Insulin Action on the Cultured Human Fibroblast

Glucose Uptake, Protein Synthesis, RNA Synthesis

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SUMMARY

After cultured human fibroblasts had been maintained in a serum-free medium for twenty-four hours, a biologic effect of physiologic concentrations of insulin was demonstrated. Insulin at physiologic concentrations (0.1 mU./ml.) stimulated glucose uptake and uridine incorporation into RNA by cultured human fibroblasts. Incorporation of leucine into protein was a less sensitive index of insulin action. Fetal fibroblasts were more sensitive than adult fibroblasts to the insulin effect on RNA metabolism. This may be in part the reason for the faster growth rate of fetal fibroblasts. DIABETES *23*:443-48, May, 1974.

The fibroblast may play an important pathogenic role in the development of the concomitants of diabetes, e.g., microangiopathy. Microangiopathy appears to be associated with an alteration in basement membrane glycoproteins in critical vessels.[1,2] The fibroblast is capable of glycoprotein synthesis, and therefore may be a key cell for the investigation of this aspect of diabetes. Towards this end, we have been interested in the effects of insulin on the cultured human fibroblast.

We have previously found that some diabetic fibroblasts do not show increased glucose conversion to CO_2 with insulin.[3] Some, but not others have also shown this lack of effect of insulin.[4,5] A decrease in the plating efficiency of fibroblasts from prediabetic individuals has been described,[6] as well as limitation of growth potential of diabetic human fibroblasts.[7]

We reported that insulin (1 to 10 mU./ml.) increased conversion of glucose to CO_2 by normal fetal and adult cultured human fibroblasts,[3] but we could demonstrate this action only when a serum-free medium was used. Since the amount of insulin in the culture medium (Eagle's minimal essential medium with 10 per cent fetal calf serum) was less than 10 μU./ml., as measured by radioimmunoassay, this effect of serum may be due to nonsuppressible insulin-like activity of serum.[8]

Because insulin definitely stimulated conversion of glucose to CO_2 only at relatively high insulin concentrations, we investigated its effect on other metabolic functions: glucose uptake, RNA synthesis (incorporation of 14-C-uridine) and protein synthesis (incorporation of 3-H-leucine). We found a significant effect of insulin on the first two of these parameters at an insulin concentration of 0.1 mU./ml.

MATERIALS AND METHODS

Cultures: Fibroblasts were established in tissue culture from minced skin obtained from the anterior chest wall of aborted human fetuses or at necropsy. Cultures were maintained in Eagle's minimal essential medium (MEM) with Earle's salt base at 37° C. in an atmosphere of 5 per cent CO_2 in air. MEM was always supplemented with nonessential amino acids (NEAA), 10 per cent (v/v) fetal calf serum (FCS, Industrial Biological Laboratories, Inc., or Microbiological Associates) and neomycin (50 μg./ml.) unless stated otherwise. All studies were on early subcultures, usually before the ninth passage and always before the fifteenth, following outgrowth from explants (each passage was a 1:2 split).

Experimental Cultures: For individual experiments, cells were loosened by incubation at 37° C. for a few minutes (usually three to five) with trypsin solution (0.25 per cent trypsin in isotonic saline), suspended in MEM, centrifuged, resuspended in MEM, counted in a hemocytometer and dispersed by micropipet (Eppendorf) in 100 to 200 μL. volumes into sterile glass

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disposable scintillation counting vials (Kimble) containing 2.0 ml. of MEM. Cell density was 2 to 3 x 10^5 cells per vial. Each vial was gassed with 5 per cent CO_2 in air, tightly capped and incubated at 37° C. overnight. MEM was removed, the cells washed gently with isotonic saline, and 2.0 ml. of experimental medium ⅜JEM) added. EM-1, used in studies of glucose uptake, was Eagle's MEM with glucose (40 mg./100 ml.), NEAA and neomycin (50 μg./ml.), but void of FCS. EM-2, used in studies of 3-H-leucine or 14-C-uridine incorporation, was Eagle's MEM, with NEAA and neomycin, but void of FCS. Cells were incubated in EM for twenty-four hours. Fresh EM was then added with various amounts of insulin, and incubation at 37° C. was continued for sixty minutes at which time the appropriate radiolabeled substrate was added. Thus, following the initial trypsinization, cells underwent several washes and medium changes prior to incubation with insulin and the radiolabeled substrate.

