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Studies on the Topography of the Fat Cell Plasma Membrane†

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ABSTRACT: Purified plasma membranes from isolated fat cells are composed of at least 13 major peptide components including two major glycopeptides, as determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis. These glycopeptides with apparent molecular weights of 94,000 and 78,000 apparently account for a substantial amount of the total membrane protein. Membrane components which are exposed on the exterior cell surface were determined by catalytic iodination of intact cells with lactoperoxidase in the presence of H_2O_2 and $Na^{125}I$. Following iodination of fat cells only 6% of the total ^{125}I incorporated into isolated membrane fractions was found associated with mitochondrial membranes, indicating little or no penetration of lactoperoxidase into fat cells. Gel electrophoresis of the isolated plasma membranes derived from catalytically iodinated intact fat cells revealed that essentially all the label was associated with the glycopeptide of 94,000 molecular weight and, to a lesser extent, the lighter glycopeptide. In contrast, exposure of isolated plasma membranes to this labeling procedure resulted in greater total incorporation of radioactivity into the membrane and a more uniform iodination of all membrane peptides. These studies indicate that the two major membrane glycopeptides represent most of the protein on the exterior of the isolated fat cell surface. Procedures used to isolate the plasma membrane fraction from these cells apparently severely disrupt the highly organized structure of the intact cell surface which may account, at least in part, for the relative insensitivity of isolated membranes to effects of insulin and other hormones.

Many of the hormones which regulate adipose cell functions are thought to interact with these cells at the level of the plasma membrane. Direct evidence for this concept was provided in the cases of insulin (Cuatrecasas, 1969) and growth hormone (Hecht *et al.*, 1972) by the demonstration that covalent linkage of these hormones to Sepharose beads larger than the fat cells does not prohibit their physiological actions. Understanding the molecular events involved in these hormone–plasma membrane interactions will probably require a detailed knowledge of the membrane components and their organization. We recently initiated studies on the fat cell plasma membrane and found that major peptide components could be reproducibly resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (Czech and Lynn, 1973a). Further characterization of some of these components is in progress.

The aim of the present studies was to obtain information about the spatial arrangement of the peptide and glycopeptide constituents of the fat cell surface. We have employed the lactoperoxidase iodination procedure which has been success-

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TABLE I: Appearance of ^{125}I in Membrane Fractions Isolated from Enzymatically Iodinated Fat Cells.[a]

Fraction	cpm/mg of Protein	Total cpm/ Fraction	% of Total cpm
Mitochondria	8.0×10^3	2.1×10^4	6
Microsomes	3.4×10^4	1.1×10^5	32
Plasma membrane	1.1×10^5	2.1×10^5	62
Plasma membrane from cells treated without lactoperoxidase	9.0×10^2	1.9×10^3	

[a] Aliquots (0.4 ml) of membrane fractions prepared as described in Methods were added to 5 ml of ice-cold 10% trichloroacetic acid and after vigorous mixing decanted onto glass fiber filters (Whatman GF/C) which had been washed in 10% trichloroacetic acid. The membranes were washed twice with 8 ml of ice-cold 10% trichloroacetic acid, and the radioactivity contained by the filters was determined.

fully used to identify membrane components exposed on the exterior of intact cells (Phillips and Morrison, 1971; Poduslo *et al.*, 1972; Phillips, 1972) and isolated membranes (Phillips and Morrison, 1971; Poduslo *et al.*, 1972). The present results indicate that most of the protein exposed on the exterior fat cell surface consists of the major membrane glycopeptides of apparent molecular weights 94,000 and 78,000.

Methods

Isolation of Fat Cells. White fat cells were obtained by enzymatic digestion of the parametrial adipose tissue from 150- to 200-g female rats (Charles River CD strain) fed laboratory chow ad libitum (Rodbell, 1964). For each experiment involving isolation of purified plasma membranes the parametrial adipose tissue (20–40 g) from 15 or more rats was pooled and cut into small pieces with scissors, blotted, and added to 1-oz plastic bottles. Each bottle contained 4 g of tissue and 8 or 10 ml of 3% albumin in phosphate buffer with 0.8–2 mg/ml of crude collagenase (*Clostridium histolyticum*, Worthington) and was incubated for 60 min at 37°. The phosphate buffer contained 128 mM NaCl, 1.4 mM $CaCl_2$, 1.4 mM $MgSO_4$, 5.2 mM KCl, and 10 mM Na_2HPO_4. The albumin buffer was made up fresh daily and the pH adjusted to 7.4 with sodium hydroxide after addition of the desired amount of bovine serum albumin fraction V powder (Armour lot No. H38311).

