Use of a novel rapid preparation of fat-cell plasma membranes employing Percoll to investigate the effects of insulin and adrenaline on membrane protein phosphorylation within intact fat-cells

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1. A rapid method was developed for the preparation of plasma membranes from either isolated rat fat-cells or intact epididymal fat-pads with the use of density-gradient centrifugation in the presence of Percoll. On the basis of 5'-nucleotidase activity, the yield of plasma membranes was about 50% and purification over 10-fold. Activities of marker enzymes indicated that contamination by mitochondria and microsomal fraction was small. 2. Incorporation of $^{32}\text{P}_i$ into proteins associated with plasma membranes within isolated fat-cells was investigated. Four major bands of labelled phosphoproteins were separated by sodium dodecyl sulphate/polyacrylamide-slab-gel electrophoresis; the apparent subunit mol.wts. were 67 000, 61 000, 26 000 and 20 000. None of these phosphoprotein bands corresponded to periodate/Schiff-staining glycoproteins. The extent of phosphorylation of the 61 000-mol.wt. phosphoprotein band was increased by about 30 and 60% after exposure of fat-cells for 15 min to insulin or adrenaline respectively.

A number of methods have been reported for the preparation of fat-cell plasma membranes. Most depend on a combination of differential and gradient centrifugation and require from 3 to 24 h to complete (McKeel & Jarett, 1970; Avruch & Wallach, 1971; Jarett, 1974; Avruch *et al.*, 1976a; Kawai & Spiro, 1977; Lewis *et al.*, 1979). An exception is the preparation method of Luzio *et al.* (1976), which depends on immunoprecipitation. This technique is undoubtedly quicker, but requires the necessary antisera and results in a product contaminated with antibody proteins. In the present paper we describe a novel method for the preparation of the plasma membranes in which the key step is separation of the membranes from mitochondria and other subcellular components in a self-forming Percoll gradient. The method appears to be a real improvement on the previously available methods in terms of simplicity, rapidity and yield.

Fat-cell plasma membranes contain a number of phosphoproteins into which ^{32}P is rapidly incorporated when fat-cells are incubated with $^{32}\text{P}_i$ (Avruch *et al.*, 1976a,b; Benjamin & Clayton, 1978). However, there is confusion over the values of subunit molecular weights after separation by

SDS/polyacrylamide-gel electrophoresis and whether or not two of the phosphoproteins are the same as the two major periodate/Schiff-staining glycoproteins in the membrane (Avruch *et al.*, 1976b; Benjamin & Clayton, 1978). A problem with these studies is the time taken to prepare the membrane fractions and the attendant possibility of changes in phosphorylation during the preparation. We have employed the rapid Percoll procedure for separating fat-cell membranes to re-assess the incorporation of $^{32}\text{P}_i$ into the membrane phosphoproteins and to explore the effects of the presence of insulin and adrenaline on the incorporation.

Experimental

Materials

Sources of biochemicals were as given by Severn *et al.* (1974, 1976). Collagenase was obtained from International Enzymes, Windsor, Berks., U.K. (lot nos. 504-19 and 504-22). Percoll was from Pharmacia Fine Chemicals (London, W5 5SS, U.K.), 2,6-dichlorophenol-indophenol was from BDH Chemicals (Poole, Dorset, U.K.) and $[2\text{-}^3\text{H}]\text{AMP}$ and $^{32}\text{P}_i$ were from The Radiochemical Centre (Amersham, Bucks., U.K.).

Abbreviations used: SDS, sodium dodecyl sulphate.

Sources of tissue, preparation and incubation of fat-cells

Epididymal fat-pads were obtained from male albino Wistar rats (body wt. 160–200 g) with free access to water and stock laboratory diet (breeding diet; Oxoid, Basingstoke, Hants., U.K.). After decapitation of the animals, the pads were removed and either used intact or incubated with collagenase to yield isolated fat-cells by the method of Rodbell (1964). Details were as given by Severson *et al.* (1974). All incubations were carried out in bicarbonate-buffered medium (Krebs & Henseleit, 1932) containing 1.25 mM-CaCl₂, albumin (10–20 mg/ml) and 1 mM-glucose plus other additions as indicated. For the determination of ³²P incorporation into fat-cell phosphoproteins, the incubation procedure of Hughes *et al.* (1977) and Brownsey *et al.* (1977, 1979) was followed. Essential details are given in Table and Figure legends.

Preparation of plasma-membrane fractions from isolated fat-cells

The essential steps in the preparation are summarized in Scheme 1.

