Effects of Insulin upon Glucose Metabolism and Protein Synthesis by the Anterior Pituitary in Vitro

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SUMMARY

The effects of insulin upon the metabolism of glucose by single rat anterior pituitary glands in vitro have been compared with the effects of raising the concentration of glucose in the suspending medium. The pattern of stimulation with insulin differed from that induced by increasing glucose. Insulin enhanced glucose oxidation, glycogenesis and lipogenesis out of proportion to the increase in glucose uptake.

The anterior pituitary exhibited relative insensitivity to insulin in vitro compared to muscle and adipose tissue. Stimulation of glucose metabolism was detectable at 1,000 uU. insulin per milliliter medium, and maximum stimulation occurred between 0.1 and 1.0 U. per milliliter. Insulin sensitivity was not altered by fasting. The effect of insulin was attenuated upon substituting phosphate for bicarbonate as buffer in the incubation medium.

The incorporation of glycine C-14 into tissue protein was stimulated by insulin and this effect was undiminished in the absence of glucose in the medium. The incorporation of C-14-alpha-amino-isobutyric acid from the medium into the anterior pituitary was greater than that reported for rat diaphragm and was not affected by insulin.

The data indicate that insulin could potentially affect hormonogenesis in anterior pituitary through stimulation of protein synthesis. The cell types responsive to insulin and the physiologic consequences of insulin activity upon the anterior pituitary remain to be established. DIABETES *15*: 115-22, February, 1966.

Previous studies provided evidence for including the anterior pituitary among the insulin sensitive tissues.[1] This evidence included in vitro responses to insulin in the metabolism of glucose by anterior pituitary and documentation of changes in the glucose metabolism of glands collected from diabetic animals. Unanswered was the relative importance of glucose in anterior pituitary metabolism as a source of energy for protein synthesis and the possible influence of insulin upon this later process. It was subsequently shown that, although 25 per cent of metabolized glucose was converted to amino acids and protein by anterior pituitary in vitro,[2] protein synthesis from glycine was little affected by fasting the donor animals or lowering the concentration of glucose in the medium.[3] These data suggested that other substrates could sustain protein synthesis and raised a question as to the potential importance of insulin as a regulator of hormonogenesis through effects upon glucose metabolism alone.

In the present study the effects of insulin upon the metabolism of glucose in anterior pituitary are examined in more detail and compared to the effects of raising medium glucose. In addition, evidence is presented for an effect of insulin upon protein synthesis which is independent of glucose. These observations permit postulation of two mechanisms whereby insulin might participate in regulation of hormonogenesis in anterior pituitary, i.e., through effects upon glucose metabolism and through direct stimulation of protein synthesis.

METHODS

Single anterior pituitary glands (AP) from male and female rats* weighing 180 to 220 gm. have been incubated in vitro by methods previously presented in detail.[1,2] In brief, glands (weighing 6 to 12 mg.) were collected within 1 min. after decapitation and incubated in 0.350 ml. Krebs-Ringer Bicarbonate buffer (KRB), *p*H 7.4, which was gassed with 95 per cent O_2, 5 per cent CO_2. The media contained 1 uc. glucose-UL-C-14 (1 mM.), 1 uc. glycine-2-C-14 (0.9 mM.) or 1 uc. Alpha-Amino Isobutyric acid-1-C-14 (AIB), (5.5 mM.)† Depending upon the experimental design, media

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*Sprague-Dawley rats purchased from Northwest Rodent Farms, Pullman, Washington.

†Glucose-UL-C-14, glycine-2-C-14 and alpha-amino-isobutyric-1-C-14 acid were purchased from the New England Nuclear Corporation, Boston. Chromatographic purity was verified in our laboratory.

were supplemented with varying concentrations of unlabeled glucose and crystalline bovine insulin.* Incubations were performed in a Dubnoff metabolic shaker (100 cycles per minute) at 37° C. for 3 hrs.

