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*AN INFLUENCE OF INSULIN ON THE SYNTHESIS OF A RAPIDLY LABELED RNA BY ISOLATED RAT DIAPHRAGM**

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Insulin, when added *in vitro* to isolated rat diaphragm, will enhance the incorporation of labeled amino acids[1–4] and of amino acid precursors[5] into protein; neither the precise mechanism nor the exact site at which the hormone acts to bring about this effect is known. The influence of insulin on protein biosynthesis in muscle does seem, however, to be separate and distinct from the action of the hormone in facilitating the entry of glucose into the cell.[3, 4] Moreover, there are observations[5–8] that, at least by indirection, support the hypothesis that insulin stimulates protein biosynthesis through an effect on an intracellular process.

To obtain information as to the identity of the intracellular process, the effect of insulin on nucleic acid synthesis was investigated. Insulin was found to enhance RNA synthesis in muscle;[9, 10] this was reflected in an increase in incorporation of C^{14} from several substrates (adenine, orotic acid, and glucose) into RNA and by an actual net synthesis of RNA. Most remarkably, the latter occurred in the absence of any added substrate.

It is a commonplace that considerable importance attaches to the isolation of the primary biochemical locus at which insulin influences metabolic processes, for that information is vital to an analysis of the mechanism of the hormone's action. Especially pertinent is the identification of the exact molecular species of RNA whose synthesis is increased by insulin. The stimulation of the production of a specific RNA, more so if that RNA were limiting for protein synthesis, as may be the case with messenger RNA,[11] would add weight to the speculation that RNA synthesis is the intracellular site of action of insulin in stimulating protein synthesis.

The recognition of the problem and its implication has led to an attempt to identify the fraction of the cellular RNA whose synthesis is stimulated by insulin. It is the purpose of this paper to report the results of that investigation.

Materials and Methods.—Nonfasted male Wistar or Sprague-Dawley rats, maintained under standard conditions[4] until they reached 110–130 gm, were killed by decapitation and the hemidiaphragms rapidly removed and prepared for incubation. The two hemidiaphragms from a single rat were distributed between control and experimental groups; six hemidiaphragms were incubated in a 50-ml conical flask with 5 ml of Krebs-Henseleit bicarbonate medium (pH 7.4) containing the additions indicated in the figures. Incubation was at 37° in a Dubnoff shaker (120 cycles/min); the gas phase was O_2-CO_2 (95:5).

The adenine-8-C^{14} (specific activity 5.06 mc/millimole) and the carrier-free P^{32}-orthophosphate were purchased from Nuclear-Chicago Corporation or the Radiochemical Centre, Amersham. The insulin (beef zinc insulin crystals, lot 719106) assaying 25.2 units/mg and containing less than 0.1% glucagon was a gift of Dr. O. Behrens of Eli Lilly and Company; it was dissolved in N/300 hydrochloric acid to form a stock solution of 20 units/ml.

Extraction of RNA: After incubation the flasks were drained, and the hemidiaphragms were washed three times with 50 ml of cold 0.05 *M* Tris buffer, pH 7.5, to remove excess isotope. The hemidiaphragms were then placed in an iced mortar; 1 ml of 10% sodium lauryl sulfate and 0.5 ml of bentonite (suspended in 0.05 *M* Tris, pH 8.1) were added immediately and the diaphragm muscle minced and ground to a slurry with addition of 10 ml of 0.05 *M* Tris buffer, pH 7.5. The slurry was transferred to glass-stoppered tubes and shaken at 20° for 8 min; 10 ml of freshly distilled water-saturated phenol was added and shaking continued for 10 min, now at 0°. The preparation was centrifuged (3,500 rpm at 0°) for 15 min and the water layer removed and retained. Water (10 ml) was added to the phenol residue which was shaken for 5 min at 0°, recentrifuged, and the water layer removed. The combined water layers were re-extracted with 10 ml of phenol. To the final water extract was added $^1/_9$ volume of 20% sodium acetate and 3 volumes of 96% ethanol. The RNA was precipitated overnight at −15° and collected by centrifugation at 10,000 rpm for 10 min at 0°. The precipitate was dissolved in 0.4 ml of buffer (0.1 *M* NaCl; 0.01 *M* acetic acid, pH 5.2); the material not dissolved in the buffer was removed by centrifugation and discarded. The RNA was precipitated twice more with ethanol, being dissolved each time in buffer, the material not dissolved in buffer being removed and discarded.