Isolated fat-cells (0.5–0.7 g wet wt.) were prepared from the epididymal fat-pads of two or three rats and separated from the bicarbonate-buffered medium by brief centrifugation (1000 g for 30 s). In experiments where the cells had been incubated with ³²P_i, 2 ml of dinonyl phthalate was added before centrifugation. This ensured that most of the incubation medium was removed as the cells were centrifuged up through the oil. The cells were removed with an automatic pipette and immediately broken by vigorous agitation in a Vortex mixer with 2–3 ml of sucrose-based extraction medium at 0°C in a conical glass centrifuge tube for 30 s. The sucrose-based extraction medium was 0.25 M-sucrose/10 mM-Tris/HCl/2 mM-EGTA, pH 7.4, with in addition 20 mM-NaF, 7.5 mM-reduced glutathione and 5 mM-EDTA when ³²P incorporation was being investigated. All subsequent operations were carried out at 0–4°C. The crude extract was defatted by centrifugation (1000 g for 30 s), and the infranatant and small pellet were aspirated with a syringe from below the fat-plug and centrifuged at 30000 g for 30 min. The 'supernatant fraction' was removed, and the pellet was suspended in 400–500 µl of the sucrose-based extraction medium by using a 'cold finger' to yield a 'particulate fraction'. The plasma membranes were separated from mitochondria and other components in the particulate fraction by centrifugation in the presence of a self-forming gradient of Percoll. Percoll was mixed with 2 M-sucrose/80 mM-Tris/HCl/8 mM-EGTA and the sucrose-based extraction medium in the proportions 7:1:32 (by vol.). The density of this solution was

1.05. A sample of the particulate fraction (usually 400 µl) was dispersed in 8 ml of the iso-osmotic Percoll solution, and the mixture was centrifuged in a fixed-angle rotor at 10000 g for 15 min. Two well-defined bands were clearly visible, one just below the surface ('plasma membranes') and one very close to the bottom ('mitochondria'). In most studies these bands were removed in a total volume of about 1 ml with an automatic pipette, but when indicated the Percoll gradient was divided into 0.5–0.6 ml fractions by inserting a long tube and aspirating from the bottom with a peristaltic pump. Usually the plasma-membrane fraction was washed by diluting approx. 5-fold in NaCl-based medium (0.15 M-NaCl/10 mM-Tris/HCl/1 mM-EGTA, pH 7.4) followed by centrifugation at 10000 g for 2 min. In particular, this wash served to remove much of the Percoll. The mitochondrial fraction was washed in a similar manner, but sucrose-based extraction medium was used rather than NaCl-based medium.

The density of Percoll-containing fractions was determined in the absence of added sample by using a refractometer (sucrose range 0–28%; Bellingham and Stanley, Tunbridge Wells, Kent, U.K.) in conjunction with the calibration curve supplied by Pharmacia.

Preparation of plasma membranes from intact epididymal fat-pads

The procedure closely followed that described above for isolated fat-cells, with the following modifications at the initial stages. The pads from two rats were blotted and immediately homogenized in 8 ml of sucrose-based extraction medium at 0°C with a Polytron PT20 homogenizer (about 3 s on position 3). The crude extract was centrifuged for 1000 g for 30 s, and the infranatant was removed by syringe from between the fat-plug and cell debris.

Assay of enzyme activities

Succinate dehydrogenase (EC 1.3.99.1) was determined as the succinate-dependent reduction of 2,6-dichlorophenol-indophenol (Bachmann *et al.*, 1966), and citrate synthase (EC 4.1.3.7) as described by Coore *et al.* (1971). NADH-cytochrome *c* reductase (EC 1.6.99.3) and NADPH-cytochrome *c* reductase (EC 1.6.2.4) were assayed in the presence of 1 µg of rotenone/ml as described by Dallner *et al.* (1966), and lactate dehydrogenase (EC 1.1.1.27) as described by Keiding *et al.* (1974).

The activity of 5'-nucleotidase was assayed as the release of [³H]adenosine from [2-³H]AMP (Avruch & Wallach, 1971; Newby *et al.*, 1975). Details were as follows. Samples (50 µl) of homogenate or subcellular fraction were incubated for 30 min at 30°C in a total volume of 500 µl containing 50 mM-Tris/HCl, 2 mM-MgCl₂ and Triton X-100

Scheme 1. Summary for the procedure employed to prepare plasma membranes from isolated fat-cells and intact rat epididymal fat-pads

For full details see the Experimental section. All operations were carried out at 0–4°C.

(1 mg/ml), pH 8.0, with [2-³H]AMP (200 μM and 0.25 Ci/mol). The incubation was terminated by addition of 100 μl of 0.15 M-ZnSO₄ followed by 100 μl of 0.15 M-Ba(OH)₂ to precipitate the un-hydrolysed AMP. After centrifugation at 10000 g for 30 s, samples of the supernatant were taken for the determination of radioactivity in a scintillation counter with the toluene/methoxyethanol-based scintillant described by Severson *et al.* (1974).

