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# Rapid Stimulation by Insulin of Ribosome Synthesis in Cultured Chick Embryo Fibroblasts†

Robert M. DePhilip, David E. Chadwick, Ronald A. Ignotz, William E. Lynch, and Irving Lieberman*

ABSTRACT: The effect of insulin on the synthesis of cytoplasmic ribosomes was studied in primary cultures of resting chick embryo fibroblasts. For obviation of problems with precursor nucleotide pools, ribosome formation was measured by the incorporation of [$^3$H]leucine into ribosomal core proteins and the results have been expressed as the quotients of the specific activities of the core and total cell proteins. The following observations were made. (1) Insulin (5 $\mu$g/mL) raised the incorporation of [$^3$H]leucine into total chick cell protein by 1.3–1.5 times whereas ribosome production was stimulated by almost fourfold. (2) The ratios of the specific activities of the core and total cell proteins rose gradually as the concentration of insulin was raised from 0 to 1 $\mu$g/mL. (3) Insulin also elevated the incorporation of [$^3$H]adenine into the 28S ribonucleic acid (RNA) of cytoplasmic ribosomes. A constant relationship was maintained between the increased labeling of the 28S RNA and the proteins of the 60S-derived cores at all the levels of the hormone tested, suggesting that insulin did not influence the specific activity of the adenosine 5′-triphosphate that served as a precursor for preribosomal RNA synthesis. (4) Insulin increased the uptake of [$^3$H]adenine into 41S preribosomal RNA by two- to threefold. (5) The hormone did not, however, affect either the speed with which the 41S rRNA was processed or the time that labeled 60S subribosomes first appeared in the cytoplasm. (6) The stimulation by insulin of the labeling of 41S preribosomal RNA and of 60S subribosomes was rapid and appeared to begin within 10 min after the resting cells were treated with the hormone. (7) Insulin increased the labeling of 41S preribosomal RNA in cells that had no exogenous source of glucose.

Most investigations on the actions of insulin have been concerned with carbohydrate, fat, and protein metabolism. The hormone is known, however, to enhance the incorporation of labeled precursors into the rRNAs of muscle (Wool, 1963), liver (Steiner & King, 1966), and cultured chick embryo fibroblasts (Baseman et al., 1974; Baseman & Hayes, 1975).

Some of these studies have taken into account possible effects of insulin on the labeling of the precursor nucleotide in the cell (Wool, 1963; Baseman et al., 1974). In these cases, however, it has been assumed that the specific activity of the appropriate nucleoside triphosphate in whole-cell extracts gives a valid picture of the status of the immediate RNA precursor. This assumption is open to question. The nucleus and the cytoplasm may have different nucleotide pools (Plagemann, 1971, 1972; Goody & Ellem, 1975; Khym et al., 1978), and there is even evidence for compartmentalization within the nucleus itself (Dämmgen & Scholtissek, 1975).

To avoid problems with precursor pools, we have measured the effect of insulin on ribosome production in resting cultures of chick embryo fibroblasts by the incorporation of [$^3$H]leucine into ribosomal proteins. The data are expressed as the quotients of the specific activities of ribosomal core and total cell proteins. The validity of the results is not influenced by the compartmentalization of free amino acids in the cell (Ward & Mortimore, 1978) but depends only on the reasonable assumption that identical pools of leucyl-tRNAs are used to make ribosomal and nonribosomal proteins.

## Materials and Methods

*Materials.* Crystalline bovine insulin (~25 IU/mg) was from Sigma. Basal medium that lacked glucose was prepared from an MEM Select-amine Kit (Grand Island Biological Co).

*Cultures.* Primary cultures of chick embryo fibroblasts were made from trypsinized minces of 11- or 12-day-old embryos. The cells were grown at 38 °C in a $CO_2$–air atmosphere in 50-mm glass dishes in 4 mL of basal medium (Minimal Essential, Eagle) supplemented with 4% calf serum. Confluency was generally reached by the third day, and the cultures were used 2 or 3 days later. At this time, each culture

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contained ~1 mg of protein, 70 μg of RNA, and 20 μg of DNA.

*[$^3H$]Leucine Incorporation into Total Cell Protein.* The medium on the primary cultures was removed, and 2 mL of basal medium was added. After 30 min, the preincubation medium was replaced with 2 mL of fresh basal medium containing 0.2 μCi of [$^3$H]leucine (40–60 Ci/mmol). After incubation, the labeled medium was removed and protein synthesis was stopped with 1 mL of 1% trichloroacetic acid. The cells were then dislodged with a rubber spatula, washed with trichloroacetic acid, ethanol, and ether, and dissolved by boiling for 60 min in 2 mL of 0.05 M NaOH. A portion of the NaOH solution was counted and another was used for the colorimetric estimation of protein (Lowry et al., 1951). Specific activities were the averages of the results with three or four cultures.