At the end of incubation, acid was added to the medium, and Hyamine† was placed in a suspended plastic cup. CO_2 was collected over a 45-min. period and analyzed for radioactivity by liquid scintillation counting.‡ With each experiment two flasks were incubated without tissue. Volatile radioactivity (200 to 400 cpm.) was consistently recovered in the Hyamine when glucose-UL-C-14 was the substrate. This small degree of contamination was constant for each lot of C-14-glucose. Since this material contributed less than 1.0 per cent of the C-14-O_2 evolved during tissue incubations, no corrections were made. The tissues were analyzed for lipid (fatty acids)[2] and glycogen radioactivity[2] when C-14-glucose was the substrate, and for protein C-14 (washed trichloroacetic acid (TCA) precipitate[4] when C-14-glycine was the substrate. The validity of the TCA method for quantifying protein radioactivity was tested by comparison with that obtained after separation of tissue proteins by electrophoresis. The two methods gave comparable radio-labeling for AP total protein. Significant coprecipitation artifact was excluded by the finding of negligible labeling of TCA precipitates after 5-min. incubations of AP with C-14-glycine. The media were analyzed for lactic acid C-14 which had been chromatographically isolated.[2] Glucose uptake from the medium was calculated from the final concentration of glucose determined enzymatically§ upon deproteinized filtrates of the incubation medium. The incorporation of glucose C-14 into CO_2, glycogen and lipid was expressed as μg. equivalents of glucose per mg. AP wet weight, calculated from the initial specific activity of the substrate glucose and the total radioactivity recovered in the product.[1,2] This method of quantifying pathways of glucose metabolism provides information about the total number of glucose carbon atoms converted to each product without regard to the specific pathways traversed during the over-all conversion. For example, the μg. glucose-UL-C-14 recovered in C-14-O_2 may have been derived from the pentose pathway, the glycolytic pathway, through pyruvate decarboxylation, through Krebs' cycle oxidation or through many possible routes of recycling of other intermediate products of glucose metabolism. The incorporation of glycine C-14 into protein was expressed as CPM (per 10^5 CPM in the system) per milligram tissue. With all radioactive substrates employed the total radioactivity in the incubation flasks ranged from $6 - 9 \times 10^5$ CPM.

In other experiments, the tissue uptake of C-14-labeled AIB was measured after 15 min. and 1 hr. incubation in KRB containing 1.0 mg. glucose per ml. At the end of the incubation period, tissues were washed three times for 15 sec. in KRB without labeled AIB, homogenized in cold distilled water and centrifuged. An aliquot of the supernatant fluid was dried on filter paper and counted in the liquid scintillation detector. Aliquots of the media were similarly counted. The slice medium (S/M) ratio of AIB radioactivity was then calculated utilizing an assumed extracellular fluid space of 20 per cent of the initial tissue wet weight. (S/M = CPM per ml. intracellular space/CPM per ml. incubation medium.)

RESULTS

1. *Comparison of the effects of increased medium glucose with the effect of insulin upon glucose metabolism in AP*

Single APs from fasted males were incubated with glucose-UL-C-14 in media containing 1.0, 2.0 or 3.0 mg. glucose per milliliter. At the same time glands were incubated in media containing 1.0 mg. glucose per ml. supplemented with 10^{-3}, 10^{-2}, 10^{-1} and 10^{-0} U. bovine insulin per milliliter. Figure 1 illustrates the changes in glucose uptake and disposition of glucose C-14 observed with increasing medium glucose concentration (above) in comparison to the effect of increasing insulin concentration (below). The data obtained at 1.0 mg. glucose per milliliter in the absence of insulin have been set at 100 per cent. These basal values as μg. per milligram were (mean ± S.E.) glucose uptake 7.3 ± 0.7, C-14-O_2 1.57 ± 0.15, glycogen C-14 .039 ± .006 and lipid C-14 .042 ± .004. Increments above this basal level associated with increasing medium glucose or increasing concentrations of insulin were expressed as per cent of the basal level. Eight to twelve glands were incubated at each concentration of glucose or insulin.

*Glucagon free crystalline bovine zinc insulin was generously supplied by Dr. O. K. Behrens of Eli Lilly Company.

†Hyamine 10X purchased from the Packard Instrument Co., Inc., La Grange, Illinois.

‡All radioactive determinations were done with conventional liquid scintillation technics in a Packard Tri-Carb liquid scintillation counter, Packard Instrument Company, Inc., La Grange, Illinois. Each analytical procedure was checked repeatedly for quenching by addition of toluene-C-14 as internal standard. Quenching was not encountered with the technics employed.