Analysis of RNA: The RNA (0.1 ml) was layered on 4.7 ml of linear sucrose gradient (20–5%) containing 0.1 *M* NaCl and 0.01 *M* acetic acid, pH 5.2. After centrifugation at 37,000 rpm for 4 hr in the SW 39 rotor of a Spinco model L ultracentrifuge, a hole was pierced in the bottom of the tube and drops collected sequentially. The extinction at 260 mμ was determined for each fraction (after addition of 1 ml of water), and their radioactivity assessed after precipitation and washing with ice-cold 0.5 *N* perchloric acid onto membrane filters.

A sample of RNA from diaphragm muscle incubated with P^{32} was washed twice with ice-cold perchloric acid (0.5 *N*) to remove nucleotides, hydrolyzed in 0.1 *N* KOH at 37° for 24 hr, neutralized with perchloric acid, and the nucleotides separated by electrophoresis[12] at pH 3.5 in 0.05 *M* ammonium formate buffer. The nucleotides were identified on the paper under ultraviolet light, the spots cut out, and the nucleotides eluted onto aluminum planchets, dried, and counted in a gas flow counter. Each of the four nucleotides contained P^{32}, indicating the labeling of the RNA was not due merely to terminal addition of radioactive nucleotides.

Adenine-8-C^{14} uptake: Six hemidiaphragms were incubated in 20 ml of Krebs-Henseleit bicarbonate medium containing 1 μc/ml of adenine-8-C^{14} and with or without insulin (0.1 unit/ml) for 2 hr at 37°. At the end of the incubation, the hemidiaphragms were rapidly weighed and then minced and ground to a slurry in ice-cold 10% trichloroacetic acid. The trichloroacetic acid-soluble supernatant was collected by centrifugation; the precipitate was washed twice with ice-cold 10% trichloroacetic acid, and the washes were retained. A 1.5-ml sample of the pooled soluble extract was transferred to a stainless steel planchet, evaporated to dryness, and counted. A 0.1-ml sample of the incubation medium was diluted to 5 ml; 1 ml was transferred to a planchet, dried, and counted in a like manner. The results are expressed, in the first instance, as the ratio of the number of counts per ml of diaphragm water (calculated on the assumption that the diaphragm tissue contains 75% water) divided by the number of counts per unit volume remaining in the incubation medium.

Uptake was reckoned as the difference between the radioactivity originally present in the medium and that remaining at the end of incubation. The results are expressed as counts per min per gm of tissue for 2 hr.

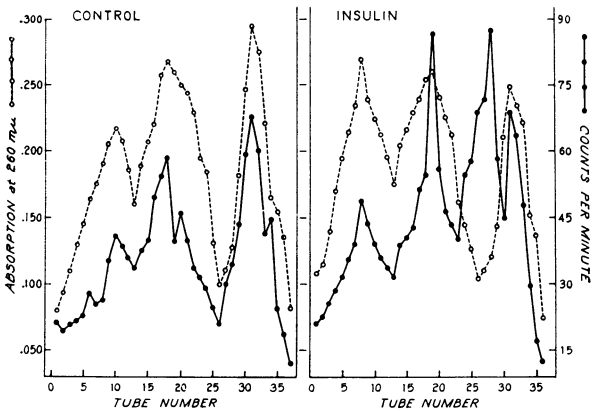

FIG. 1.—Sedimentation of C^{14}-adenine-labeled RNA from rat diaphragm muscle: effect of insulin. Six paired hemidiaphragms were incubated for 2 hr at 37° in 5 ml of Krebs-Henseleit bicarbonate buffer containing 1 μc/ml of adenine-8-C^{14} and with or without insulin (0.1 unit/ml); no glucose was added. At the end of the incubation the RNA was extracted and analyzed on linear sucrose gradients as described in *Materials and Methods*.

Samples of the acid-soluble tissue extract were evaporated to dryness and the residue redissolved in 1 ml of 1 *N* HCl. The nucleotides were separated by ascending paper chromatography[13] on Whatman No. 3 filter paper in methanol-ethanol-HCl-H_2O (50:25:6:19). The bases were located on the paper with an ultraviolet light and a radiochromatogram scanner. The adenine spot was cut out and eluted with 1.5 ml of 0.1 *N* HCl, the absorption determined at 260 and 280 mμ, and the molar concentration calculated; 1 ml of the eluate was plated, dried, and counted. The specific activity of the adenine in the intracellular pool was then calculated.

