METABOLIC AND ULTRASTRUCTURAL CHANGES INDUCED IN ADIPOSE TISSUE BY INSULIN

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ABSTRACT

The addition *in vitro* of insulin to rat adipose tissue (epididymal) produces marked metabolic changes which may be followed by measurement of the net gas exchange of the tissue. Using this method to monitor the metabolic action of insulin, concomitant observations with the electron microscope on the tissue have been made. These reveal that pronounced morphological changes are induced by insulin. The plasma membranes of the adipose cells become invaginated at many sites to form minute finger-like indentations. Numerous tiny, membrane-bounded vesicles are also present and arranged in relationship to the plasma membrane in such a way as to suggest that their formation occurred when a recessed fold was pinched off. Deeper in the cytoplasm, especially in specimens that had been incubated a longer time, numerous large, smooth, membrane-limited vesicles are seen. Finally, in these incubated specimens the cytoplasmic matrix has lost much of its granular nature, small lipid droplets are frequently found in the cytoplasm and suggestive changes have occurred in mitochondria. In control specimens, incubated without insulin for identical periods of time, indentations and vesicles in the plasma membrane are sparse at best and no vesicles or membrane-bound spaces appear deeper in the cytoplasm. The metabolic and morphologic changes induced by insulin seem to be interdependent events. Both changes appear to be initiated rapidly and concomitantly in the tissue. Both processes are initiated by insulin at concentrations considered to be physiological, 0.004 μg. (100 μunits) per ml. Insulin treated with alkali fails to initiate either process. It is concluded that insulin initiates pinocytosis in rat adipose tissue and the possible significance of this process in the mode of action of insulin is discussed.

INTRODUCTION

It is now well known that addition of insulin to rat epididymal fat pad incubated in a bicarbonate-buffered medium causes a marked stimulation of the uptake of glucose by the adipose cells and the rapid synthesis of fat from glucose (1, 2). This synthesis is accompanied by a pronounced change in the total gas exchange of the adipose tissue as a considerable excess of CO_2 output occurs over oxygen consumption (3, 4). This increase in CO_2 output as a consequence of fat synthesis may be

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measured manometrically, and such a procedure offers the opportunity to compare possible ultrastructural changes in adipose cells with monitored metabolic changes in function. Thus, it was deemed important to examine with the electron microscope adipose cells that had been stimulated by insulin *in vitro* and to compare them with similar cells that had not been stimulated. This paper, therefore, deals largely with the submicroscopic changes in adipose cells attending insulin stimulation, and with a discussion of a possible mechanism of action of insulin based on the morphological findings. The submicroscopic changes have been reported briefly elsewhere (5, 6).

MATERIALS AND METHODS

The present report is based upon 53 experiments on the epididymal fat pad of young (160 to 250 gm.) male albino rats. The tissues of this series were examined with the electron microscope in 38 experiments and in all except one, the experiments were monitored by means of manometric studies, which have been published (3).

The rats, fed *ad libitum* on Purina chow, were killed by decapitation and the epididymal fat pads were quickly removed with a minimum of handling, and pieces cut therefrom as described previously (4). The pieces were weighed on a torsion balance (average 125 mg.) and placed directly into a Warburg vessel containing the medium to be employed for the experiment. Warburg vessels of 8 to 10 ml. capacity, without a center well, but containing a side arm with a vented stopper were used. The basic bicarbonate medium employed was that of Krebs and Henseleit (7) to which glucose (4 mg./ml.) and sometimes gelatin (2 mg./ml.) were added. (As shown previously (3), gelatin protects against the loss in activity encountered in very dilute solutions of insulin, presumably due in part to absorption of insulin on glass surfaces.) Solutions of insulin (30 units/ml.) were made by dissolving crystalline zinc insulin[1] in water containing two drops of 0.1 N HCl/5 ml. Such stock solutions were kept cold until used, diluted with the appropriate basic bicarbonate medium, and placed in the side arm of the Warburg vessel. The vessels were mounted on their manometers and placed in a constant temperature bath (37.2°C.) and gassed with slow shaking. The gas phase was a mixture of 5 per cent CO_2—95 per cent air. After an equilibration period of from 5 to 10 minutes, the pH of the medium was 7.35. Readings, taken at 5 or 10 minute intervals, were begun within 10 to 15 minutes after insertion of the flasks in the water bath, and continued for an hour.

[1] Crystalline zinc insulin, Lot 466368 (which assayed 25 units/mg.) was kindly furnished by Eli Lilly and Company.

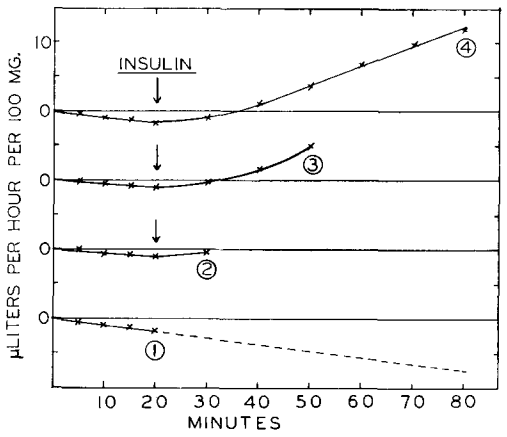


FIGURE 1

An example of the manometric monitoring of insulin action on adipose tissue employed for microscopic studies. (See text for details.)

A monitoring experiment is illustrated in Fig. 1. Here each of four pieces of tissue (2 proximal and 2 distal halves of the epididymal adipose tissue from a single rat) were incubated separately under the conditions described above. The net gas exchange expressed as μl. of CO_2 per hour per 100 mg. for each of the four pieces of tissue is shown. As can be seen during the first 20 minutes, the pressure change in all four flasks was negative. At 20 minutes, flask No. 1 was removed and the tissue in it immediately fixed for electron microscopy. At this same time, as indicated by the arrow, insulin was tipped from the side arm into the other flasks to yield a final concentration of 0.1 unit/ml. As a result, the characteristic change in gas pressure from a negative to a positive value promptly occurred in each of these flasks. At 10, 30, and 60 minute intervals after the addition of insulin, one of the remaining flasks was removed and the tissue fixed. Thus, evidence of the biochemical action of insulin on the tissue was obtained before its fixation.

In most experiments only two pieces, both distal portions of the epididymal fat pad, from a single rat, were employed; one piece serving as a control. Gelatin in bicarbonate buffer or the buffer alone was placed in the side arm of the control flask and these contents were tipped into the center well at the same time that insulin was tipped into the experimental flask. In such experiments, the control piece was incubated for the same length of time as the piece treated with insulin. Thus, it would be

removed at some point along the dotted line shown for flask 1 in Fig. 1 which corresponded in total time of incubation with the flask to which insulin had been added.

Although most of the specimens were incubated for a 20- or 30-minute period after the addition of insulin, some were incubated 10, 15, 50, or 60 minutes. In one case, the tissue was incubated for only 2 minutes without monitoring after the addition of insulin. In this instance, the proximal portion of the fat pads were monitored manometrically to indicate that the tissue was responsive.

Several concentrations of insulin were tested. In addition to 0.1 μ/ml., 10^{-3} μ/ml. (10^3 micro units) or 10^{-4} μ/ml. (10^2 micro units) were assayed in incubations as described above for 20 or 30 minutes. It should be noted that 10^3 micro units/per ml. of insulin, produced an effect which was 95 to 100 per cent of that obtained with 10^5 micro units/ml. (3). One hundred micro units per ml. of insulin (a physiological concentration) caused a response that was approximately 60 per cent of that obtained with 10^5 micro units/ml.

Experiments were also performed in which insulin was added to tissue incubated in the absence of glucose. The control tissue for these experiments was incubated with insulin in the presence of glucose. Tissue was also incubated in the presence of glucose and inactivated insulin. The control tissue for these experiments was incubated with glucose and the same amount of insulin which had not been inactivated. Insulin was inactivated by dissolving 10 mg. in 20 ml. of 0.05 N NaOH and letting the solution stand overnight at 38° (8). The solution was then brought to a slightly acid condition by the addition of 5 N HCl and diluted appropriately with Krebs-Ringer bicarbonate for use. No activity was observed manometrically on the epididymal fat pad with this preparation at concentrations which corresponded to 0.1 unit of active insulin per ml. Pieces of adipose tissue were also removed from freshly killed rats and immediately fixed to serve as unincubated controls.

At the end of the incubation period, the fat pads were removed from the vessels, spread on wax plates in a few drops of osmium tetroxide, and pieces, 1 to 2 mm.^{3}, were cut from the thinnest portions of the pads. These pieces were fixed for 1 or 2 hours in 1 or 2 per cent osmium tetroxide, buffered to pH 7.4 in an acetate-veronal mixture containing enough sucrose to raise the over-all osmolar concentration to 0.44 M. After fixation, the small blocks were washed briefly in sucrose, dehydrated in increasing concentrations of alcohol, embedded in *n*-butyl methacrylate, and sectioned with a Porter and Blum microtome. The sections, made at two different levels, periphery and center of the blocks, were mounted on carbon-coated grids and viewed with an RCA(EMU-2E, or EMU-3C) electron microscope.

