Complement-Mediated Production of Plasma-Membrane Vesicles from Rat Fat-Cells

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1. Rat isolated fat-cells were coated with rabbit anti-(rat erythrocyte) antibody and incubated with fresh guinea-pig serum for 25 min at 37°C, which resulted in a more than 95% release of the cytosolic enzyme lactate dehydrogenase. 2. Under these conditions fragmentation of the plasma membrane was examined by following the plasma-membrane markers 5'-nucleotidase, adrenaline-sensitive adenylate cyclase and membrane-bound rabbit immunoglobulin G through a differential-centrifugation fractionation procedure. 3. Approx. 50% of the plasma-membrane markers remained associated with triacylglycerol. Of the remainder more than half was pelleted by centrifugation at 10000g for 30 min. 4. The 10000g supernatant was fractionated by centrifugation on a sucrose density gradient (15–50%, w/w). This procedure resulted in the production of two visible white bands on the density gradient. The bands consisted of vesicles derived from the plasma membrane, since they coincided with peaks of 5'-nucleotidase activity, contained membrane-bound immunoglobulin G and the denser one had adenylate cyclase activity. The phospholipid and protein contents of the vesicles were determined and compared with those in purified plasma membrane. 5. It is suggested that complement-mediated lysis of rat fat-cells causes the production of plasma-membrane vesicles that differ in composition from the whole plasma membrane.

Complement proteins have been extensively studied in recent years to determine the molecular sequence of the complement cascade (Müller-Eberhard, 1975; Lachmann, 1975; Fearon & Austen, 1976; Porter & Reid, 1978). The effects of this cascade on cell membranes have been studied by using artificial phospholipid membrane systems (Kinsky, 1972; Lachmann *et al.*, 1973; Humphries & McConnell, 1975; Michaels *et al.*, 1976; Henry *et al.*, 1978), enucleate mammalian erythrocytes (Iles *et al.*, 1973; Nakamura *et al.*, 1976) and bacterial cells (Brunner *et al.*, 1976; Inoue *et al.*, 1977). Although these studies have led to a greater understanding of the biochemical events associated with complement-mediated cell lysis, relatively little information is available on the effects of complement on the membranes of nucleate cells.

Abbreviations used: p[NH]ppG, guanosine 5'-[β -imido]triphosphate; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; IgG, immunoglobulin G; SDS, sodium dodecyl sulphate.

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In the present study we have investigated complement-mediated lysis of rat isolated fat-cells, in order to examine its effects on the plasma membrane. The rat fat-cell plasma membrane is particularly suitable for such a study, since it has been purified by a number of techniques (Rodbell, 1967; McKeel & Jarett, 1970; Avruch & Wallach, 1971; Luzio *et al.*, 1976), is widely studied as a hormonally sensitive membrane system (Rodbell, 1967; Stein & Hales, 1972; Harwood *et al.*, 1973) and contains a well-defined ectoenzyme, 5'-nucleotidase (Newby *et al.*, 1975).

Anti-(rat erythrocyte) serum was used to initiate the complement sequence. This cross-reacted with rat fat-cells, but did not affect several membrane activities, including 5'-nucleotidase, adenylate cyclase and hormonal sensitivity (Luzio *et al.*, 1976).

Experimental

Materials

Collagenase was obtained from Worthington Biochemical Corp., Freehold, NJ, U.S.A.; bovine serum albumin (fraction V) was from Armour Pharmaceutical Co., Eastbourne, Sussex, U.K.; rabbit IgG (Cohn Fraction II) was from Koch-Light

Laboratories, Colnbrook, Bucks., U.K.; adrenaline was from Hopkin and Williams, Chadwell Heath, Essex, U.K.; NADH, creatine kinase and creatine phosphate were from Boehringer Corp., (London) Ltd., Lewes, Sussex, U.K.; Swinny filter holder was from Millipore Corp., Bedford, MA, U.S.A.; Kieselguhr H (Merck), dinonyl phthalate, *NNN'*-tetramethylethylenediamine, SDS and 2-mercaptoethanol were from BDH, Poole, Dorset, U.K.; [2-³H]AMP (15–20 Ci/mmol), cyclic [2,8-³H]AMP (32 Ci/mmol) and Na ¹²⁵I were from The Radiochemical Centre, Amersham, Bucks., U.K.; acrylamide, *NN'*-methylenebisacrylamide and riboflavin were from Bio-Rad, Richmond, CA, U.S.A.

