^{125}I-Insulin Binding to Cultured Human Lymphocytes

INITIAL LOCALIZATION AND FATE OF HORMONE DETERMINED BY QUANTITATIVE ELECTRON MICROSCOPIC AUTORADIOGRAPHY

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ABSTRACT Morphologic and biochemical studies indicate that the initial action of insulin is binding to a cell surface receptor. Whether further translocation of the hormone, or a product of the hormone, occurs is unclear and has not been investigated by direct means. To determine the fate of ^{125}I-insulin bound to its receptor, we have examined the distribution of radioactivity by quantitative electron microscopic autoradiography. Cultured lymphocytes of the IM-9 cell line were incubated with 0.1 nM ^{125}I-insulin at 15° and 37°C for incubation periods extending from 2 to 90 min. At 15°C, grains localize to the plasma membrane and there is no translocation as a function of time. At 37°C, grains predominantly localize to the plasma membrane but there is a small shift in distribution to a distance of 300–700 nm from the plasma membrane. This small additional band component of irradiation extends to ≅10–15% of the cell radius. When a morphometric analysis is applied to grains extending 300 nm and beyond from the plasma membrane, we find no preferential localization to any intracellular organelle.

We interpret these data to indicate that in the cultured lymphocyte, labeled insulin initially localizes to the plasma membrane but as function of time and increasing temperature there is a small but definite translocation of the hormone or a product of the hormone to a highly limited aea of the cell periphery.

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INTRODUCTION

Several lines of evidence suggest that the initial interaction of insulin with the cell is binding to a receptor which is an integral protein of the plasma membrane (1). Subfractionation of cells incubated with labeled insulin demonstrates that the binding of the hormone is predominantly localized to the plasma membrane fraction (2, 3) and detergents are, under most circumstances, necessary to free the binding protein from the membrane (4, 5).

Other studies suggest that the receptor site is at the external or exposed face of the membrane. Anti-insulin serum will partially reverse the action of insulin on the diaphragm (6) and fat cells (7). Trypsin abolishes the fat cells response to insulin stimulation without affecting overall cellular integrity, other hormonal responses, or the glucose transport system per se (8). Fat cell membranes that have been reversed to expose the inner surface of the membrane do not bind insulin (9). Further support for this concept comes from the binding of ferritin-insulin conjugates to cell surfaces. Ferritin-insulin conjugates have been localized by conventional electron microscopy and freeze etching techniques to the surface of liver membranes (10) and by conventional electron microscopy to the surface of fat cells and fat cell membranes (2, 11, 12).

Direct binding studies with ^{125}I-insulin have demonstrated that the receptor-bound hormone is in rapid equlibrium with its external environment (1). These techniques have further been used to extensively characterize the specificity, physicochemical properties, kinetics, and regulation of the receptor as well as degradation of the labeled hormone. The mechanism, however, by which insulin activates intracellular processes remains largely unknown.

Recently, it has been shown that insulin (13) and

other polypeptide hormones (14) may become less dissociable as a function of time at higher incubation temperatures. Other studies have shown in disrupted cells that insulin is not only bound to plasma membrane receptors but also to sites on isolated Golgi (15), nuclei (16), and endoplasmic reticulum (17). Still other studies have suggested that insulin degradation is linked to binding by a first order rate constant and that this specific degradation is likely to be an intracellular process (18, 19). In the present study we have coupled direct binding studies, of the insulin receptor of the well-characterized IM-9 human lymphocyte (20–22), with quantitative electron microscopic autoradiography and morphometry in an attempt to gain further insight into the following questions: (*a*) Is the distribution of autoradiographic grains from labeled insulin consistent with cell surface localization? (*b*) If so, is there a change in this distribution as a function of time of incubation or increasing temperature? (*c*) Does labeled insulin when incubated with intact cells preferentially localize to intracellular organelles?

METHODS

Cells and reagents. Human-cultured lymphocytes of the IM-9 cell line were used for all experiments. Cells were grown at 37°C in RPMI 1640 medium containing 10% fetal calf serum and 25 mM Hepes (International Biological Laboratories, Inc., Rockville, Md.). Glutamine (0.29 mg/ml) was added just before feeding. Cells were "fed" three times a week by dividing the culture 1:3 and adding fresh media. Cells late in log phase or in early stationary phase of growth were split 1:2 in fresh medium 24 h before use. ^{125}I-insulin was prepared at a sp act of 200 μCi/μg by a modification (21, 23) of the chloramine T method (24). The labeled insulin was purified by filtration on G-50 Sephadex (Pharmacia Fine Chemicals, Inc., Uppsala, Sweden) at 4°C before each experiment.

Incubation conditions. Cells were removed from the growth media by centrifugation at 600 *g* for 10 min and washed once by resuspension in the buffer used for the incubation (*vide infra*). Approximately 4–5 × 10^6 cells/ml were incubated in assay buffer (100 mM Hepes, 120 mM NaCl, 1–2 mM magnesium sulfate, 1 mM EDTA, 10 mM glucose, 15 mM sodium acetate, and 10 mg/ml bovine serum albumin pH 7.6)[1] with 0.25 nm ^{125}I-insulin at 15° and 37°C for varying periods of time in 17 × 100 mm plastic Falcon tubes (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.). Identical incubations were carried out in the presence of 1.67 μM unlabeled insulin to determine nonspecific binding. At appropriate time intervals, 1.5 ml of cells were gently aspirated into conical plastic tubes, centrifuged at 200 *g* at 4°C for 15 min and the supernate aspirated.[2] 4% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, was then added to the cell pellet and fixed for a minimum of 4 h at room temperature.[3] The glutaraldehyde was then aspirated and replaced with 0.1 M phosphate buffer, pH 7.4, until further processing. Radioactivity in both fixed and unfixed pellets was similar indicating that fixation did not dissociate the labeled insulin from the cell.

Insulin degradation in the media was measured by trichloracetic acid precipitation (28). Dissociation of labeled insulin from the lymphocyte was determined by dilution (100-fold), dilution in the presence of an excess of unlabeled insulin (0.167 μM), and dilution at pH 6.5 as described (22).

Preparation for electron microscopy. The glutaraldehyde or phosphate buffer was discarded and the pellet washed three times with fresh phosphate buffer and postfixed in 0.1 M osmium tetroxide, pH 7.4, at room temperature for 2 h. The pellet was then progressively dehydrated through 30–100% alcohol and embedded in Epon (Shell Chemical Co., Houston, Tex.). Sections ≅70 nm thick, were cut on a LKB ultramicrotome (LKB Instruments, Inc., Rockville, Md.) and placed on grids coated with parlodion and carbon.

Preparation for autoradiography. Grids were prepared for autoradiography by the method of Caro et al. (29). Grids containing the sections were mounted on clean glass slides. Ilford L4 emulsion (Ilford Ltd., Ilford, Essex, England) was mixed with distilled water in a water bath at 40°C for 10 min and in an ice bath for 3 min with constant gentle stirring. When the emulsion reached the proper consistancy it was applied to the grids by a wire loop. An attempt was made to apply a thin- or mono-layer emulsion (140 nm) on half the grids and a somewhat thicker emulsion to enhance sensitivity on the remaining half. The grids were dried overnight at room temperature and then stored at 4°C for 3–5 wk.