§Glucostat reagent, Worthington Biochemical Company, Freehold, New Jersey.

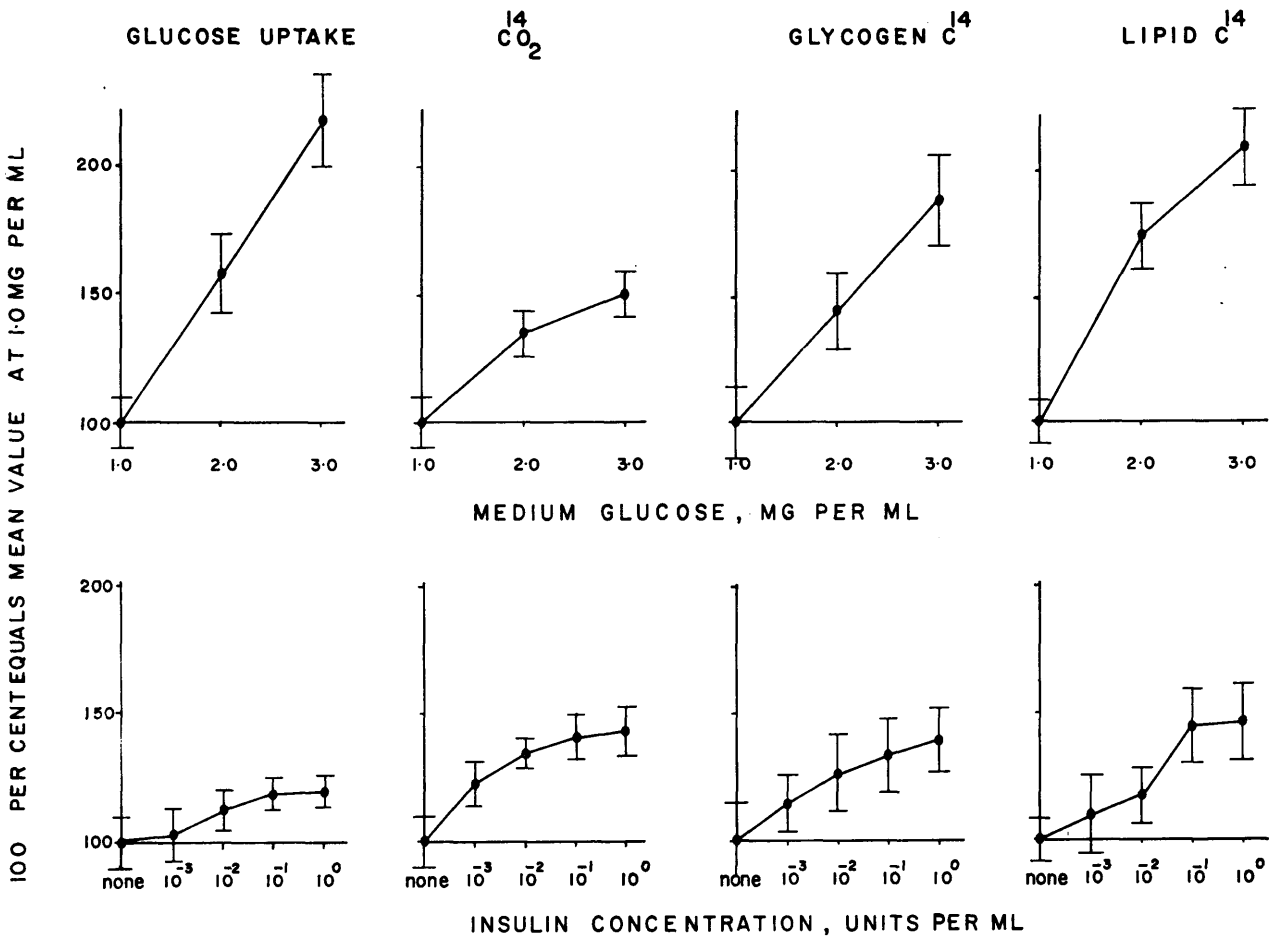

FIG. 1. Comparison of the effects of increasing medium glucose concentration with the effect of insulin upon glucose-UL-C-14 metabolism by single anterior pituitaries from fasted male rats.

