essential that AdoHcy be removed as it is formed, a function performed in mammalian cells by AdoHcy hydrolase. However, the equilibrium constant (K_{eq}) for hydrolysis greatly favors AdoHcy synthesis from adenosine and L-homocysteine, the products of AdoHcy cleavage (*31*). Under physiologic conditions AdoHcy hydrolysis proceeds because these products are efficiently removed, preventing the thermodynamically favored accumulation of AdoHcy.

Kredich and Martin (*7*) have shown that in ADA-inhibited mouse lymphoblasts, toxic concentrations of adenosine in fact caused both a marked accumulation of AdoHcy and inhibition of DNA methylation, findings we have extended in studies with human lymphoblasts (*32*). The toxicity of adenine may also be related to inhibition of AdoHcy cleavage. Studies showing that adenosine blocks ribosomal RNA maturation in a human plasma cell line also point to inhibition of methylation as a mechanism for its cytotoxicity (*33*). We believe that the affinity of AdoHcy hydrolase for adenosine may be an important factor in determining in which tissues AdoHcy might accumulate when other routes of adenosine metabolism are interrupted. In this regard it is interesting that the Michaelis constants reported for both the rat and beef liver enzymes for adenosine are from 0.4 to 1.5 m*M* (*34, 35*), in marked contrast to those of the human placental and lymphoblast enzymes, ~1 μM.

Whether or not accumulation of AdoHcy and inhibition of methylation contribute to the immune dysfunction in ADA-deficient individuals must await studies of affected individuals. Nevertheless, understanding of the regulation of nucleic acid and other methylase reactions is of considerable importance to several areas of investigation, including control of differentiation, gene expression, viral replication, and neoplastic transformation. Tight binding of adenosine to AdoHcy hydrolase in some tissues but not others may permit selective methods for modifying growth of tumor or virus-infected cells, and may provide a means for better defining the importance of various methylation reactions.

Note added in proof: We have recently found that adenosine-binding activity in extracts of mouse liver copurifies with AdoHcy hydrolase activity.

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Entry of Insulin into Human Cultured Lymphocytes: Electron Microscope Autoradiographic Analysis

Abstract. *Electron microscope autoradiographs were prepared of IM-9 human cultured lymphocytes incubated with iodine-125-labeled insulin. With the use of [^{125}I]insulin and Ilford L-4 emulsion, the technique had a resolution half-distance of approximately 0.085 micrometer. Autoradiographs revealed a time-dependent entry of insulin into the cell interior that was maximal after 30 minutes of incubation. At this time point nearly 40 percent of the [^{125}I]insulin was in the interior of the cell at a distance 1 micrometer or greater from the plasma membrane. Grain distribution and volume density analyses revealed that the intracellular insulin was concentrated in the endoplasmic reticulum and nuclear membrane.*

Studies at this laboratory in which IM-9 human cultured lymphocytes were incubated with [^{125}I]insulin and then examined by either cell fractionation or light microscope autoradiography, have indicated that insulin can enter the interior of these cells and bind to the nucleus (*1*). These techniques, however, lack the sensitivity to define precisely where insulin is located in the cell. Because of their greater sensitivity, electron microscope autoradiographs have the potential of defining more precisely the intracellular organelles with which insulin or other hormones interact. Only recently have analytical techniques become available for quantitating electron microscope autoradiographs of labeled hormones (*2*). An important advance has been the development by Salpeter *et al.* of grain density histograms to determine the resolution of ^{125}I or other isotopes (*2*). Accordingly, we have prepared and analyzed electron microscope autoradiographs of human cultured lymphocytes incubated with [^{125}I]insulin.