Grids were developed in freshly prepared Microdol X (Eastman Kodak Co., Rochester, N. Y.) at 20°C for 4 min, fixed in Kodak F 24 (Eastman Kodak Co.) for 5 min and successively washed in four baths of distilled water. The grids were carefully removed from the glass slides, dried for 24 h and stained with uranyl acetate for 15 min followed by lead citrate for 8 min. Samples were examined on a Phillips EM 300 electron microscope (Phillips Electronic Instruments, Eindhoren, The Netherlands) and grains were photographed on all cells that were judged to be well-preserved. Pictures were taken at a magnification of 11,000 calibrated with a reference grid, 2,160 lines/mm (E. F. Fullam, Inc., Schenectady, N. Y.).

Sampling and analysis of data. For each time point of incubation, at least two separate samples of cells provided by two different Epon blocks were used. From each sample, four grids were prepared for autoradiography and one control grid was prepared without emulsion. Thus, eight separate grids from each time point were examined. Except where noted, ≅150 photographs of consecutive grains were taken

[1] This buffer is hypertonic (410 mosmol/liter). The salts used have been shown to be optimal for binding (20) but recent studies (unpublished observations) have demonstrated the need for greater buffering capacity because of the sharp alkaline pH optimum for insulin binding. Thus, 100 mM Hepes which has been empirically shown to be optimal for insulin binding to cultured lymphocytes and circulating monocytes has been used for these and other recent studies (21, 22, 25–27). The cell diameter measured in our assay buffer differs from the diameter measured in the growth media by <7%.

[2] In our initial experiments the final centrifugation was carried out for 1 min in a Beckman microfuge exactly as previous binding studies (20–22). Under these circumstances the cells are well-preserved but more tightly packed. Because the nonspecific binding is no greater with the more gentle centrifugation this procedure is prefered. Because the cells were in a 1.5-ml volume, to minimize perturbing the cells, no further wash step was performed. To determine the influence of washing an aliquot of cells was resuspended in fresh buffer at 4°C and centrifugation repeated. Under these circumstances there is a mean 11% reduction in either total binding or specific binding when compared to the unwashed cells.

[3] For comparison, cells were allowed to remain in 4% glutaraldehyde for 48–72 h. and the final results were essentially identical.

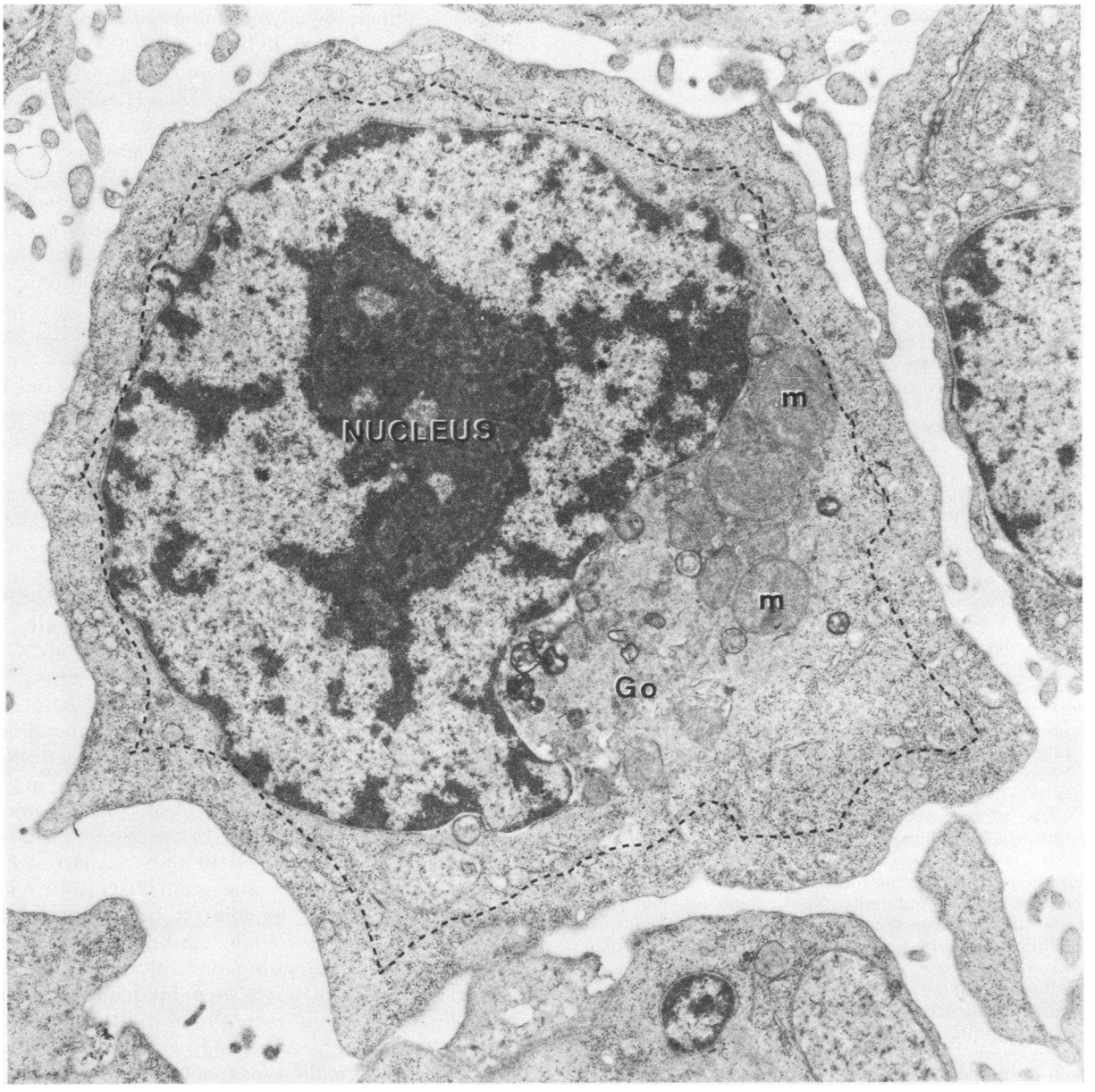


FIGURE 1 Thin section of an IM-9 lymphocyte. The cell is ≅11.2±0.1 μm in diameter in the assay buffer and 11.7±0.1 in the growth medium. The nucleus occupying a large volume of the cell is surrounded by the cytoplasm rich in ribosomes and containing well-preserved organelles: mitochondria (*m*). Golgi area (*Go*). The dashed line diagramatically illustrates the approximate maximum penetration of the additional band component of irradiation as extrapolated from Fig. 5B. The following assumptions were made for this calculation: (*a*) that the mean diameter of the cell is 11 μm in fixed section which agrees well with measurements made in wet preparations (36); (*b*) Because the additional component does not extend beyond 900 nm on the histogram (Fig. 5B), the source of irradiation ends at least two-half distances before 900 nm or at 700 nm; this represents 10–15% of a cell with an average radius of 5,500 nm. The dashed line has, therefore, been drawn to represent 15% of the cell radius. ×12,000.