*[$^3H$]Leucine Incorporation into Cytoplasmic Ribosomal Core Proteins.* Twenty-four resting cultures were preincubated and labeled as for the estimation of the specific activity of total cell protein except that each culture received 10 μCi of [$^3$H]leucine (sp act. 40–60 Ci/mmol). After incubation, the radioactive medium was replaced with 2 mL of ice-cold, unlabeled basal medium and the cells were immediately scraped and collected by centrifugation (900*g*, 1 min, 3 °C). The pooled cells, suspended in 7 mL (0 °C) of a solution of 0.01 M Tris[1]-HCl (pH 7.8), 1 mM $MgCl_2$, 0.3 M sucrose, and 0.05% Triton X-100 (Perry & Kelley, 1968), were broken with 10 strokes of a tight Dounce pestle. To the homogenate was added 1 mL of 0.2 M Mops (pH 7.8), and particulates were discarded by centrifugation at 12000*g* for 10 min (3 °C). After the addition of 0.9 mL of 10% sodium deoxycholate to the supernatant fraction, it was layered over 1 mL of 14% sucrose and ribosomes were sedimented at 105000*g* for 4 h at 3 °C. Under these conditions of centrifugation, ~90% of free 40S subribosomes and all free 60S subribosomes were also collected in the pellet.

The procedures used to dissociate the ribosomes and to separate the subunits were based on those of Martin et al. (1969). The ribosomes were suspended in 0.5 mL of a solution of 0.5 M KCl, 3 mM $MgCl_2$, and 10 mM Mops (pH 7.8), and 10 μL of 10 mM puromycin was added. The suspension was incubated at 37 °C for 20 min and then layered on 12 mL of a 5–20% linear sucrose gradient in the KCl, $MgCl_2$, and Mops solution. 40S- and 60S-derived cores were separated by centrifugation for 2 h at 273000*g* at 25 °C. Fractions (0.45 mL) were collected by upward displacement through a UV flow monitor, and the subunits were located by their absorbance at 254 nm. Absorbing fractions were pooled, and the subunits were precipitated overnight at −20 °C with 3 volumes of ethanol.

Finally, the 40S- and 60S-derived subribosomes were each suspended in 0.5 mL of a solution of 20 mM KCl, 20 mM EDTA, and 1 mM potassium phosphate (pH 7.0). The final purification of the cores was achieved by sedimentation in 12 mL of a 5–30% linear sucrose gradient in the KCl and potassium phosphate buffer at 273000*g* (3 h, 20 °C).

Specific activities of the proteins of the subribosomal cores that had been treated with KCl and puromycin and then with EDTA were the averages of the results with the three richest gradient fractions (identified by absorption at 254 nm). Protein was measured by the procedure of Lowry et al. (1951) for dilute solutions.

[1] Abbreviations used: EDTA, ethylenediaminetetraacetate; Mops, 3-(*N*-morpholino)propanesulfonic acid; $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

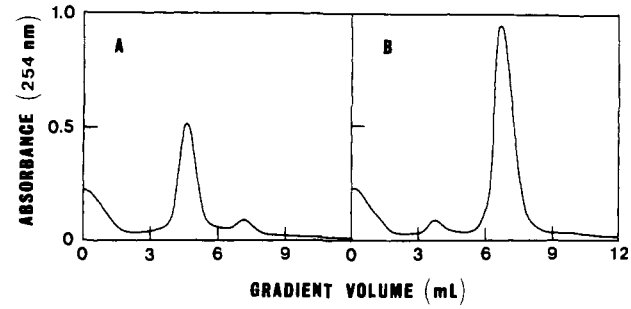


FIGURE 1: Sedimentation patterns of subribosomal cores in sucrose gradients. Cores were prepared by treating ribosomes from 24 resting chick fibroblast cultures with puromycin and 0.5 M KCl and then with EDTA, as described under Materials and Methods. Sedimentation was from left to right. Panel A: 40S-derived cores. Panel B: 60S-derived cores.