Previously, we demonstrated that when IM-9 human cultured lymphocytes are incubated with [^{125}I]insulin in a complete culture medium supplemented with 10 percent fetal calf serum at 37°C the cellular uptake of insulin is maximal within 1 minute, and a steady state can be maintained for 90 minutes or longer (*1*). Under these conditions, cell viability remains greater than 97 percent of total,

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and insulin degradation is minimal (*1*). After the uptake of insulin into the cell, the subsequent binding to the nucleus is approximately one-half maximal after 5 minutes (*1*).

When autoradiographs are examined with the electron microscope after short incubation periods, that is, 30 seconds, the majority of the silver grains representing insulin appear over the plasma membrane (Fig. 1A). In contrast, after 30 minutes of incubation, a large number of grains are seen over various organelles in the cell interior including the nucleus (Fig. 1B). Since the grains seen in the electron microscopic autoradiographs are located in an emulsion layer that is above the structures of the cell, there can only be a statistical probability that an observed grain is actually located above the structure seen in the micrograph. Thus, the possibility exists that grains seen over intracellular structures could result from the scatter of [^{125}I]-insulin bound only to the plasma membrane. To demonstrate clearly that insulin does enter the intact cell, we prepared grain distribution profiles from cells at different time points and compared them to a line source of ^{125}I.

A thin layer of [^{125}I]insulin was embedded in plastic resin, sectioned, and covered with Ilford L-4 emulsion and developed as described by Salpeter *et al.* without modification (*2*). The location of grains around this line source was noted and a grain density histogram for ^{125}I was constructed (Fig. 2A). As expected, the number of grains fell off rapidly as the distance from the source increased. The half-distance was approximately 0.085 μm [this half-distance value is very similar to the half-distance values of 0.08 to 0.09 μm reported by Salpeter *et al.* for ^{125}I-labeled albumin (*2*)]. Less than 2 percent of the radioactivity was found at a distance of 1 μm or greater from the source. This analysis indicates that if a cell has a source of radioactivity localized only at the plasma membrane, less than 2 percent of the total cellular grains will be found at a distance of 1 μm from the surface of the cell. Grain distribution profiles were then prepared of the autoradiographs of human cultured lymphocytes. After 30 seconds of incubation, a plot of the number of grains as a function of their distance from the plasma membrane showed that approximately 14 percent of the grains were found at a distance 1 μm or more from the plasma membrane, indicating that even at very early time points insulin had moved into the interior of the cell. In contrast, outside of the cell at a distance of 1 μm or more from the plasma membrane essentially no grains were detected. The amount of intracellular insulin increased with time, and after 30 minutes of incubation nearly 40 percent of the grains were inside the cell. At later periods of incubation, there was no further intracellular translocation. When cells incubated for 30 minutes with labeled insulin were treated with 1*M* acetic acid, 85 to 90 percent of the total cellular radioactivity was extracted. Analysis of the radioactivity both on Sephadex G-50 columns and in rebinding studies with

Table 1. Grain distribution analysis of [^{125}I]insulin in lymphocytes. Autoradiographs were analyzed with the electron microscope, the location of grains noted, and organelles credited. When grains were located over two or more organelles, partial credit was given to each organelle. Extracellular grains were assigned to the plasma membrane. Abbreviations: PM, plasma membrane; CY, cytoplasm; ER, endoplasmic reticulum; GO, Golgi; MI, mitochondria; NM, nuclear membrane; and NU, nucleus.

Time	Grains counted	Cellular location (percentage of total grains)						
		PM	CY	ER	GO	MI	NM	NU
30 seconds	356	70.6	19.8	3.5	0.3	1.7	0.8	3.1
5 minutes	394	55.8	21.9	10.9	0.5	2.2	0.9	7.4
30 minutes	406	39.1	26.4	11.4	0.5	3.6	3.3	15.6

Table 2. Comparison of volume densities of intracellular organelles with intracellular grain distributions. The volume densities of the organelles were determined by the method of Weibel and Bolender (*3*). The intracellular grain distributions after 30 minutes of incubation were obtained from Table 1. Volume densities for ER, GO, and NM were determined by considering both their membranes and cisternal spaces.