At the end of the digestion period cells were filtered through two layers of cheesecloth and washed twice with warm albumin buffer followed by one wash with warm 0.25 M sucrose, 1 mM EDTA, 5 mM Tris (pH 7.5, buffer A).

Preparation of Plasma Membrane Fraction. Plasma membranes were prepared from isolated rat cells essentially according to previously reported methods (McKeel and Jarett, 1970; Laudat *et al.*, 1972). Fat cells derived from approximately 10 g of tissue were added to a prechilled glass homogenizer with 25 ml of ice-cold buffer A and homogenized (Teflon pestle) with five up and down strokes. The homogenate was centrifuged for 10 min at 20,000*g* and the resulting pellet was resuspended in buffer A by hand homogenization. The resuspended pellet was layered on a 24.5-ml linear sucrose gradient (27.6–54.1%, w/w) containing 1 mM EDTA and 5 mM Tris–HCl (pH 7.5). The gradient was centrifuged in a Beckman 25.1 rotor at 24,000 rpm for 1 hr at 0°, and the milky plasma membrane fraction was collected. This fraction was diluted with 6–10 volumes of 1 mM EDTA (pH 7.5) and centrifuged at 30,000*g* for 20 min. Membranes were washed at least three times with 30 ml of ice-cold 1 mM EDTA. Alternatively, plasma membranes were prepared by layering the resuspended 20,000*g* pellet over 25 ml of 30% sucrose and centrifuging in a Beckman 25.1 rotor for 1 hr at 24,000 rpm. The plasma membranes at the sucrose interface were subsequently treated as described above. No difference in the peptide components of membranes prepared by these methods could be found (Czech and Lynn, 1973a). The mitochondrial fraction from the gradient was also collected, diluted with six volumes of 1 mM EDTA, and centrifuged at 30,000*g* for 15 min. "Microsomes" were prepared (McKeel and Jarett, 1970) by centrifuging the supernatant above the first 20,000*g* pellet at 160,000*g* for 45 min.

Plasma membranes were stored at −20° for several weeks with no detectable change in the mobilities or relative amounts of any of the peptide components upon sodium dodecyl sulfate polyacrylamide electrophoresis. Protein was estimated (Lowry *et al.*, 1951) with bovine serum albumin as the standard.

Iodination of Cells or Isolated Membranes. Lactoperoxidase-catalyzed iodination of intact fat cells and isolated membranes was performed (Phillips, 1972) with several additions of H_2O_2 aliquots. Fat cells prepared for iodination were quickly washed twice with Krebs–Ringer buffer in the absence of albumin instead of the washing medium described above. The fat cells derived from 10 to 15 g of adipose tissue were incubated at 25° in 20 ml of Krebs–Ringer phosphate buffer containing 1 mCi of carrier-free $Na^{125}I$ and 0.5 mg of lactoperoxidase. The reaction was commenced by five 10-μl aliquots of 5×10^{-2} M H_2O_2 added at 30-sec intervals. The iodination of cells was inhibited by addition of 8 ml of 3% albumin medium and centrifugation of cells. The cells were washed in warm 3% albumin buffer four times followed by one wash with 0.25 M sucrose, 5 mM Tris–HCl, and 1 mM EDTA (pH 7.5). Plasma membranes were prepared as described above and washed three times in 1 mM EDTA (pH 7.5). Plasma membranes derived from control cells treated similarly but without lactoperoxidase contained 100 times less label after this washing procedure (Table I). Further washings reduced this residual label to essentially zero cpm.

Isolated plasma membranes (0.3–0.5 mg of protein) were catalytically iodinated in 1 ml of Krebs–Ringer phosphate buffer (pH 7.4) with 0.05 mg of lactoperoxidase, 25 μCi of $Na^{125}I$, and five additions of 5 μl of 5×10^{-2} M H_2O_2 at 30-sec intervals. The iodination of membranes was inhibited by addition of ice-cold 1 mM EDTA (pH 7.5) and centrifuging at 50,000*g* for 20 min. The membranes were washed four times in 1 mM EDTA (pH 7.5) before solubilization in dodecyl sulfate. Membranes treated similarly, but without lactoperoxidase, contained essentially no incorporated label.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis. Gel electrophoresis of plasma membranes and Coomassie Blue staining of gels were performed (Weber and Osborn, 1969) using 8.5% acrylamide gels. Membranes suspended in 1 mM EDTA were routinely diluted with an equal volume of 2% sodium dodecyl sulfate, 8 M urea, and 5% mercaptoethanol in phosphate buffer and were shaken at room temperature or 37° for 15–30 min with no difference in results. The reduced, solubilized membranes were applied to