All enzyme activities are expressed as units, where 1 unit represents the conversion of 1 μmol of substrate to products in 1 min.

Assay of protein and DNA

Protein was assayed by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. The presence of Percoll can cause turbidity in the assay mixture, but this problem was circumvented by a brief period of centrifugation (1000 g for 1 min)

before the measurement of absorbance without appreciably affecting the assay of added albumin.

DNA was assayed by the method of Burton (1956), with calf thymus DNA as standard.

Separation of proteins, glycoproteins and [³²P]phosphoproteins by SDS/polyacrylamide-gel electrophoresis

Separation was carried out in 1 cm tracks on 10% (w/v) slab gels (14 cm × 14 cm) by using the solutions described by Laemmli (1970). Proteins in the defatted whole-cell homogenate and supernatant fractions were precipitated with 10% (w/v) trichloroacetic acid, and then redissolved in stacking gel buffer (pH 6.8) containing 62.5 mM-Tris/HCl, SDS (40 mg/ml), sucrose (200 mg/ml), Bromophenol Blue (0.2 mg/ml) and 100 mM-2-mercaptoethanol by heating at 100°C for 5 min. Other samples for analysis were dissolved directly in this

solution; it should be noted that Percoll is precipitated by trichloroacetic acid. Electrophoresis was carried out at 20°C for 2–3 h at 40 mA/slab. The slab gel was then treated for 1 h with 1 M-trichloroacetic acid in 50% (w/v) methanol to fix the proteins before staining with either Coomassie Brilliant Blue for protein or by the periodate/Schiff method (Zaccharius *et al.*, 1969) for carbohydrate and, particularly, sialic acid-bearing proteins. The gels were laid on cellophan and dried on boards under vacuum before either densitometric scanning or, in the ^{32}P -incorporation studies, radioautography. Subunit molecular weights were assigned by using the following markers: rabbit skeletal-muscle phosphorylase (mol.wt. 98 000), bovine serum albumin (mol.wt. 68 000), subunits of purified pig heart pyruvate dehydrogenase (mol.wts. 74 000, 52 000, 42 000 and 37 000), chymotrypsinogen (mol.wt. 26 000) and cytochrome *c* (mol.wt. 12 000).

[^{32}P]Phosphoproteins were detected by exposing the dried gels to Kodak Kodirex KT X-ray film for 2–7 days and scanning the radioautographs. Exposure of the film was such that peak heights did not exceed an absorbance value of 1.0. Peak heights were measured from the base-line taken as the lowest absorbance on that track (Brownsey *et al.*, 1979). Densitometric scanning was performed at 625 nm for Coomassie Blue-stained bands and radioautographs, or at 525 nm for periodate/Schiff-stained bands, with a Gilford spectrophotometer linked to a Hewlett Packard 9845S computer.

Results and discussion

Separation of fat-cell plasma membranes with the use of Percoll

In preliminary experiments with Percoll, gradients were preformed by centrifugation, a particulate fraction from fat-cells was carefully layered on the top of the gradient and a second centrifugation was carried out. Such procedures indicated that Percoll gradients could be a useful way of separating plasma membranes (density about 1.04) from mitochondria (density about 1.08). It was then realized that the preparation of a preformed gradient was unnecessary and that an excellent separation of mitochondria and plasma membranes could be achieved simply by briefly centrifuging a particulate fraction that had been dispersed in medium containing sufficient Percoll to give a density of 1.05. On centrifugation (10 000 *g* for 15 min), a density gradient from about 1.08 to 1.04 was set up, and the plasma membranes and mitochondria formed visible bands close to the top and the bottom of the gradient respectively. The excellent and reproducible separation achieved is well illustrated in Fig. 1, which shows the mean distribution of 5'-nucleotidase (plasma-membrane marker enzyme), succinate de-

Fig. 1. Distribution of 5'-nucleotidase (■), succinate dehydrogenase (●) and citrate synthase (□) activities after separation of the particulate fraction derived from isolated fat-cells

Particulate fraction (400 μl) derived from the fat-cells of two or three rats was mixed with 8 ml of sucrose medium containing Percoll (initial density 1.05) and centrifuged at 10 000 *g* for 15 min. The resulting gradient was fractionated, and the fractions (0.6 ml) were assayed for enzyme activity (see the Experimental section for details). Results are expressed as percentages of the total activity recovered, and are the means $\pm$ S.E.M. for six separate experiments (b). The Figure also shows the density gradient (O, a) measured in five separate experiments in the absence of added particulate fraction.

hydrogenase (mitochondrial-inner-membrane marker enzyme) and citrate synthase (mitochondrial-matrix marker enzyme) obtained in six separate experiments. The top 2 ml of gradient contained over 75% of the recovered 5'-nucleotidase but only about 15% of the two mitochondrial enzymes. In contrast, the bottom 2 ml of the gradient contained

50–60% of the mitochondrial marker enzymes but only about 10% of the 5'-nucleotidase. An equally satisfactory separation could also be achieved with a crude particulate preparation obtained from intact epididymal fat-pads. It has been demonstrated that 5'-nucleotidase is almost exclusively an ectoenzyme in fat-cells (Newby *et al.*, 1975), and its distribution follows closely that of other plasma-membrane markers (Luzio *et al.*, 1976).