Glands were incubated in 0.350 ml. KRB at 37° C. for three hours with increasing concentrations of glucose or insulin in the medium. The glucose uptake and conversion of glucose-C-14 to CO_2, glycogen and lipid at 1.0 mg. glucose per milliliter medium without added insulin were set at 100 per cent and the changes with increasing glucose (above) or insulin (below) have been depicted as per cents ± S.E. of these basal values. Eight to twelve glands were incubated at each condition. Insulin stimulated glucose oxidation and, to a lesser extent glycogenesis and lipogenesis, out of proportion to its effect upon glucose uptake.

With increasing concentration of medium glucose, glycogenesis and lipogenesis were enhanced in parallel with glucose uptake, while glucose oxidation (C-14-O_2) did not rise proportionately. By contrast, with insulin, the percentage increases in glucose oxidation, glycogenesis and lipogenesis were relatively greater than the increase in glucose uptake.

An effect of insulin upon glucose oxidation was evident at an insulin concentration of 1,000 μU. per milliliter (10^{-3}U.). At this concentration, the increase in glucose oxidation accounted for virtually all of the small additional glucose uptake. The maximal effect of insulin upon glucose oxidation approached that induced by a threefold increase of glucose concentration. For the other products and for glucose uptake, the stimulatory effect of increased glucose was much greater than that produced by maximal insulinization.

The effects of incubation with a wider range of medium glucose concentrations upon the oxidation of glucose C-14 and incorporation of glucose into other products were examined further as illustrated in figure 2. At the lowest concentration of glucose (with the lowest rate of glucose uptake) most of the assimilated glucose underwent oxidation to CO_2. With increasing medium glucose concentration, as more glucose uptake occurred, the amount of glucose undergoing oxidation approached a maximum (as did lipogenesis), while the remainder of assimilated glucose was converted to lac-