Glucose Uptake: Cells were incubated at 37° C. with 14-C-glucose for thirty minutes. Vials were then transferred to an ice-water bath, radioactive medium was quickly removed, cells were rinsed with 5 ml. isotonic saline (4° C.) three times and 0.5 ml. NCS (Amersham/Searle) or Protosol (New England Nuclear) was added to digest the cells. Liquifluor (10 ml., New England Nuclear) was added and radioactivity was measured. Thus uptake measured not only transport of glucose into the cells but also subsequent metabolic conversion and accumulation of metabolites.

3-H-Leucine Incorporation into Protein: Cells were incubated at 37° C. with 3-H-leucine for sixty minutes. Vials were then transferred to an ice-water bath, radioactive medium was removed, and the cells were rinsed twice with isotonic saline (4° C.) and three times with 5 per cent trichloroacetic acid (TCA, 4° C.), the last for ten minutes. Cells were heated with 5 per cent TCA at 90° C. for fifteen minutes, rinsed twice with 80 per cent ethanol, dried under an air stream, and digested with 0.5 ml. NCS or Protosol. Liquifluor (10 ml.) was added and radioactivity measured.

14-C-Uridine Incorporation into RNA: Cells were incubated at 37° C. with 14-C-uridine for sixty minutes. Vials were then transferred to an ice-water bath and radioactive medium was removed. Cells were rinsed twice with isotonic saline (4° C.), followed by three washes, each for thirty minutes, with 2 per cent perchloric acid (PCA, 4° C.), a ten-minute wash with 80 per cent ethanol, and two rinses with 80 per cent ethanol. Cells were dried and digested in 0.5 ml. NCS or Protosol. Liquifluor (10 ml.) was added and radioactivity determined.

In some experiments with 14-C-uridine, the 2 per cent PCA washes were pooled, neutralized with 1.0 N KOH, dried, and redissolved in 250 μL. distilled water. Aliquots (10 μL.) were spotted on thin layer chromatography sheets (Polygram Cel 300 PEI, Brinkman Instruments, Inc.), along with carrier uridine-5′-monophosphate (UMP), uridine-5′-diphosphate (UDP), and uridine-5′-triphosphate (UTP) and developed with 1.0 M LiCl. The nucleotides were identified by ultraviolet lamp scanning. They were cut out of the chromatogram, extracted with 0.7 M $MgCl_2$-2.0 M pH 7.4 Tris HCl (100:1 v/v) for thirty minutes at 22° C., and an aliquot of the extract was assayed for radioactivity in Aquasol (10 ml., New England Nuclear).

Other: Protein content was determined on 0.1 N NaOH digests of cultures run in parallel with the radiolabeled cultures.[9]

Isotopes and Chemicals: Crystalline porcine insulin (single peak, glucagon-free) was from Eli Lilly. U-14-C-D-glucose, 4,5-3-H-L-leucine and 2-14-C-uridine were from New England Nuclear.

RESULTS

The time course of uptake of D-glucose is shown in figure 1. A clear increase in glucose uptake was seen with insulin at twenty, forty and sixty minutes incubation with 14-C-glucose. In all subsequent studies of glucose uptake, cells were incubated with 14-C-glucose for thirty minutes.

The uptake of glucose was also increased by high glucose concentrations (table 1). At each glucose level, insulin (100 mU./ml.) stimulated glucose uptake. The basal uptake of glucose was slightly, but significantly, reduced by 0.1 mM phlorizin and substantially decreased by 1.0 mM phlorizin. Insulin-stimulated glucose uptake was not affected by 0.1 mM phlorizin, but was reduced by 1.0 mM phlorizin (table 2). As little insulin as 0.1 mU./ml. stimulated glucose uptake (figure 2).