The complete method for the preparation of plasma membranes involved, in turn, cell disruption, preparation of a crude particulate fraction, centrifugation in the presence of Percoll and, lastly, removal of Percoll from the plasma-membrane and mitochondrial fractions by dilution and further brief centrifugation (Scheme 1). The techniques used for cell disruption differed for the isolated fat-cells and intact epididymal fat-pads; in both cases they were based on those developed previously for the preparation of coupled intact mitochondria from the two preparations (Martin & Denton, 1970; Severson *et al.*, 1976). The entire procedure for the preparation of membranes could be completed within 60 min.

Details of the recovery of protein, 5'-nucleotidase, the mitochondrial marker enzymes and lactate dehydrogenase (cytoplasmic marker enzyme) are given in Table 1. The washed plasma-membrane fraction contained about 55% of the 5'-nucleotidase activity present in the original whole-cell homogenate but only about 4% of the protein. This represented an increase in specific activity of over 10-fold. In contrast, the recovery of the two

mitochondrial enzymes was between 4 and 6%, and that of lactate dehydrogenase was less than 1%. In addition, the recovery of DNA in the membrane preparation was determined in seven separate preparations. The content of DNA in the defatted whole-cell homogenate was $7.5 \pm 0.9 \mu\text{g}/\text{mg}$ of protein, and the recovery of DNA into the washed plasma-membrane fraction was $16.6 \pm 3.7\%$ of the total. (Results are given as means $\pm$ S.E.M. for seven observations.) We have also attempted to assess the contamination of the plasma-membrane preparation by endoplasmic-reticulum membranes by determining the recovery of NADH-cytochrome *c* reductase and NADPH-cytochrome *c* reductase (Dallner *et al.*, 1966). The mean activities of these enzymes in the defatted whole-cell homogenate were 374 ± 38 and 27 ± 0.5 units/mg of protein respectively (means $\pm$ S.E.M. for three separate preparations). The recoveries of activities into the washed plasma-membrane preparations were $4.2 \pm 0.1\%$ and less than 10% respectively.

Percoll did not have any apparent adverse effects on fat-cell mitochondria. When defatted bovine serum albumin (30 mg/ml) was added to the sucrose-based medium used for the preparation of the initial crude extract and for resuspending the crude particulate fraction, the purified mitochondria exhibited coupling ratios of about 5 when oxoglutarate and malate were substrates and of about 3 when succinate was the substrate. These values are similar to values obtained with mitochondria prepared without purification with Percoll (Martin & Denton, 1970; Severson *et al.*, 1976).

Table 1. Recovery of subcellular markers and protein in subcellular fractions of fat-cells produced during the preparation of fat-cell plasma membranes

Samples of fat-cells were fractionated as summarized in Scheme 1 and described in detail in the Experimental section. All values are given as means $\pm$ S.E.M. of at least five observations made with separate preparations of fat-cells and are expressed as percentages of values in the defatted whole-cell homogenate. The specific activities of the marker enzymes in this fraction were: 5'-nucleotidase, 10.3 ± 1.03 (15) munits/mg of protein; succinate dehydrogenase, 5.33 ± 0.87 (5) munits/mg of protein; citrate synthase, 132 ± 23 (9) munits/mg of protein; lactate dehydrogenase, 780 ± 44 (6) munits/mg of protein. N.D., Activity not detectable.

Fraction	Values (% of value in defatted whole-cell homogenate)							
	5'-Nucleotidase		Succinate dehydrogenase		Citrate synthase		Lactate dehydrogenase	
	Protein content	Specific activity	Recovery of activity	Specific activity	Recovery of activity	Specific activity	Recovery of activity	Specific activity
30000g supernatant	72 ± 3	7.1 ± 4.1	6 ± 3	N.D.	N.D.	16 ± 2	11 ± 2	133 ± 12
30000g particulate	18 ± 2	393 ± 37	83 ± 3	533 ± 48	103 ± 8	329 ± 84	70 ± 9	31 ± 4
Plasma membranes	9.1 ± 0.8	580 ± 11	70 ± 7	189 ± 33	17 ± 4	130 ± 23	21 ± 4	23 ± 3
Washed plasma membranes	3.8 ± 0.6	1130 ± 100	55 ± 7	114 ± 31	4 ± 1	184 ± 21	6 ± 2	32 ± 18
Mitochondria	8.9 ± 1.5	154 ± 70	11 ± 2	568 ± 130	41 ± 2.5	553 ± 123	76 ± 13	13 ± 1
Washed mitochondria	4.6 ± 1.0	138 ± 30	7 ± 1	1100 ± 160	34 ± 1	759 ± 165	45 ± 8	28 ± 1
								0.2 ± 0.01