Lubrol 12A9 was a kind gift from Mr. J. D. O. Baird, Organics Division, ICI, Blackley, Manchester, U.K.

Antisera

Anti-(rat erythrocyte) sera were raised in mixed-strain rabbits as described by Luzio *et al.* (1976) Guinea-pig anti-(rat liver 5'-nucleotidase) serum, which does not inhibit guinea-pig serum 5'-nucleotidase, was raised to purified 5'-nucleotidase (Widnell, 1975) by multiple intradermal injection, and was a kind gift from Dr. K. K. Stanley of this Department. Both antisera were heated at 56°C for 30 min to inactivate complement (Lachmann, 1975). Donkey anti-(rabbit globulin) precipitating serum was obtained from Wellcome Reagents, Wellcome Research Laboratories, Beckenham, Kent, U.K.

Preparation of fat-cells

Male Wistar rats (130–150 g) were obtained from Ralph Tuck, Rayleigh, Essex, U.K., and given free access to food and water. Rats were killed by decapitation and the fat-cells isolated from the epididymal fat-pads by the method of Rodbell (1964), as described by Luzio (1979).

The isolated cells were resuspended in a medium of Krebs–Ringer/Hepes buffer (1.3 mM-Ca²⁺, 20 mM-Hepes), pH 7.4, as described by Newby *et al.* (1975), containing 5 mM-glucose and 4% (w/v) bovine serum albumin.

Complement-mediated lysis of fat-cells

Fat-cells (30–50 mg dry wt./ml) were coated with rabbit anti-(rat erythrocyte) serum by incubation with 1:100 (v/v) dilution of antiserum at 37°C for 15 min. The cells were washed once with 10 ml, and then resuspended in 20 ml of Krebs–Ringer/Hepes buffer at 37°C containing a 1:10 (v/v) dilution of fresh guinea-pig serum as a source of complement. In some experiments, 0.5 ml samples were taken during the incubation, centrifuged at 10000g for 30 s with 0.2 ml of dinonyl phthalate and the infranatants assayed for the presence of cytosolic and plasma-membrane enzymes activities. The action of

complement on the fat-cells was halted after 25 min by centrifuging the cell suspension with 1 ml of dinonyl phthalate at 300g for 1 min at 37°C. The oil layer and fat cake were removed, and the total infranatant (called the lysate) was cooled at 4°C.

Disruption of fat-cells by other techniques

Mechanical disruption of fat-cells was achieved at 37°C by repeated aspiration (20 times) into a plastic syringe fitted with a Swinny filter holder containing a stainless-steel photo-etched support screen (pore size approx. 200 μm) without a filter (Avruch & Wallach, 1971). Osmotic lysis of fat-cells was achieved by suspending them in a 1:50 (v/v) dilution of Krebs–Ringer/Hepes buffer (pH 7.4) at 37°C, followed by repeated inversion (40 times). The homogenized and osmotically lysed mixtures were centrifuged at 300g for 1 min with 1 ml of dinonyl phthalate, the oil and fat layers removed, and the infranatants cooled at 4°C, before subsequent fractionation.

Fractionation of the lysate

The lysate was fractionated by centrifugation at 1000g for 10 min at 4°C in a MSE bench centrifuge. The supernatant was then centrifuged at 10000g for 30 min at 4°C in a Sorvall RC-5 Super-Speed centrifuge. In some experiments the 10000g supernatant was centrifuged at 100000g for 1 h at 4°C in a SW27 rotor in a Beckman L5-65 ultracentrifuge. The 10000g supernatant was concentrated by ultrafiltration over an Amicon PM10 membrane under N₂ at 450 kPa; the concentrate (4–5 ml) was then layered on top of a 26 ml continuous sucrose gradient (15–50%, w/w, i.e. 1.063–1.238 g/ml at 4°C) in 20 mM-Hepes/NaOH, pH 7.4. The gradient was centrifuged at 100000g for 24 h at 4°C in a Beckman SW27 rotor, after which fractions were collected and the sucrose concentration in each was determined by refractometry. Fresh guinea-pig serum, diluted in Krebs–Ringer/Hepes buffer, pH 7.4, and the concentrated to the same extent as the lysate, was layered on a sucrose gradient and centrifuged as a control in all experiments.

Preparation of fat-cell membranes

Fat-cell plasma membranes were prepared from isolated rat fat-cells by the method of McKeel & Jarett (1970) as described by Jarett (1974).