*Specific Activity of Cytoplasmic 28S rRNA.* Twenty-four resting cultures were labeled with [$^3$H]adenine, the labeled cells were harvested, and 60S-derived subribosomal cores were prepared with 0.5 M KCl and puromycin in the usual manner. Sucrose gradient fractions (0.45 mL) of the EDTA-treated cores were each collected in 0.1 mL of 10% $NaDodSO_4$, and the peak fractions were pooled and diluted to 10 mL with a solution of 0.025 M Mops, pH 7.6, 0.025 M KCl, and 0.01 M $MgCl_2$. RNA was extracted twice with water-saturated phenol (55 °C). The RNA was then precipitated with ethanol, dissolved in 0.5 mL of NETS buffer (0.1 M NaCl, 0.01 M EDTA, 0.01 M Tris-HCl, pH 7.6, and 0.2% $NaDodSO_4$), and sedimented in 12 mL of a 5–20% linear sucrose gradient in NETS buffer for 5 h at 246000*g* (20 °C). RNA was estimated by absorbance at 260 nm.

*41S Preribosomal RNA.*[2] RNA was extracted from whole cells (5 cultures/group) essentially as described by Wolf & Schlessinger (1977). The labeled medium was removed, and the attached cells were immediately lysed by the addition to each dish of 1 mL of 1% $NaDodSO_4$ (55 °C). The lysates were poured into 10 mL of water-saturated phenol (55 °C), and 5 mL of a solution (pH 5.4) of 0.2 M Tris-HCl and 0.02 M EDTA was added. The mixture was homogenized, and the extracted RNA was precipitated with ethanol and air-dried. The dry RNA was dissolved in 0.12 mL of NETS buffer from which the NaCl had been omitted, and 0.28 mL of a mixture of 86% dimethyl sulfoxide (v/v) and 14% dimethylformamide (v/v) (Edmonds et al., 1976) was added. After being heated at 70 °C for 3 min, the sample was cooled and diluted with 0.4 mL of NETS buffer and 0.25 mg of RNA was layered on 12 mL of a 15–30% linear sucrose gradient containing 100 mM NaCl, 10 mM Tris-HCl, and 0.2% $NaDodSO_4$, pH 7.4. Centrifugation was for 4 h at 273000*g* (20 °C). Fractions (0.45 mL) were collected by upward displacement.

## Results

*Sedimentation Patterns of Subribosomal Core Particles.* The sedimentation patterns of the 40S- and 60S-derived core particles that had been treated with puromycin and 0.5 M KCl and then with EDTA are shown in Figure 1. Incubation of the chick cells with insulin had no effect either on the recoveries or the sedimentation properties of the core particles.

*Kinetics of Labeling of Total Cell Protein.* Confluent cultures of the chick fibroblasts in fresh basal medium in-

[2] In unpublished work by L. Villareal that was quoted by Weiss et al. (1977), the preribosomal RNA of chicken was assigned a sedimentation coefficient of 41S on the basis of comparison with the 45S preribosomal RNA of HeLa cells.

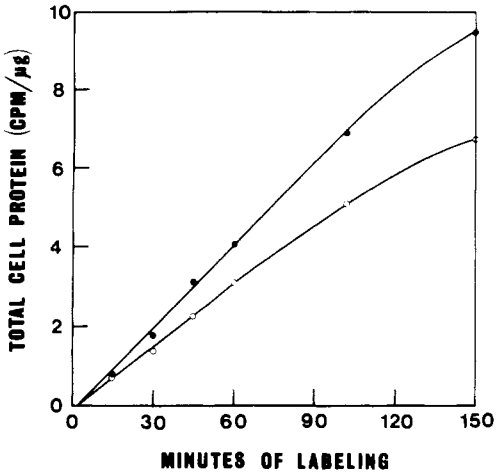


FIGURE 2: Kinetics of labeling of total cell protein. After preincubation in basal medium for 30 min, confluent cultures of chick fibroblasts were labeled with [$^3$H]leucine (0.2 μCi/culture) in 2 mL of fresh basal medium for the times shown. Specific activities of total cell protein were estimated as described under Materials and Methods. Each value represents the average of the results with three cultures. (○) No insulin; (●) 5 μg/mL insulin.