Item	Intracellular organelles					
	CY	ER	GO	MI	NM	NU
Volume density*	49.0	4.8	1.9	6.4	1.2	36.7
Intracellular grains*	43.4	18.7	0.8	5.9	5.4	25.6

*Percentage of total.

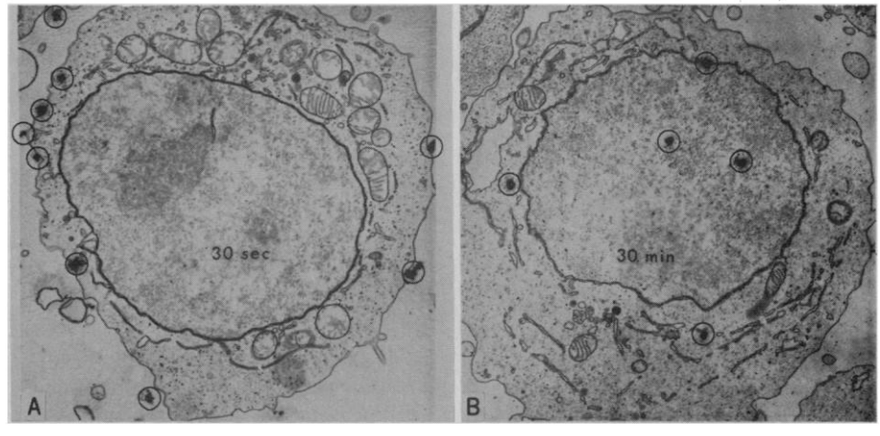

Fig. 1. Electron microscope autoradiographs of IM-9 human lymphocytes incubated with [^{125}I]insulin. Cells (10^6 per milliliter) were incubated with 1.6 n*M* [^{125}I]insulin (*1*) in Eagle's complete medium supplemented with 50 m*M* Hepes (*p*H 7.5) and 10 percent fetal calf serum at 37°C. The cells were then washed twice at 4°C in a buffer containing 154 m*M* NaCl, 20 m*M* tris, *p*H 7.5, and fixed for 1 hour at 23°C in 0.8 percent paraformaldehyde and 2.7 percent glutaraldehyde in 80 m*M* sodium cacodylate, *p*H 7.4. The fixed cells were collected on 0.22-μm Millipore filters and postfixed for 1 hour at 23°C in 1 percent osmium tetroxide with 1.5 percent potassium ferrocyanide. Cells were dehydrated and embedded in Epon 812 epoxy resin (Ladd Research). For size analysis, 1-μm sections were stained with toluidine blue and random fields were photographed with an AO series microscope. The average diameter of the cells was 17.4 μm (*3*). Sections at 0.09 μm for electron microscopic autoradiography were cut on a Sorvall MT2-B ultramicrotome. Sections were collected on 300-mesh copper grids, coated with Parlodion and carbon, mounted on glass slides, and a monolayer of Ilford L-4 emulsion, approximately 1.5 μm in thickness (*4*), was applied with a wire loop. Grids were exposed for 4 to 8 weeks at 4°C, developed in Kodak D-19 developer at 16°C for 4 minutes, fixed in Kodak Rapid Fixer for 4 minutes, and washed for 30 minutes. Grids were then stained for 3 minutes with lead citrate and photographs were obtained at a ×4500 magnification on a Phillips 201 transmission electron microscope. (A) After 30 seconds of incubation. (B) After 30 minutes of incubation.