A significant difference in response to insulin was seen in 14-C-uridine incorporation between fetal vs. adult fibroblasts (figure 3). In another study, cells were incubated with or without insulin (10 mU./ml.) for 120 minutes; 14-C-uridine was added at ninety minutes. On assaying the 2 per cent PCA wash for incorporation of radioactivity into UMP, UDP and

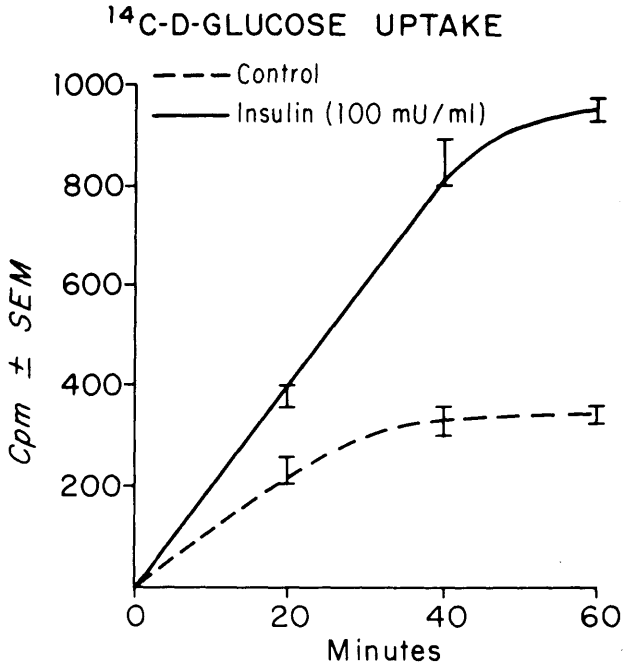

FIG. 1. Cells were preincubated with or without insulin (100 mU./ml.) for sixty minutes, then U-14-C-D-glucose was added (specific activity 56.3 μCi/mMole) for a second incubation of twenty, forty or sixty minutes. Each point is the mean ± SEM from quadruplicate culture vessels.

UTP (table 3), insulin was found to increase incorporation of 14-C-uridine into UDP and UTP the most (2- and 2.7-fold increase), into RNA next (1.7-fold), and into UMP least (1.5-fold).

When cells were first incubated with 14-C-uridine for sixty minutes, then washed free of radioactive medium and incubated with various insulin concentrations for ninety minutes, incorporation of 14-C-uridine into RNA was still enhanced by insulin (table 4).

Insulin (100 mU./ml.) increased incorporation of 3-H-leucine into protein, causing a linear response for

TABLE 1

14-C-D-Glucose Uptake
(picomoles/culture ± SEM)

	No Insulin	Insulin (100 mU./ml.)
Glucose (40 mg./100 ml.)	1.31 ± 0.05	2.06 ± 0.14
Glucose (100 mg./100 ml.)	1.50 ± 0.05	3.06 ± 0.18
Glucose (300 mg./100 ml.)	3.84 ± 0.05	5.95 ± 0.48

Each value is the mean ± SEM of triplicate culture vessels.

TABLE 2

14-C-D-Glucose Uptake
(cpm/culture ± SEM)

	No Insulin	Insulin (100 mU./ml.)
Control	131 ± 5	206 ± 14
Phlorizin (0.1 mM.)	99 ± 8	203 ± 21
Phlorizin (1.0 mM.)	72 ± 5	105 ± 10

Each value is the mean ± SEM of triplicate culture vessels. Specific activity of U-14-C-D-glucose was 56.3 μCi/mMole.

six hours (figure 4). An effect of insulin was not seen below concentrations of 1.0 to 10 mU./ml. (figure 5). When insulin (100 mU./ml.) was included in the culture medium for twenty-four hours, there was a 25 per cent increase in total protein synthesis (table 5).

DISCUSSION

Human fibroblast cultures are easily established and have a relatively long life span (about fifty generations).[10] Therefore, these cultures are useful for many investigations, including studies of inherited metabolic disorders, even though the fibroblast is a relatively undifferentiated cell in comparison to many