Fig. 2. SDS/polyacrylamide-gel electrophoresis of the proteins in subcellular fractions of (a) intact fat-pads and (b) isolated fat-cells

Proteins in samples of washed plasma-membrane fractions were separated along parallel tracks by SDS/polyacrylamide-slab-gel electrophoresis. The Figure shows densitometric traces after staining with Coomassie Blue. The black blocks (■) indicate the position of the periodate/Schiff-staining bands. Also indicated are the positions of marker proteins: (i) rabbit skeletal-muscle phosphorylase (mol.wt. 98 000), (ii) bovine serum albumin (mol.wt. 68 000), (iii) α -subunit of pig heart pyruvate dehydrogenase complex (mol.wt. 42 000), (iv) chymotrypsinogen (mol.wt. 26 000) and (v) cytochrome *c* (mol.wt. 12 000). The dye front (Bromophenol Blue) was allowed to migrate 10 cm. Results are typical for six different preparations.

Proteins and periodate/Schiff-staining glycoproteins in the washed plasma-membrane fractions prepared from isolated fat-cells and intact epididymal fat-pads

Typical densitometric traces of the Coomassie Blue-staining protein bands that were separated by SDS/polyacrylamide-gel electrophoresis are shown in Fig. 2. Although there were qualitative differences in the relative amounts of certain protein bands, the overall protein compositions of the plasma-membrane fractions prepared from isolated fat-cells and intact epididymal fat-pads appeared to be rather similar. There was no evidence that the collagenase treatment involved in the preparation of the isolated

fat-cells resulted in the loss of any protein band or the appearance of new low-molecular-weight protein bands. Indeed, there was appreciably more of the low-molecular-weight proteins in the membranes prepared from intact fat-pads. The array of protein bands in the membrane preparations was clearly different from that found in the washed mitochondrial fractions from both isolated fat-cells and intact fat-pads (not shown). In agreement with other studies (Czech & Lynn, 1973; Avruch *et al.*, 1976*a,b*; Kawai & Spiro, 1977), plasma membranes derived from fat-cells appear to contain two prominent periodate/Schiff-staining glycoproteins (subunit mol.wts. 94 000 and 80 000), which coincide with major protein bands (Figs. 2 and 4). In

contrast, these glycoprotein bands were absent from the mitochondrial fractions.

Incorporation of $^{32}\text{P}_i$ into plasma-membrane phosphoproteins in isolated fat-cells

In these studies, fat-cells were incubated with $^{32}\text{P}_i$ for 75 min. Previous studies have shown that this period of time is sufficient to achieve maximum steady-state labelling of the major bands of ^{32}P -labelled phosphoproteins in the defatted whole-cell homogenate that can be separated by SDS/polyacrylamide-gel electrophoresis (Avruch *et al.*, 1976a,b; Brownsey *et al.*, 1977). The ^{32}P -labelled phosphoprotein bands observed at the various stages of a typical preparation of plasma membranes from fat-cells previously incubated with $^{32}\text{P}_i$ are indicated in Fig. 3. Fig. 3(a) shows the bands present in the defatted whole-cell homogenate together with the numbers assigned to these bands in this and other studies in this laboratory (Hughes *et al.*, 1977, 1980; Brownsey *et al.*, 1977, 1979; Belsham *et al.*, 1980). The washed plasma-membrane fraction (Fig. 3d) contained four major phosphoprotein bands derived from bands 4, 5, 8 and 9A of the original whole-cell homogenate. The approximate subunit mol.wts. were 67 000, 61 000, 26 000 and 20 000 respectively. The width of band 8 in this and other plasma-membrane fractions was somewhat greater than the other three bands, and this may indicate that this band is composed of more than one phosphoprotein. The peak heights of the densitometric traces of phosphoprotein bands 4, 5, 8 and 9A in the defatted whole-cell homogenate do not represent solely incorporation into the corresponding plasma-membrane phosphoproteins since all four of these bands contained ^{32}P -labelled components that remained in the 30 000 g-supernatant fraction (Fig. 3b). These are unlikely to be of plasma-membrane origin, since this fraction contained less than 10% of the fat-cell 5'-nucleotidase activity (Table 1). Moreover, with bands 4, 5 and 9A, the phosphoprotein bands remaining in the supernatant fraction clearly have slightly different subunit molecular weights to those in the particulate fraction (Fig. 3c).