It should be noted here that adipose cells are difficult to prepare for electron microscopy, owing mainly to the large quantity of lipid which accumulates in them as a single vacuole. This lipid, which is very osmiophilic, is present in far greater quantity than the protoplasm of the cell and could presumably react sufficiently with the osmium tetroxide so as to decrease appreciably its concentration in the fixing medium. In addition, the rate of penetration of the fixative, which is notoriously slow, could be further retarded by the quantity of avidly reactive lipid. For these reasons, we found as the investigation proceeded that large volumes (20 cc.) of higher concentration of osmium tetroxide (2 per cent) for longer times (2 hours) improved the results. Even then, the polymer-like lipid that resulted from reaction with the osmium tetroxide (9) was difficult to section and frequently produced shredding and ripples in the tissue sections.

Another difficulty was imposed by the normal distribution of cytoplasm of the adipose cell which surrounds the central lipid vacuole as a thin rim. Only in the region of the nucleus of most adipose cells is a rather thickened area of cytoplasm observed. The thin portions not only contained sparse organelles such as mitochondria, but in some areas were so thin (less than 2000 A) so as to be non-informative. Therefore, the major portion of the study was conducted on regions of the cytoplasm selected because of their thickness.

In addition to the biochemical monitoring experiments described, other analyses were performed on the water, lipid, and lipid phosphorus content. For this purpose two pieces, distal and proximal, were cut from each fat pad and weighed on a torsion balance. They were then transferred to tared sintered glass funnels, floated in a small volume of isotonic NaCl, and carefully spread over the sintered glass surface with forceps. The NaCl was removed by gentle suction and blotting with filter paper, and the tissues were dried *in vacuo* over P_2O_5 at 38° overnight in a desiccator which had been flushed 3 times with nitrogen prior to the final evacuation. They were weighed the next morning. In two experiments the tissues were returned to the desiccator and left *in vacuo* over P_2O_5 at 38° for 4 more days. No further weight loss occurred.

The dried pieces of tissue were extracted 5 times with 2 ml. portions of 2:1 $CHCl_3$—CH_3OH. Each time the tissue and the solvent were stirred together on the sintered glass funnel with a glass rod and the solvent removed by gentle suction. The extracts

were transferred to tared evaporating dishes and the solvent evaporated off on a steam bath. The extracted material was dried *in vacuo* over P_2O_5 and paraffin chips overnight and weighed the next morning. The residue remaining on the sintered glass funnels after the extraction was determined by weighing after drying *in vacuo* over P_2O_5.

Lipid residues were redissolved in 2:1 $CHCl_3$—CH_3OH, transferred to digestion tubes, and the solvent evaporated off on a steam bath. They were then heated over micro burners with 1.5 to 2.5 ml. of 10 N H_2SO_4, with the addition from time to time of 1 drop of concentrated HNO_3, until the solution cleared. This usually required 6 to 8 hours. The solutions were cooled and transferred to volumetric flasks. Phosphorus was determined by the method of Fiske and Subbarow (10). A blank, 2.5 ml. of 10 N H_2SO_4, treated in the same manner, contained no measurable P.

RESULTS

Electron Microscopic Studies

Two pronounced morphological findings qualified the activity of insulin (10^5 micro units for 20 to 30 minutes) on adipose tissue. The first of these relates to the plasma membrane, a thin, uniform, continuous membrane, approximately 70 to 80 A in diameter, that encloses the cell surface. This membrane was invaginated at many sites to form numerous minute indentations (Figs. 2, 3, 6, 9, 12). These invaginations were globular, tubular, or tear-drop in shape and the smallest of them measured approximately 150 A in diameter at the widest part. Their length usually exceeded their width and the neck of the opening was frequently the narrowest part; in some cases not more than 50 A in diameter. They were surrounded by a membrane which was continuous with the plasma membrane, but in some cases the invaginated portion appeared thinner or less opaque than the non-invaginated parts. These indentations appeared to pinch off from the surface as evidenced by the narrow necks, as well as by the frequent occurrence of small, completely closed-off vesicles touching or very close to the under surface of the plasma membrane of the adipose cell (Figs. 2, 9). In some cases of the relatively long (300 to 400 A) tubular invaginations, the pinching off occurred at the distal portion of the tube. The indentations and vesicles were randomly scattered at the surfaces of the affected cells, but sometimes they occurred evenly spaced with gaps of several hundred A of unindented membrane between them (Figs. 6, 12). Frequently, they were bunched with 5 or 6 vesicles or indentations occurring along a length of membrane of about 1000 A, and as often there were similar stretches of surface membrane without indentations or vesicles (Figs. 2, 5). The adipose cells of the periphery of the blocks generally showed more of the described surface activity than those at the center of the block. However, there was some degree of variation of these findings from cell to cell even at the periphery.

The second most notable change in those adipose cells subjected to insulin was the occurrence of numerous, smooth, membrane-bounded vesicles throughout the cytoplasm (Figs. 2, 3, 9, 12). The vesicles were not of uniform size in different regions of the cytoplasm, but tended to be progressively larger in diameter as one proceeds into the deeper portions of the cytoplasm. Some of the larger ones were at least 4 or 5 times the diameter of those at the surface. However, this progression in size as a function of depth of cytoplasm was not uniform since some larger vesicles occurred moderately close to the surface membrane, and in the distal portions of the cytoplasm where the larger vesicles usually occurred, some smaller ones (500 to 600 A) were evident. The membrane surrounding the vesicles deep in the cytoplasm appeared, for the most part, similar to that enclosing the vesicles near the surface of the cell. However, the surface membrane of some of the large vesicles appeared fuzzy, less opaque, and sometimes incomplete. The occurrence and disposition of these cytoplasmic vesicles is compatible with the formation of a discontinuous reticulum formed by migration and swelling and/or coalescence of the surface vesicles.

It should be emphasized that the morphological changes induced by insulin occurred regardless of the presence or absence of either glucose or gelatin in the incubation medium (*cf.* Figs. 8 and 9 with Figs. 4 and 10).

Additional information, relating to the occurrence of indentations of the plasma membrane and cytoplasmic vesicles, was obtained in the experiments in which the amount of insulin in the media was varied, or the duration of the incubation with a fixed concentration of insulin was altered. Since adipose tissue incubated briefly in insulin, washed, and then incubated in the glucose medium without insulin, will synthesize fat from glucose (11), it was decided to determine

how early the morphological changes described could be noted. It was found that soaking the tissue in insulin (10^5 micro units per ml.) for 2 minutes produced invaginations of the plasma membrane and formation of small vesicles in relation to the surface membrane, but not in the deeper portions of the cytoplasm (Fig. 5). The affected cells were noted only in the extreme periphery of the blocks; the centers appearing like the control tissues to be described. When the duration of the incubation period in insulin-containing media was increased to 10 or 15 minutes, the process of invagination and vesiculation in the adipose cells had increased in amount and extent. However, even here, only the cells at the periphery of these blocks appeared to contain the degree of membrane reaction similar to that already described for specimens incubated for 20 or 30 minutes. For these latter times of incubation no obvious morphological differences could be observed.

The morphological changes were more striking in those tissues which had been incubated in media containing insulin and glucose for an hour. It was, indeed, surprising to find cells so markedly altered morphologically by the long incubation still showing invaginations of the plasma membrane and cytoplasmic vesicles (Fig. 7). In a few instances, the invagination of the plasma membrane was of considerable extent and was not seen in any of the other specimens. These sometimes extended almost the entire width of the cytoplasm and small vesicles were present in relationship to the distal half of the invagination.

Concerning the findings that accompanied the experiments in which the concentration of insulin was varied, 10^5 micro units/ml. and 10^3 micro units/ml. produced very similar results for the same duration of incubation so that they could not be distinguished from each other. However, 10^2 micro units/ml. (a physiological concentration) produced fewer changes (*cf.* Figs. 6 and 9). A 30-minute incubation of adipose tissue in media containing this concentration of insulin produced an effect similar to that obtained by incubation for 10 to 15 minutes in media containing insulin at a concentration of 10^5 micro units/ml.