(resulting in ≅200 grains) for each time point on well-preserved cells. Grain centers were determined by overlying the grain with a series of concentric circles. The distance from the grain center to the nearest plasma membrane was then measured either on positive prints at a final magnification of 33,000 or on the negatives projected on a table projector unit at a final magnification of 57,000. In each case, the distance from the grain center to the plasma membrane meas-

TABLE I

Percent TCA Precipitable ^{125}I-Insulin in Incubation Medium

Time, *min*	0	30	60	90
	%	%	%	%
15°C	94	92	90	90
37°C	94	90	85	—

ured in millimeters was calculated in nanometers with the use of the calibration grid. The normalized number of grains was plotted as a function of the distance of the grain center from the plasma membrane and histograms constructed according to the method of Salpeter et al. (30–32). Because the surface was very large compared to the number of grains, normalized grain number was used instead of grain density.

To apply the universal curve for ^{125}I irradiation, we have assumed a half distance for our data of 90 nm. The half distance represents the distance from the irradiation source that contains 50% of the developed grains (30).

Morphometry. Cell diameter was measured from semithin sections (1 μm thick) and photographed with a phase-contrast microscope (Carl Zeiss, Oberkochen, West Germany). A total of 1,000 cells were measured at a final magnification of 1,300. Cell diameter was evaluated by overlying the cell with circles of an increasing known diameter. The mean diameter obtained was corrected by $4/\pi$ according to Gieger and Riedwyl (33). To determine the volume density of organelles, 60 cells were randomly photographed at two different magnifications (nucleus at ×4,500, the mitochondria and Golgi area at ×9,000). Morphometric determinations were made, with the "multipurpose test system" used on a table projection unit (34). The volume density, Vv, of organelles was determined by point counting (34). Vv = Pp organelle/Pp cell where Pp represent the test points enclosed by a given profile.

Because mitochondria and the Golgi area were photographed at a higher magnification it was first necessary to relate this volume density to one unit of volume of cytoplasm before extrapolating to the total cell.

The percentage of autoradiographic grains beyond 300 nm of the plasma membrane which were related to each of the intracellular organelles previously characterized by morphometry was evaluated by superimposing on each grain a circle with a diameter of the grain. When this circle overlay a single organelle this structure was given one point, whereas two or three organelles occupying a single circle were given ½ or ⅓ of a point. The total number of points accumulated by a given organelle was then calculated and expressed as the percentage of the total number of grains counted.

RESULTS

General characteristics of the cultured lymphocyte. A representative example of the IM-9 lymphocyte used for these experiments is shown in Fig. 1. These cells have general morphologic features of other human lymphocytes that grow in continuous culture (35). The cell diameter was calculated to be 11.2±0.1 μm in the assay buffer and 11.7±0.1 μm in the growth media and is similar to an earlier study of this cell in an unfixed preparation (36).[4] Plasma membrane and intracellular organelles i.e. mitochondria and the Golgi region are easily identified and demarcated.

Time-course binding and dissociation experiments. When IM-9 lymphocytes are incubated with 0.25 nM ^{125}I-insulin at 15°C steady-state binding is reached between 30 and 60 min and continues for 90 min; at 37°C steady-state binding is reached by 2 min and continues for 60 min (for example of these binding curves, see Fig. 4, ref. 20, and inset to Fig. 6).

When the labeled insulin was allowed to bind to cells at 15° or 37°C and diluted to infinity, the amount of hormone dissociated for any given time was the same for both binding temperatures; under conditions of dilution plus the addition of cold insulin or decreased pH ≅90% of the labeled hormone was dissociated from the cell by 90 min regardless of the time or temperature of association (22) (data not shown).

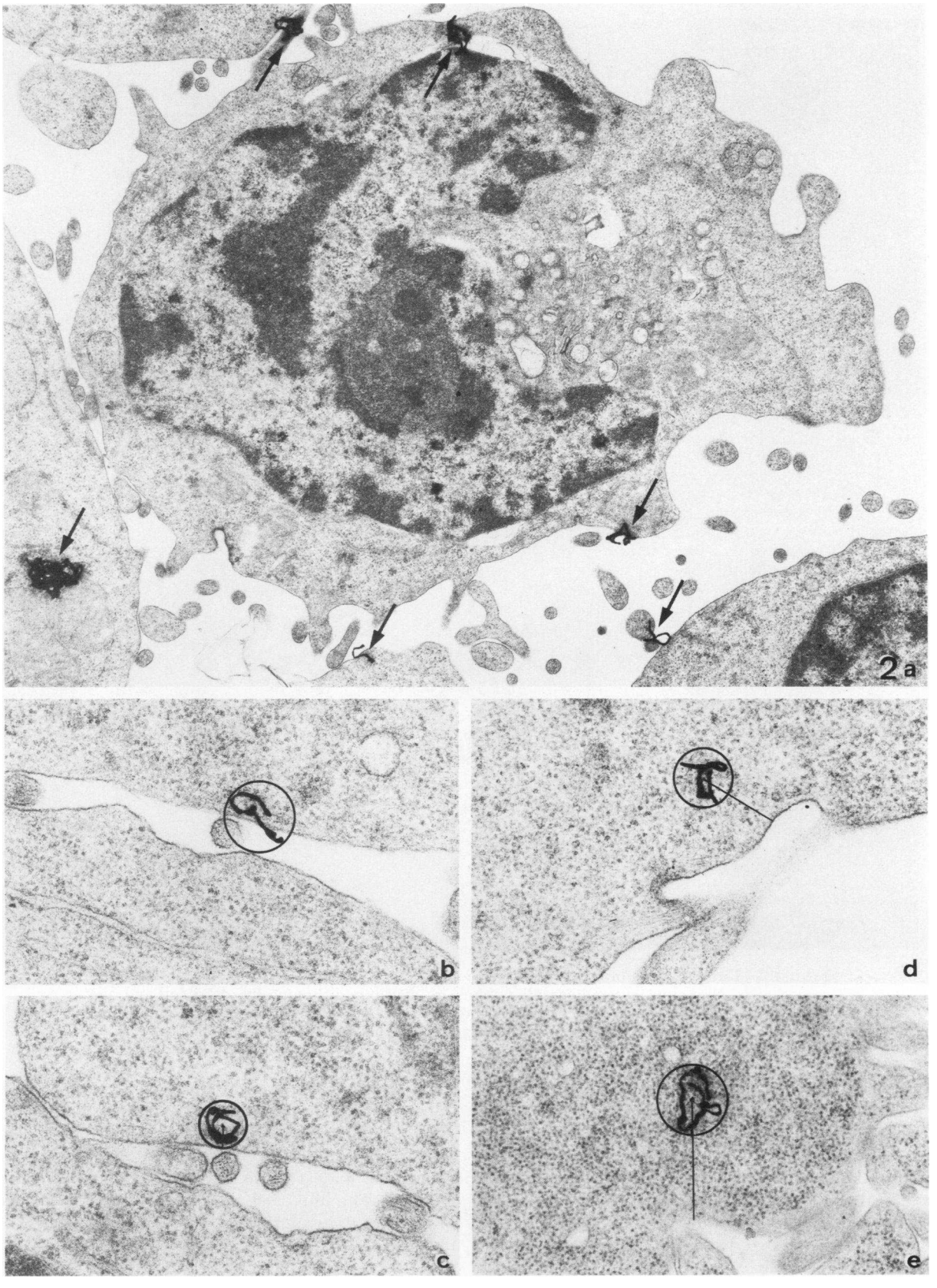
Using criteria of trichloroacetic acid precipitability there was very little degradation of labeled insulin in the incubation media at 15°C and no >15% at 37°C (Table I).

