Biosynthesis of Growth Hormone in the Rat Anterior Pituitary Gland

STIMULATION OF BIOSYNTHESIS *IN VITRO* BY INSULIN

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The effect of insulin on the incorporation of radioactive leucine into growth hormone was investigated by using rat anterior pituitary glands incubated *in vitro*. A 50% stimulation over control values was observed at insulin concentrations above $2\mu\text{M}$ (280 munits/ml). The effect was specific for growth hormone biosynthesis, over the range $1\text{--}5\mu\text{M}$ -insulin (140–700 munits/ml). Lower more physiological concentrations had no significant effect in this system. Above $10\mu\text{M}$ (1.4 units/ml) total protein synthesis was also increased. The stimulation of growth hormone synthesis could be partially blocked by the addition of actinomycin D, suggesting that RNA synthesis was involved. Insulin was found to stimulate the rate of glucose utilization in a similar way to growth hormone synthesis. 2-Deoxyglucose and phloridzin, which both prevented insulin from stimulating glucose utilization, also prevented the effect of insulin on growth hormone synthesis. If glucose was replaced by fructose in the medium, the effect of insulin on growth hormone synthesis was decreased. We conclude that the rate of utilization of glucose may be an important step in mediating the effect of insulin on growth hormone synthesis.

The factors controlling the secretion of protein hormones by the anterior pituitary gland have been widely investigated. Control is believed to be exercised via a number of releasing and inhibiting hormones produced by the hypothalamus (Guillemin, 1967, 1971; Harris, 1970). A hypothalamic releasing hormone for growth hormone has been characterized (Schally *et al.*, 1971) though some doubts remain as to whether this is the true (or only) physiological growth hormone releasing hormone (Sandow *et al.*, 1972).

The hypothalamic releasing hormones probably act by causing their target cells in the pituitary to discharge the contents of their storage granules into the pituitary blood stream by fusion with the plasma membrane (Couch *et al.*, 1969; Farquhar, 1961). In the case of most anterior pituitary hormones this causes the depletion of the hormone content of the gland to a low amount and results in an increased rate of synthesis to restore the deficiency. With growth hormone, however, the normal pituitary content (about $40\mu\text{g}/\text{mg}$ of wet tissue in 2-month-old male rats, increasing to about $65\mu\text{g}/\text{mg}$ at 4 months; Birge *et al.*, 1967) is more than sufficient to cope with the amounts released by physiological stimuli *in vitro* (approximately 1–2% of the total content per h; Frohman *et al.*, 1971; A. Betteridge, unpublished results). Thus there may not be a close linkage between control of secretion and biosynthesis for growth hormone; the two processes may be controlled quite dif-

ferently and metabolic factors that affect growth hormone release indirectly, via the hypothalamus, may affect growth hormone synthesis directly.

A number of reports have been published recently showing that the rate of incorporation of amino acids into rat and bovine growth hormones *in vitro* can be influenced specifically. Yamamoto *et al.* (1970), by using a disc-electrophoresis method to purify the labelled product, and Burek & Frohman (1970), by using an immunoprecipitation technique, both investigated the effects of age and sex on growth hormone synthesis and agreed that the rate was higher in males than females and was highest during the period of maximum growth. The effects of the oestrous cycle (Ieiri *et al.*, 1971) and the administration of sex hormones (MacLeod *et al.*, 1969) have also been studied. It has been observed by MacLeod & Abad (1968) that the presence of pituitary tumours secreting growth hormone, and also pituitary iso-grafts (MacLeod, 1970), causes a decrease in the rate of growth hormone synthesis, but does not affect the rate of secretion of growth hormone *in vitro*.

The purpose of the present study was to determine the effect *in vitro* of insulin on growth hormone synthesis by rat pituitary explants. This is of particular interest because of the observed interrelation of these two hormones (Korner, 1961; 1970). It is generally believed that insulin has no direct influence on growth hormone release from the anterior pituitary.