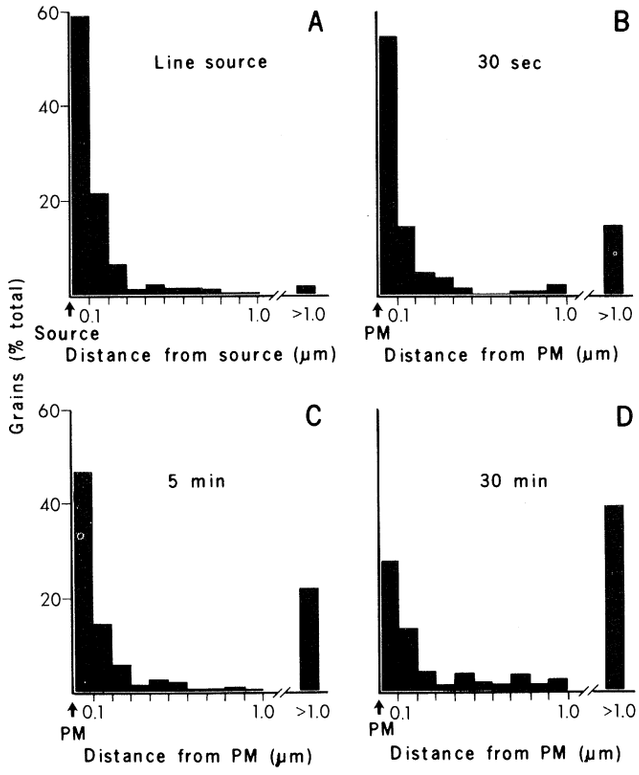

Fig. 2. Grain distribution profiles. (A) Grain density histogram for [^{125}I]insulin in Ilford L-4 emulsion prepared by the method of Salpeter *et al.* (*2*); 289 grains were counted. (B, C, and D) Incubation of lymphocytes with [^{125}I]-insulin for 30 seconds (235 grains), 5 minutes (385 grains), and 30 minutes (273 grains). Micrographs were prepared as described in Fig. 1. A Graphic Data Digitalizer (Ladd Research) was used to measure the distance from the center of the grains to the nearest plasma membrane (*PM*).

fresh lymphocytes revealed that more than 95 percent of the radioactivity was associated with intact insulin. These results indicate that insulin enters these cells in a time-dependent manner and then penetrates to a distance of 1 μm or more.

The cellular and intracellular distribution of the grains was calculated (Table 1). After 30 seconds of incubation, the majority of the grains were found on the plasma membrane and the rest of the grains were distributed among various cellular organelles and cytoplasm. After 5 minutes and 30 minutes of incubation there was a progressive decrease in grains on the plasma membrane, and an increase in grains over all cellular components, especially the nucleus and endoplasmic reticulum. As was seen in earlier studies (*1*), uptake of insulin into the nucleus was approximately one-half of the maximum after 5 minutes of incubation and maximal at 30 minutes. Incubation of cells with labeled insulin plus a large excess of unlabeled insulin (16 μ*M*) reduced the number of grains present to less than 5 percent of that seen with labeled hormone alone. These few grains were found to be randomly distributed throughout the cells and thus no background corrections were made.

To determine whether the grains in the cell interior at steady state were associated with the various intracellular compartments in proportion to the volume of these compartments, we conducted volume density studies (Table 2). After 30 minutes of incubation, the percentage of grains associated with both the endoplasmic reticulum and nuclear membrane was greater than their volume densities, whereas the percentage of grains associated with the cytoplasm, Golgi apparatus, and nucleus was less. These analyses suggested that the interaction of insulin with intracellular organelles is not a random process.

The present studies confirm and extend prior studies at this laboratory indicating that insulin can enter the interior of human cultured lymphocytes and bind to intracellular structures such as the nucleus. The time course of nuclear binding as determined by electron microscope autoradiography very closely resembles the time course of insulin binding to the nucleus as determined by cell fractionation studies (*1*). Carpentier and co-workers (*5*) have also obtained electron microscope autoradiographs of insulin associated with human cultured lympho cytes. These authors confirm our findings that insulin can enter these cells. In contrast to the present studies, however, they calculate that insulin penetrates lymphocytes no further than 0.9 μm and is not associated with intracellular organelles. In the present study lymphocytes were incubated in growth media with serum, whereas Carpentier *et al.* (*5*) incubated cells in a hypertonic, serum-free buffer devoid of Ca^{2+}, K^+, and PO_4^{2-}. It is possible, therefore, that the differences in the intracellular localization of insulin reported in these two studies may reflect differences in the incubation solutions employed.