Autoradiography. When cells at each time point are fixed and the thin sections processed for autoradiography, the developed grains are easily identified (Fig. 2). At a higher magnification the distance from the grain center to the nearest point of the plasma membrane can be measured (Fig. 2). In the intact cell, with the small insulin tracer used in these experiments, there is rarely more than one grain associated with each section of the cell. By contrast, in damaged cells several grains may be found distributed throughout the cell (Fig. 3). In evaluating over 4,000 photographs, this pattern has only been seen in damaged cells. Similar findings have been reported in other types of lymphocytes (38). Therefore, only cells judged to be intact were photographed for quantitative studies.

Quantitative autoradiographic studies. We have assumed that the initial-binding step should most

[4] A previous report that this cell is 20–30 μm in diameter (37) has been reevaluated and recent estimates indicate that the diameter is less than previously reported (I. D. Goldfine, personal communication).

FIGURE 2 (*a*) General view at low magnification of an IM-9 lymphocyte with developed autoradiographic grains. The grains (arrows) are mainly localized at the periphery of the cell and in some instances seems to be more closely related to microvilli. ×13,000 (*b–e*), Autoradiographic grains seen at the magnification chosen for the quantitative determination of their distance from the plasma membrane. The mode of evaluation of this distance is illustrated: each grain is superimposed by a circle with the diameter of the grain and the distance between the circle center and the nearest plasma membrane is measured. The four examples illustrate respectively (*b*) <45 nm (*c*) 150 nm (*d*) 350 nm (*e*) 600 nm. ×33,000.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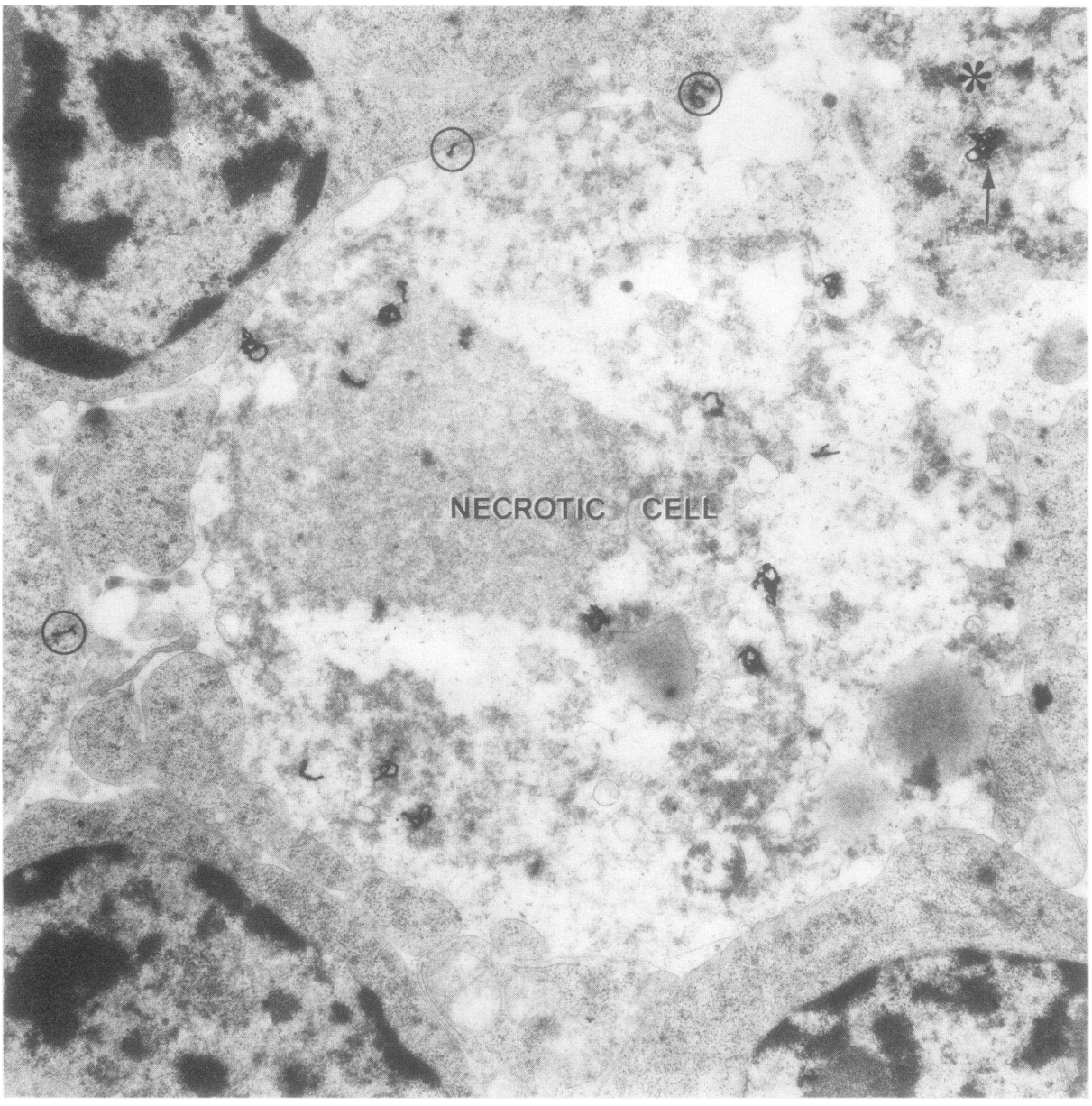


FIGURE 3 General view of a dead cell surrounded by a damaged cell (*) and six well-preserved cells. Numerous autoradiographic grains are distributed through the dead cell and one grain is seen within the cytoplasm of the damaged cell (arrow). Well-preserved lymphocytes show autoradiographic grains localized close to the plasma membrane (circles). ×13,000.

accurately be reflected by the earliest time point of the incubation and at the lowest temperature tested. Therefore, the distance from each grain center to the nearest point on their respective plasma membrane for the 2 min time point at 15°C (Fig. 2) was measured and the grain distribution plotted according to the method of Salpeter et al. (30–32). In this form of analysis, the normalized number of grains are plotted as a function of their distance from the plasma membrane and a histogram constructed (Fig. 4A). The shape of the histogram demonstrates that the grains are generally symetrically distributed around the plasma membrane. The long tail to the right presumably represents the widest angle of radiation scatter and background. The universal curve for ^{125}I irradiation (distribution of grains around a known line source [32]) is superimposable on this histogram (Fig. 4A). We interpret this to mean that the grain distribution at 2 min