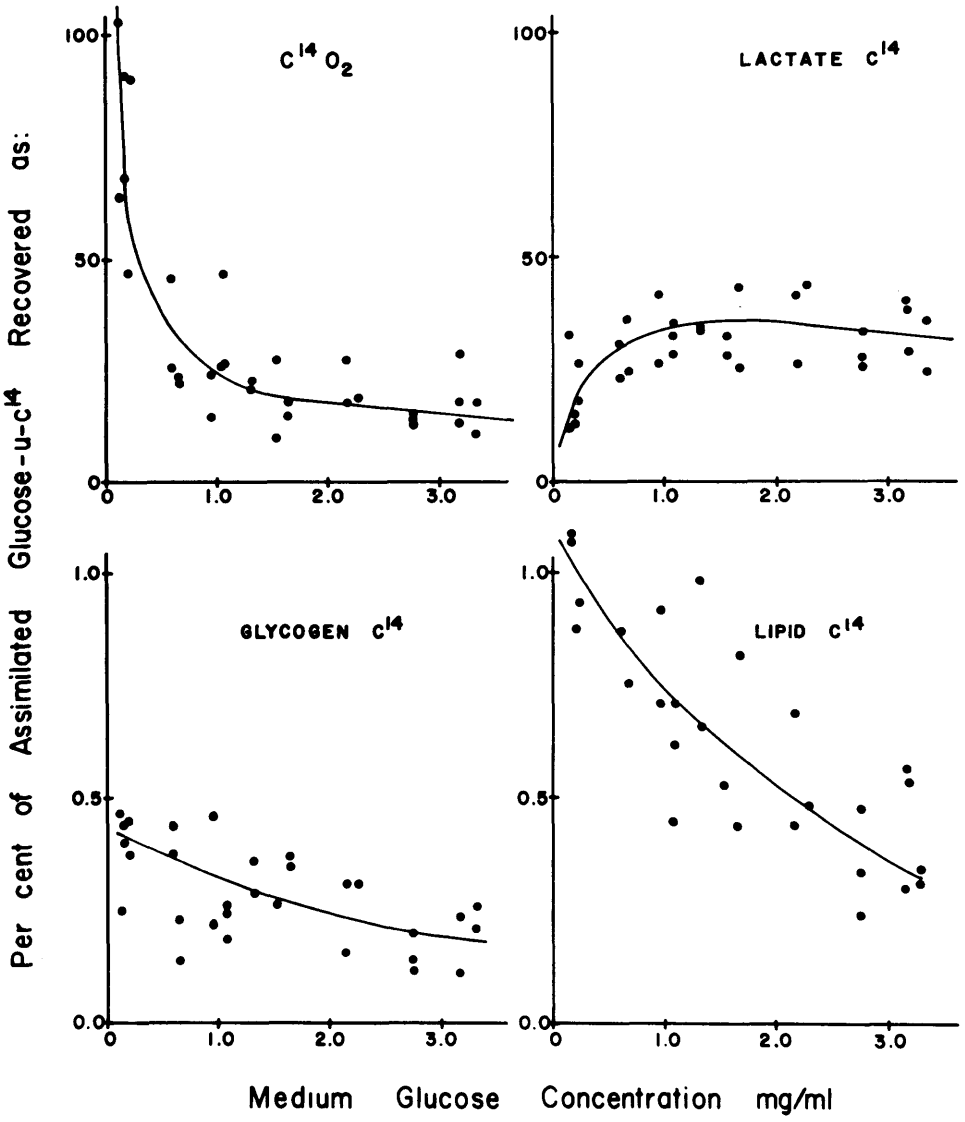

FIG. 2.

The effect of increasing medium glucose concentration upon the fraction of assimilated glucose (glucose uptake) converted to CO_2, glycogen, lipid and lactic acid by rat anterior pituitary.

Single glands were incubated as in figure 1. Individual data are presented. The per cent of assimilated glucose-UL-C-14 recovered in the products listed changed as the concentration of glucose in the medium was raised. At the lowest concentrations of medium glucose, glucose uptake was low and the largest fraction underwent oxidation to CO_2. The sum of total recoveries in individual glands at the lowest concentrations of medium glucose ranged from 78 to 115 per cent. As the glucose concentration was increased, glucose uptake rose and the fraction undergoing oxidation declined, while the fraction appearing as lactic acid increased.

tic acid and other products. It is of interest to note that the shift in the pattern of glucose metabolism from predominant oxidation to predominant glycolysis occurred at medium glucose concentrations within the normal plasma glucose range between 0.5 and 1.0 mg. per milliliter (50 to 100 mg. per 100 ml.).

2. *The metabolism of glucose and the response to insulin in Krebs-Ringer Phosphate buffer*

Glands were incubated in Krebs-Ringer Phosphate buffer (KRP) *pH* 7.4 with and without insulin (0.1 U. per ml.) in the presence of glucose-UL-C-14 and 1.0 mg. per milliliter glucose in the media (table 1). Whereas in KRB, glucose uptake is regularly enhanced about 20 per cent by insulin, glucose uptake was not increased in KRP. The fraction of C-14-glucose converted to C-14-O_2 was reduced in KRP to approximately 60 per cent of that found in KRB. Lipogenesis from glucose was somewhat reduced in KRP compared to KRB and not significantly changed by insulin. The only product displaying a significant increase with insulin in KRP was glycogen C-14.

3. *The effect of insulin upon glycogen content of AP*

For each experiment, AP glands from fifteen rats were collected and distributed into three groups. Five glands were placed directly in alkali for initial glycogen content. Five glands were incubated for 3 hrs. in 1.2 ml. KRB, with or without 0.1 U. insulin per milliliter, in the presence of 1.0 mg. glucose per milliliter medium.

TABLE 1

The effect of phosphate buffered medium upon glucose metabolism and insulin effects in anterior pituitary

Number of glands	Krebs-Ringer phosphate: No insulin 8	(p difference) Insulin vs. no insulin	Insulin 8	(p difference) KRP vs. KRB no insulin	Krebs-Ringer bicarbonate: No insulin 8
	ug./mg.±S.E.		ug./mg.±S.E.		ug./mg.±S.E.
Glucose uptake	7.4±2.1	(NS)	7.5±1.8	(NS)	8.1±1.4
C-14-O_2	1.1± .09	(NS)	1.4± .11	(p .02)	1.8± .11
Glycogen C-14	.032±.004	(p .05)	.054±.007	(NS)	.038±.008
Lipid C-14	.021±.003	(NS)	.026±.004	(p .05)	.049±.006

*Single glands from fasted male rats were incubated with 1 uc glucose-UL-C-14 at 1.0 mg. glucose per milliliter for three hours. The insulin concentration was 0.1 U. per milliliter.