In comparison, control preparations which had been incubated for the same length of time in media containing glucose, gelatin, or inactivated insulin did not show the above described changes, or did not show them to anywhere near the extent of the tissues incubated in media containing insulin (Figs. 4, 10, 11). In fact, the differences between experimental and control tissues was sufficiently great that they could be and were selected accurately in several blind studies. Although minute indentations of the plasma membrane were seen in the control specimens, especially those that had been incubated for a longer duration, they were rare. In fact, in only one of ten control tissues examined, was the amount of invagination of the plasma membrane significant, and even then, it was much less than in the experimental tissues. In the remaining ones, it was usual to find relatively long stretches of the plasma membrane of adipose cells showing no signs of morphological change. (It should be reiterated at this point, that the monitoring of the tissues manometrically not only gave an indication of the biochemical differences between controls and experimental fat pads, but provided a certain degree of selection. This selection was dictated by the over-all gas exchange during the period before the contents of the side arm was dumped. In several instances, a slight positive pressure response was evident from the beginning, possibly because of intrinsic factors, and these tissues were discarded. A positive pressure response was shown by none of the control tissues in the present report.) In addition, whenever invaginations or vesicles were seen in the control specimens they were always confined to the cell surface. No internal system of smooth membrane-bounded vesicles were seen in any of these preparations. All of the control tissues appeared similar regardless of whether the incubating media contained glucose and gelatin or inactivated insulin. It should be pointed out at this time that the occurrence of endoplasmic reticulum as short profiles or as circular cross-sections in either unincubated or incubated control adipose cells was exceedingly sparse; in fact, in most areas of the cytoplasm of these cells, it was lacking. Even within the control tissues differences were noted between the adipose cells, on the one hand, and the endothelial cells and the cells of connective tissue, on the other. In the latter cells invaginations of the plasma membrane and the occurrence of membrane-bound vesicles in the cytoplasm were relatively common.

At the surface of the plasma membrane of both experimental and control cells, and with an intervening space of about 150 A which appeared of light density, was deposited a layer of ground

substance somewhat thicker than the plasma membrane (Figs. 3, 4, 10, 12). This layer of basement membrane appeared as a homogeneous, amorphous, or lightly granular accumulation of material that did not have sharp boundaries but tended to spread as wisps of the same material from the regions of its greatest density. The basement membrane showed no gaps or indentations that accompanied the invaginations of the plasma membrane, and showed no detectable differences between control and experimental preparations.

Three other differences were noted in the comparison of the experimental and control tissues. They are presented here, as though of secondary importance, because they were found only in some of the specimens, or their significance has a questionable status. The first of these concerns the cytoplasmic matrix which appeared homogeneous or granular in unincubated tissue with the granular material occurring in several sizes, shapes, and densities. In addition to single granules, approximately 80 A in diameter of both light and heavy density, the granules of lighter density sometimes were accumulated into irregular small bodies or were oriented to form short thread-like strands. Some of this granular material was presumably glycogen deposits (Fig. 2). In the incubated control tissue, some spotty extraction of the granular hyaloplasm was noted especially in sections taken from the periphery of the blocks. In comparison, tissues subjected to insulin (with or without glucose) appeared more extracted than their controls which had been incubated for the same period of time. This was most marked in the blocks incubated for 1 hour in the insulin-containing media. Here, almost complete absence of the cytoplasmic matrix was apparent in most of the cells; the membranes of the cytoplasmic vesicles standing out in bold relief (Fig. 7).

The second difference concerns the small droplets of osmiophilic lipid, several thousand A in diameter, which were relatively uncommon in the cytoplasm of unincubated cells or of cells incubated in media containing either glucose or insulin alone. However, these droplets were comparatively plentiful in adipose cells incubated in both these reagents for 20 or 30 minutes (Figs. 2, 9, 12), but not 1 hour. The lipid droplets were not membrane bounded, but in tissues exposed to insulin and glucose, they were frequently disposed alongside of large vesicles.

Finally, although it is difficult to screen and compare mitochondrial populations of control and experimental tissues, there was a suggestion of mitochondrial differences that is being investigated further. The mitochondria were normally numerous in the thicker portions of the cytoplasm adjacent to the nucleus, but quite sparse in the thinner portions of the cytoplasm. They typically revealed double external membranes and contained a moderately dense homogeneous matrix. The cristae, however, were noted in some instances to stretch the entire width of the organelle. Incubation, of any variety, caused some distortion of mitochondrial structure, such as swelling, disruption, and spotty extraction of the matrix, but the distortions appeared to be more common in the control than in the experimental tissues subjected to insulin, with or without glucose. The occurrence in insulin-treated tissues of occasional very long mitochondria (Fig. 2), Y-shaped ones (Fig. 3), and relatively dense aggregates of them (Figs. 2, 3, 8, 9) suggested mitochondrial activity or growth. In the case of the aggregates, it was not unusual to find a number of smaller mitochondria in the vicinity of larger ones (Figs. 2, 3). In addition, some of the mitochondria of tissues subjected to insulin contained shorter cristae that did not extend across the entire organelle (Figs. 3, 8, 9). Although this latter finding might have been due to the plane of section, the frequency of this occurrence in any one section, or in the sections in general, suggests that the observation is worthy of note. However, these latter findings were only apparent in the specimens incubated for 20 to 30 minutes. They were absent in the short as well as the long incubations; distortions of the mitochondrial structure being common in the latter.

Water, Lipid, and Lipid Phosphorus Content

In order to permit the calculations that will be presented it was necessary to obtain the data on the relative lipid and water content of the epididymal fat pad given in Table I. Some 83 to 87 per cent of the total wet weight of the tissue is material soluble in $CHCl_3$-CH_3OH mixture. The distal portions contain 20 to 30 per cent more water and thus presumably more protoplasm per unit wet weight than the proximal portions. This difference is reflected to some extent in the relative metabolic activities of the two portions of the tissue when expressed in terms of total wet weight (4). If the total amount of phosphorus found in the lipid fraction is assumed to be due to phospholipid, then it can be calculated that phospholipid represents only 0.38 per cent (25×0.015) of the total lipid present in these cells.

The morphological changes observed are such that the question arises as to whether tissue incubated in the presence of insulin has accumulated water. In order to study this point we have weighed tissue both before and after the incubation period.

Experiments were performed in which paired distal portions of the fat pad were removed from the animal, weighed, and then incubated in Krebs-Ringer bicarbonate glucose medium in the usual manner. After 30 minutes of incubation, insulin dissolved in Krebs-Ringer bicarbonate gelatin medium was added to one flask to yield a final concentration of 10^3 micro units/ml. A similar amount of bicarbonate gelatin medium was added to the other flask. After an additional hour, during which the characteristic response to insulin was seen in the one flask, the tissue was removed, blotted gently to remove excess medium, and again weighed. In a series of five such experiments tissue which had been incubated with insulin showed a gain in weight which averaged 1.2 per cent. Calculations indicate that weight gains due to fat synthesis should amount to about 0.2 per cent. The average gain in weight for the control tissue was 6.3 per cent. Thus, control tissue which shows no significant morphological changes shows increases in weight which are five to six times those seen in tissue treated with insulin. We have not ascertained what weight changes ensue after the preliminary 30 minutes incubation, so that it is not possible to say whether the action of insulin is to prevent water intake or cause its extrusion. In any case, it would appear that the morphological changes produced by insulin cannot be ascribed to any great retention of water. It perhaps should be pointed out that the above figures apply to changes in the weight of the total fat pad. If the gain in weight observed is due to water influx, then all the change would presumably occur in the non-lipid portion of the fat cell. As shown in Table I, the water content of the distal portion of the fat pad accounts for only some 14 per cent of the total. Thus, the weight gain observed expressed in terms of this cellular compartment amounts to some 9 per cent for the insulin-treated tissue as compared to 44 per cent for the control.

DISCUSSION

The insulin-induced morphological changes in adipose tissue which have been described here are those characteristic of the process which W. H. Lewis (12) in 1931 termed "pinocytosis" or cell drinking. In this process the cell surface and adjacent cytoplasm are thrown into a state of vigorous activity, with the flow of vesicles derived from indentations of the plasma membrane into the interior of the cytoplasm. The process thus resembles phagocytosis but differs from it in that the cell engulfs small droplets of the fluid surrounding it rather than solid particles. Lewis observed the occurrence of pinocytosis in normal (12) and malignant (13) tissue culture cells. His observations have been confirmed on other cultured cells (14, 15) and the process has been studied extensively in amebae (16–21). In all the studies just cited the process has been observable with the aid of the light microscope. It is only

TABLE I

Lipid and Water Content of the Epididymal Fat Pad

	Per cent of wet weight			
	Left distal	Left proximal	Right distal	Right proximal
Water	14.4 (11.6–16.4)	10.9 (9.6–12.1)	14.3 (11.5–16.7)	11.9 (10.9–13.4)
$CHCl_3$—CH_3OH soluble	83.8 (80.5–87.3)	87.0 (86.0–88.2)	82.9 (80.6–85.0)	85.8 (83.0–88.0)
$CHCl_3$—CH_3OH insoluble	2.5 (2.2–2.9)	2.3 (1.9–2.7)	2.4 (2.1–2.9)	2.3 (1.5–2.9)
Total	100.7	100.2	99.6	100.2

Values are the mean of five experiments with the range given in parentheses. Total phosphorus was determined on the $CHCl_3$—CH_3—OH soluble fractions from the distal portions of tissue. The mean value for seven pieces of tissue was 0.015 mg. P per 100 mg. of lipid.

recently that studies with the electron microscope (22–27) have revealed that pinocytosis may occur in which the vesicles formed are of such minute dimensions that they escape detection with the ordinary light microscope. The observations recorded here obviously fall into this category. It is of additional interest that many other electron microscopic studies have indicated that a variety of small particulate materials enter cells by pinocytosis (28–34).