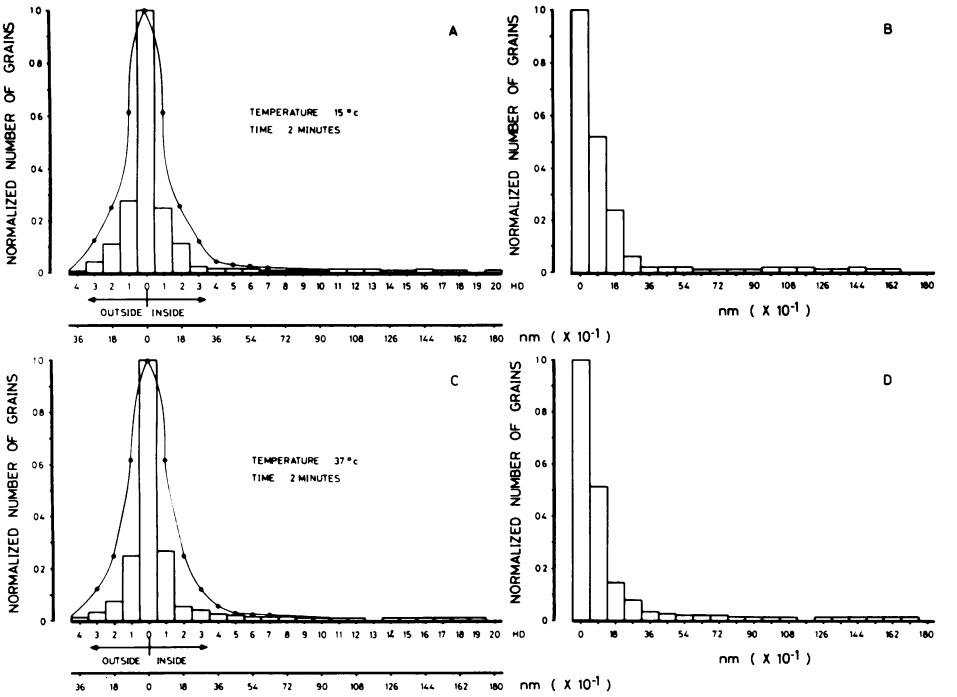


FIGURE 4 Autoradiographic profiles of ^{125}I-insulin binding to IM-9 lymphocytes after 2 min of incubation with 0.1 nM ^{125}I-insulin at 15° (A) and 37°C (C). The normalized number of grains are plotted as a function of the distance of the grain center from the plasma membrane. This distance is expressed both in nanometers and in half distance (distance from a linear source of ^{125}I that contains 50% of the developed grains). The half distance mode of expression of the results allows us to draw the universal curve for ^{125}I (—— in A and C) and to compare this curve to our histograms. The universal curve shown here describes the distribution of ^{125}I around a known line source of irradiation. Because for some time points, lymphocytes were not completely separated it was impossible to plot the grains both outside and inside the plasma membrane. We have, to maintain uniformity, constructed histograms for these same 2 min time points with all grains plotted on or inside the plasma membrane (B and D). The histograms A and B are constructed from the same data and likewise C and D are constructed from the same data. Grains appearing ≅900 nm and beyond are assumed to represent the widest angles of radiation scatter and background.

of incubation at 15°C is consistent with a line source of irradiation which, in this case, is the plasma membrane. Though this technique does not have sufficient sensitivity to resolve the inner and outer surfaces of the membrane, we assume that the grain distribution represents binding to sites on the external surface of the membrane. A similar distribution of grains and relationship to the universal curve of ^{125}I irradiation is seen for the 2 min time point at 37°C (Fig. 4C). To follow the fate of the labeled hormone as a function of time and increasing temperature, it was necessary to slightly modify the construction of the histogram. For some of the time points, cells are not sufficiently separated to allow plotting grains both outside and inside the cell. We have, therefore, plotted all grains as either on or inside the membrane (Fig. 4B and D). We have moreover verified that there are no differences between grain distribution from the same time point whether cells are separated or more tightly compact (data not shown). When histograms are constructed for each time point, as Fig. 4B, at 15°C there is essentially no difference in grain distribution from 2 to 90 min of incubation (Fig. 5A). A similar pattern is seen for a second incubation (data not shown). At 37°C, though there is no systematic change in grain distribution as a function of time, there is an additional component appearing at each time point at a distance of 300–900 nm from the plasma membrane (Figs. 5B, 6, and Table II). In all three experiments there is a small but definite increase in grains from 300 to 900 nm when the earliest time point is compared to the latest time point.[5]

[5] A preliminary study, where incubation was carried out at 37°C for 30 min. in growth media instead of the assay buffer, demonstrates essentially the same results as shown in Fig. 6B. Under these conditions, however, a threefold increase in the concentration of labeled insulin is necessary for adequate binding.