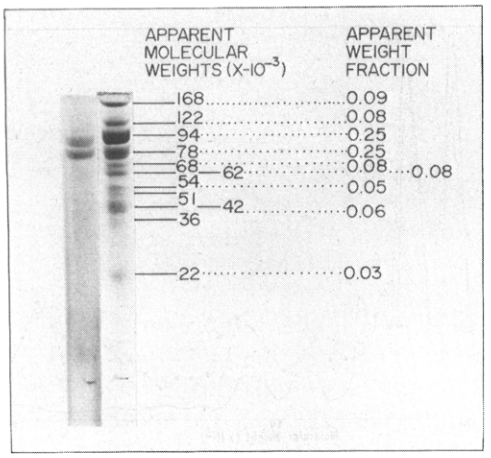

FIGURE 1: Dodecyl sulfate gel electrophoresis of fat cell plasma membranes. Membranes (90 μg of protein/gel) were solubilized and electrophoresed as described in Methods, and gels were stained for carbohydrate (A) or protein (B). Several Coomassie Blue stained gels were scanned at 550 nm and the mean areas under absorbance peaks corresponding to the various bands were taken as the apparent weight fraction of each region.

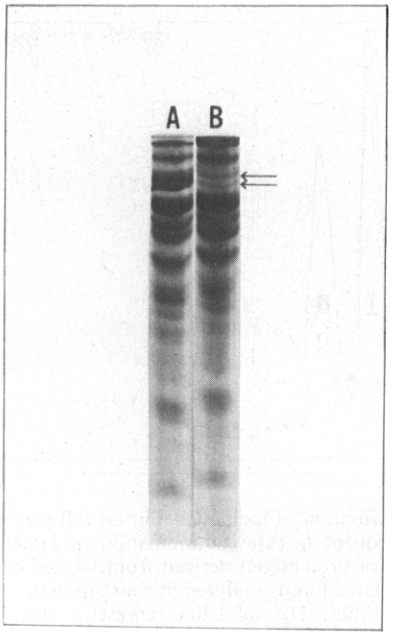

FIGURE 2: Dodecyl sulfate gel electrophoresis of fat cell membranes solubilized in the presence (gel A) or absence (gel B) of mercaptoethanol. Membranes (110 μg of protein/gel) were electrophoresed in the presence or absence of reducing agent and the gels stained for 8 hr in Coomassie Blue dye before destaining. Two peptide bands (arrows, gel B) appear in the region (94,000 apparent molecular weight) occupied by the larger glycopeptide when reduced.

120 × 8 mm columns containing polyacrylamide gels with the normal amount of cross-linker (Weber and Osborn, 1969). A current of 4 mA/gel for electrophoresis performed overnight or 8 mA/gel for 6–8-hr runs was applied.

Following immersion in Coomassie Blue stain (1.25 g of Coomassie Blue dissolved in 456 ml of 50% methanol plus 46 mg of glacial acetic acid) for 3–6 hr the gels were electrophoretically destained in a Canalco destainer. Gels were stained for carbohydrate essentially according to an earlier method (Zacharius *et al.*, 1969) with the following modifications. Following electrophoresis each gel was immersed in 50 ml of 12.5% trichloroacetic acid for 2–5 hr, rinsed in deionized water, and placed in 50 ml of fresh 12.5% trichloroacetic acid for a second 2–5-hr period before the periodic acid step. Destaining of gels was performed in a large excess of 50% methanol over a 24-hr period. Absorbance scans of gels was carried out on a Gilford 2000 spectrometer at 550 nm for Coomassie Blue and 560 nm for Schiff reagent.

Molecular weight estimations were made on gels using bovine serum albumin, ovalbumin, lactate dehydrogenase, concanavalin A, and cytochrome *c* as standards. Human immunoglobulin was run as a high molecular weight standard (160,000) in the absence of mercaptoethanol. The values presented in Figure 1 are based on four separate experiments performed with several different preparations of membranes. Gels run with one-half and twice the normal concentration of cross-linkers resulted in essentially the same values. Gels run with labeled membranes were stained with Coomassie Blue as above, scanned, and cut into 2-mm lateral slices (Hubbard and Cohn, 1972). Gel slices were incubated at 90° with 1 ml of ammonium hydroxide for several hours followed by evaporation of this reagent after a strip of filter paper was placed upright into each vial. The radioactivity was counted in a Triton–Omnifluor–toluene fluid (Czech *et al.*, 1973). In more recent experiments gel slices have been counted directly in a gamma scintillation spectrometer with no significant difference in labeling pattern observed.