Assays

Lactate dehydrogenase (EC 1.1.1.27) was assayed as a cytosolic marker by the method of Keiding *et al.* (1974). Cytochrome oxidase (EC 1.9.3.1) was assayed as a mitochondrial marker by the method of Cooperstein & Lazarow (1951). Adenylate cyclase (EC 4.6.1.1) was assayed for 8 min at 37°C by the method of Salomon *et al.* (1974). The [α -³²P]ATP was made by the method

of Martin & Voorheis (1977) and was a kind gift from Dr. B. R. Martin (Department of Biochemistry, University of Cambridge). 5'-Nucleotidase (EC 3.1.3.5) was assayed by a modification of the radioassay of Avruch & Wallach (1971), as described by Luzio *et al.* (1976). Inhibition of fat-cell-derived 5'-nucleotidase was performed by incubating 100 μ l samples at 37°C for 15 min with a 1:5 (v/v) dilution of guinea-pig anti-(rat liver 5'-nucleotidase) serum before assay for 5'-nucleotidase activity. Cell-bound anti-(rat erythrocyte) IgG was measured, after solubilization of membranes with 2% (v/v) Lubrol, by immunoradiometric assay as described by Beck & Hales (1975). The presence of Lubrol did not affect the standard curve, which allowed detection of 1.2 ng of IgG/ml. Protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. Phospholipids were extracted by the method of Folch *et al.* (1957) and analysed by the method of Rouser *et al.* (1970), and the phosphorous content of each spot was determined by the method of Bartlett (1959).

Electron microscopy

Samples (0.5 μ l) were taken from the gradient, diluted with 0.5 μ l of 20 mM-Hepes/NaOH, pH 7.4, and fixed for 10 min at 20°C with 1 μ l of 2% (w/v) phosphotungstic acid or ammonium molybdate on carbon-coated grids. The grids were then examined under a transmission electron microscope.

SDS/polyacrylamide-gel electrophoresis

Sucrose-gradient samples and fat-cell plasma membranes were freeze-dried, resuspended in 50 mM- Na_2CO_3 and incubated with 2% (w/v) SDS and 10% (v/v) 2-mercaptoethanol at 100°C for 15 min. Electrophoresis was performed on 5.5 cm disc gels by the method of Neville & Glossmann (1971). Rat erythrocyte-membrane proteins were used as molecular-weight reference markers (Glossmann & Neville, 1971). After being stained with

Coomassie Brilliant Blue, the gels were scanned at 600 nm in a Gilford spectrophotometer with linear transport.

Results

In the initial experiments, conditions were chosen to allow reproducible complement-mediated lysis of fat-cells, as measured by release of lactate dehydrogenase. It was found that coating fat-cells with 1:100 (v/v) dilution of anti-(rat erythrocyte) serum for 15 min at 37°C, washing in buffer, and subsequently incubating with 1:10 (v/v) dilution of fresh guinea-pig serum for 25 min resulted in release of more than 95% of the lactate dehydrogenase. As in the experiments of Newby *et al.* (1975), between 15 and 20% of the total lactate dehydrogenase was present in the medium at the start of each incubation.

The release of lactate dehydrogenase was reduced to between zero and 10% above the basal values if either the initiating antibody or the guinea-pig serum was omitted from the incubation medium. Heating the guinea-pig serum at 56°C for 30 min to destroy the complement activity (Lachmann, 1975), before addition to the fat-cells, reduced the release of the lactate dehydrogenase to 5% above basal values and the release of 5'-nucleotidase to basal values.

With the conditions used to achieve 95% release of lactate dehydrogenase, more than 50% of the 5'-nucleotidase and adenylate cyclase activities were found in the medium, after its separation from damaged cells and triacylglycerol by centrifugation through dinonyl phthalate.

Fractionation of the lysate

After rupture of the cells with antibody and complement, the lysate was fractionated by differential centrifugation at 1000g for 10 min and 10000g for 30 min. The amount of the plasma-membrane enzymes 5'-nucleotidase and adenylate cyclase present in each fraction was measured (Table 1); 14% of the 5'-nucleotidase and 12% of the adenylate cyclase were not pelleted by centrifuga-

Table 1. Recovery of plasma-membrane activities during differential centrifugation of the fat-cell lysate produced by complement action

After removal of the fat cake (see the Experimental section) the lysate was centrifuged at 1000g for 10 min at 4°C in a bench MSE Minor centrifuge. The supernatant was recentrifuged at 10000g for 30 min at 4°C in a Sorvall RC5 SuperSpeed centrifuge. Pellets were resuspended in 1 ml of incubation medium and frozen in solid CO_2 . Results are expressed as percentage of total cell activity (for 5'-nucleotidase and adenylate cyclase) and percentage of total cell-bound IgG, and are means $\pm$ S.E.M. for the numbers of experiments shown in parentheses.