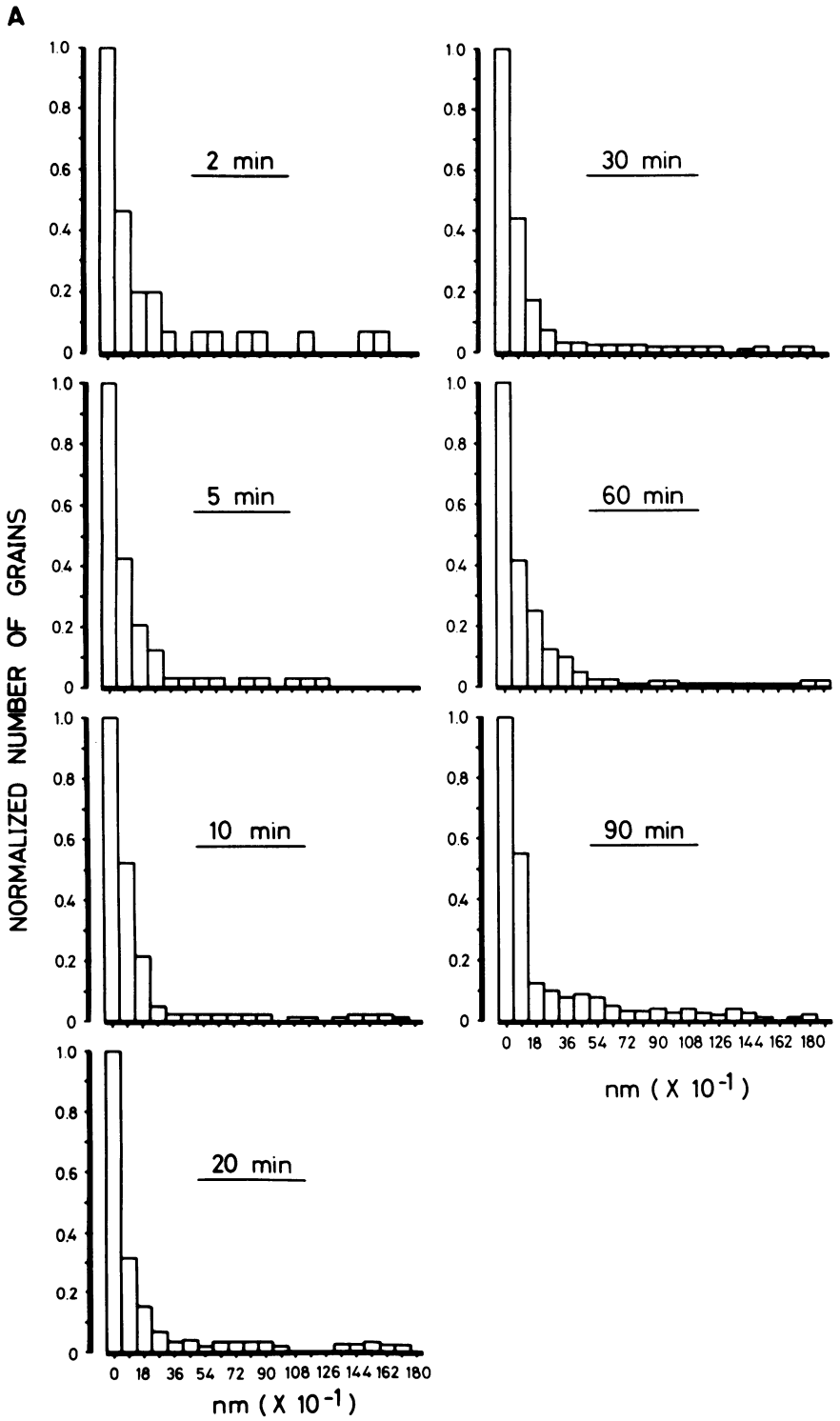
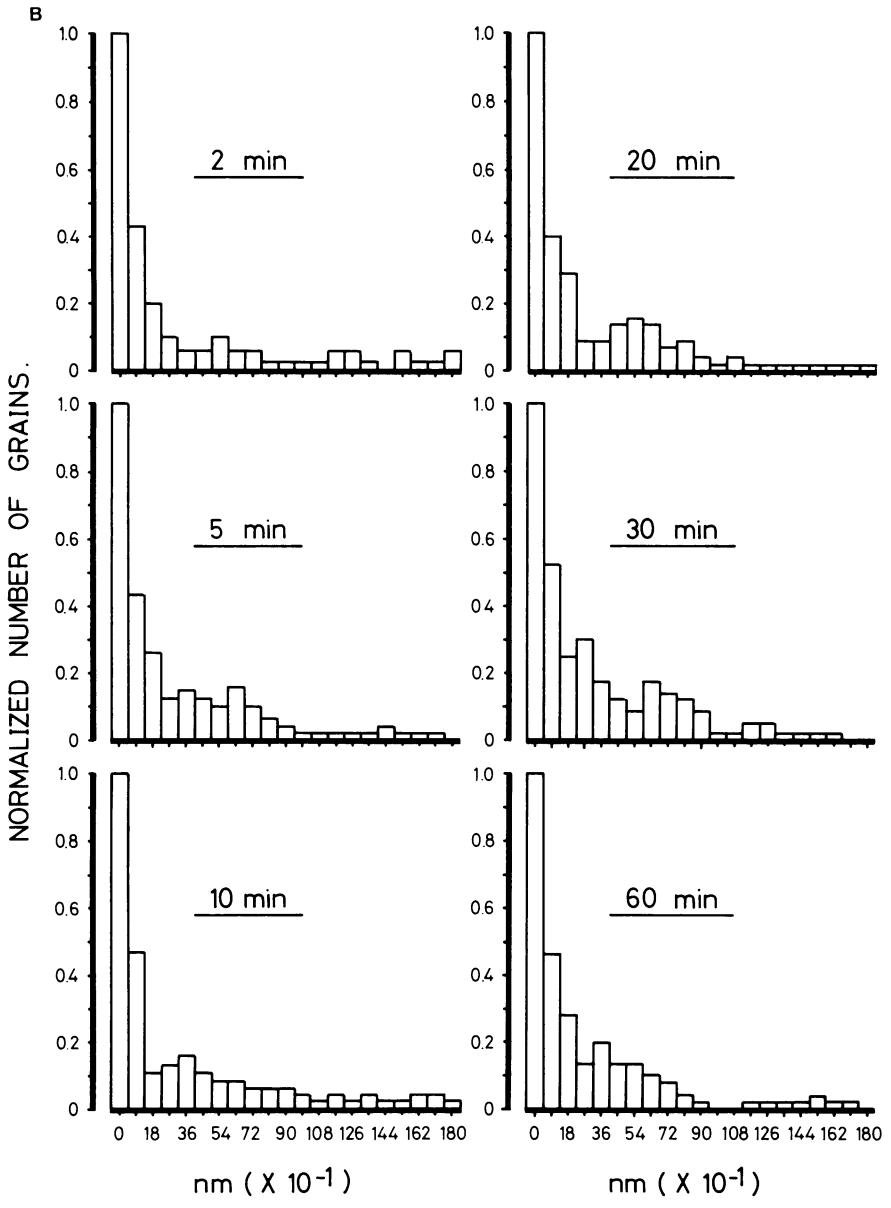


FIGURE 5A Autoradiographic profiles of ^{125}I-insulin binding to IM-9 lymphocytes after incubation with 0.1 nM insulin at 15°C. Binding became maximal between 30 and 60 min of incubation and a steady state was maintained through 90 min. Nonspecific binding was 2% of total binding at 90 min of incubation. The histograms were constructed according to the format shown in Fig. 4B and D: number of grains measured: 2 min, 38; 5 min, 53; 10 min, 175; 20 min, 178; 30 min, 209; 60 min, 242; 90 min, 198.



5B Autoradiographic profile of ^{125}I-insulin binding to IM-9 lymphocytes after incubation with 0.1 nM insulin at 37°C. Binding became maximal by 2 min of incubation and was maintained at steady state through 30 min followed by a slow decrease in binding up to 60 min. Nonspecific binding was 12% of total binding at 60 min of incubation. In addition to the major source of irradiation shown in the 2 min time point, there is an additional component appearing at each time point of incubation at a distance of 300–900 nm from the plasma membrane: number of grains measured: 2 min, 84; 5 min, 134; 10 min, 126; 20 min, 128; 30 min, 142; 60 min, 132.

Nonspecific binding. In all histograms presented, the grains evaluated correspond to total binding (Figs. 4, 5, 6).

The nonspecific binding (cell associated radioactivity in the presence of a large excess of unlabeled insulin) represents a small proportion of the total.

When the nonspecific binding is considered per se, the number of grains per grid is very low but when grains from a total of 16 grids were pooled, a total of 173 grains could be analyzed. Of these, 45 grains (26%) were on damaged cells and are not included in the histogram (Fig. 7). In contrast to the total binding a larger proportion of the grains appear at 200 nm from the plasma membrane and beyond. This illustrates that