Reagents. Lactoperoxidase was obtained from Calbiochem and had an absorbance 412 nm/absorbance 280 nm ratio of 0.6. $Na^{125}I$ was purchased from New England Nuclear. Reagents used for the preparation and use of polyacrylamide gels were obtained from Eastman.

Results

Figure 1 shows the dodecyl sulfate polyacrylamide gel patterns of fat cell plasma membranes after staining with Schiff reagent (gel A) and Coomassie Blue (gel B). Two major carbohydrate positive bands correspond to peptides with apparent molecular weights of 94,000 and 78,000. A much fainter Schiff reagent positive reaction is seen corresponding to the 62,000 molecular weight peptide, and a diffuse positive band appears just above the dye front suggesting the possible presence of glycolipids. Analysis of the areas under the absorbance peaks of the Coomassie Blue stained gels in several experiments revealed the apparent weight fractions of the various peptide regions. The two major glycopeptides accounted for about 50% of the total staining intensity of the membrane peptides (Figure 1). The 168,000 molecular weight peptide(s) represented about 9% of the total fixed stain and the 122,000, 68,000, and 62,000 molecular weight peptides about 8% each.

We had previously found (Czech and Lynn, 1973a) that the major difference in the gel pattern of membranes which were solubilized without reducing agent was the strikingly reduced mobility of the 94,000 molecular weight glycopeptide. In the present experiments (Figure 2), using improved staining technique, we found two additional Coomassie staining bands (arrows, gel B) in the 94,000 molecular weight region which is vacated by the glycopeptide in the absence of mercaptoethanol. Schiff reagent staining confirmed the presence of the glycopeptide at the top of the gel (not illustrated).

The appearance of label in various membrane fractions when intact fat cells are catalytically iodinated with lactoperoxidase, H_2O_2 and $Na^{125}I$ is summarized in Table I. In this representative experiment the plasma membrane fraction was substantially enriched in label; 62% of the total radioactivity appeared in the plasma membrane fraction, 32% in the micro-

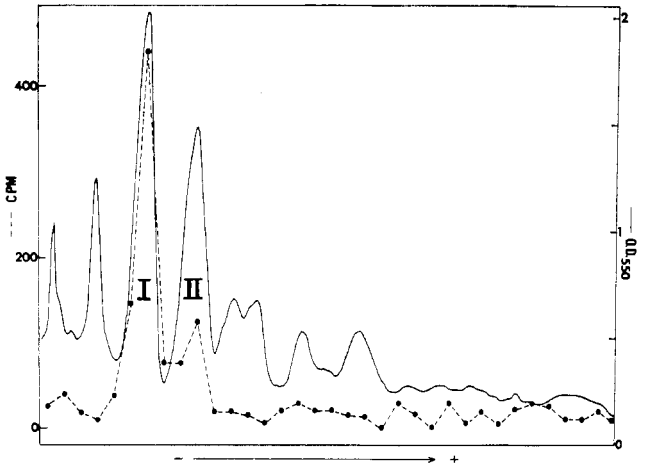

FIGURE 3: Distribution of iodine label in fat cell plasma membrane peptides subsequent to catalytic iodination of intact cells. Membranes (60 μg of protein/gel) derived from labeled cells were electrophoresed, stained and analyzed for distribution of label as described in Methods. The solid line represents absorbance at 550 nm and the dotted line shows the counts per minute observed in each 2-nm gel slice. Bands I and II represent the 94,000 and 78,000 molecular weight glycopeptides, respectively.

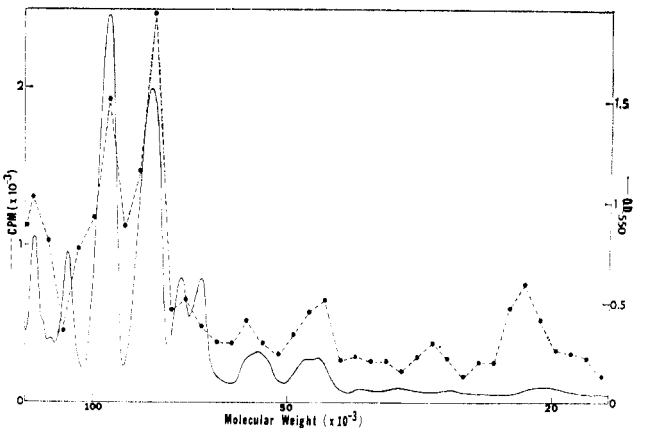

FIGURE 4: Distribution of iodine label in fat cell plasma membrane peptides subsequent to catalytic iodination of isolated membranes. Membranes were enzymatically iodinated (see Methods) and applied (70 μg of protein/gel) to 8.5% acrylamide gels. The gels were stained for protein (—) and analyzed for labeled peptides (- - - - -).