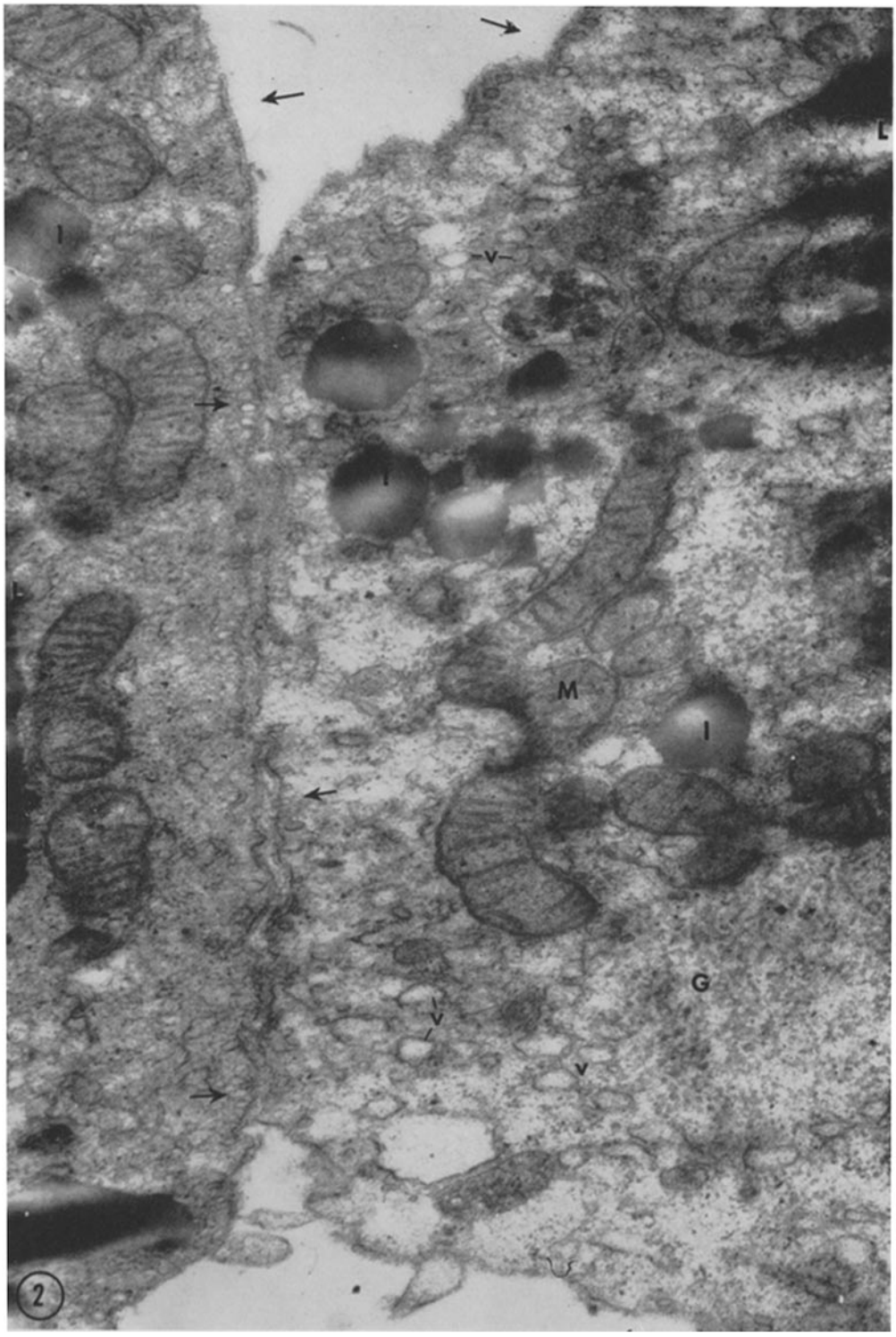
To the best of our knowledge insulin is the only hormone which has so far been demonstrated to initiate pinocytosis. We are not the first to observe that insulin possesses this property. Paul and Pearson, using the light microscope, have observed a stimulation of pinocytosis by insulin in cultures of HeLa cells. This was documented in a film which was shown at the International Congress for Cell Biology in 1957. Paul (35) has informed us that the phenomenon is difficult to reproduce. This is in marked contrast to the effect observed by us which is readily reproducible.

The ability of insulin to initiate in adipose tissue both the uptake of glucose and the process of pinocytosis raises the question as to the interdependence of the two events. Several facts suggest that such an interrelationship does exist. First, the two processes appear to be initiated nearly concomitantly. Metabolic changes can be observed to occur within 5 minutes after the addition of 10^5 micro units of insulin per ml. of incubating medium, and the full extent of such changes has been reached within 10 minutes (3). The initial stages of pinocytosis are evident morphologically within 2 minutes after the addition of insulin and are well advanced by 10 minutes. Second, both processes can be initiated by insulin at concentrations which are extremely small and considered to be physiological, namely 0.004 μg. (100 micro units) per ml. of incubating medium. Indeed, at this specific concentration the intensity of the metabolic response is on the average about 60 per cent of the maximum obtainable (3) when insulin is present in excess. Pinocytosis also appears to be less marked at this specific level than at higher insulin concentrations. This suggests that the magnitude of the response for both processes is, in part, a function of the insulin concentration at these levels. This point is, however, in need of further documentation. Third, if insulin is treated with alkali in a manner which has been shown by others (8) to destroy its biological activity *in vivo*, then it simultaneously loses its ability to induce pinocytosis and to produce metabolic changes in the epididymal fat pad *in vitro*.

The possibility that pinocytosis may be involved in the mode of action of insulin has a definite amount of appeal in the light of current concepts of the manner in which this hormone functions. Levine and coworkers (36) were the first to provide evidence that insulin acts by regulating the entry of glucose into cells. The evidence for this viewpoint is inferred from studies (36, 37) of the volume of distribution in eviscerated animals of sugars that are not metabolized. When D-galactose, D-xylose, or L-arabinose are injected intravenously into such an animal, their concentration in the blood stream reaches a plateau value that is higher than that to be expected if they were distributed throughout the total body water. If insulin is administered the blood concentration of these sugars rapidly drops as though the volume of distribution had been increased. These results are interpreted to mean that insulin has increased the permeability of certain cell membranes to these sugars and thereby facilitated their entry into

FIGURE 2

Micrograph of portions of the cytoplasm of two adjacent adipose cells which had been incubated in a bicarbonate medium containing glucose (4 mg./ml.) and gelatin (2 mg./ml.) and insulin (10^3 micro units) for 20 minutes. Numerous infoldings of the plasma membrane as well as small vesicles just under the cell surface (arrows) are apparent in both cells. The cytoplasm contains many smooth, membrane-bounded vesicles (*v*) which are larger in the deeper regions than at the periphery. The cytoplasmic matrix contains many granular deposits, presumably glycogen (*G*) and appears washed out in several regions. Numerous small lipid droplets (*l*) are scattered throughout the cytoplasm and the edges of the central lipid vacuoles (*L*) are shown in both cells. Many mitochondria are apparent and one of them (*M*) shows unusual length and tortuosity. × 37,000.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cells. Thus, this type of approach has provided indirect evidence that insulin affects the cell membrane. The finding reported here that insulin induces pinocytosis provides direct evidence that insulin can act upon the cell membrane. The two experimental findings taken together provide strong support for the hypothesis that insulin acts by regulating the entry of glucose into cells by bringing about a change in the properties or the activity of the cell membrane.

On the basis of what is known regarding pinocytosis in amebae and the action of insulin on mammalian cells the following conjectural picture of the mode of action of insulin may be presented. The first step would be adsorption of the insulin molecule on the cell surface. This would be a process similar to that described in ameba by Brandt (21). In Brandt's experiments γ-globulin which is capable of initiating pinocytosis in amebae, was labeled with a fluorescent dye so that the fate of the protein molecule could be visualized. The first step was the adsorption of the protein to the surface membrane or plasmalemma of the ameba. Invagination of the membrane then occurred at the site where the protein was adsorbed. The adsorption of insulin on cell surfaces as a first step in its action would be in keeping also with the observations of Stadie *et al.* (38) on the fixation of insulin to diaphragm muscle. These workers observed that when diaphragm muscle was briefly dipped into a medium containing insulin, removed, thoroughly washed, and then placed in a glucose-containing medium an enhanced glucose uptake occurred. Observations of a similar nature have been made upon adipose tissue (11).