Table I: Effects of Insulin on the Incorporation of [$^3$H]Leucine into Proteins of Various Cell Fractions[a]

| fraction | cpm/μg of protein | | x-fold increase |
|---|---|---|---|
| | control | insulin | |
| homogenate | 291 | 396 | 1.36 |
| 12000*g* pellet | 296 | 389 | 1.31 |
| soluble | 258 | 374 | 1.45 |
| 40S-derived cores | 48 | 200 | 4.17 |
| 60S-derived cores | 33.5 | 123 | 3.67 |

[a] Resting cultures of primary chick cells (28 dishes/group) were preincubated in 2 mL of basal medium for 30 min. At zero time, the medium was replaced with 2 mL of fresh basal medium containing 5 μg/mL insulin, as shown, and [$^3$H]leucine (5 μCi/mL). Labeling was for 2.5 h. Twenty-four of the cultures were used for the isolation of subribosomal cores with KCl–puromycin and EDTA, and the remainder was used for the preparation of the other cell fractions. The soluble fraction was obtained by sedimenting the 12000*g* supernatant fraction at 105000*g* for 2 h.

corporated [$^3$H]leucine into total cell protein at a linear rate for ~90 min, whereupon the rate began to decline (Figure 2). Supplementation of the basal medium with insulin stimulated the initial rate of incorporation by about 1.3-fold and delayed the onset of the decline. The stimulation of [$^3$H]leucine incorporation by insulin was due to an elevated rate of protein synthesis. Thus, the specific activities of [$^3$H]leucyl-tRNA in control and insulin-treated cells, as measured with [$^{14}$C]-5-dimethylaminonaphthalene-1-sulfonyl chloride (dansyl chloride) (McKee et al., 1978), were indistinguishable both at 15 and 45 min of labeling (T. Gansler and I. Lieberman, unpublished experiments).

*Effects of Insulin on [$^3$H]Leucine Incorporation into Proteins of Various Cell Fractions.* Insulin enhanced the incorporation of [$^3$H]leucine into the proteins of all the fractions of the chick cells that were examined (Table I). As the table shows, the predominant effect, by far, was on the labeling of the ribosomal core proteins.

*Purity of Subribosomal Core Particles.* The possibility was examined that some of the radioactivity of the purified ribosomal cores was in adsorbed, nonribosomal protein. To this end, 60S-derived cores were prepared from cells that had been labeled with [$^3$H]leucine in the presence of actinomycin D (Table II). When rRNA synthesis was completely blocked, the radioactivity associated with the 60S-derived cytoplasmic

Table II: Comparison of Effects of Actinomycin D on the Labeling of the Protein and 28S RNA of 60S-Derived Subribosomal Cores[a]

| insulin | actinomycin (μg/mL) | core protein | | 28S RNA | |
|---|---|---|---|---|---|
| | | cpm/μg of protein | % of control | cpm/μg of RNA | % of control |
| – | 0 | 41 | 100 | 54 | 100 |
| | 1.0[b] | 2.5 | 6.1 | 0 | 0 |
| + | 0 | 135 | 100 | 165 | 100 |
| | 0.005 | 60 | 44 | 71 | 43 |
| | 0.01 | 39 | 29 | 30 | 18 |
| | 1.0 | 7.5 | 5.6 | 0 | 0 |

[a] Resting cultures of primary chick cells (24 dishes/group) were preincubated for 30 min in 2 mL of basal medium. At zero time, the medium was replaced with 2 mL of fresh basal medium containing 5 μg/mL insulin and actinomycin D, as shown, and [$^3$H]-leucine (5 μCi/mL) or [$^3$H]adenine (1 μCi/mL; sp act. 27.5 Ci/μmol). Labeling was for 2.5 h. 60S-derived cores were purified with puromycin–KCl and EDTA, and the specific activities of the protein and 28S RNA of the cores were estimated as described under Materials and Methods. [b] Even at a concentration of 1 μg/mL, the antibiotic reduced the incorporation of [$^3$H]leucine into the total cell protein of the control and insulin-treated cells by only 25%.

particles was reduced by almost 95%. Newly made ribosomal proteins that can exchange with the proteins of preformed ribosomes (Warner & Udem, 1972; Lastick & McConkey, 1976) may have accounted for a portion of the counts in the cores from the actinomycin D treated cells.

The same measurements were made with three different preparations of 40S-derived cores. The radioactivity of the [$^3$H]leucine-labeled cores was reduced by only 60–70% even when rRNA synthesis was completely blocked with actinomycin D, indicating contamination with nonribosomal proteins. It was assumed that the contaminants were responsible for the greater specific activity of the protein of the small subunit than the large subunit (Table I) that was invariably found.

*Titration of Insulin.* The labeling of 60S-derived subribosomal cores with [$^3$H]leucine was examined as a function of insulin concentration (Figure 3). The quotient specific activity of core protein/specific activity of total cell protein was increased by as little as 0.1 μg/mL insulin and was almost maximal with 1 μg/mL hormone.