Experimental

Materials

L-[4,5-³H]Leucine (specific radioactivity 40–50 Ci/mmol), Na¹²⁵I (specific radioactivity >14 mCi/μg) and D-[¹⁴C]glucose (specific radioactivity 268 mCi/mmol) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Rat growth hormone (NIAMD-Rat GH-B-1) was obtained from Dr. A. Parlow under the auspices of the National Institutes of Health Endocrine Study Programme. Actinomycin D was from Nutritional Biochemical Co., Cleveland, Ohio, U.S.A., phloridzin dihydrate was from Fluka A.G. Chemische Fabrik, Buchs, Switzerland. 2-Deoxy-D-glucose and Naphthol Blue Black (Amido Black) were obtained from Sigma (London) Chemical Co., London S.W.6, U.K. Hyamine hydroxide was obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K. Crystalline ox insulin (23.7 i.u./mg) and insulin-binding reagent were from Wellcome Reagents Ltd., Beckenham, Kent, U.K.

Incubation method

Anterior pituitary glands were collected immediately upon death from 3–4-month-old male Sprague-Dawley rats (fed *ad libitum* on standard rat pellets). The glands were then bisected and the lobes placed in Krebs-Ringer bicarbonate medium (118 mM-NaCl – 1.2 mM-MgSO₄ – 1.2 mM-KH₂PO₄ – 4.7 mM-KCl – 2.5 mM-CaCl₂ – 25 mM-NaHCO₃) containing 11.2 mM-glucose, equilibrated with 5% CO₂ in air to give a final pH of 7.4.

After 15 min the lobes were transferred individually to sterile polystyrene tubes containing 0.4 ml of the incubation medium. Control medium was the Krebs-Ringer buffer containing 5 μCi of [³H]leucine/ml. Unless otherwise stated, the incubations lasted for 5 h at 37°C in 5% CO₂ in air humidified atmosphere.

Measurement of amino acid uptake and incorporation

At the end of the incubation period, the explant was removed from its medium, washed in ice-cold saline, weighed to the nearest 0.02 mg and then homogenized in 0.4 ml of 0.1 M-Tris, adjusted to pH 9.5 with glacial acetic acid. Portions (10 or 20 μl) of the media and homogenates were precipitated with 5% (w/v) trichloroacetic acid and the insoluble material was collected on glass-fibre filters for counting in a toluene scintillator [3.5 g of 2,5-diphenyloxazole and 50 mg of 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene in 1 l of toluene; giving 35% efficiency for ³H in a Beckman LS.100 liquid-scintillation counter]. If uptake of labelled amino acid

into the tissue was to be measured, portions of the homogenate were counted in Triton-toluene scintillator (300 ml of Triton X100–700 ml of toluene–5 g of 2,5-diphenyloxazole; giving 30–35% efficiency). This gave an estimate of total [³H]leucine in the tissue. Subtracting the amount of labelled material precipitated gave the trichloroacetic acid-soluble content of the radioactive material in the explant.

Separation of labelled growth hormone

The rest of the media and homogenates were dialysed overnight at 4°C against 50 mM-Tris, adjusted to pH 8.0 with conc. HCl. Portions (50 or 100 μl) of the non-diffusible materials were subjected to disc electrophoresis on gels formed by the polymerization of 8% (w/v) acrylamide and 0.2% (w/v) methylene-bisacrylamide in glass tubes of internal diameter 6.2 mm. The buffer system was that of Davis (1964) but sample and spacer gels were not employed. The sample was mixed with 10 μl of a saturated solution of Bromophenol Blue in glycerol and the gels were run at 5 mA/tube until the blue marker band reached the bottom of the tube (6–7 cm). The gels were removed and stained in 0.004% Amido Black in 7.5% (v/v) acetic acid until the predominantly growth hormone-containing band (Lewis *et al.*, 1965) was completely stained. The stained gels were scanned with a Joyce-Loebl Chromoscan and the growth hormone content was calculated by using a standard curve previously prepared for the machine with purified rat growth hormone (NIAMD-Rat GH-B-1) under the same conditions as outlined above.