Table 2 summarizes the results of three experiments, one with males and two with females as AP donors. The glucose uptake (μg. per milligram) by the pooled glands in the incubated flasks is given in parenthesis. The glycogen content of AP declined during incubation in vitro. The final glycogen content was higher in each experiment in the presence of insulin than it was after incubation in buffer alone. The previously described[2] higher glycogen content of AP from males is evident.

4. *The effect of added insulin upon incorporation of glycine-2-C-14 into tissue protein*

TABLE 2

The effect of insulin upon the glycogen content of anterior pituitary during incubation

Experiment	Sex	Glycogen content in ug. per mg. wet weight AP: Before incubation	After incubation for three hours: No insulin	Insulin
1	Male	0.99	0.51(8.7)*	0.62(10.6)
2	Female	0.49	0.12(6.7)	0.20(8.0)
3	Female	0.26	0.09(6.9)	0.19(8.1)

*Glucose uptake during incubation is given in parentheses. Five glands were incubated in each flask. Incubation was carried out at 37° C. for three hours in 1.2 ml. KRB with 1.0 mg. glucose per milliliter with or without 0.1 U. insulin per milliliter.

Single glands were incubated in KRB (glucose concentration of 1.0 mg. per milliliter) with or without 0.1 U. per milliliter added insulin. Table 3 summarizes the results of nineteen control and nineteen insulin experiments on fasted male rats.* The incorporation of glycine into protein was significantly enhanced in the presence of insulin. Stimulation of glucose uptake by insulin was proportionally greater than the effect upon protein synthesis. Oxidation of glycine to C-14-O_2 was not significantly increased in the presence of insulin.

An effect of insulin of equal magnitude upon protein synthesis could be demonstrated in experiments performed with no glucose present in the media (table 4). Without glucose in the medium, oxidation of glycine to CO_2 was increased. Again insulin did not significantly alter this pathway.

The effect of increasing medium glucose upon glycine incorporation into protein is summarized in table 5. No significant change in protein synthesis from glycine occurred over the range zero to 1.6 mg. glucose per milliliter medium. The effect of increasing competition

*A series of experiments comparing the insulin sensitivity of APs from fed and fasted rats or of males and females revealed no significant differences in stimulation of glucose metabolism or amino acid incorporation.

TABLE 3

The effect of insulin upon incorporation of glycine-2-C-14 into tissue proteins in anterior pituitary

	Number of glands*	Glucose uptake ug. per mg.	Protein C-14 cpm/10^5 cpm/mg.	C-14-O_2 cpm/10^5 cpm/mg.
No insulin	19	8.3±0.5†	133±6.0	7.4±2.6
(p difference)		(.025)	(.05)	(NS)
Insulin	19	10.0±0.4	155±8.1	8.1±3.0

*Single APs from fasted male rats were incubated for three hours at 37° C. in 0.350 ml. KRB containing 1.0 mg. glucose per milliliter, 1 uc glycine-2-C-14 (0.9 mM) with or without 0.1 U. insulin per milliliter.
†S.E.

between glucose and glycine for oxidative pathways is again evident in the declining oxidation of glycine as unlabeled glucose is added to the system.

5. *The lack of effect of insulin upon the transport of alpha-amino-isobutyric acid in AP*

Slice: medium ratios (S/M) for the distribution of AIB C-14 after 15 and 60 min. of incubation are presented in figure 3. Two experiments, each with eight control and eight insulin flasks, were performed for each interval of incubation and the mean values are presented for these four experiments. Insulin (0.1 U. per milliliter) did not alter the distribution of AIB after either period of incubation. The ratios of distribution of AIB achieved by AP after 60 min. were higher than those reported for unstimulated rat diaphragm muscle (S/M up to 1.0) but less than maximum ratios obtained with insulin stimulation in diaphragm (approximately 3.0).[5]