Results.—The results were conditioned by several parameters, but especially by the duration of the experiment, the nature of the radioactive substrate, and whether the tissue was exposed continuously to the substrate or a pulse of radioactivity was employed. But no matter the experimental conditions, three extinction peaks were obtained with sedimentation coefficients of 4, 19, and 28 S (Figs. 1–3). The first is probably transfer RNA while the other two are of ribosomal origin. This finding is constant and is uninfluenced by insulin. The specific radioactivity of the three species of RNA is conditioned by the duration of incubation, the transfer RNA being labeled first and the ribonucleoprotein RNA later (experiments not shown); usually, but by no means always, the 19 and 28 S peaks have similar radioactivity.

In the first experiment (Fig. 1), diaphragm was incubated with adenine-8-C^{14} (1 μc/ml) and with or without insulin (0.1 unit/ml) for 2 hr; no glucose was added. The fractionation on sucrose gradients of RNA from muscle in the control experiment (no insulin) revealed the transfer RNA (4 S) to be most radioactive, the other two fractions to be less so. Analysis of RNA from insulin-treated diaphragms disclosed the following: The specific activity of at least one species of ribonucleoprotein RNA (19 S) was increased by the hormone. But perhaps the most remarkable finding was the appearance of a new peak of radioactivity of extremely high specific activity (i.e., high counts, low extinction) and of a sedimentation constant (between 4 and 18 S) that suggests it is messenger RNA. The latter finding, however,

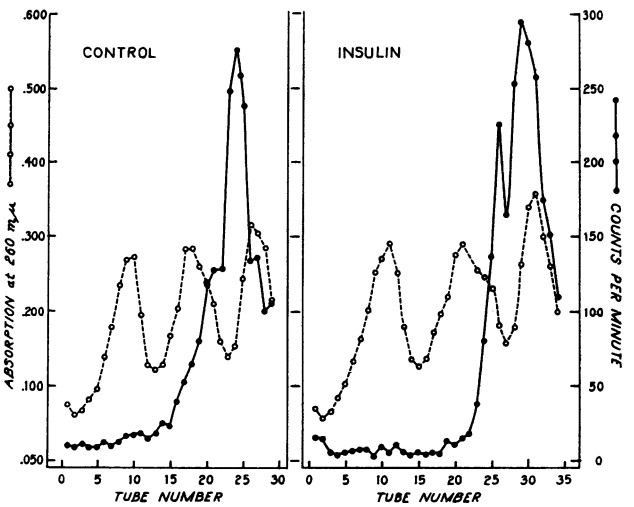

FIG. 2.—Sedimentation of P^{32}-labeled RNA from rat diaphragm muscle: effect of insulin. Six paired hemidiaphragms were incubated for 5 min at 37° in 5 ml of Krebs-Henseleit bicarbonate buffer (from which KH_2PO_4 was omitted) containing 0.5 mc of P^{32}-orthophosphate and with or without insulin (0.1 unit/ml); no glucose was added. The RNA was prepared and analyzed as described in *Materials and Methods*. Note that only 28 drops were collected in the control experiment, whereas 35 were obtained in the insulin experiment.

has not been entirely constant. Although this fraction is not seen in experiments of this design without added hormone, it is not uniformly present in experiments with insulin.

In the next experiment (Fig. 2), diaphragm was incubated for only 5 min with 0.5 mc of P^{32} and with or without insulin (0.1 unit/ml); no glucose was added. Most of the radioactivity was in the peak that precedes transfer RNA; this rapidly labeled RNA fraction has an S value similar to that described for messenger RNA of *E. coli*.[14] The amount of rapidly labeled RNA was increased by insulin.

When diaphragm is incubated with P^{32} for a longer period, i.e., 2 hr (Fig. 3), one no longer sees a peak corresponding to messenger RNA (which is presumably buried in the other peaks) and notes instead that insulin increases the specific activity of ribonucleoprotein RNA as in the instance of the experiment with C^{14}-adenine (cf. Fig. 1).

One interpretation of the results is that insulin increases the rate and magnitude of transport into the muscle cell interior of the RNA precursors. If that were so, then the RNA isolated would be more radioactive, even if there were no change in the rate of RNA synthesis. This possibility must be given credence since insulin increases the transport of a number of substrates[15] and, in fact, there are recorded preliminary observations[16] that insulin accelerates phosphate uptake by muscle, an observation of great pertinence for the present experiments. This being the case, we felt it necessary to determine if insulin altered adenine-8-C^{14} uptake by isolated diaphragm and, more to the point, if the hormone changes the specific activity of the intracellular adenine pool. Neither parameter was influenced by insulin