*The Stimulatory Activity Resided in Insulin.* The possibility was ruled out that the action of insulin on ribosome synthesis was due to a contaminant in the hormone preparation (Table III). Thus, the properties of the stimulatory agent were the same as those of insulin. Treatment of the hormone with acid had no effect on its ability to increase ribosome formation whereas exposure to alkaline pH or to a reducing agent, procedures that are known to inactivate insulin (Haugaard & Marsh, 1953; Bewley & Li, 1969), destroyed the activity.

*Comparison of Effects of Insulin on Labeling of the 28S rRNA and Protein of 60S-Derived Subribosomal Core Particles.* The relative increases in the incorporation of [$^3$H]adenine into the RNA and of [$^3$H]leucine into the protein of 60S-derived cores were indistinguishable at all of the levels of insulin tested (Figure 4). These results were taken to mean that the hormone did not affect the specific activity of the nuclear [$^3$H]ATP pool that served as a substrate for the formation of 41S preribosomal RNA.

*Rapid Stimulation of Labeling of 41S Preribosomal RNA by Insulin.* The incorporation of [$^3$H]adenine into 41S preribosomal RNA was measured during three consecutive 10-min intervals after the resting chick cultures were treated with insulin (Figure 5). As the figure shows, the hormone

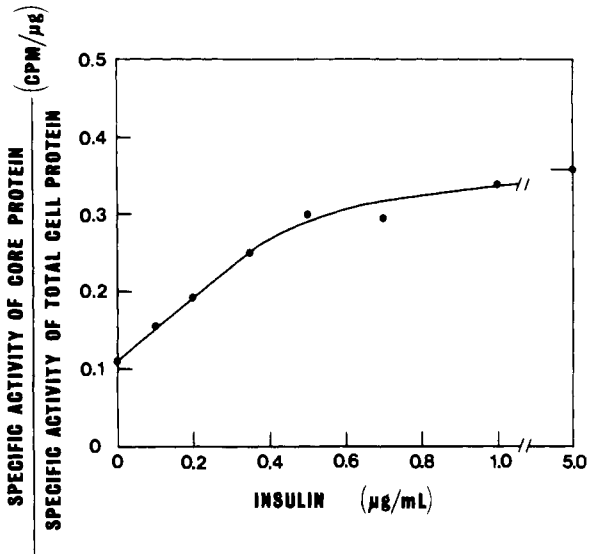


FIGURE 3: Specific activity of 60S-derived ribosomal core protein relative to total cell protein as a function of concentration of insulin. After preincubation for 30 min, confluent cultures of primary chick cells were labeled for 2.5 h with [$^3$H]leucine (10 $\mu$Ci/culture) in 2 mL of fresh basal medium supplemented with insulin, as shown. The preparation of the cores and the estimation of the specific activities of the core and total cell proteins were as detailed under Materials and Methods. The specific activities of the total protein of cells exposed to 0, 0.1, 0.2, 0.35, 0.5, 0.7, 1.0, and 5.0 $\mu$g/mL insulin were 222, 243, 249, 257, 279, 279, 287, and 304 cpm/$\mu$g, respectively.

Table III: Effect of Insulin That Had Been Treated with Acid, Base, or Dithiothreitol on Incorporation of [$^3$H]Leucine into Proteins of 60S-Derived Ribosomal Cores[a]

| addn to culture medium | ribosome synthesis, (cpm per $\mu$g of core protein)/(cpm per $\mu$g of total cell protein) |
|---|---|
| none | 0.117 |
| insulin | 0.274 |
| acid-treated insulin[b] | 0.280 |
| NaOH-treated insulin | 0.118 |
| dithiothreitol-treated insulin | 0.109 |

[a] Resting cultures of primary chick cells that had been preincubated in 2 mL of basal medium for 30 min were given 2 mL of fresh basal medium containing 5 $\mu$g/mL untreated or treated insulin and [$^3$H]leucine (5 $\mu$Ci/mL). Labeling was for 2.5 h. 60S-derived subribosomal cores were isolated with puromycin-KCl and EDTA, and the specific activities of the core and total cell proteins were measured as described under Materials and Methods. [b] Acid treatment of insulin was with 1 M $H_2SO_4$ for 3 h at 0 °C, and alkaline treatment was with 0.03 M NaOH for 3 h at 34 °C (Haugaard & Marsh, 1953). Reduction was with 0.6 mM DL-dithiothreitol for 1 h at 23 °C (Bewley & Li, 1969). Residual dithiothreitol was removed by dialysis with an Amicon membrane whose nominal exclusion weight limit was 1000 daltons.