somal fraction, and only 6% in the mitochondrial fraction (Table I). It was not feasible to monitor ^{125}I incorporation into soluble cytoplasmic components since the albumin in the buffer used for washing the iodinated cells becomes labeled and contaminates this cell fraction. However, the very low level of label found in the mitochondrial fraction attests to the inability of the lactoperoxidase labeling system to significantly penetrate fat cells. Plasma membranes derived from cells treated with reagents but without lactoperoxidase contained only about 2000 cpm compared with 210,000 cpm when the enzyme was present. This small amount of residual label could be essentially eliminated after two further washings of the membranes (not illustrated).

Analysis of plasma membranes derived from enzymatically iodinated fat cells on dodecyl sulfate polyacrylamide gels revealed a remarkably selective distribution of label (Figure 3). Essentially all the label was found in the region containing the major membrane glycopeptides (I and II). The specific activity of the 94,000 molecular weight glycopeptide (I) was consistently observed to be four to five times greater than that of the lighter glycopeptide. Little or no label was found near the dye front (lipid region) in any of the experiments with membranes which were carefully washed free of unreacted iodide.

When isolated membranes from fat cells were exposed to catalytic iodination the resulting labeling pattern differed markedly from that observed after iodination of intact cells (Figure 4). Essentially all the membrane peptides were significantly labeled to a degree roughly proportional to protein weight fraction. The 22,000 molecular weight peptide appeared more heavily labeled on a weight basis compared to other peptides. The region corresponding to the 78,000 molecular weight glycopeptide contained significantly more label compared to the 94,000 molecular weight region, in contrast to the results obtained upon labeling intact cells. In addition to the more uniform labeling pattern observed after iodination of isolated membranes, the total amount of ^{125}I incorporated into the membrane was markedly higher than that obtained by labeling intact cells (see Methods).

Discussion

Since isolated fat cells are prepared by digestion of tissue with a crude collagenase preparation known to contain protease activity, some surface peptides may be removed by this process and others which remain may represent partially degraded fragments of native membrane proteins. Nevertheless, the adipocyte surface appears particularly sensitive to the actions of a large number of hormones and other agents. For example, we have studied the effects of thiols (Czech and Fain, 1972) and lectins (Czech and Lynn, 1973b) on fat cell metabolism. These agents mimicked the action of insulin on fat cell glucose oxidation and lipolysis, apparently secondary to interactions at the level of the plasma membrane. Thus, membrane components and structure necessary for mechanisms involved in hormone action survive the procedure used to isolate fat cells and this system is well suited for studies on a hormonally responsive cell surface. The present studies were designed to continue our analysis of the fat cell surface components and their organization with the ultimate aim of understanding these mechanisms.

The purity of fat cell plasma membranes as prepared in this report has been studied in detail (McKeel and Jarett, 1970) using enzyme markers and morphology. These workers concluded that the plasma membranes were contaminated by about 10% by both microsomes and mitochondria. This fat cell plasma membrane fraction was found to consist of 11 major peptides ranging in apparent molecular weights from 168,000 to 22,000 (Czech and Lynn, 1973a). The findings presented in this report indicate that the major glycopeptides (94,000 and 78,000) account for a large fraction of the total membrane protein (Figure 1). The evidence available which supports the concept that the glycopeptides represent most of the Coomassie Blue staining material in the 94,000 and 78,000 molecular weight regions is: (1) Schiff reagent positive regions are consistently associated with these Coomassie Blue bands when membranes are subjected to dodecyl sulfate gel electrophoresis on gels containing normal, one-half normal, or twice normal amounts of cross-linker; (2) when membranes were solubilized in the absence of reducing agent most of the Coomassie Blue stain in the 94,000 molecular weight region remains at the top of the gel (Figure 2); schiff reagent staining under these conditions results in a strong positive reaction

associated with this large molecular weight band at the top of the gel as well as the usual positive band associated with the 78,000 molecular weight region); and (3) analysis of fat cell plasma membranes showed that both the Coomassie Blue and Schiff reagent staining material in the 94,000 and 78,000 molecular weight regions were relatively stable to trypsin treatment of intact cells while three other major peptides were digested (Czech and Lynn, 1973a). When isolated membranes were trypsinized in 1 mM EDTA, the loss of material stained by Schiff reagent paralleled the loss of Coomassie Blue staining material in these regions.