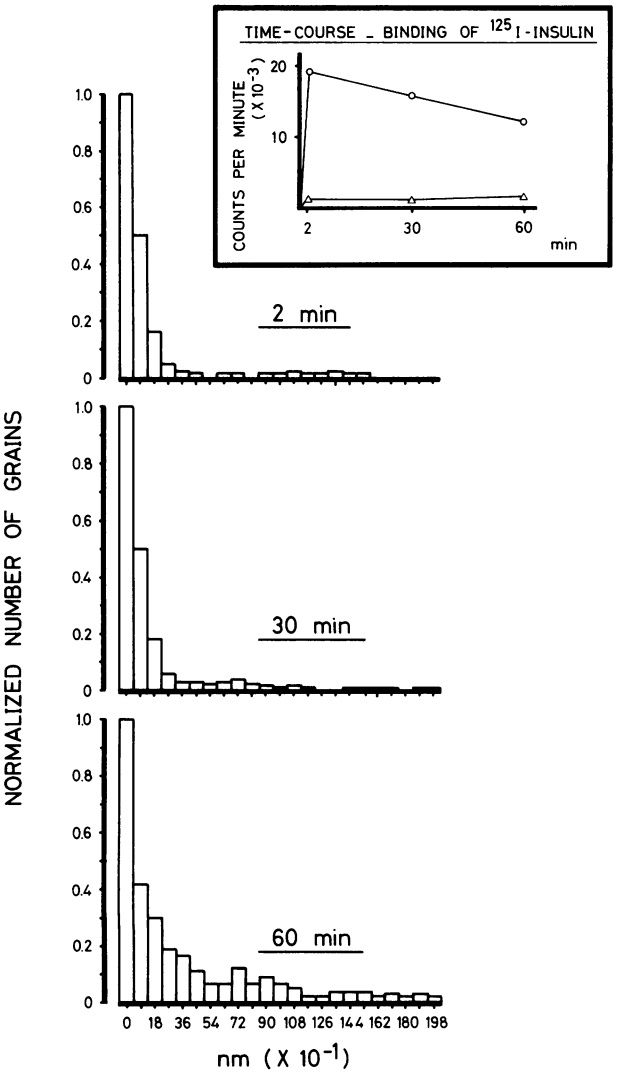


FIGURE 6 Autoradiographic profile of ^{125}I-insulin binding to IM-9 lymphocytes after incubation with 0.1 nM insulin at 37°C. Once again, when the 60 min histogram is compared to the one at 2 min, an additional component appears at a distance from 300 nm to 900 nm from the plasma membrane. Number of grains measured: 2 min, 229; 30 min, 246; 60 min, 186.

when the nonspecific binding is a significant proportion of the total binding, it must clearly be taken into account in the analysis of the histogram.

Morphometry. To determine if the grains appearing beyond 300 nm from the plasma membrane have a specific localization within the cell, we have determined by morphometric means the volume density of the nucleus, Golgi area, mitochondria, and the residual cytoplasm of IM-9 lymphocytes as well as the percentage of grains related to these organelles (Figs. 1 and 2). The ratio of volume density of organelles to the percentage of grains related to organelles is very close to one in all instances indicating that these grains do not preferentially localize to these organelles (Table III).

DISCUSSION

We have drawn several conclusions from the present study: (*a*) After 2 min of incubation at 15°C, the autoradiographic grain distribution is consistent with a line source of irradiation. We interpret this to mean that insulin initially localizes to the plasma membrane, presumably its external surface. (*b*) At 15°C there is no significant shift in this grain distribution over a 90-min period of incubation. We interpret this to mean that once the physical process of binding has occurred, there is no further translocation of the hormone at this temperature. (*c*) After 2 min of incubation at 37°C, the grain distribution is consistent with a line source of irradiation which again suggests that these grains are localized to the plasma membrane. (*d*) With increasing time of incubation at 37°C, grains remain predominantly localized to the plasma membrane but a small additional band component of irradiation appears between 300 and 900 nm of the plasma membrane. This band component is apparent either by comparing the total grain distribution at 15°C and 37°C or by comparing the earliest time point (2 min) with the latest time point (30 or 60 min) at 37°C (Table II). We interpret this to mean that after the initial binding step there is a translocation of labeled material to a highly limited area of the peripheral cytoplasm. This additional band component of irradiation does not extend beyond 15% of the cell radius (Fig. 1).

TABLE II
Relationship of Grain Distribution to Nonspecific Binding

	Translocation of grains at 37°C beyond 300 nm*	Δ Nonspecific binding‡
	%	%
Experiment 1 (from Fig. 5)	25§ (60 min)	6.0
Experiment 2 (from Fig. 6)	30 (60 min)	2.3
Experiment 3 (data not shown)	23 (30 min)	4.1

* For this calculation we have used the data from Fig. 4, 2 min at 15°C to represent the initial localization. Percent of grains from 0 to ±300 nm = 85% of the total number of grains therefore grains beyond 300 nm (100% − 85%) = 15% of the total number of grains. Thus for each experiment, the percent translocation is percent of grains beyond 300 nm minus 15%.

‡ Difference in nonspecific binding for two conditions that were compared.

§ If percent grains beyond 300 nm for all time point at 15°C (Fig. 5A) are compared to percent grains at 37°C beyond 300 nm (Fig. 5B) the percent change = 17%.

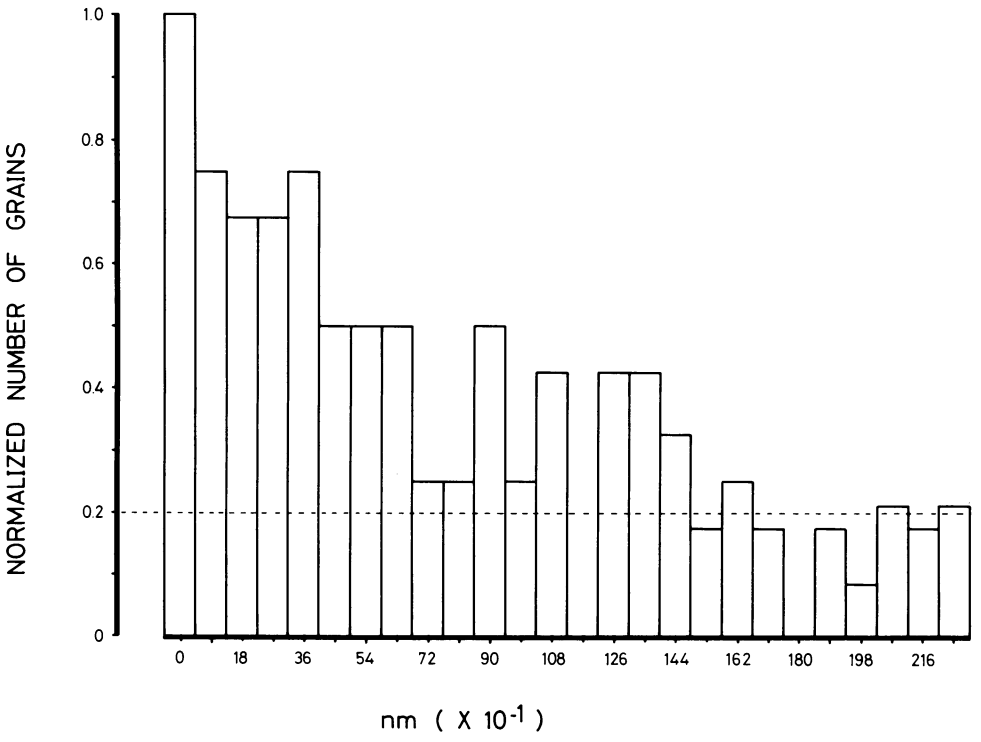


FIGURE 7 Autoradiographic profile of ^{125}I-insulin binding to IM-9 lymphocytes in the presence of an excess of unlabeled insulin (nonspecific binding). The number of grains on each grid was very low and it was, therefore, necessary to pool grains from a total of 16 grids. Though background is very low for all previous histograms shown (≅0.006 grain /μm^2 or ≅1–2 grains/ histogram column), it becomes significant when there are very few grains per histogram column. Thus, (– – – –)shows that the area of the histogram below the line could be attributed to background alone. Even if the area below the dashed line is substracted it is apparent that more grains are appearing to 200 nm and beyond from the plasma membrane.