The results of our present studies bear similarities to recent studies of the entry of insulin into liver cells. Bergeron and colleagues (*6*) injected insulin into the portal vein of rats, and then prepared electron microscope autoradiographs of liver at various time points (*6*). They found that 1 to 3 minutes after the injection of insulin, most of the grains were located on the plasma membrane (*6*). In contrast, at later time points, they found that most of the insulin had entered the interior of the cell (*6*). Studies from our laboratory confirm the results of Bergeron *et al.* in intact liver (*7*).

The biological significance of the uptake of insulin into target tissues is unknown. We and others have described specific binding sites for insulin on intracellular structures (*8*) including the nucleus and nuclear membrane (*8–10*), smooth and rough endoplasmic reticulum (*10, 11*), and Golgi apparatus (*12*). Since insulin has a variety of effects on intracellular organelles, and since insulin enters cells and binds to these organelles, we have postulated that intracellular insulin participates in the regulation of insulin-dependent intracellular functions (*1, 13*).

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Growth of Infective Forms of *Trypanosoma rhodesiense* in vitro, the Causative Agent of African Trypanosomiasis

Abstract. *A new approach to the culture of African trypanosomes led to the growth of the infective forms of the causative agent of human African trypanosomiasis. Infective cultures of* Trypanosoma rhodesiense *were initiated and maintained in vitro on Chinese hamster lung cells. By changing daily one-third of the Hepes-buffered RPMI 1640 medium containing 20 percent fetal bovine serum, the trypanosome numbers increased to 3 × 10⁶ to 5 × 10⁶ cells per milliliter. After 80 days in vitro at 37°C, the cultured trypomastigotes are infective for mice and rats and morphologically similar to bloodstream trypomastigotes in having a subterminal kinetoplast and a surface coat. In addition, they possess* L-α-*glycerophosphate oxidase, the predominant steady-state terminal oxidase of bloodstream trypomastigotes.*

Trypanosomiasis has been placed by the World Health Organization high on the list of the ten major health problems facing mankind today. Sleeping sickness constitutes a permanent and serious risk to the health and well-being of at least 35 million people. Biochemical and immunological studies of *Trypanosoma rhodesiense* and *Trypanosoma gambiense*, the causative agents of the disease in humans, would be facilitated if the infective stages of these organisms could be grown in vitro.

The development of a medium and conditions under which infective trypanosomes can be maintained in vitro has been attempted for numerous years (*1*), and the descriptions by Hirumi *et al.* (*2*) of conditions that will allow the development of infective forms of *Trypanosoma brucei* in culture have stimulated further studies in this area. Using bovine fibroblast-like cells in Hepes-buffered RPMI 1640 medium (*3*) containing 20 percent inactivated fetal bovine serum (FBS), Hirumi *et al.* were able to grow trypanosomes that were infective for mammalian hosts and also retained the morphological characteristics of long, slender bloodstream trypomastigotes with the surface coat (*2*).

Our studies were directed toward identifying established tissue culture cell lines that would support the growth of *T. rhodesiense* at high yield and toward developing simple procedures for establishing infective trypomastigotes in culture. In addition, we wanted to determine the biochemical similarities between *Trypanosoma rhodesiense* infective trypomastigotes maintained in culture and the slender bloodstream trypomastigotes isolated from humans. In this report, we show that it has been possible to initiate and maintain infective forms of *T. rhodesiense* in vitro on Chinese hamster (*Cricetulus griseus*) lung cells (ATCC CCL 16 Don). The trypanosomes maintained on these tissue culture cells are infective to mammalian hosts and possess the L-α-glycerophosphate oxidase system characteristic of bloodstream forms.