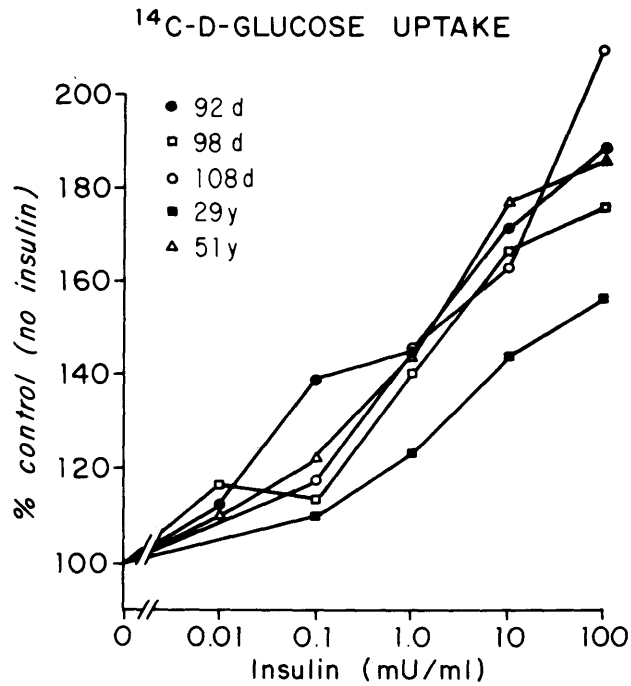

FIG. 2. U-14-C-D-glucose was added to a specific activity of 56.3 μCi/mMole. Each point is the mean from quadruplicate culture vessels.

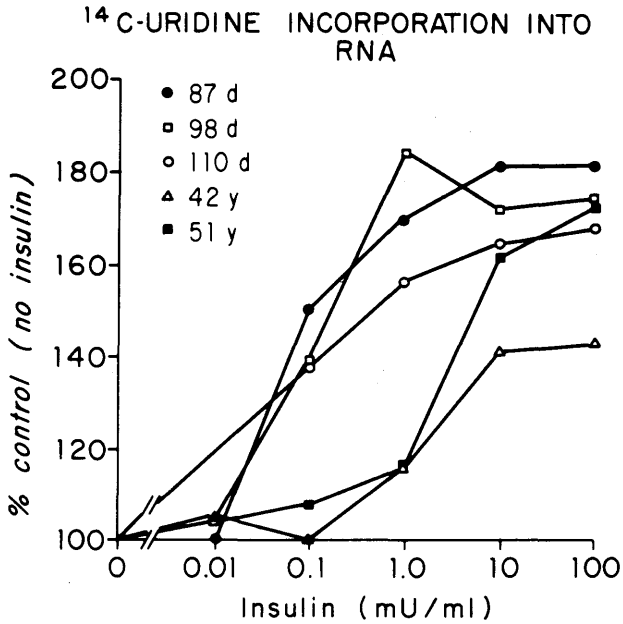

FIG. 3. Specific activity of 2-14-C-uridine added in tracer amounts was 52.7 μCi/mMole. Each point is the mean from quadruplicate culture vessels.

others, e.g., the isolated adipocyte. Specifically, in the case of insulin, a maximal effect on glucose conversion to CO_2 by the isolated adipocyte is seen at 1.0 mU./ml., with a 50 per cent maximal response seen at 9 μU./ml.[11] On the other hand, the cultured human fibroblast is relatively insensitive to insulin. We found that 1 to 10 mU./ml. insulin affected conversion of glucose to CO_2, but a maximal effect was not attained even with 100 mU./ml.[3] Others have had similar results.[4,5] Insulin (10 mU./ml.) accelerated protein synthesis in primary cultures of chick embryo fibroblasts.[12] Insulin affected several growth and

TABLE 3

14-C-Uridine Incorporation in RNA (cpm ± SEM)

	No Insulin	Insulin (10 mU./ml.)
UMP[a]	48 ± 3	72 ± 3
UDP[a]	72 ± 3	146 ± 4
UTP[a]	26 ± 2	69 ± 10
RNA[b]	458 ± 18	778 ± 32

Specific activity of 2-14-C-uridine added in tracer amounts was 52.7 μCi/mMole. Each value is the mean ± SEM from quadruplicate culture vessels.

a—Measured on aliquot of PCA wash.

b—Measured on cell pellet.

TABLE 4

14-C-Uridine Incorporation in RNA in Fetal Fibroblasts (108 d.)*

Insulin	cpm ± SEM	% Control
0	1,917 ± 71	100
0.1	2,039 ± 40	104
1.0	2,349 ± 45	123
10	2,218 ± 29	116
100	2,429 ± 67	127

*Cells were prelabeled with 14-C-uridine for sixty minutes. Radioactive medium was washed off, and cells were incubated with various insulin concentrations for ninety minutes. Specific activity of 2-14-C-uridine added in tracer amounts was 52.7 μCi/mMole. Each value is the mean ± SEM from quadruplicate culture vessels.