The growth hormone band was then excised and treated for scintillation counting by dissolving in H₂O₂ at 50°C overnight as detailed by Moss & Ingram (1965). Triplicate gels were run for each sample of medium or homogenate and the mean value was determined. Results from a number of experiments were pooled and Student's *t* test was used to assess the difference between various treatments and control values.

Measurement of the rate of glucose oxidation

The rate of oxidation of D-[U-¹⁴C]glucose, by anterior pituitary lobes, was measured by a modification of the method of Ashcroft *et al.* (1972). Bisected anterior pituitaries were placed in 10 ml Ehrlenmeyer flasks together with 0.5 ml of medium containing 1.25 μCi of [¹⁴C]glucose. The flasks were gassed with 5% CO₂ in air, stoppered and shaken at 37°C for 3 h. The reaction was stopped by the addition of 0.2 ml of 0.2 M-HCl and 0.4 ml of Hyamine hydroxide was placed in a small plastic cup suspended inside the flask. The flasks were then shaken for a further hour. The Hyamine was then transferred to a counting

bottle containing toluene scintillator and counted for radioactivity. The pituitary tissue was removed from the flask and weighed.

Radioimmunoassay for insulin

The amount of immunoreactive insulin present in the medium was measured by a double-antibody radioimmunoassay system, essentially that of Hales & Randle (1963). Crystalline ox insulin was used as standard. The medium was diluted to produce approx. 100 μ units/ml and this was assayed by the procedure outlined in the Wellcome 'Insulin Binding Reagent for Radioimmunoassay' kit.

Results

Effect of various concentrations of insulin on incorporation into growth hormone

To investigate the effect of insulin on the rate of incorporation of labelled precursor amino acid into growth hormone, ox insulin was added to the incubation medium at concentrations ranging from 1–50 μ M. The results are expressed in Fig. 1. To calculate total growth hormone synthesis the results from tissue and medium are combined and expressed in terms of d.p.m. of radioactivity incorporated/mg wet wt. of pituitary tissue. The effect of the additive is expressed in terms of mean d.p.m. ($\pm$ S.E.M.)/mg as a percentage of the control value.

It was observed that insulin, at a concentration greater than 1 μ M (140 munits/ml) caused a 40–60% stimulation of [3 H]leucine incorporation over control values. Although there is possibly an increase in stimulation with increased insulin concentration, the effect is by no means linear. This may be due to the rather high concentrations, physiologically, being used at the upper end of the scale. In addition, the stimulation by insulin, of incorporation into growth hormone released into the medium, was similar to that observed for total growth hormone synthesis. The specific radioactivity (d.p.m./ μ g) of both total and released growth hormone was increased by insulin. The total amount of growth hormone released (expressed as μ g of growth hormone in the medium/mg of wet tissue) was not affected by the inclusion of insulin in the medium. From this it seems that insulin was not exerting any direct effect on the release mechanism. In subsequent experiments the results from media and homogenates were pooled to give a measurement of total incorporation.

It was possible that the insulin was being rapidly degraded under the conditions used, which would explain the need for such high initial concentrations. To test this possibility samples of the medium were taken at various times during the incubation and subjected to a radioimmunoassay to determine the rate of degradation. It was found that the amount of

Fig. 1. Effect of insulin concentration on the rate of growth hormone synthesis in rat anterior pituitary lobes *in vitro*

Anterior pituitary lobes were incubated in the Krebs-Ringer buffer containing [3 H]leucine and various concentrations of insulin, for 5 h at 37°C. The rate of growth hormone synthesis by rat pituitary *in vitro* was measured as described in the text and the results are expressed as the percentage stimulation over the control value for each insulin concentration. Each experimental point is the mean $\pm$ S.E.M. for the numbers of observations shown in parentheses. Results significantly different from control values ($P < 0.05$) are shown by an asterisk.

degradation was typically about 20% over 5 h (maximum 25%), which corresponds to a half-life of 11.5 h for insulin (initially at 5 μ M) under these conditions. Obviously this rate of degradation would not account for the need to add insulin at a concentration 100 times the normal serum concentrations at the beginning of a 5 h incubation.