	5'-Nucleotidase	Adenylate cyclase	Bound IgG
Lysate	54 $\pm$ 3 (12)	58 $\pm$ 8 (8)	43 $\pm$ 3 (4)
After centrifugation at 10 ³ g for 10 min			
Pellet	12 $\pm$ 1 (18)	8 $\pm$ 3 (8)	10 $\pm$ 2 (4)
Supernatant	33 $\pm$ 1 (18)	36 $\pm$ 2 (8)	33 $\pm$ 4 (4)
After centrifugation at 10 ⁴ g for 30 min			
Pellet	15 $\pm$ 1 (18)	12 $\pm$ 3 (8)	18 $\pm$ 3 (4)
Supernatant	14 $\pm$ 1 (18)	12 $\pm$ 4 (8)	14 $\pm$ 5 (4)

tion at 10000g for 30 min. This remaining activity was further analysed by density-gradient centrifugation.

The 10000g supernatants were concentrated by ultrafiltration and layered on continuous sucrose gradients (15–50%, w/w), which were centrifuged at 100000g for 24 h at 4°C. Two sharp white bands (designated I and II) were observed at sucrose densities 1.18 and 1.13 g/ml (Fig. 1) and their compositions investigated. Antibody-coated fat-cells broken by mechanical homogenization or osmotic lysis and subsequently fractionated in the same way produced no visible bands on the sucrose gradient.

The gradient profiles of 5'-nucleotidase, adenylate cyclase and cell-bound anti-(rat erythrocyte) IgG were determined and are shown in Figs. 2 and 3. After subtraction of the activity on the gradient derived from guinea-pig serum (Fig. 2b), both visible bands were associated with peaks of 5'-nucleotidase activity, and there was, in addition, a third peak of 5'-nucleotidase activity at a lower density (1.08 g/ml (Fig. 2a). All three peaks of 5'-nucleotidase activity were inhibited by more than 95% by guinea-pig anti-(rat liver 5'-nucleotidase) serum (which does not neutralize the guinea-pig serum enzyme), indicating that they were derived from the fat-cells (Table 2). Only one peak of adenylate cyclase was observed on

the gradient, corresponding to the denser white band (band I) (Fig. 2a). The profile of IgG on the gradient was broader than that obtained by loading free IgG and showed a major component associated with the 5'-nucleotidase activity of visible band II (Fig. 3). The presence of a large amount of apparently free IgG on the gradient was due to the incomplete washing procedure, after the cells had been coated with antibody. Although this wash removed $99.3 \pm 0.3\%$ ($\pm$ S.E.M.) (5) of unbound IgG, the remainder was sufficient to account for the free IgG seen on the gradient.

To test whether the fractionation enzyme profiles were dependent on the duration of complement action, coated cells were incubated with the same amount of fresh guinea-pig serum for 1 h at 37°C. Both the differential-centrifugation and sucrose-gradient profiles of 5'-nucleotidase activity were similar to those obtained after 25 min of complement action (results not shown). No adenylate cyclase activity was detectable on the gradient after this 1 h incubation at 37°C, presumably owing to inactivation of the enzyme at this temperature (Birnbaumer *et al.*, 1969).

Further characterization of the plasma-membrane fragments released during complement-mediated cell lysis

Band I. Electron microscopy of negatively stained samples from band I showed a population of vesicles of mean diameter 84.0 ± 1.5 nm ($\pm$ S.E.M.) (270) (Plate 1a). The association of the plasma-membrane marker enzymes adenylate cyclase and 5'-nucleotidase with band I suggests that the vesicles originate from plasma membrane. Less than 0.2% of the total cell cytochrome oxidase was observed in band I.