metabolic parameters of a human fibroblast strain (WI-38), but 1 U./ml. was required.[13] The effects of insulin (1 to 100 mU./ml.) and of nonsuppressible insulin-like activity (NSILA) of serum on growth and on glucose and DNA metabolism of chick embryo fibroblasts have been recently described.[8] The 3T6 mouse fibroblast line has been used as a target cell to show a possible "abnormal" insulin from diabetic pancreas.[14]

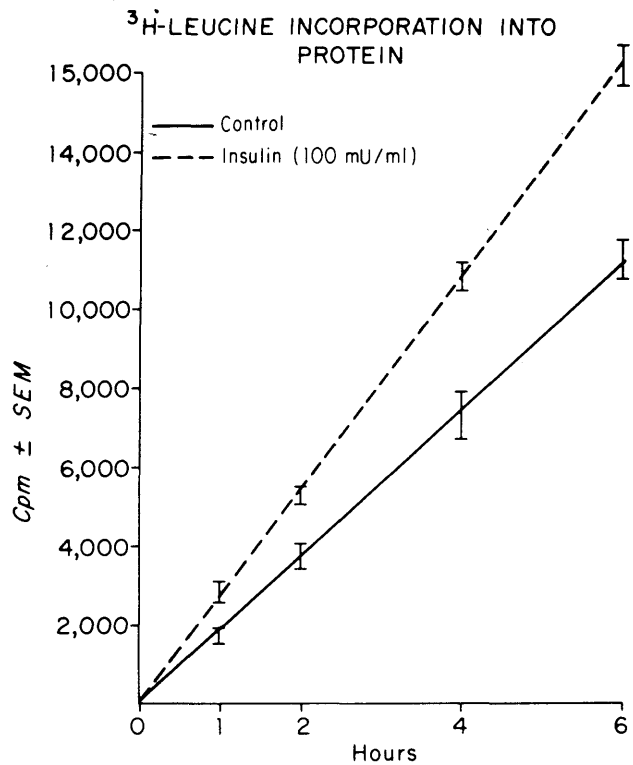

FIG. 4. 4,5-3-H-L-leucine was added to a specific activity of 3.13 mCi/mMole. Each point is the mean ± SEM from quadruplicate culture vessels.

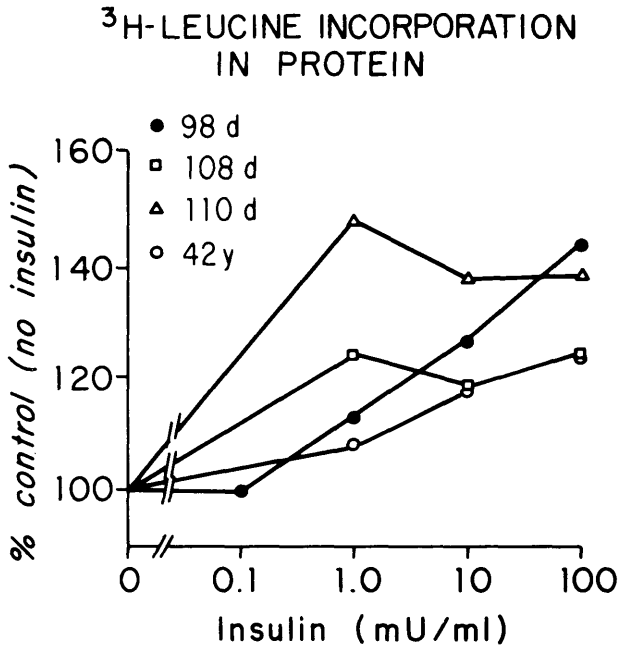

FIG. 5. 4,5-3-H-L-leucine was added to a specific activity of 3.13 mCi/mMole. Each point is the mean from quadruplicate culture vessels.

We had previously found that the presence of serum obscured any protential effect of insulin on glucose conversion to CO_2.[3] Thus in our present studies, we deleted serum from the culture medium for twenty-four hours prior to testing insulin effect. By so doing, we were able to demonstrate a clear effect at an insulin concentration of 0.1 mU./ml. on glucose uptake and on uridine incorporation into RNA.

The time course of uptake of glucose exhibits a plateau. In part this may be explained by metabolism of the glucose; a small portion is converted to CO_2 but a major part is metabolized to lactate.[15] Another explanation is that transport is bi-directional: Glucose is transported both into and out of the cell. The height of this plateau reflects the steady state accumulation of glucose and glucose metabolites within the cell. Thus, insulin clearly increases the net rate of uptake of glucose and the height of the plateau reached (figure 1). We have not determined the fate of this glucose.