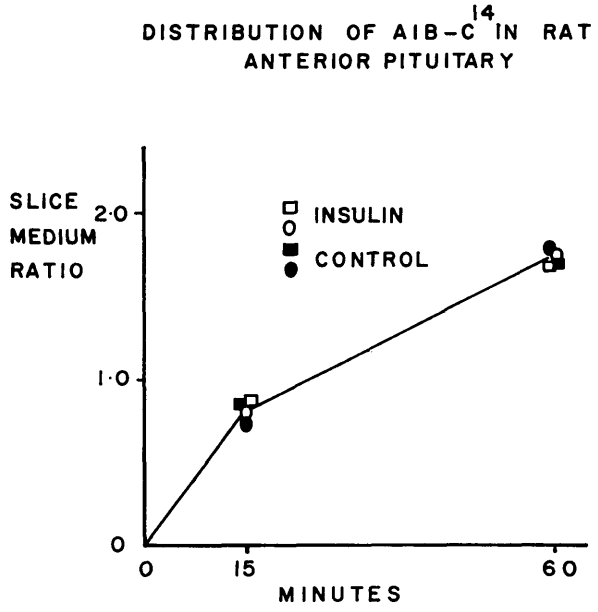

FIG. 3. The lack of insulin effect upon the distribution of the nonutilized amino acid (AIB-C-14) between medium and tissue in anterior pituitary.

The slice: medium ratios for AIB were not influenced by insulin after 15 or 60 min. incubation of single APs from fasted male rats. Two experiments each comprising eight glands incubated with and eight without insulin were performed at each time interval. The mean values for each group of eight have been plotted.

DISCUSSION

In anterior pituitary in vitro, insulin enhanced all of the pathways of glucose metabolism measured including glucose uptake, oxidation of glucose, glycogenesis and lipogenesis from glucose. These effects of insulin were not mimicked completely by increasing glucose in the medium. The most striking difference noted was a disproportionate increase in glucose oxidation in the presence of insulin. The pathways for oxidation of glucose to CO_2 in AP are apparently limited, since the fraction of assimilated glucose undergoing oxidation, as shown in figure 2, declined as glucose uptake was accelerated by raising the medium glucose concentration. The μg equivalents of glucose converted to CO_2 were comparable after insulin to those obtained with the higher medium glucose concentrations alone. Thus, insulin induced a nearly maximal rate of glucose oxidation, while glucose uptake was stimulated relatively little. Both the percentage increase of glycogenesis and lipogenesis were stimulated more by insulin than was glucose uptake. In adipose tissue[6] and rat diaphragm[7] such disparities between the effects of glucose and insulin have been interpreted as evidence for sites of insulin activity beyond the transport step. In muscle, the demonstration of insulin stimulation of protein synthesis from amino acids in the absence of glucose[8] and under conditions where transport of amino acids was not rate limiting[9] has also been interpreted in this way. As in muscle, the synthesis of protein from glycine

TABLE 4

Preservation of the effect of insulin upon glycine-2-C-14 incorporation into tissue protein in the absence of glucose in the medium

	Number of glands*	Protein C-14 cpm/10^5 cpm/mg.	C-14-O_2 cpm/10^5 cpm/mg.
No insulin	8	127±3.3†	19.8±4.2
(p difference)		(.025)	(NS)
Insulin	7	145±5.2	20.2±2.1

*Single APs from fasted male rats were incubated for three hours at 37° C. in 0.350 ml. KRB containing 1 uc glycine-2-C-14 (0.9 mM) with or without 0.1 U. insulin per milliliter.
†S.E.

TABLE 5

The lack of effect of glucose as substrate upon glycine-2-C-14 incorporation into tissue protein

Number of glands*	Medium glucose concentration mg./ml.	Protein C-14 cpm/10^5 cpm/mg.	C-14-O_2 cpm/10^5 cpm/mg.
4	0	205±22†	22±10
4	0.5	244±7	13±1.2
4	0.9	216±29	9±0.4
4	1.6	245±19	8±1.0

*Single APs from fasted male rats incubated for three hours at 37° C. in 0.350 ml. KRB containing 1 uc glycine-2-C-14 (0.9 mM).
†S.E.

in AP was stimulated by insulin both in the presence and in the absence of glucose. Unlike diaphragm, however, insulin did not affect the distribution of the nonutilized amino acid (AIB). It is tempting to interpret these data in AP as reinforcing the concept of multiple sites for insulin activity, but an alternative explanation must be considered.