The relative incorporation of $^{32}\text{P}_i$ into the four major [^{32}P]phosphoprotein bands in the plasma-membrane fraction was rather consistent with fat-cell preparations incubated in the absence of hormones. Overall in eight separate experiments, the proportions of peak heights of the densitometric traces of phosphoprotein bands 4:5:8:9A were $1:0.97 \pm 0.04:0.64 \pm 0.05:0.61 \pm 0.07$ (values as means $\pm$ S.E.M.).

The mitochondrial fraction (Fig. 3e) contained only two phosphoprotein bands of approximate subunit mol.wts. 48 000 and 42 000. Other studies have indicated that the latter (band 6) is the

Fig. 3. Subcellular fractionation of ^{32}P -labelled phosphoproteins in isolated fat-cells

Fat-cells were incubated for 75 min with $^{32}\text{P}_i$ (0.2 mM, 1000 d.p.m./pmol) in the absence of hormones. Subcellular fractions were prepared as given in the Experimental section in the presence of added EDTA (5 mM) and NaF (20 mM). Proteins from fractions derived from equivalent amounts of cells (20 mg dry wt.) were separated by SDS/polyacrylamide-slab-gel electrophoresis along parallel tracks on the same slab. The Figure shows densitometric traces of the radioautograph of the dried-down slab locating the ^{32}P -labelled phosphoproteins in the following fractions: (a) defatted whole-cell homogenate; (b) 30 000 g supernatant; (c) 30 000 g pellet; (d) washed plasma membranes; (e) washed mitochondria. The numbering of the bands of phosphoproteins used in the text is indicated in (a). The dye front (Bromophenol Blue) was allowed to migrate 10 cm. Results are typical for six different experiments.

phosphorylated α -subunit of the pyruvate dehydrogenase complex (Hughes *et al.*, 1980), whereas the former (band 5A) may be the corresponding subunit in the branched-chain 2-oxo acid dehydrogenase complex (Hughes & Halestrap, 1980). The plasma-membrane fraction contained very little of these mitochondrial phosphoproteins. Table 2 summarizes the results obtained in nine separate experiments similar to the one illustrated in Fig. 3. In the washed plasma-membrane fraction the ^{32}P associated with either band 4 or 5 was more than 5 times that in band 6, whereas in the washed mitochondrial fraction the ^{32}P associated with band 6 was 2–3 times that into either band 4 or 5. This further illustrates the excellent separation of plasma membranes and mitochondria that can be achieved by the Percoll centrifugation step.

None of the four major ^{32}P -labelled phosphoprotein bands in the plasma-membrane fraction coincided with the two prominent periodate/Schiff-staining glycoprotein bands (Fig. 4). On the other hand, phosphoprotein bands 4 and 5 did co-migrate with two distinct Coomassie Blue-staining protein bands (Fig. 4).

Effects of insulin and adrenaline on the incorporation of $^{32}\text{P}_i$ into the four major labelled phosphoprotein bands in plasma membranes of isolated fat-cells

When either insulin or adrenaline was present for the final 15 min of a 75 min incubation of fat-cells with $^{32}\text{P}_i$, incorporation of $^{32}\text{P}_i$ into the phosphoprotein band 5 in the plasma membranes was significantly increased after correction had been made for differences in protein associated with the band (Table 3). The increase after exposure to insulin was only about 30%, whereas with adrenaline the increase was about 60%. Smaller increases in incorporation into phosphoprotein bands 4, 8 and 9A may also have occurred, but the apparent increases were not statistically significant (Table 3). Densitometric scans showing the effects of insulin and adrenaline on the $^{32}\text{P}_i$ incorporation into washed plasma membranes, in one typical experiment, are shown in Fig. 5.

As found in other studies, both insulin and adrenaline increased the phosphorylation of phosphoprotein band 2 in the defatted whole-cell homogenate but had no appreciable effect on the phosphorylation of band 3 (Table 3; Benjamin & Singer, 1975; Avruch *et al.*, 1976b; Hughes *et al.*, 1977, 1980; Brownsey *et al.*, 1979). Phosphoprotein band 2, which has a subunit mol.wt. of 130000, may be the cytoplasmic protein ATP-citrate lyase (EC 4.11.3.9) (Alexander *et al.*, 1979; Janski *et al.*, 1979). Little or no effect of insulin or adrenaline was observed on the peak height of the densitometric trace of band 5 in the defatted