Throughout these experiments it was noted that the specific radioactivity of the released growth hormone was nearly always 2.5–3 times higher than that of the growth hormone retained in the tissue, suggesting that newly synthesized growth hormone is

preferentially released from the pituitary. This observation could be important in the context of the suggestion by Collu & Martini (1972) that the pituitary may contain separate pools for storage and for release of growth hormone. If this is the case, it is possible that newly synthesized growth hormone passes preferentially into the latter pool.

Effect of insulin on the incorporation into total protein

The effect of insulin on incorporation into growth hormone could be merely the result of a general increase in the rate of protein synthesis in the pituitary tissue. To investigate this hypothesis, media and homogenates were analysed for radioactive material which was insoluble in trichloroacetic acid and an estimate of the amount of 'free' radioactive leucine in the incubated tissue was also carried out. As can be seen from Fig. 2, in the control incubations, the intra-

Fig. 2. Effect of $5\mu\text{M}$ -insulin on the rate of uptake and incorporation into protein of $[^3\text{H}]$ leucine by rat anterior pituitary lobes *in vitro*

Anterior pituitary lobes were incubated at 37°C in the Krebs-Ringer buffer, containing $[^3\text{H}]$ leucine and in the presence or absence of $5\mu\text{M}$ -insulin for various periods of time. The amount of trichloroacetic acid-soluble radioactivity in the control (○) and insulin-treated (△) pituitary lobes was measured as described in the text. The trichloroacetic acid-insoluble radioactivity in the tissue was also measured. Control (□), insulin-treated (●). Each experimental point is the mean of three separate determinations.

tissue concentration of $[^3\text{H}]$ leucine reaches a plateau value before the 2h time-point and remains at that value for at least another 3h. This is in agreement with the results of Samli *et al.* (1971). We also confirmed that total protein synthesis is approximately linear with time over the period studied. The rate of total protein synthesis was not significantly affected by the presence of $5\mu\text{M}$ (0.7 units/ml) of insulin in the incubation medium and the intracellular amount of $[^3\text{H}]$ leucine seems to plateau at a slightly lower value, if anything, in the presence of the hormone.

In addition, a time-course of incorporation into growth hormone was carried out and the results are shown in Fig. 3. The rate of total growth hormone synthesis is linear for up to at least 6h. There is a lag period of approx. 2h before radioactive growth hormone appears in the medium.

Fig. 4 shows the rate of incorporation of labelled amino acid (L-leucine) into trichloroacetic acid-insoluble protein released into the incubation medium. There is very little difference between the curves for control and insulin-treated tissue. After an initial lag period, which is roughly the time required for synthesis of new protein and its processing

Fig. 3. Time-course of incorporation of $[^3\text{H}]$ leucine into growth hormone by rat anterior lobes *in vitro*

Anterior pituitary lobes were incubated at 37°C in the Krebs-Ringer buffer, containing $5\mu\text{Ci}$ of $[^3\text{H}]$ leucine/ml, for various lengths of time. The rate of incorporation into growth hormone, both that retained in the tissue and that released into the medium was measured as described in the text. The rate of total growth hormone synthesis (○) and the rate of appearance of newly synthesized growth hormone in the medium (●) are shown. Each experimental value is the mean of three separate observations.

Fig. 4. Effect of $5 \mu\text{M}$ -insulin on the rate of incorporation of $[^3\text{H}]$ leucine into protein released into the medium by rat anterior pituitary lobes in vitro

Anterior pituitary lobes were incubated at 37°C in the Krebs-Ringer buffer, containing $[^3\text{H}]$ leucine and in the presence or absence of $5 \mu\text{M}$ -insulin, for various periods of time. The amount of trichloroacetic acid-insoluble radioactivity released into the medium by the control (○) and insulin-treated (●) pituitaries was measured as described in the text. Each experimental point is the mean of three separate determinations.

before release into the medium, the rate of release increases up to about 4 h and then slows down slightly. The exact cause of this is unknown but it could be due to the exhaustion of factors needed for protein release.

