsorbent [which could be prepared from 2 ml of donkey anti-(rabbit globulin) serum] for the preparation of 1 mg plasma membrane protein. The specificity of the method may be manipulated by the use of purified antibodies directed against individual membrane proteins. For example, it has been shown that antibodies may be prepared which are capable of neutralizing the ectoenzyme 5′-nucleotidase (Newby *et al.*, 1975).

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