Fig. 4. Densitometric traces showing (a) Coomassie Blue-staining proteins, (b) the periodate/Schiff-staining glycoproteins and (c) the ^{32}P -labelled phosphoproteins in the same washed plasma-membrane fraction from isolated fat-cells

Washed plasma-membrane fraction was prepared from fat-cells incubated with $^{32}\text{P}_i$ as given in Fig. 3. Proteins were separated by SDS/polyacrylamide-slab-gel electrophoresis. *Protein bands corresponding to the two major periodate/Schiff-staining glycoproteins; †protein bands corresponding to the two major [^{32}P]phosphoproteins.

whole-cell homogenate (Table 3). This was presumably because of the supernatant phosphoprotein that contributes to the peak height in this fraction (see Fig. 3). No useful value for the effect of adrenaline on the $^{32}\text{P}_i$ incorporation into band 4 of the defatted whole-cell homogenate could be obtained, because, in the presence of adrenaline, there is a very large increase in the phosphorylation of an adjacent phosphoprotein referred to as 4A in an

Table 2. Recovery of [^{32}P]phosphoprotein bands 4–6 in washed plasma-membrane and mitochondrial fractions

Fat-cells were incubated with $^{32}\text{P}_i$ in the absence of hormones, and the [^{32}P]phosphoprotein bands in subcellular fractions were separated as given in Fig. 3. Results are the means $\pm$ s.e.m. for nine separate preparations of fat cells.

Fraction	^{32}P in band 4	^{32}P in band 5
	$\frac{\text{ratio}}{^{32}\text{P} \text{ in band 6}}$	$\frac{\text{ratio}}{^{32}\text{P} \text{ in band 6}}$
Particulate	1.81 ± 0.21	1.86 ± 0.17
Washed plasma membranes	5.50 ± 0.62	5.36 ± 0.55
Washed mitochondria	0.375 ± 0.06	0.41 ± 0.06

Table 3. Effects of insulin and adrenaline on the incorporation of $^{32}\text{P}_i$ into the major phosphoproteins associated with the plasma membranes within intact fat-cells

Fat-cells were incubated with $^{32}\text{P}_i$ in the presence or absence of hormones as given in Fig. 5. Defatted whole-cell homogenate and washed plasma-membrane fractions were prepared, and the proteins were separated by SDS/polyacrylamide-slab-gel electrophoresis. Changes in incorporation of $^{32}\text{P}_i$ into the indicated phosphoprotein bands were determined from the peak heights of densitometric traces of radioautographs. Differences in protein loading between samples of defatted whole-cell homogenate from control and hormone-treated cells were negligible and no correction was applied. Unavoidable differences between protein loading of samples of washed plasma membranes were corrected on the basis of densitometric scanning after staining with Coomassie Blue. For phosphoprotein bands 4 and 5 correction was based on the peak height of the corresponding Coomassie Blue-staining protein band; since phosphoprotein bands 8 and 9A have no clearly defined corresponding protein band, correction was based on the integral of the area under the densitometric scan corresponding to mol.wts. between 140000 and 40000 (which represents the most heavily stained region of the gels). Results are given as means $\pm$ s.e.m. calculated from observations on eight separate preparations of fat-cells. Significance of effect of hormone: ** $P < 0.01$; * $P < 0.05$.

Incorporation of $^{32}\text{P}_i$ (as % of no-hormone control)

Fraction	Phosphoprotein band no.	Approx. mol.wt.	Effect of hormone	
			Effect of insulin	Effect of adrenaline
Defatted whole-cell homogenate	2	130 000	$155 \pm 7^{**}$	$141 \pm 6^{**}$
	3	85 000	105 ± 5	101 ± 10
	4	67 000	103 ± 6	—
	5	61 000	$118 \pm 7^*$	116 ± 10
Washed plasma membranes	4	67 000	118 ± 7	119 ± 16
	5	61 000	$132 \pm 10^*$	$160 \pm 19^{**}$
	8	26 000	117 ± 8	116 ± 8
	9A	20 000	117 ± 9	106 ± 8

earlier study (Brownsey *et al.*, 1979). This phosphoprotein appeared almost exclusively in the supernatant fraction.

As an alternative means of demonstrating a specific effect of adrenaline on the phosphorylation of phosphoprotein band 5 in the fat-cell plasma membrane, we have calculated the ratio of the incorporation of $^{32}\text{P}_i$ into band 5 to the incorporation into (band 4 + band 8 + band 9A) in the washed plasma-membrane fraction. The calculation of this ratio does not involve any allowance for differences in protein loading on the gels. The value of this ratio in membranes from fat-cells incubated in the absence of hormones was 0.43 ± 0.026 . After exposure to adrenaline the ratio was significantly increased ($P < 0.01$, Student's *t* test) to 0.52 ± 0.024 . Although insulin also increased this ratio (to

0.47 ± 0.026), the increase was not statistically significant. (Results given as means $\pm$ s.e.m. for eight observations taken from experiments reported in Table 3.)