In spite of the above evidence, further proof is required to document this substantial contribution of these glycopeptides to the total membrane protein. Diverse proteins often stain very differently, and the possibility that other peptides contaminate the glycoprotein bands cannot be ruled out. On the other hand, the apparent weight fraction data presented in Figure 1 may represent an underestimate for the glycopeptides since these species are known to bind Coomassie Blue poorly compared to peptides without carbohydrate (Fairbanks *et al.*, 1971). Of further interest was our finding that two other peptides occupy the 94,000 molecular weight region (arrows, Figure 2), raising the number of major membrane peptides to at least 13.

The considerable enrichment of iodination in the plasma membrane fraction of catalytically labeled fat cells supports the utility of this procedure for identification of surface components (Table I). It has been conclusively demonstrated that lactoperoxidase does not penetrate erythrocytes (Phillips and Morrison, 1970). This also appears to be the case in the present studies since only 6% of the total label was incorporated into the fat cell mitochondrial fraction. The distribution of label in the microsomal fraction probably reflects contamination of this fraction by plasma membranes. This conclusion is strengthened by the finding that gel electrophoresis of the mitochondrial and microsomal fractions showed the label to be associated with the same glycopeptides which contain the label present in the plasma membranes (not illustrated). These glycopeptides (94,000 and 78,000 molecular weight) were previously shown to be present in the mitochondrial and microsomal fractions but were considerably enriched in the plasma membrane fraction (Czech and Lynn, 1973a). The present data thus indicate that a considerable amount of plasma membranes contaminates the fat cell microsomal fraction. In this regard, substantial adenylate cyclase activity, a marker for the plasma membrane, was found associated with microsomes in the presence of fluoride (McKeel and Jarett, 1970). In contrast, the plasma membrane fraction appears relatively pure according to enzyme marker and morphology studies (McKeel and Jarett, 1970) and our observation that the mitochondrial and microsomal fractions contained considerable amounts of low molecular weight peptide species (30,000–20,000) which were absent in the plasma membranes (Czech and Lynn, 1973a).

The highly selective distribution of label among the peptides composing the plasma membranes derived from iodinated fat cells (Figure 3) is in concert with the notion that this procedure only labels accessible peptides. Apparently the glycopeptide of 78,000 molecular weight is more shielded from the lactoperoxidase than is the larger glycopeptide since the specific activity of the former was several fold higher (Figure 3). That the highly selective nature of the labeling in these experiments is not due to an intrinsic inability of the membrane peptides to become labeled is supported by the uniform pattern of labeling of isolated membranes (Figure 4). We conclude that most of the exposed protein on the exterior of the fat cell surface consists of the larger glycopeptide and, to a lesser degree, the 78,000 molecular weight glycopeptide.

We have consistently observed a greatly increased incorporation of ^{125}I into isolated plasma membranes using the lactoperoxidase method compared to ^{125}I incorporation into intact fat cells. Similar results have been previously reported with erythrocytes (Phillips and Morrison, 1971) and fibroblasts (Poduslo *et al.*, 1972). Although preparation of fat cells involves digestion with crude collagenase which contains protease activity (Kono, 1969), the surface of isolated fat cells apparently represents a tightly organized structure. It is not clear as yet whether the isolation of membranes alters the accessibility of protein components to lactoperoxidase or changes their susceptibility to iodination. In any case, the disruption of the organization or structures of membrane proteins which apparently accompanies the preparation of the plasma membrane fraction may explain, in part, the reduced or lost sensitivity of membrane activities to hormones. For example, although addition of insulin to intact cells has been reported to result in increased glucose transport in the subsequently isolated plasma membranes, no effect of insulin added directly to isolated membranes could be observed (Martin and Carter, 1970).

Our recent finding that concanavalin A binds to isolated fat cells as evidenced by marked agglutination of cells by this lectin (Czech and Lynn, 1973c) is in concert with the conclusion of the present findings that glycopeptides occupy the exterior cell surface. Studies designed to demonstrate whether one or both of the surface glycopeptides described in this report are indeed concanavalain A receptor(s) are in progress.

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