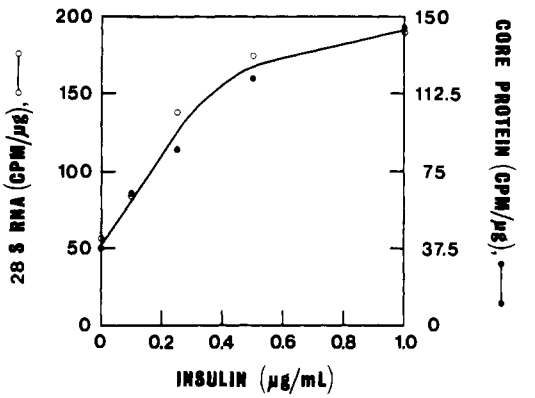


FIGURE 4: Comparison of effects of insulin on specific activities of the 28S RNA and protein of 60S-derived cores. After preincubation for 30 min, confluent cultures of primary chick cells in 2 mL of fresh basal medium supplemented with insulin, as shown, were labeled for 2.5 h with [$^3$H]adenine (1 $\mu$Ci/mL; sp act. 27.5 $\mu$Ci/$\mu$mol) or [$^3$H]leucine (5 $\mu$Ci/mL). The isolation of 60S-derived ribosomal cores and the estimation of the specific activities of the [$^3$H]adenine-labeled 28S RNA (○) and the [$^3$H]leucine-labeled protein (●) were as described under Materials and Methods.

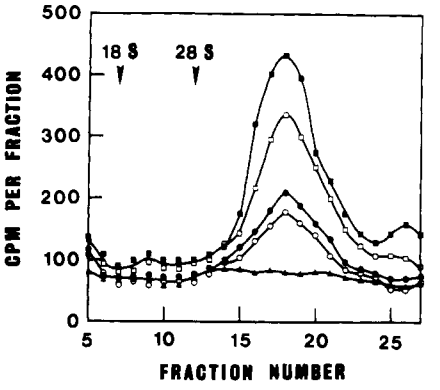


FIGURE 5: Incorporation of [$^3$H]adenine into 41S preribosomal RNA as a function of length of exposure of cells to insulin. Resting cultures of primary chick cells were preincubated in 2 mL of basal medium for 30 min. The medium was then replaced with 2 mL of fresh basal medium containing 5 $\mu$g/mL insulin, as shown. Labeling with [$^3$H]adenine (0.4 $\mu$Ci/culture, 19 Ci/mmol) was for 10 min beginning at 0, 10, or 20 min after replacement of the preincubation medium. The cultures with actinomycin D (0.04 $\mu$g/mL) were exposed to the antibiotic both during the preincubation period and afterwards. Purification and sedimentation of total cell RNA were as detailed under Materials and Methods. Sedimentation was from left to right. (○) No insulin, labeled at 0–10 min after the preincubation period (the same results were obtained at the later times); (●, □, ■) with insulin, labeled at 0–10, 10–20, or 20–30 min after the preincubation period, respectively; (▲) with insulin and actinomycin D, labeled between 10 and 20 min after the preincubation period. The results with actinomycin D were the same with the control and insulin-treated cultures.

caused a small increase in the labeling of 41S RNA during the first 10-min period and, between 10 and 20 min, the stimulation was more than half of the maximum. The figure also shows that actinomycin D (0.04 $\mu$g/mL) blocked completely the appearance of the radioactive 41S RNA.

*Lack of Effect of Insulin on Rate of Processing of Preribosomal RNA.* [$^3$H]Methylmethionine was used to determine whether insulin speeded up the rate of processing of 41S RNA (Figure 6). With both the control and insulin-treated cells, the radioactivity in 32S and 18S RNA was at best negligible after 15 min of labeling and appeared during the next 5-min period.

*Kinetics of Appearance of 60S Subribosomes in the Cytoplasm.* In view of the speed with which insulin increased the labeling of 41S preribosomal RNA, it became of interest to learn whether the production of cytoplasmic ribosomes was also rapidly stimulated by the hormone (Figure 7). The figure shows, first, that radioactive 28S rRNA began to appear in the cytoplasm of both control and insulin-treated cells at about the same time (40 min) and, second, that insulin raised the rate of synthesis of cytoplasmic 60S subunits with little or no lag period.

*Glucose-Free Medium.* Insulin stimulated the incorporation of [$^3$H]adenine into 41S RNA by cells labeled in a basal medium that lacked glucose (Figure 8). The action of the hormone on the labeling of the RNA was not, therefore, dependent upon an increase in the cellular rate of glucose transport.