To release the contents of any sealed vesicles, the gradient fractions containing band I were pooled, resuspended in a hypo-osmotic medium (2 mM-Hepes/NaOH, pH 7.4) and relayered on to the same continuous sucrose gradient (15–50%, w/w). Both the 5'-nucleotidase and IgG peaks remained associated with a white band, but its characteristic density was altered to 1.16 g/ml (Fig. 4). Adenylate cyclase activity did not survive the hypo-osmotic treatment and recentrifugation. In separate experiments it was found that band-I 5'-nucleotidase was removed from the 10000g supernatant by centrifugation at 100000g for 1 h. To characterize further the adenylate cyclase associated with this band, a 100000g pellet prepared from the 10000g supernatant of the cell lysate was resuspended in hypo-osmotic medium (2 mM-Hepes/NaOH, pH 7.4) and centrifuged into a sucrose gradient. The adenylate cyclase activity remained associated with the 5'-nucleotidase and with the visible band (Fig. 5). The basal adenylate cyclase activity was increased 4-fold by 1 μ g of adrenaline/ml, and 20-fold by 20 μ M-

Fig. 1. Photograph of sucrose gradient (15–50%, w/w) after loading of complement-derived fat-cell lysate and centrifugation at 100000g for 24 h at 4°C

The lysate was prepared, treated and centrifuged as described in the Experimental section. Two white bands, I and II, are visible.

EXPLANATION OF PLATE 1

Transmission electron micrograph of negatively stained band-I (a) and band-II (b) vesicles

Magnification $\times 54\,400$

Fig. 2. Distribution of plasma-membrane enzyme activities after centrifugation on a continuous sucrose gradient. Samples (4–5 ml) were layered on 26 ml continuous sucrose gradients (15–50%, w/w) and centrifuged at 100 000 *g* for 24 h at 4°C in a Beckman SW27 rotor. The gradients were separated into 1 ml fractions and the sucrose density of each fraction was determined by refractometry. Results have been pooled according to sucrose density and are expressed as percentages of total activity centrifuged. $\cdots$, Sucrose-density profile; $\downarrow$ positions of visible bands. (a) 5'-Nucleotidase (●) and adenylate cyclase (▲) activities in 4.5 ml of the concentrated 10000 *g* supernatant prepared from the lysate. Results are percentages of total activity centrifuged (means $\pm$ S.E.M. for nine experiments) after subtraction of 5'-nucleotidase activity arising from the guinea-pig serum (b). Total gradient recovery of 5'-nucleotidase was $93.7 \pm 1.4\%$, and that of adenylate cyclase was $86.8 \pm 8.2\%$. (b) 5'-Nucleotidase activity in 4.5 ml of concentrated fresh guinea-pig serum. Results are expressed as percentages of total activity on the corresponding lysate gradient (means $\pm$ S.E.M. from eight experiments). Recovery was $95.1 \pm 5.0\%$. This represents $31 \pm 3\%$ of the total lysate activity centrifuged.

p[NH]ppG, showing that it was also associated with a hormone receptor.

The phospholipid/protein ratio of the band-I vesicles was lower than that of purified plasma membranes. The phospholipid composition is shown in Table 3, the major difference from purified plasma membrane being the absence of phosphatidylserine. The protein composition, determined by SDS/polyacrylamide-gel electrophoresis, was radically dif-

ferent from that of purified plasma membrane, since it had only one prominent band of apparent mol.wt. 130 000, with very few other proteins (Fig. 6a).

Band II. Electron microscopy of negatively stained samples from band II showed a population of vesicles of mean diameter 111 ± 4.5 nm ($\pm$ S.E.M.) (90) (Plate 1b). The presence of 5'-nucleotidase and membrane-bound IgG, and the absence of cytochrome oxidase (less than 0.25% of total cell

Fig. 3. Distribution of rabbit IgG on continuous sucrose gradients

■, Rabbit IgG profile from 4.5 ml of the concentrated 10000g supernatant derived from the lysate. Results are means $\pm$ S.E.M. from five experiments. Recovery was $87.0 \pm 10\%$. □, IgG profile from 4.5 ml of a 2 μ g/ml solution of rabbit IgG. Results are means $\pm$ range of two experiments. Recovery was $93.6 \pm 2.5\%$.

Table 2. Inhibition of 5'-nucleotidase by guinea-pig anti-(rat liver 5'-nucleotidase) serum

Samples (100 μ l) were preincubated for 15 min at 37°C with a 1:5 (v/v) dilution of guinea-pig anti-(rat liver 5'-nucleotidase) serum, and then assayed for 5'-nucleotidase activity. Results are expressed as percentage inhibition, and are means $\pm$ S.E.M. for the numbers of determinations indicated in parentheses.