General conclusion

The preparation of plasma membranes from fat-cells with the use of centrifugation in the presence of Percoll appears to be both more rapid and convenient than previously available methods (McKeel & Jarett, 1970; Avruch & Wallach, 1971; Luzio *et al.*, 1976; Avruch *et al.*, 1976a; Kawai & Spiro, 1977; Lewis *et al.*, 1979). The yield of plasma membranes, on the basis of the recovery of 5'-nucleotidase, was over 50%, and appreciably

Fig. 5. Effects of insulin and adrenaline on the incorporation of $^{32}\text{P}_i$ into phosphoproteins of fat-cell plasma membranes. Fat-cells were incubated with $^{32}\text{P}_i$ for 75 min with no hormone (—), 0.5 μg of insulin/ml (----, a) or 1 μg of adrenaline/ml (----, b) for the final 15 min of incubation. Washed plasma-membrane fractions were prepared, and the proteins from equivalent samples were separated by SDS/polyacrylamide-slab-gel electrophoresis in parallel tracks. The Figure shows densitometric traces of the radioautograph.

larger than that reported for the other methods. The purity, as judged by the increase in 5'-nucleotidase specific activity, the activity of mitochondrial and endoplasmic-reticulum marker enzymes and the spectrum of ^{32}P -labelled phosphoproteins, appeared to be satisfactory, and at least comparable with that achieved by the other techniques. However, the washed plasma-membrane fraction in the present studies appeared to contain appreciably greater amounts of DNA than that found with the plasma-membrane preparation developed by McKeel & Jarett (1970), but the amounts were comparable with that reported for plasma membranes prepared by the immunological procedure described by Luzio *et al.* (1976).

It seems reasonable to expect that fractionation in the presence of Percoll along the lines used in this study will find wide applicability not only for the rapid purification of plasma membranes from tissues but also for the preparation of intact coupled mitochondria free of plasma membranes and other cell organelles.

The studies on the incorporation of $^{32}\text{P}_i$ into plasma-membrane phosphoproteins indicate that incorporation occurs into four major bands of phosphoproteins separable by SDS/polyacrylamide-gel electrophoresis with subunit mol.wts. of about 67000, 61000, 26000 and 20000. None of these coincide with the two major glycoproteins,

which have apparent mol.wts. of 94000 and 80000. These results are in general agreement with those obtained by Avruch *et al.* (1976a,b), but not with those obtained by Benjamin & Clayton (1978), who concluded that the two glycoproteins corresponded to two of the major phosphoprotein bands.

We observed a small increase in phosphorylation of the phosphoprotein band with subunit mol.wt. about 61000 in the plasma-membrane fraction after brief exposure of fat-cells to insulin and a larger increase after exposure to adrenaline. These observations differ from those made by Avruch *et al.* (1976b), who found no significant effects of either hormone on the incorporation of $^{32}\text{P}_i$ into any of the phosphoprotein bands in the plasma-membrane fraction except for an increase into the band of mol.wt. 26000 after exposure of the fat-cells to adrenaline. A possible explanation of the discrepancies between our studies and those made by Avruch *et al.* (1976b) may be that changes in phosphorylation occurred during the 3–4 h required to prepare the plasma-membrane fraction in the procedure of Avruch *et al.* (1976b). We cannot rule out the possibility that some alterations in phosphorylation also occurred in the present studies, despite the presence of high concentrations of EDTA and NaF throughout the preparation of the plasma-membrane fraction. However, the effects of adrenaline on the phosphorylation of the plasma-

membrane phosphoprotein band with mol.wt. 61000 could be observed in the 30000 g-particulate fraction, which required only 30 min to prepare.

Seals *et al.* (1979a,b) have reported that direct exposure of fat-cell membranes to insulin leads to decreased incorporation of ^{32}P from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ into a plasma-membrane phosphoprotein of subunit mol.wt. about 120000 in the presence of a low concentration of Mg^{2+} . No evidence was found in the present study for the phosphorylation of a plasma membrane phosphoprotein of this subunit molecular weight within intact fat cells.

There are a number of enzymes and transport processes associated with fat-cell plasma membranes that are known or are widely held to be regulated by insulin and adrenaline. Although it is not possible to assign a physiological role to any of the phosphoproteins in fat-cell membranes at present, the modest effects of insulin and adrenaline on the phosphorylation of the 61000-mol.wt. phosphoprotein band suggest that this major protein band in fat-cell plasma membranes is worthy of particular attention.

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