TABLE 5

Total protein synthesis per culture vessel
(μg. ± SEM)

Age	Control	Insulin (100 mU./ml.)	% Control
62 d.	137 ± 2	165 ± 6	120
62 d.	145 ± 2	172 ± 4	119
97 d.	93 ± 2	125 ± 6	134
98 d.	129 ± 4	148 ± 6	115
100 d.	93 ± 5	122 ± 4	131
115 d.	170 ± 10	231 ± 1	131
140 d.	143 ± 10	183 ± 3	128
30 y.	141 ± 2	176 ± 4	125
36 y.	198 ± 4	265 ± 2	134

Cells were incubated in EM-2 with or without insulin (100 mU./ml.) for twenty-four hours.

An effect on glucose uptake is apparent with 10 per cent of the insulin needed to detect increased glucose oxidation. The latter is often employed as an index of glucose uptake; however, in the cultured fibroblast, since only a very small proportion of the glucose is converted to CO_2, this is not a good measure of glucose uptake. This explains why the actual measure of glucose uptake is a more sensitive index of insulin action.

Phlorizin inhibits glucose transport. At a low concentration of phlorizin (0.1 mM.), basal glucose uptake is inhibited in part, but insulin-stimulated uptake is not. With 1.0 mM. phlorizin, both basal and insulin-stimulated glucose uptake are inhibited to a similar extent; insulin-stimulated uptake is still 1.5-fold increased over basal uptake, which is quite similar to the increase seen in the absence of phlorizin. Thus at the lower concentration, phlorizin appears to no longer inhibit glucose transport if insulin is present. However, since uptake as measured in these experiments reflected not only transport of glucose into the cell but also accumulation of glucose metabolites within the cell, we cannot exclude a possible site of action of phlorizin or insulin at some point other than transport of glucose into the cell.

We did not observe that insulin produced a maximal dose response in glucose uptake, but on using 10 mU./ml. it caused a maximal response in uridine incorporation into RNA. This is still tenfold higher than the maximal effect by insulin (1.0 mU./ml.) on glucose conversion to CO_2, by isolated fat cells.[6] However, in fetal fibroblasts a near maximal effect on uridine uptake into RNA is seen at 1.0 mU./ml. Furthermore, with fetal fibroblasts this parameter is clearly more responsive to insulin than adult fibroblasts; such an age difference was not seen in glucose uptake or in conversion of glucose to CO_2. These results suggest why fibroblasts from younger donors grow faster than those from older donors;[16] fibroblasts from younger subjects are more responsive to growth factors such as insulin. Increased uridine incorporation into RNA is therefore an index of this faster growth rate.

Our findings that insulin increased incorporation of 14-C-uridine proportionately more into UDP and UTP than into RNA are consistent with previous reports that insulin enhanced uridine incorporation into RNA of isolated bone cells, primarily by enhancing its uptake and/or phosphorylation.[17] However, we did not determine the relative pool sizes of the uridine nucleotide before and after insulin. Thus these studies in themselves can only suggest an effect of insulin on uridine uptake and/or phosphorylation rather than on RNA synthesis. However, the pulse-chase experiment (table 4) showed a significantly smaller effect of insulin on incorporation of uridine into RNA despite a longer period of incubation with insulin, as compared to results depicted in figure 3. These results also suggest that the stimulatory effect of insulin on uridine incorporation into RNA was secondary to an insulin effect in a step or steps prior to RNA synthesis.

Although an effect of insulin on incorporation of 3-H-leucine was demonstrated, this was not as sensitive an index of insulin action as were glucose uptake or uridine incorporation. An effect was seen at 1.0 to 10 μM./ml. insulin; this finding is similar to that described for the effect of insulin on chick embryo fibroblast primary cultures in serum-free medium.[9] That total protein content of fibroblast cultures is increased by insulin in the absence of serum is also confirmed (table 5).

Thus we have been able to demonstrate that insulin stimulates glucose uptake, incorporation of uridine into RNA, and protein synthesis in cultured human fibroblasts, maintained in a serum-free medium. Insulin (0.1 mU./ml.) is effective on both glucose uptake and RNA metabolism. Protein synthesis is a less sensitive index of insulin action. Contrary to other reports, the cultured human fibroblast is responsive to physiologic concentrations of insulin.

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