The Chinese hamster lung cells were obtained from the American Type Culture Collection, Rockville, Maryland. They were maintained in RPMI 1640 (Gibco) supplemented with 25 m*M* Hepes buffer, 20 m*M* sodium bicarbonate, and 20 percent heat-inactivated FBS (KC Biological) and, per 100 ml, penicillin (10,000 U), fungizone (25 μg), streptomycin (10,000 mg), and kanomycin (12.5 μg). The morphology of the tissue cell line was fibroblast-like. An inoculum of 1.8×10^6 viable tissue culture cells multiplied one to three times in 4 days in the described culture medium at 37°C in an atmosphere of 5 percent CO_2 and 95 percent air. The tissue culture cells were used just prior to becoming confluent, usually 3 to 4 days after a flask was inoculated.

The preparation of the trypanosomes from rats was simplified over the procedures described by Hirumi *et al.* (*2*). Successful cultures were established with a pleomorphic strain of *T. rhodesiense* EATRO 1895 from normal or lethally irradiated (800 rads) male rats (weighing 200 g) that were inoculated intraperitoneally with 2×10^6 trypanosomes. When the parasitemia reached a level of 1×10^8 to 5×10^8 trypanosomes per milliliter 3 to 4 days later, the rats were bled by cardiac puncture. From 90 to 95 percent of the trypanosomes present had the morphology of the slender forms, but some stumpy trypanosomes were also observed. The trypanosomes were centrifuged at 1025*g* at 4°C and the buffy coat was removed. Special precautions were taken to prevent the removal of red blood cells below the buffy coat. The trypanosomes were washed twice with sterile Hanks balanced salt solution with 5 percent

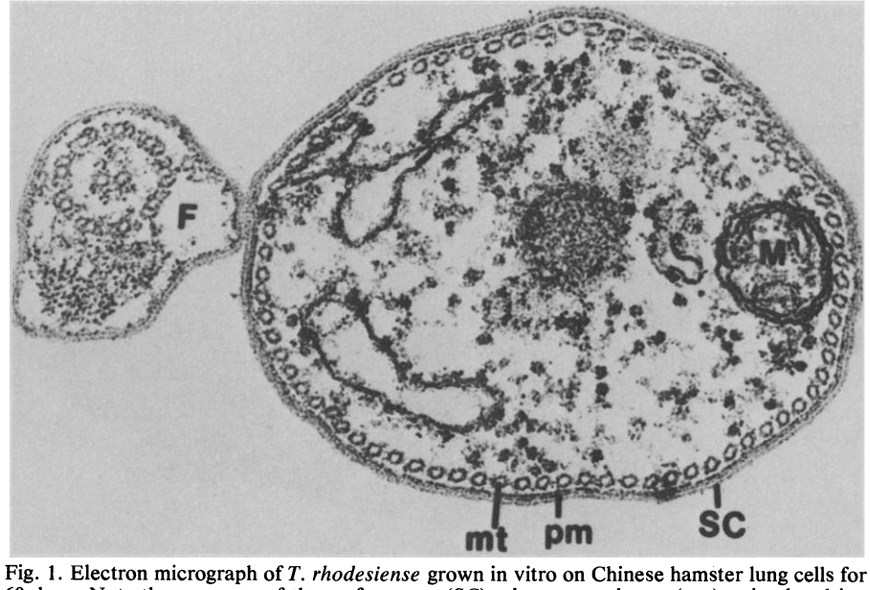

Fig. 1. Electron micrograph of *T. rhodesiense* grown in vitro on Chinese hamster lung cells for 60 days. Note the presence of the surface coat (*SC*), plasma membrane (*pm*), mitochondrion (*M*), and the microtubules (*mt*). The surface coat also surrounds the flagellum (*F*) (×100,000).

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