The second step would be invagination of the membrane at the point of attachment of the insulin molecule. Vesicle formation would then follow by the pinching off of the recessed fold of membrane. The adsorbed insulin molecule, presumably as occurs with the labeled γ-globulin in ameba, would be carried into the cell still attached to that part of the membrane which has now become the envelope surrounding the vesicle.

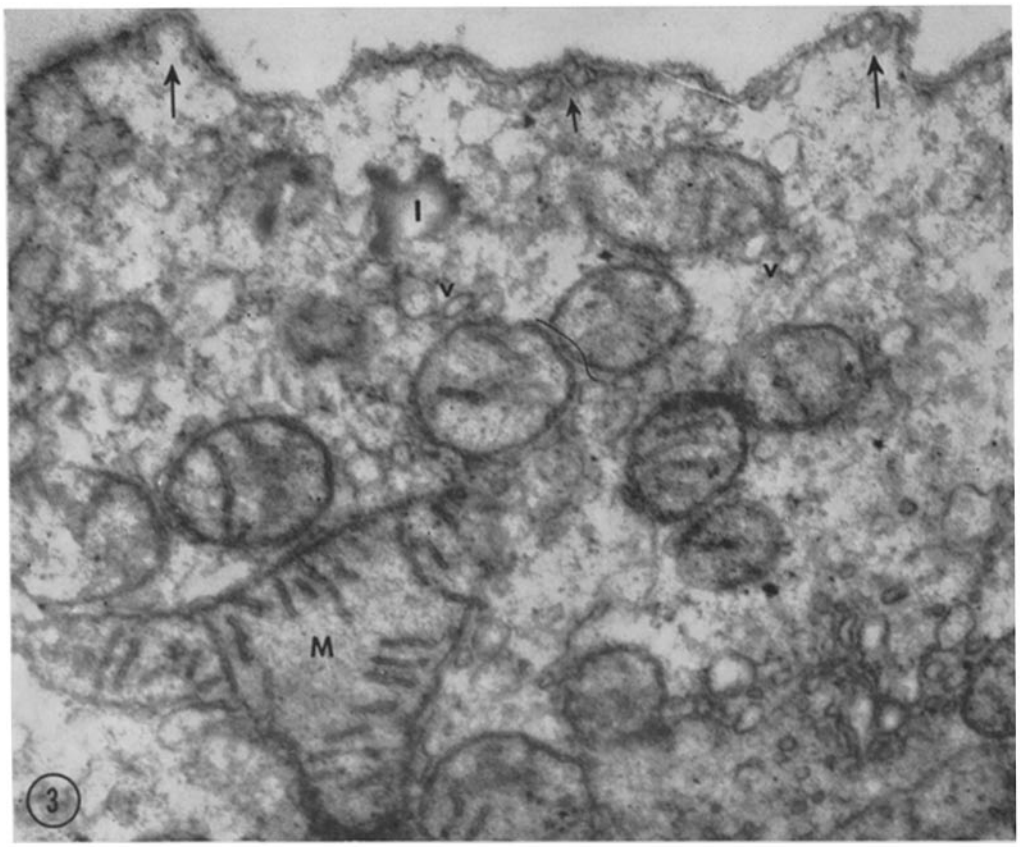
Two possibilities now exist to explain the increased entry of glucose into the cell. First, it may be carried in by the vesicles since they contain some of the fluid that bathed the cell. The amount of glucose that could be carried into the cell in this fashion is hard to estimate since we have no knowledge of the dynamic aspects of pinocytosis in adipose tissue. It has been reported (17) that amebae may ingest up to 10 per cent of their own volume of fluid in 1 hour. Some calculations may, however, be made as to the volume of fluid that the fat pad would need to ingest if all the glucose it consumes is presumed to enter by the process of pinocytosis. Hagen and Ball (39) have found that the glucose uptake of the epididymal fat pad is negligible when immersed in a medium containing

FIGURE 3

Micrograph of the cytoplasm of adipose cell incubated in basic medium containing 10^5 micro units of insulin for 20 minutes. Many invaginations of the plasma membrane are apparent as well as small vesicles just underlying the surface (arrows). A basement membrane is present at the surface of the plasma membrane and does not follow the contour of the indentations. Smooth, membrane-bound vesicles (v) of various sizes are abundant in the cytoplasm. The membrane surrounding these vesicles appears similar to that of the plasma membrane but in some of the large vesicles it appears fuzzy and in some places incomplete. Though large vesicles are common deep in the cytoplasm, some small ones are present also. A few lipid droplets (l) are apparent. Numerous small mitochondrial profiles are evident as well as one large Y-shaped mitochondria (M). Most of the cristae in these mitochondria are short. × 49,000.

FIGURE 4

Micrograph of the cytoplasm of control adipose cell incubated in the basic medium without insulin for 20 minutes. At the surface of the cell the basement membrane and plasma membrane are apparent. The latter shows only a few indentations. The hyaloplasm is granular and mottled but no vesicles are present in the cytoplasm. A mitochondria (M) shows cristae that extend the entire diameter of the organelle. × 60,000.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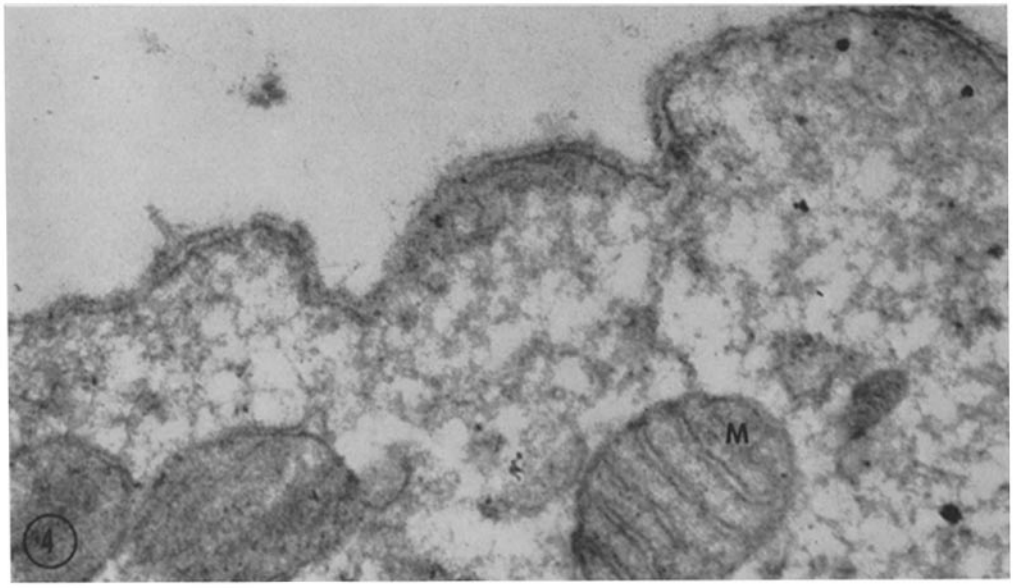
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5 μmoles of glucose per ml. In the presence of insulin, glucose uptake is markedly increased and mean values of 1.5 μmoles per 100 mg. of tissue per hour are observed. This means that 100 mg. of fat pad consumes in 1 hour the glucose contained in 0.30 ml. of medium. Now, as shown here, the average water content of the fat pad is about 15 per cent of its total wet weight. Thus, in 100 mg. of this tissue the weight of the nonlipid fraction is 15 mg. and assuming a specific gravity of one, the volume of the protoplasm is roughly 0.015 ml. Hence the metabolically active portion of this tissue is consuming the glucose contained in $\frac{0.30}{0.015}$, or twenty times its own volume of medium, each hour. If all of the glucose enters by way of vesicle formation, then the fluid turnover of the fat cell that is induced by insulin is of considerable magnitude.