Because the unit membrane is <10 nm in width, we assume that the presence of labeled insulin or a product of labeled insulin within a band 900 nm beyond the plasma membrane indicates that the labeled material has traversed the plasma membrane. Another possible interpretation would be deep infoldings of the membrane. After examining multiple samples, however, we have been unable to relate grains to such structures. (*e*) When a morphometric analysis is applied to grains that extend 300 nm and beyond the plasma membrane, we find no preferential localization of these grains to intracellular organelles.

To relate our studies to the extensive work previously carried out in the IM-9 lymphocytes (20–22, 26, 27) similar experimental conditions i.e.: phase of cell cycle, buffers, incubation conditions, physiological concentration of labeled insulin. . . . were used throughout. In this regard, the binding characteristics found in this study are very similar to what has been previously reported i.e. temperature dependence, time-course, dissociation of labeled hormone by dilution or dilution plus unlabeled insulin, and degradation of the labeled hormone.

Because the autoradiographic approach at the ultrastructural level does not make it possible to localize the source of each specific developed grain, it is essential to use a quantitative technique to analyze the grain distributions. The method chosen was described by Salpeter et al. (30–32); only minor modifications were made in certain instances to accomodate special features of our data. Salpeter et al. (30–32) have pointed out that the most important determinant of resolution

TABLE III
Morphometric Evaluation

Organelle	Volume density organelle	Grains related to organelle*	Ratio
		%	
Nucleus	32.9	32.4	1.02
Mitochondria	11.1	11.7	0.95
Golgi area	10.5	11.7	0.90
"Residual cytoplasm"‡	45.5	44.1	1.03

* 360 nm or beyond the plasma membrane.
‡ Cytoplasm area minus mitochondria and Golgi area.

for ^{125}I is the size of the silver halide crystal of the emulsion. Other photographic or geometric factors that may lead to variations in resolution for other isotopes, such as emulsion or section thickness, are less important for ^{125}I.

The superimposition of the universal curve for ^{125}I to the earliest time points of incubation at 15° and 37°C lead us to interpret our results as consistent with a line source model of irradiation. Our interpretation of an additional component appearing at 37°C with increasing time of incubation does not depend, however, on a specific model of analysis but rather to the comparison of the 37°C data to the 15°C data serving as control or as the comparison of the later time point to the early time point serving as control. Thus, although the nature of the additional component i.e. a band source . . . , is speculative, the phenomenon is reproducible and unexplained by any apparent artifact of the method.

Several factors could influence the interpretation of these results. Background is presumably due to spontaneous events that are randomly distributed. In each experimental incubation background was found to be very low and had no specific influence on any particular component of the histogram. Under certain circumstances where background comprises a significant proportion of grains from each histogram column, it must be considered in the analysis (Fig. 7). The second factor that is intrinsic to studies of labeled polypeptide hormone binding to cells or subcellular localization of labeled hormone is nonspecific binding. In autoradiography, total binding is measured because it is not possible to distinguish "specific" from "nonspecific" binding. It is apparent from Fig. 7 that if nonspecific binding becomes a significant proportion of the total binding, it could influence grain distribution. We have therefore considered whether nonspecific binding could account for the additional component seen at 37°C. It can be seen from Table II that although nonspecific binding may influence the magnitude of the additional component it is unlikely at these levels of nonspecific binding to account for it completely. By contrast to the nonspecific binding, the ability to distinguish damaged cells is a very positive feature of the electron microscopic autoradiography method. This distinction is indeed not possible by classic subcellular fractionation techniques. Thus, in measuring the cellular uptake of a labeled polypeptide hormone by fractionation of individual components of the cell there is no way to distinguish the contribution by damaged cells. Furthermore, it is impossible to be certain whether the hormone is bound in a specific or a nonspecific fashion to the damaged cell. In our study we have, therefore, avoided damaged cells in our analysis.

When cells are disrupted and the organelles purified, specific insulin binding has been demonstrated in Golgi membranes (15), endoplasmic reticulum (17), and nuclei (16). When we analyzed autoradiographic grains most likely to represent intracellular penetration (i.e. grains 300 nm and beyond from the plasma membrane) we were unable to show preferential localization to these organelles. Thus, in intact cells we find no evidence that labeled insulin localizes to specific intracellular organelles. Bergeron et al. (15), has made the intriguing suggestion that binding sites on Golgi membranes might represent newly synthetized receptors enroute to the plasma membrane. In this case one would not expect insulin in the external milieu of the cell to localize to this organelle. Recently, Goldfine et al. (37) presented data at the light microscopic level derived from the incubation of labeled insulin with the IM-9 lymphocyte. They interpret their data to indicate that labeled insulin enters the intact cell and subsequently binds to nuclei. It is not possible at the light microscopic level to localize grains to the highly limited area of the cell periphery as suggested by our study and as previously mentioned we find no evidence for preferential localization of labeled insulin to intracellular organelles. Thus, it is not possible to directly compare the data from the study of Goldfine et al. (37) to our own because of the differences in the techniques. Clearly, however, there are conceptual differences which may be resolved by further study.

At an early phase of this study we suggested that labeled insulin localized to the plasma membrane of the cultured lymphocytes and there was no further translocation (39). We now conclude that there is a small but definite translocation of labeled insulin or a product of labeled insulin[6] to a highly limited area of the cell periphery after the initial binding step. This conclusion is based on our observations in three separate incubations of lymphocytes and after a careful consideration of the potential pitfalls of the methodology. More importantly, however, is our observation that this same highly limited translocation of auto-

[6] Because most of the labeled insulin remains associated with the cell membrane we have not attempted to analyze the nature of the cell-bound radioactivity. It is known, however, that degraded products that are found in the incubation media do not bind to the receptor (28). Furthermore, the typical degradation products of insulin, small iodotyrosyle peptides or iodide, would be expected to be removed from the preparation during the dehydratation procedure required for preparing the tissue for electron microscopy and therefore, would not contribute significantly to developed grains. Whether receptor-bound insulin is translocated intracellularly and degraded is under investigation in the isolated liver cell. Preliminary studies indicate that when isolated hepatocytes, that have been incubated with labeled insulin under similar conditions to the cultured human lymphocytes are separated from the incubation media and extracted, approximately 80% of the labeled material maintains the molecular size of native insulin (P. Gorden and P. Freychet. Unpublished observations).

radiographic grains (within a distance of 15% of the cell radius) occurs in isolated hepatocytes but to a quantitatively much higher degree (40).

If as we have suggested labeled insulin traverses the plasma membrane, then the mechanism by which this occurs is of considerable interest. A mechanism such as pinocytosis or possibly other mechanisms is not apparent in the lymphocyte and hopefully the hepatocyte, where we have a quantitatively more significant change, will provide some clue. As in previous studies the cultured human lymphocyte has provided a valuable model system for studying hormone receptor interaction and receptor regulation (20–22, 26, 27, 36). These cells, however, have not been shown to have specific insulin biologic responses (see ref. 20 for discussion) and no direct physiologic implications can be drawn from these studies. The combination of direct binding studies and quantitative electron microscopy should provide a valuable tool for the further study of receptor-linked processes that may relate to hormone action and (or) degradation in biologically responsive cells such as hepatocytes (40).

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