Sample	Inhibition by antiserum (%)
Fat-cell homogenate	97 ± 1 (3)
Guinea-pig serum	5 ± 2 (5)
Band I	96 ± 2 (3)
Band II	97 ± 3 (4)
Hypo-osmotically treated band-II fractions; activity at	
1.16 g/ml	100 ± 1 (3)
1.10 g/ml	61 ± 8 (4)

activity), suggest that these vesicles are also derived from the plasma membrane.

The sucrose-gradient fractions containing band-II 5'-nucleotidase activity were pooled, resuspended in a hypo-osmotic medium (2 mM-Hepes/NaOH, pH 7.4) and relayered on to the same continuous sucrose gradient (15–50%, w/w). The resulting profiles of 5'-nucleotidase activity and rabbit IgG (Fig. 7) show that hypo-osmotic treatment caused approx. 90% of the layered band-II 5'-nucleotidase and IgG

Fig. 4. Distribution of plasma-membrane markers of hypo-osmotically treated sucrose gradient fractions containing band I, on a continuous sucrose gradient

The same (15–50%, w/w) continuous sucrose gradients were used as those described in the legend to Fig. 2. 5'-Nucleotidase (●) and IgG (■) after centrifugation of 4.5 ml of hypo-osmotically treated band-I fractions are shown. Results are percentages of total activity centrifuged (means $\pm$ S.E.M. for three determinations). Recovery of 5'-nucleotidase was $97.5 \pm 2.5\%$ and that of IgG was $74.0 \pm 4.5\%$.

Fig. 5. Distribution of plasma-membrane markers of the 100000g pellet on a continuous sucrose gradient

5'-Nucleotidase (●) and adenylate cyclase (▲) profiles are shown after centrifugation, on the 15–50% (w/w) continuous sucrose gradient, of 4.5 ml of the hypo-osmotically treated 100000g pellet derived from the lysate. Results are means $\pm$ S.E.M. for three determinations. Recovery of 5'-nucleotidase was $77.0 \pm 3.0\%$ and that of adenylate cyclase was $40.0 \pm 10\%$.

to float at a density of 1.10 g/ml, as opposed to the characteristic band-II density of 1.13 g/ml. The white band was visible at a density of 1.16 g/ml and was associated with 8% of the band-II 5'-nucleotidase and 7% of the band-II IgG. In order to identify

Table 3. *Phospholipid composition of fat-cell plasma membranes and gradient fractions corresponding to bands I and II*
Results are expressed as percentage of total recovered lipid phosphorus. The plasma-membrane results are means $\pm$ s.e.m. for t.l.c. analyses of four separate membrane preparations. Bands I and II results are t.l.c. analyses of gradient fractions pooled from four experiments. Abbreviation: N.D., not detectable.

	Plasma membranes	Band I	Band II
Total phospholipid (μ g/mg of protein)	794 $\pm$ 73	294 $\pm$ 15	560 $\pm$ 15
Phosphatidylcholine + choline plasmalogen	32 $\pm$ 1	46	46
Lysophosphatidylcholine		N.D.	27
Phosphatidylethanolamine + ethanolamine plasmalogen	26 $\pm$ 1	16	<10
Sphingomyelin	23 $\pm$ 1	20	13
Phosphatidylserine	11 $\pm$ 1	N.D.	N.D.
Phosphatidylinositol	7 $\pm$ 1	18	N.D.

Fig. 6. *SDS/polyacrylamide-gel-electrophoresis profiles (approx. 60 μ g of protein/gel) of plasma-membrane and hypo-osmotically treated band-I and band-II samples*
Samples were solubilized and electrophoresed as described in the Experimental section and the gels stained with Coomassie Blue. (a) Band I; (b) band II; (c) purified plasma membranes.

Fig. 7. *Distribution of IgG and 5'-nucleotidase activity after centrifugation of hypo-osmotically treated band-II fractions on a continuous sucrose gradient*
The same (15–50%, w/w) continuous gradients were used as those described in the legend to Fig. 2. IgG (■) and 5'-nucleotidase (●) profiles after centrifugation of 4.5 ml of hypo-osmotically treated band-II fractions are shown. Results are percentages of total activity centrifuged (means $\pm$ range from two experiments). Recovery of IgG was 99.0 $\pm$ 1.0%, and that of 5'-nucleotidase was 98.0 $\pm$ 2%.