This problem may be approached in another manner. If the average diameter of a vesicle at the moment of its formation is taken as 200 A, then assuming each vesicle is a sphere, it can be calculated to have a volume of 4.2×10^{-18} ml. This would mean that in order for 100 mg. of tissue to take in 0.3 ml. of fluid per hour it would need to form $\frac{0.30}{4.2 \times 10^{-18}}$, or 7×10^{16} vesicles. We may now in turn calculate the number of insulin molecules available to 100 mg. of tissue. In our experiments each 100 mg. of tissue is on the average bathed by 1 ml. of fluid. Maximum metabolic activity may be elicited in these experiments at insulin concentrations of 10^3 micro units per ml. If the molecular weight of insulin is taken as 6,000 and the activity of the sample as 25 units per mg., then it may be calculated that 4.0×10^{12} molecules of insulin are present per ml. of medium. This then means that each insulin molecule would be required to initiate the formation of approximately 17,500 vesicles under the described conditions. Even if the assumption is made that the vesicle diameter can reach 1000 A, then a figure of 140 vesicles per insulin molecule is obtained. Moreover, it should be stressed that these calculations apply only to 1 hour of incubation and that this tissue will respond to such an amount of insulin with unabated activity for at least 5 hours (3). Hence, one is forced by these calculations to the conclusion that either all the glucose does not enter by vesicle formation or if it does, then one insulin molecule triggers the formation of a large

FIGURE 5

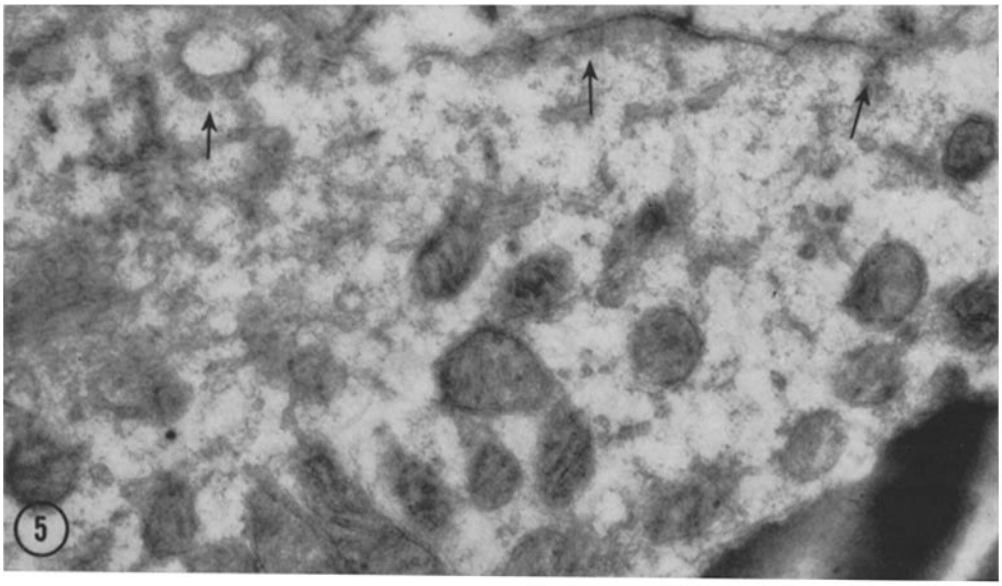
Micrograph of the cytoplasm of adipose cell in the extreme periphery of a block incubated in the basic solution containing insulin (10^5 micro units) for 2 minutes. Note that accumulation of vesicles or indentations that occur in relation to the plasma membrane or near the cell surface (arrows). Although some small vesicles appear to have gained a position deeper in the cytoplasm, no large cytoplasmic vesicles occur. $\times$ 37,000.

FIGURE 6

Micrograph of adipose cell incubated in the basic medium containing a physiological concentration (10^2 micro units) of insulin for 20 minutes. Note that the cell surface is studded with small vesicles but few have gained the interior of the cytoplasm. The plasma membrane does not appear as a sharp line because of the obliquity of the section. $\times$ 42,000.

FIGURE 7

Micrograph of cytoplasm of adipose cell incubated with the basic medium containing 10^3 micro units of insulin for 1 hour. It was indeed surprising to find many cells in these specimens completely devoid of granular hyaloplasm yet showing evidence of invagination of the plasma membrane and formation of cytoplasmic vesicles. The group of vesicles on the right of the micrograph shows clearly the progression in size of vesicles from plasma membrane to interior of cytoplasm. $\times$ 32,000.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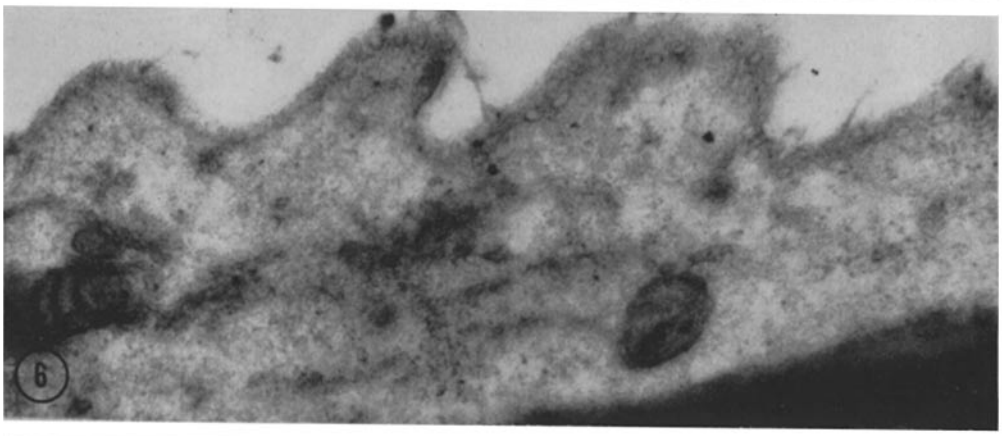
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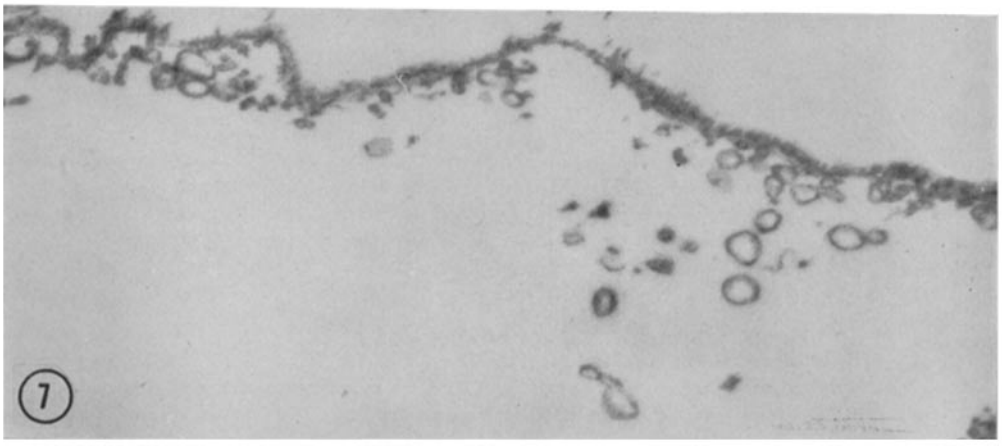
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number of vesicles by exciting the activity of a unit area of surface membrane.

In regard to the entrance of glucose into the cell by means of vesicle formation, the question can be raised not only as to the amount that may enter but also as to its availability for metabolic processes after it has so entered. The argument can be made that the vesicle is bounded by an envelope which was derived from a cell membrane which was impervious to glucose in the first place and hence should remain so. Chapman-Andresen and Holter (18) have shown that this argument is not valid in the case of ameba. They have immersed ameba in medium containing radioactive glucose and then initiated pinocytosis. By the use of autoradiographs they have shown that radioactivity is present in the vesicle upon its formation but that shortly thereafter radioactivity may be detected in the cytoplasm surrounding the vesicle. It thus appears that in amebae the membrane bounding the vesicle is permeable to glucose.

This brings us to the consideration of another possible means by which glucose entry may be facilitated by pinocytosis, namely that the permeability of the cell membrane itself may become altered. It is possible that the non-invaginated surface membrane may acquire permeability properties similar to that of the membrane surrounding the vesicles. This change in permeability could be brought about by reason of vesicle formation which results in a loss of surface membrane. This loss would require the cell to either replace its membrane by the synthesis of new or to make some other adjustment. In either case, an alteration in permeability to glucose and other small molecules might be established. Thus, the ability of insulin to alter the cell permeability would be an indirect one. Changes of this sort in the permeability of the cell membrane would be attractive on the basis that it might still permit a certain selectivity with regard to the molecules to be admitted. Certainly, it is difficult to see how any selective admission, at least as far as small molecules are concerned, could be achieved when material is transported into the cells by means of vesicles. Until more information is available it will be necessary to consider the possibility that glucose and other compounds may enter both by way of vesicles and altered permeability of the surface membrane.