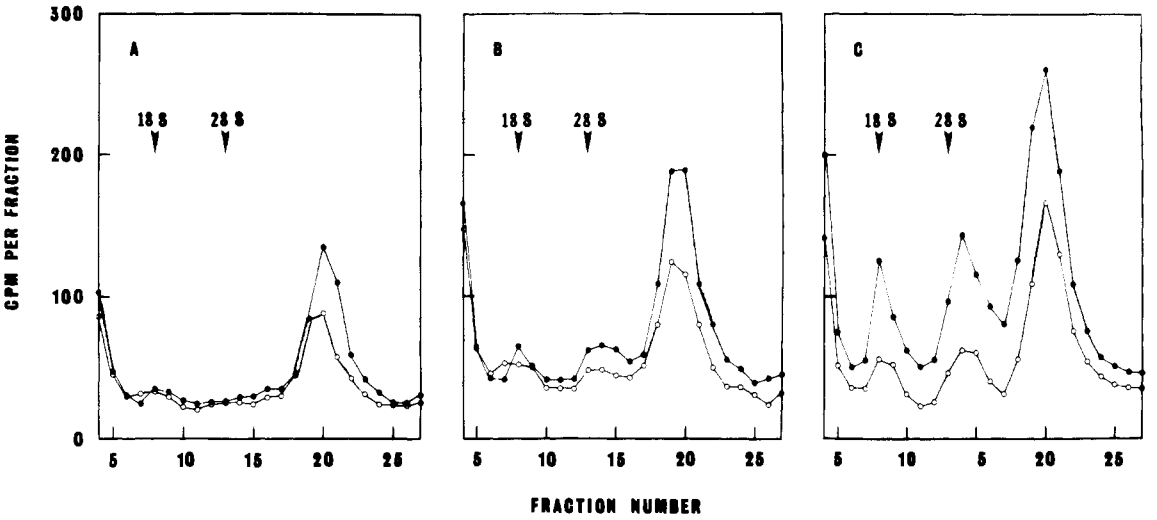


FIGURE 6: Rates of processing of 41S preribosomal RNA. Resting cultures of primary chick fibroblasts (5 cultures/group) were preincubated in 2 mL of basal medium for 30 min. At zero time, the medium was replaced with 2 mL of fresh basal medium containing 5 $\mu$g/mL insulin, as indicated, and 10 $\mu$Ci of [$^3$H]methylmethionine (15 Ci/mmol). Extraction and sedimentation of total cell RNA were as described under Materials and Methods except that, before sedimentation, each sample of RNA was treated with 100 $\mu$g of proteinase K (37 °C, 30 min) and the RNA was then precipitated with ethanol. Sedimentation was from left to right. Panel A: labeling was from 0 to 15 min. Panel B: labeling was from 0 to 20 min. Panel C: labeling was from 0 to 25 min. (○) No insulin; (●) with insulin.

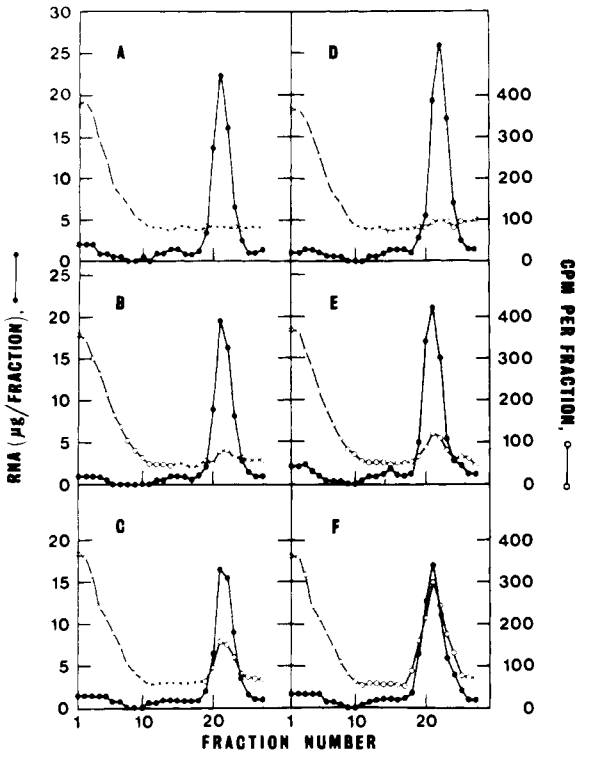


FIGURE 7: Kinetics of appearance of 60S subribosomes in the cytoplasm. Confluent cultures of primary chick cells were preincubated in 2 mL of basal medium for 30 min. The medium was then replaced with 2 mL of fresh basal medium containing [$^3$H]adenine (1.0 $\mu$Ci/mL; sp act. 110 $\mu$Ci/$\mu$mol). Labeling was for 35, 40, or 45 min. Cytoplasmic 60S-derived subribosomal cores were prepared with KCl–puromycin and EDTA, and the RNA was extracted with hot phenol. Sedimentation of 28S RNA, from left to right, was in a sucrose gradient as described under Materials and Methods. The radioactivity in the top portion of the gradient was considered to represent contaminating nuclear RNA (Perry & Kelley, 1968). Panels A–C: no insulin, labeled between 0 and 35, 0 and 40, and 0 and 45 min, respectively. Panels D–F: with insulin, labeled as for the control cultures.