Unlike muscle and adipose tissue, the AP is composed of a mixed cell population. If insulin were to affect a single cell type, to the exclusion of the others, stimulation of the particular metabolic pattern in this smaller population might give rise to many of the findings cited above. Thus, raising the medium glucose concentration would be expected to enhance the general or integrated metabolic pattern of all of the cells, while insulin would bring out the special pattern of the sensitive population.* At the moment, such a construction is purely speculative, but the possibility warrants caution in interpreting biochemical data obtained from the whole anterior pituitary as supporting particular sites of insulin action.

Compared to diaphragm[10] and adipose tissue,[11] anterior pituitary is quite insensitive to insulin in vitro. The effects of insulin were just detectable at a concentration of 1,000 uU. per milliliter and the dose response curve did not reach a plateau until 100 mU. per milliliter were present in the flask. Such in vitro insensitivity might reflect true cellular insensitivity or be the result of experimental artifacts. Leaching of insulin degrading enzymes from the tissue[12,13] or of contra-insulin hormonal factors[14] into the medium during incubation are two such possibilities. Our previous demonstration[1] that the in vitro metabolism of glucose by AP is reduced in diabetic rats and restored by insulin therapy suggests, however, that the AP is sensitive to physiologic levels of insulin in vivo.

In 1959, Beloff-Chain et al.[15] reported unsuccessful attempts to demonstrate insulin effects on pituitary tissue in vitro. These studies were done on whole rat pituitaries, incubated in phosphate buffered media. In the present study, incubation in phosphate buffer resulted in less oxidation of glucose and diminished lipogenesis in AP compared to the results in bicarbonate buffered medium. The effects of insulin upon the metabolism of AP in phosphate buffer were limited to stimulation of glycogenesis. The failure of Beloff-Chain and associates to obtain an effect of insulin upon glycogenesis might be explicable by the presence of posterior pituitary in their preparations or by the nonspecific chromatographic method employed for quantifying glycogen.[16]

The role of glucose as one substrate for the protein synthetic function of the anterior pituitary is reflected biochemically in the large fraction (about 25 per cent) of glucose C-14 converted to amino acids and protein in vitro.[2] However, the constancy of incorporation of glycine into protein in vitro in APs from fasted and fed animals[3] and in glands incubated in the absence of glucose (table 5) suggests that energy for protein synthesis is available from alternate substrates. One possible endogenous substrate is glycogen. The store of glycogen in AP, however, is limited. In normal, fed male rats the mean glycogen content was 0.64 μg. per milligram wet weight.[2] Thus the glycogen store in AP is sufficient to maintain basal glucose metabolism (glucose uptake at 1.0 milligram glucose per milliliter medium) for only 15 or 20 min. Therefore, glycogen is probably not an important endogenous substrate for sustained protein synthesis, although it might provide energy for an acute requirement. The activity of phosphorylase in AP is reflected in the consistent depletion of glycogen found during incubation of glands in vitro (table 2). The addition of insulin resulted in less depletion of AP glycogen and stimulated incorporation of glucose C-14 into glycogen. These data suggest a role for insulin in maintenance of tissue glycogen. However, Dixit and Lazarow[18] found that the glycogen content of AP is actually elevated in diabetic animals which led them to conclude that the glycogen content of AP is primarily conditioned by the height of the blood glucose. By exclusion the tissue lipid stores appear to be the most likely source of energy for protein synthesis in the absence of glucose.

The potential role of insulin in regulating protein synthetic pathways in AP is twofold by virtue of its ability to stimulate both glucose metabolism and protein synthesis from glycine in the absence of glucose as substrate. Whether the effect of insulin upon protein synthesis as measured in vitro is reflected in control of the specialized pathways of hormone synthesis remains to be determined. Three approaches to the resolution of this problem warrant further investigation; a search for effects of insulin upon in vitro synthesis of specific trophins, the study of insulin effects upon isolated pituitary cell lines and re-examination of pituitary function in diabetic animals and man.

*If one postulated an insulin sensitive mass one fifth the total tissue mass, then the calculated increase in glucose uptake or protein synthesis for this smaller mass would be 100 per cent rather than 20 per cent as found with maximal insulin stimulation.

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