The observations reported here have been confined to adipose tissue and it will be of interest to learn whether insulin is capable of initiating pinocytosis in other tissues, such as muscle, where glucose entry is enhanced by its action. It will be of equal interest to learn whether other hormones,

FIGURE 8

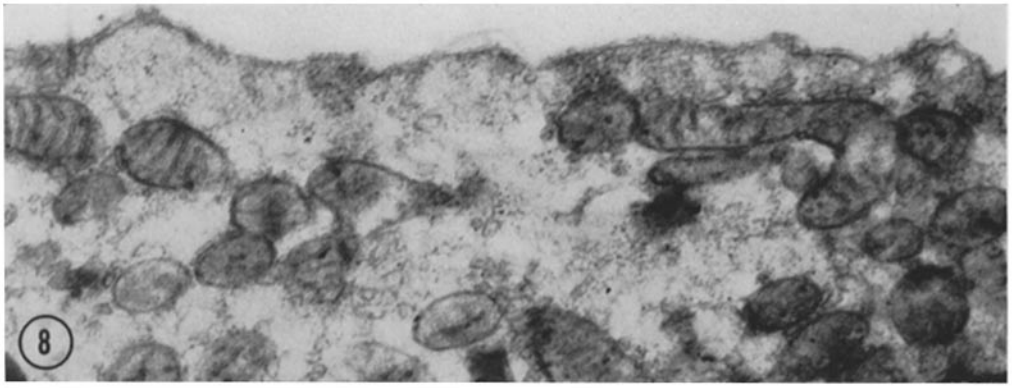
Micrograph of adipose cell incubated in a medium containing 10^3 micro units of insulin but no glucose for 20 minutes. Numerous vesicles appear in relation to the cell surface as well as in the interior of the cytoplasm. In this specimen there is not too great a progression in size of vesicles from surface to interior. Numerous mitochondria containing short cristae are in evidence and a number of smaller ones appeared clustered about larger ones. × 26,000.

FIGURE 9

Micrograph of adipose cell incubated in basic solution containing glucose and insulin (10^3 micro units) for 20 minutes. Periphery of the cell abounds with indentation of the plasma membrane and tiny vesicles. Deeper in the cytoplasm larger and frequently irregularly shaped vesicles are apparent. Some of these large vesicles are also quite close to the cell surface. A few lipid droplets are apparent with small vesicles adhering to their sides. Mitochondria are plentiful and contain short cristae. × 32,000.

FIGURE 10

Micrograph of control adipose cell incubated for 20 minutes in a medium containing glucose but no insulin. There are very few indentations of the plasma membrane or vesicles within cytoplasm. Mitochondria are large and contain cristae which extend the entire width of the organelle. A shortprofile of endoplasmic reticulum is apparent at the upper left. × 49,000.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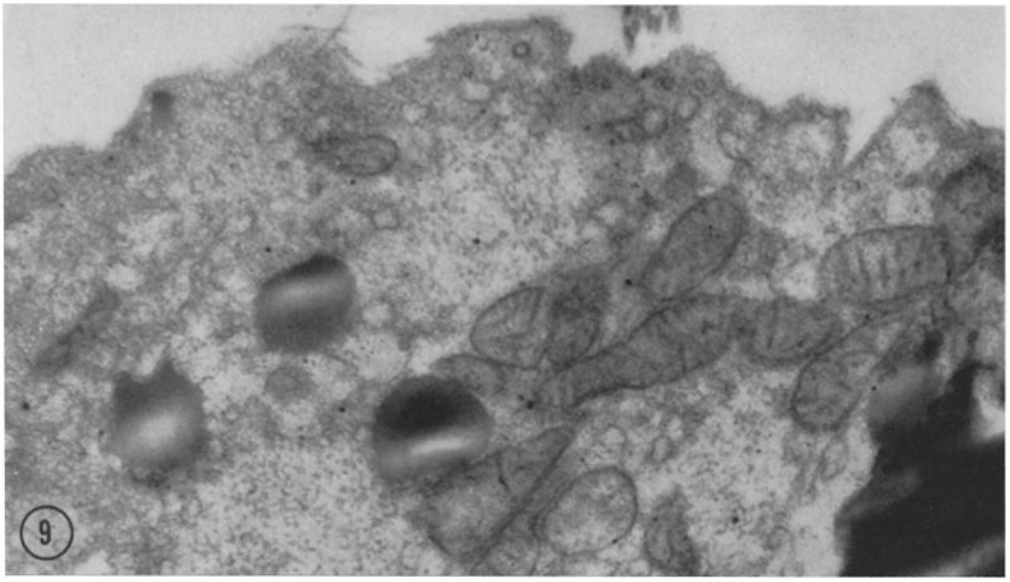
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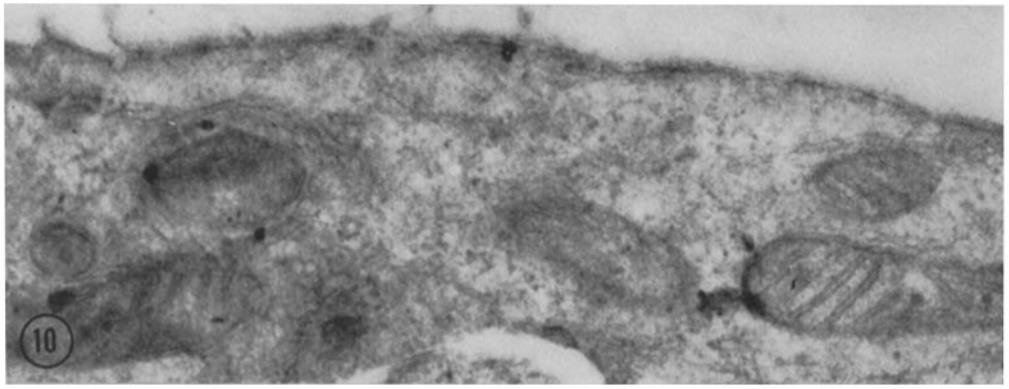
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especially those that are proteins of low molecular weight, have the ability to initiate pinocytosis in their target organs or to inhibit this process. The analogy of the lock and key is often applied to an enzyme and its substrate. Does this analogy also fit in certain cases, a hormone and the cell surface of its target organ?

In addition to vesicle formation several other morphological findings were noted in adipose tissue as a potential consequence of insulin action which deserve comment. One of these is the appearance of fat droplets in the cytoplasm of adipose cells which had been incubated in the presence of insulin and glucose. This finding is suggestive morphological evidence of lipid synthesis which was shown to occur by the manometric monitoring experiments. Another striking change is the loss of the granular matrix of the cytoplasm of tissue that occurs after it has been in contact with insulin for 30 minutes or more. All the chemical evidence indicates that the tissue is in metabolic high gear at this time and continues so for many hours to follow. Are this loss of matrix and the possible changes in the mitochondria a reflection of the extremely active metabolic state of this tissue? Though no answer can be given, it is of interest to raise this question especially in relation to the state of the enzymatic, structural, and other components of the cell. It seems reasonable to assume that during periods of high metabolic activity, an accelerated degradation and synthesis of many of the cellular components occurs. Not the least of these components might be those that comprise the cell's surface membrane which, as a result of vesicle formation, might be undergoing constant renewal. In this connection it should be noted that the addition of insulin *in vitro* has been found to increase the rate of incorporation of labeled amino acids into proteins (40–43) and of radioactive phosphorus into phospholipids and nucleic acids (35). In the case of amino acid incorporation into protein, the accelerating action of insulin has been demonstrated under conditions in which an increased entry of the amino acid into the tissue has been excluded as an explanation for this effect (43). It would not be surprising to find that an increased turnover of other cellular components accompanies the action of insulin. Such changes may well reflect not a primary action of insulin, but one directly attendant on the increased metabolic activity of the cell that follows in the wake of insulin's action upon the cell membrane.

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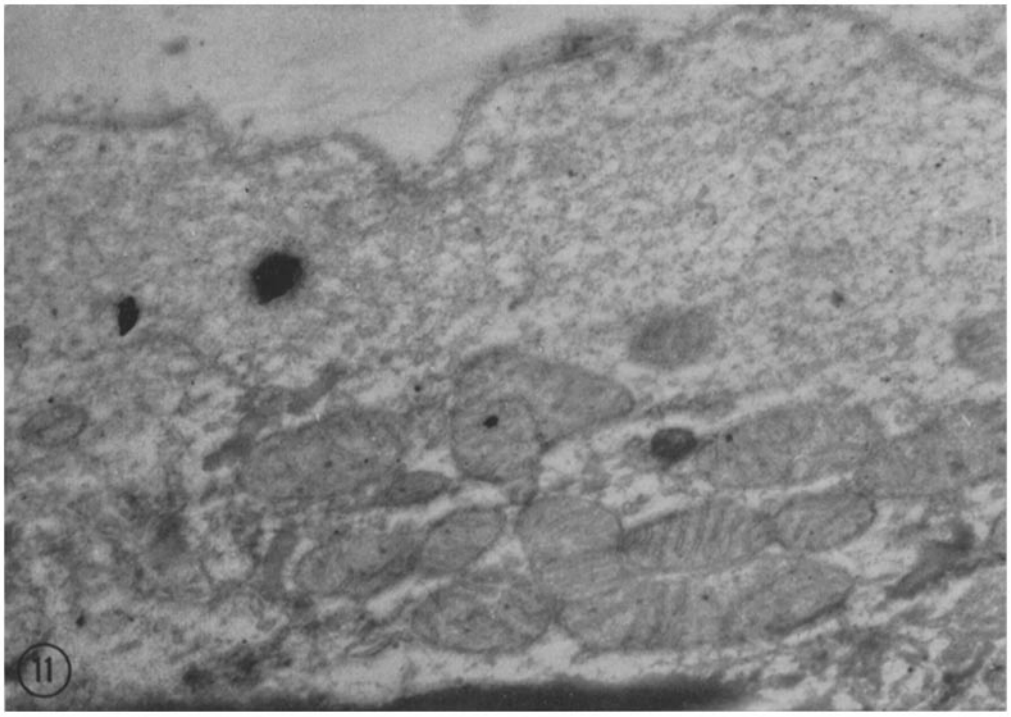
FIGURE 11

Section of adipose cell incubated for 30 minutes in the basic medium containing insulin which had been inactivated. Little indication of surface activity (plasma membrane indentations or vesicles) is apparent. To all intents and purposes, the section appears like those which had been incubated without insulin. × 37,000.