the source of this 5'-nucleotidase activity floating at a density of 1.16g/ml, gradient fraction corresponding to this activity were incubated with the inhibiting guinea-pig anti-(rat liver 5'-nucleotidase) serum. The enzyme activity in these fractions was 100.0 $\pm$ 0.4% (3) inhibited by the antiserum (Table 2), indicating that the activity was derived from the fat-cell. The 5'-nucleotidase activity at density 1.16g/ml after hypo-osmotic treatment corresponds to approx. 0.6% of the initial fat-cell activity, and the IgG remaining amounts to 0.8% of the total IgG bound. Of the band-II 5'-nucleotidase released by hypo-osmotic treatment and floating at a density of 1.10g/ml, 61% was derived from the fat-cell and

Table 4. Summary of membrane fragments produced during complement-mediated cell lysis

	5'-Nucleotidase (% of total activity)	Adenylate cyclase (% of total activity)	Bound IgG (% of total)	Diameter of vesicles (nm)	Density (g/ml)	Protein composition	Phospholipid composition
1. Membrane associated with triacylglycerol	46 ± 3	41 ± 8	57 ± 3	—	—	—	—
2. Membrane pellet produced by centrifugation at 1000 g for 10 min	12 ± 1	7 ± 3	10 ± 2	—	—	—	—
3. Membrane pellet produced by centrifugation at 10 000 g for 30 min	14 ± 1	12 ± 3	18 ± 3	—	—	—	—
4. Membrane in the 10 000 g supernatant band I	5 ± 1	13 ± 2*	2	84.0 ± 1.5	1.18	One major protein approx. mol.wt. 130 000	No phosphatidylserine
band II	10 ± 2	0	12 ± 2	111.0 ± 4.5	1.13	Lacking some high- mol.wt. plasma- membrane proteins	Enriched in ceramide and lysophosphatidyl- choline

* Adrenaline-sensitive.

39% from the guinea-pig serum [defined by inhibition with guinea-pig anti-(rat liver 5'-nucleotidase) serum] (see Table 2). The phospholipid composition of the band-II vesicles (Table 3) had some important differences from that of isolated plasma membranes. There was no phosphatidylserine or phosphatidylinositol detectable, but there was a large amount of lysophosphatidylcholine present. In addition, a spot co-chromatographing with ceramide was observed. SDS/polyacrylamide-gel electrophoresis gave a profile (Fig. 6b) similar to that of isolated plasma membranes, although some high-molecular-weight proteins, e.g. the major band-I protein, and some low-molecular-weight proteins were missing.

A summary of the properties of the vesicles produced during complement-mediated cell lysis is given in Table 4.

Association of IgG with 5'-nucleotidase in bands I and II

To ensure that the coincidence of the 5'-nucleotidase activity and rabbit IgG in bands I and II was not due to the initiating antibody binding to the enzyme, the bound antibody and enzyme were separated by detergent solubilization and precipitation of bound antibody with an anti-(rabbit IgG) serum. This experiment was possible, since detergent conditions may be chosen that do not interfere with antibody-antigen binding. Fat-cells, precoated with rabbit anti-(rat erythrocyte) antibody, were mechanically homogenized, and the fat cake was removed. The homogenate was incubated with a precipitating anti-(rabbit IgG) serum in the presence of a non-ionic detergent (2% Lubrol), and then centrifuged at 12 000g for 4 min. This resulted in 99.97 ± 0.04% (±s.e.m.) (9) of the rabbit IgG and less than 0.10 ± 0.02% (±s.e.m.) (12) of the 5'-nucleotidase activity being pelleted, showing that the rabbit anti-(rat erythrocyte) serum does not bind to rat fat-cell 5'-nucleotidase.

Discussion

The orderly sequence in which the complement components act has largely been determined (Barkas, 1978; Porter & Reid, 1978), but less is known about the fate of the plasma membrane during complement-mediated cell lysis, especially during lysis of nucleate mammalian cells. In the present study we have investigated the fragmentation of the plasma membrane caused during guinea-pig complement-mediated lysis of antibody-coated rat isolated fat-cells.

After complete release of the cytosolic marker enzyme lactate dehydrogenase by the action of guinea-pig complement, approx. 50% of plasma-membrane marker enzymes remained associated with triacylglycerol. More than half of the plasma-membrane enzyme activity released from triacylgly-

cerol during complement-mediated cell lysis was pelleted by centrifugation at 10000 *g* for 30 min. The remaining activity was analysed on sucrose density gradients, allowing the separation of visible white bands (associated with peaks of plasma-membrane enzyme activity), which could not be obtained by similar treatment of fat-cell homogenates prepared by mechanical disruption or osmotic lysis of isolated fat-cells.