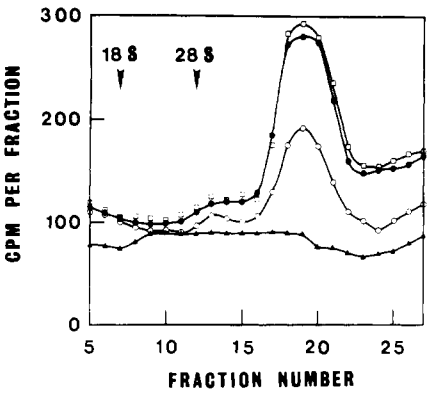


FIGURE 8: Stimulation of incorporation of [$^3$H]adenine into 41S preribosomal RNA by insulin in glucose-free medium. The conditions were the same as for Figure 5 except that, after preincubation for 30 min in the complete basal medium, some of the cultures were washed 3 times (total of 6 mL) with Hanks' balanced salt solution and were then incubated in basal medium that lacked glucose. Labeling was with [$^3$H]adenine at 15–25 min after the preincubation period. (●) Insulin with no glucose; (□) insulin and glucose; (○) no insulin with glucose; (▲) insulin with glucose and actinomycin D (0.04 $\mu$g/mL).

## Discussion

To circumvent problems with nucleotide pools, we have studied the effect of insulin on ribosome production by estimating the ratios of [$^3$H]leucine incorporated into ribosomal and total cell protein. Insulin stimulates ribosome formation in resting chick embryo fibroblasts by several-fold.

The ratios of the specific activities of ribosomal and total cell protein are analogous to the $\alpha_r$ of Schlief (1967) in studies with bacteria (quotients of rates of synthesis of ribosomal and total cell protein). The term $\alpha_r$ could not be applied here, however, because it is not known whether the fibroblasts use all molecules of newly formed ribosomal protein to make ribosomes and whether the pools of free ribosomal proteins are as negligible in the chick cells as they are, for example, in rat liver (Wool & Stöffler, 1974) and HeLa cells (Phillips & McConkey, 1976).

As a next step in the study of insulin and ribosome formation, it could be shown that the hormone has no detectable effect on the conversion of [$^3$H]adenine into the ATP that is used to synthesize preribosomal RNA. This observation entitled us to compare the incorporation of [$^3$H]adenine into the rRNAs of control and insulin-treated cells.

Kinetic measurements with [$^3$H]adenine of the labeling of 41S preribosomal RNA and of cytoplasmic 28S rRNA showed that the impact of insulin on ribosome formation in the fibroblasts is very rapid. The speed of action of the hormone in the cultured cells is reminiscent of results with isolated rat diaphragm (Wool, 1961). An increase in the incorporation of [$^{14}$C]phenylalanine into protein could be detected as early as 5 min after the addition of insulin to the tissue. A prior study had weakened considerably the possibility that the hormone enhances amino acid incorporation into diaphragm protein by raising the rate of transport or activation of amino acids (Manchester & Krahl, 1959).

The results with the cultured fibroblasts do not offer any insight into the mechanism by which insulin enhances ribosome production. One critical question is whether the hormone causes, directly or indirectly, an increase in the rate of synthesis of ribosomal proteins rather than a salvage of molecules that are already being made in excess in the resting cells. Should increased synthesis prove to be a component of the response to the hormone, it will be of great interest to learn how ribosomal protein formation is preferentially, and rapidly, stimulated. It would be difficult to invoke a role for newly made mRNAs since radioactive poly(A) messages only begin to emerge from the nucleus of insulin-treated chick cells after 10 min of labeling (R. M. DePhilip and I. Lieberman, unpublished experiments).

The enhancement of ribosome formation in the cultured fibroblasts by insulin is not dependent upon an increase in glucose transport into the cell. Similar conclusions were reached in studies of amino acid incorporation into the proteins of the isolated rat diaphragm (Manchester & Young, 1958; Wool & Krahl, 1959).

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