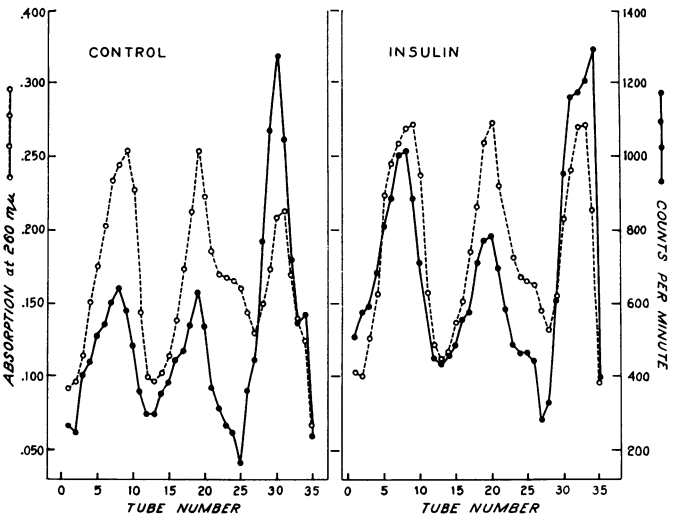

FIG. 3.—Sedimentation of P^{32}-labeled RNA from rat diaphragm: effect of insulin. Conditions of incubation and analysis of RNA were the same as in Fig. 2 except that incubation was for 2 hr rather than 5 min.

(Table 1), making it most unlikely that insulin accelerates RNA synthesis by an effect on substrate transport. It is noteworthy, however, that insulin increases the utilization of adenine-8-C^{14} (Table 1) and, inasmuch as it does so without changing the ratio of the concentration of the base in the cell interior to that in the extracellular space (incubation medium), and without changing the specific activity of the intracellular adenine pool, this may be taken as being in accord with a direct stimulatory effect of insulin on RNA synthesis.

Discussion.—A number of factors complicate the interpretation of experiments of the type recorded here. First, in the absence of added insulin, muscle does synthesize protein; since it is the rate of synthesis that appears to be accelerated by the hormone, one would expect all the components of the synthesizing machinery, including messenger RNA, to be present. One would predict an effect of insulin on the rate of a particular reaction rather than on the appearance of some new reaction. This accords then with the observation that there is synthesized in muscle a rapidly labeled nucleic acid, having the characteristics of messenger RNA, and that the synthesis of this fraction is in some circumstances increased by insulin (cf. Fig. 2). However, it is also true that in other instances insulin appears to

TABLE 1

UPTAKE AND ACCUMULATION OF ADENINE-8-C^{14} BY ISOLATED RAT DIAPHRAGM AND SPECIFIC ACTIVITY OF INTRACELLULAR ADENINE: INFLUENCE OF INSULIN

	Accumulation cpm/ml of tissue water / cpm/ml of medium	Uptake cpm/gm of tissue/2 hr	Specific activity intracellular adenine cpm/$10^{-5}M$
Control	0.035 ± 0.004	57,100	5,650 ± 423
Insulin (0.1 unit/ml)	0.037 ± 0.002	118,600	4,960 ± 632

Six hemidiaphragms were incubated in 20 ml of Krebs-Henseleit bicarbonate medium containing 1 μc/ml of adenine-8-C^{14} and with or without insulin (0.1 unit/ml) for 2 hr at 37°; no glucose was added. The analyses were made as described in *Materials and Methods*. The values for adenine utilization are from a single observation done in triplicate; the values for accumulation and specific activity are the mean ± S.E. of six separate observations.

be crucial in conditioning the appearance or persistence of messenger RNA (cf. Fig. 1). It is no less significant that insulin consistently increases the specific activity of ribonucleoprotein RNA.

This brings one to a second point, namely, that for mammalian cells there is no assurance that the RNA that is rapidly synthesized in the nucleus serves exactly the same role as it does in bacteria. The absence of this assurance considerably restricts analysis of the significance of the effect of insulin on RNA synthesis. Despite this restraint, the finding that insulin leads, in some experiments and under some conditions, to an increase in the synthesis of messenger RNA and an increase in the specific activity of ribosomal RNA has led to the postulation that RNA synthesis is the molecular site of action of insulin in promoting protein synthesis and that perhaps the same action accounts for the other biochemical and physiological effects of the hormone.[17, 18] The theory and its implications are discussed *in extenso* in another place,[17,18] but, briefly, it is proposed that insulin coordinates the synthesis of protein and other molecules by initiating the transcription of a series of messages, perhaps, by directly combining with a repressor molecule, or, alternatively, by leading to the formation of a specific metabolite that combines with the repressor. By a mechanism of this sort, insulin might initiate an increase in the rate of synthesis of a series of specific proteins, which proteins (enzymes) might condition the several anabolic actions of the hormone.

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