The two visible white bands consisted of small (approx. 100 nm diam.) membrane vesicles having the properties summarized in Table 4. These vesicles appeared to be formed as a direct consequence of complement action on intact cells. Both bound IgG and 5'-nucleotidase have been used as plasma-membrane markers in rat fat-cells (Luzio *et al.*, 1976). The association of these markers with the vesicles and absence of cytochrome oxidase indicates that the vesicles are derived from plasma membrane. However, neither vesicle reflects the overall composition of purified fat-cell plasma membrane. The denser vesicle, while retaining a hormone-sensitive adenylate cyclase, is highly enriched in plasma-membrane protein of apparent mol.wt. 130 000 with very little other protein present. The less-dense vesicle population contains no adenylate cyclase, is enriched in lysophosphatidylcholine, contains ceramide and has a protein composition that is similar to plasma membrane, though lacking some high-molecular-weight (> 100 000) bands. The third peak of 5'-nucleotidase activity observed on the sucrose gradients (density 1.08 g/ml) may represent free solubilized enzyme [mol.wt. 70 000–140 000 (Evans & Gurd, 1973), consistent with its position on the gradients] or a very light vesicle. We have not further investigated this peak of 5'-nucleotidase activity.

Specific membrane damage as a result of complement action has been reported in other systems, including in one case the production of vesicles during complement-mediated damage to *Escherichia coli* (Wilson & Spitznagel, 1968), which were similar in size to those observed in the present study. Other reported changes in membrane structure caused by complement action include the formation of small lesions (10 nm in diam.) due to the insertion of the terminal components of the complement cascade (Humphrey & Dourmashkin, 1969; Lachmann *et al.*, 1973; Hammer *et al.*, 1975; Dourmashkin, 1978), membrane thickening (Humphrey & Dourmashkin, 1969; Polley, 1971), phospholipid release (Inoue *et al.*, 1977; Shin *et al.*, 1977), loss of membrane proteins (Zimmermann & Müller-Eberhard, 1973) and protein rearrangement (Bhakdi *et al.*, 1974).

The mechanism by which complement causes specific membrane damage is not clear. Evidence has been presented suggesting that complement damage

results from neither phospholipase nor proteinase activity (Lachmann *et al.*, 1973; Inoue *et al.*, 1977), but may be due to a detergent-like effect (Kinsky, 1972). It is widely agreed that complement-mediated cytolysis is a two-stage process, during the first of which ions and small molecules pass through a trans-membrane channel resulting in an increase in colloid osmotic pressure because of the Donnan effect. Hence the cell swells, leading to the second stage, with bursting of the cell membrane and release of macromolecular contents (see, for example, Mayer, 1977). The results described in the present paper suggest that such a simplistic view may be inadequate to explain the nature of the membrane damage, which may be a direct biochemical consequence of complement action. In particular, we observed that plasma-membrane vesicles formed during complement-mediated damage of fat-cells were not produced by mechanical homogenization nor osmotic lysis and had a very different composition from isolated purified plasma membranes.

Although Lauf (1974) and Valet & Opferkuch (1975) have shown in erythrocytes that cell swelling and lysis can occur without a colloid osmotic swelling phase, it remains possible that the specific membrane vesiculation caused by complement is a reflection of the early ionic fluxes. It is of interest that formation of vesicles similar in size to those reported here and by Wilson & Spitznagel (1968) has also been observed when intracellular Ca^{2+} concentrations are elevated by the ionophore A23187 in erythrocytes (Allan *et al.*, 1976) and in the myelin sheath of peripheral rat nerve (Schlaepfer, 1977). The vesicles formed by the action of ionophore on nucleated erythrocytes were rich in ceramide (Allan & Michell, 1977), like the band-II vesicles observed in the present work. The early ionic events stimulated by complement action (Green *et al.*, 1959; Michaels *et al.*, 1976; Stephens & Henkart, 1979) include the rapid stimulation of Ca^{2+} influx (Campbell *et al.*, 1979; Luzio *et al.*, 1979), which may be particularly important in view of the normally extremely low cytosolic concentration of Ca^{2+} and its known role in the control of many metabolic processes. It would be of interest to investigate whether the complement-mediated vesiculation of fat-cell plasma membrane described in the present paper may be mimicked by the action of Ca^{2+} ionophores.

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