FIGURE 12

Section of adipose cell incubated for 30 minutes in the basic medium containing insulin (10^3 micro units). The plasma membrane is invaginated at fairly regular intervals. The deeper portions of the cytoplasm contains many large vesicles and in some cases they are incompletely closed by a surrounding membrane. There is little granular hyaloplasm and several large lipid droplets are present. The mitochondria contain short cristae and in some of them the matrix is partially extracted. × 37,000.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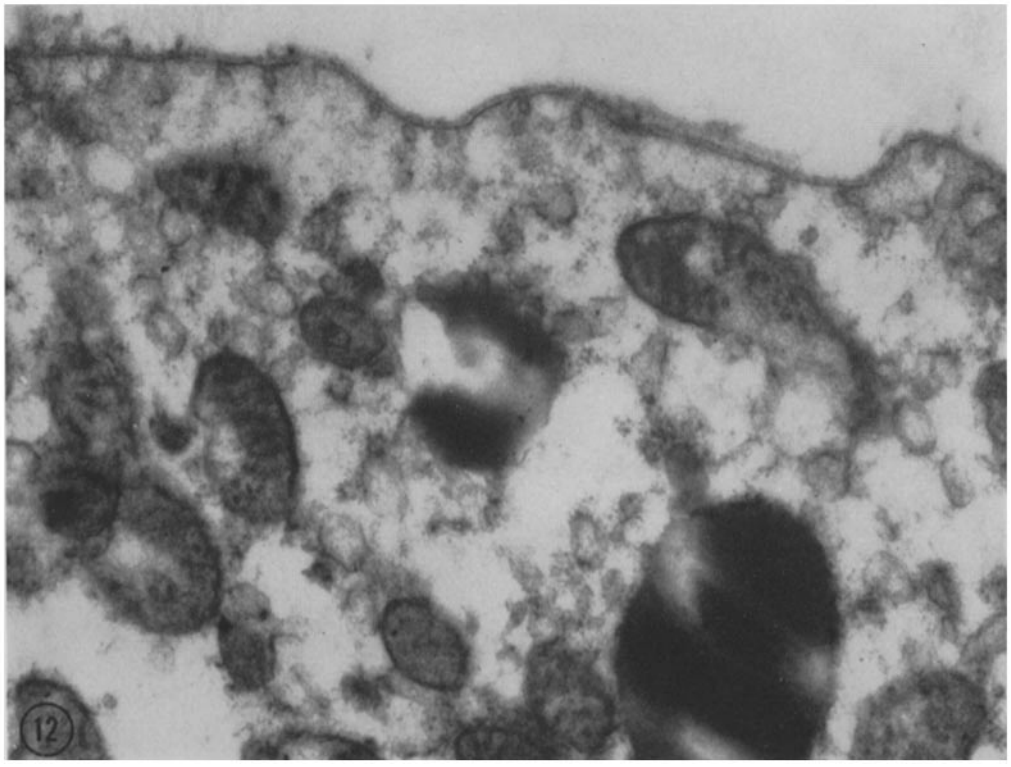
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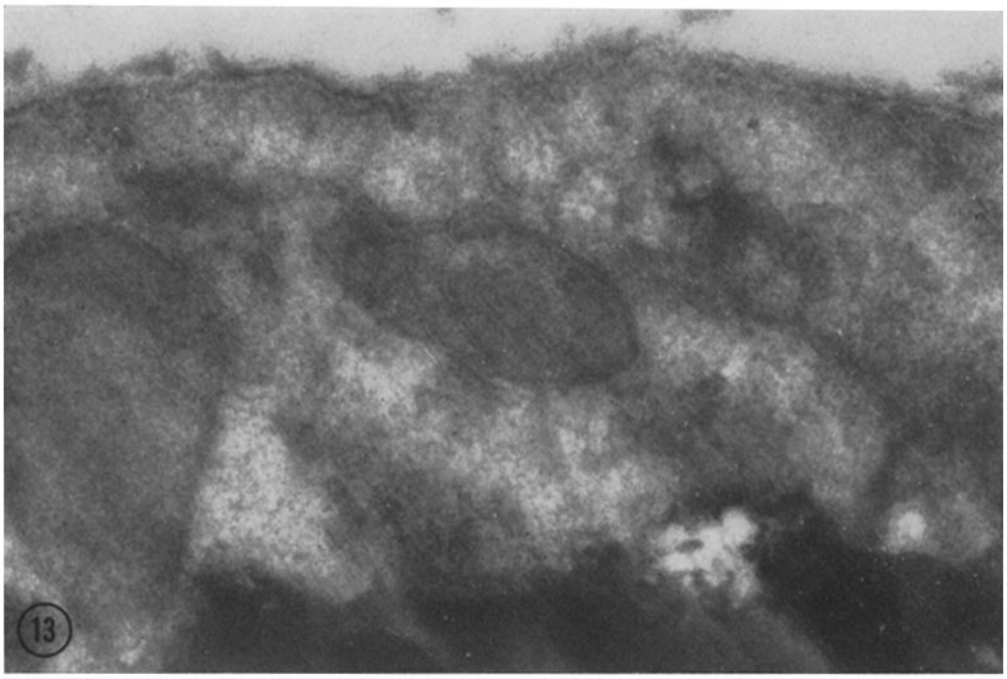
FIGURES 13 and 14

Comparable thick sections of adipose cells both incubated for 20 minutes in the basic medium. The control tissue (Fig. 13) was not subjected to insulin and the experimental tissue (Fig. 14) was subjected to 10^3 micro units of insulin. These thick sections dramatically demonstrate the changes induced by incubation in a solution containing insulin. Although the sections are too thick to make out the membranes surrounding the vesicles that crowded the peripheral cytoplasm of the cell in Fig. 14, the control cell (Fig. 13) contains no comparable structures. × 42,000.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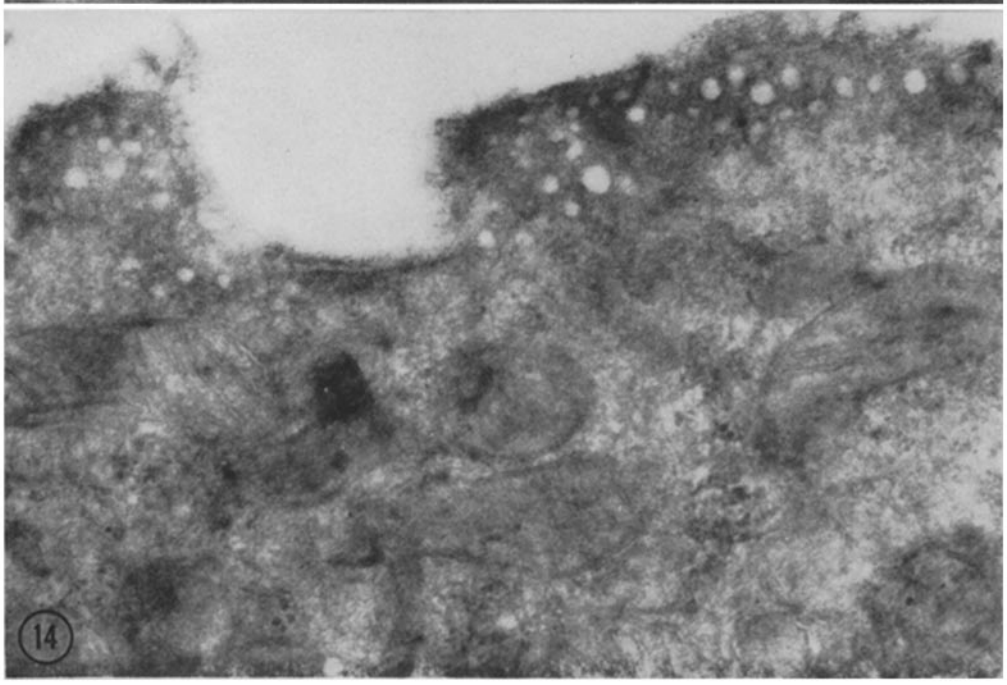
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