If the results from Fig. 1 are expressed in terms of d.p.m. incorporated into growth hormone as a percentage of total incorporation into trichloroacetic acid-insoluble material, they give the graph shown in Fig. 5. The addition of $2 \mu\text{M}$ -insulin to the medium causes a sharp rise in the amount of growth hormone synthesized compared with total protein synthesis, which is also the concentration at which the absolute amount of growth hormone synthesis was shown to increase by about 50% (Fig. 1). From 2 – $10 \mu\text{M}$ -insulin (0.3 – 1.4 units/ml) in the medium had no additional effect on the relative rate of growth hormone synthesis. The addition of between 10 and $50 \mu\text{M}$ -insulin (1.4 – 7.0 units/ml) caused a drop in the relative rate of growth hormone synthesis although the absolute rate increased quite sharply. This would

Fig. 5. Effect of insulin concentration on the rate of growth hormone synthesis in rat anterior pituitary lobes in vitro relative to the total rate of protein synthesis

Anterior pituitary lobes were incubated for 5 h at 37°C in the Krebs-Ringer buffer containing $[^3\text{H}]$ leucine and various concentrations of insulin. The rate of growth hormone synthesis was measured as described in the text and expressed as a percentage of the incorporation of $[^3\text{H}]$ leucine into trichloroacetic acid-insoluble material in the tissue. Each experimental point is the mean $\pm$ S.E.M. for the numbers of observations shown in parentheses. Results significantly different from control ($P < 0.05$) are indicated by an asterisk.

suggest that insulin at these higher concentrations, is affecting protein synthesis non-specifically.

From these observations it can be concluded that insulin, when added to the incubation medium to a concentration of $2\mu\text{M}$ (284 munits/ml), has a specific effect on the biosynthesis of growth hormone by the rat anterior pituitary.

Sensitivity of the insulin stimulation to actinomycin D

The stimulatory effect of insulin on growth hormone synthesis could be mediated by an increase in ribosomal or messenger RNA synthesis as has been suggested for insulin (Pilkis & Salaman, 1972) and growth hormone (Oravec & Korner, 1971; Salaman *et al.*, 1972) in rat liver.

To test this hypothesis, the antibiotic actinomycin D was used. This has been shown to inhibit DNA-dependent RNA synthesis in many systems and we were able to confirm the observations of Labrie *et al.* (1971) that $25\mu\text{g}$ of actinomycin D/ml in the medium inhibited incorporation of [^3H]uridine into total anterior pituitary RNA by at least 90%.

As can be seen in Table 1, actinomycin D did not significantly inhibit growth hormone synthesis, either in terms of actual incorporation, or relative to total protein synthesis, when compared with the control values. When $5\mu\text{M}$ -insulin was included in the incubations it was apparent that the stimulatory effect was decreased by about half. The effect of actinomycin D was never complete, so it must be deduced that the action of insulin cannot be completely explained in terms of an effect on RNA synthesis.

Role of glucose in the stimulatory effect of insulin

Since the action of insulin could not be wholly explained in terms of an effect on RNA synthesis, various other possible modes of action were contemplated. Since insulin did not have a marked effect on total protein synthesis or leucine uptake into the tissue, it was decided to investigate the possible role of glucose.

To determine whether insulin affected glucose utilization in rat anterior pituitaries *in vitro*, the rate of oxidation of glucose to CO_2 was measured in the presence of various concentrations of insulin. The results from these measurements are shown in Fig. 6. Both 1 and $2\mu\text{M}$ -insulin (140 and 280 munits/ml) in the medium caused a significant stimulation of about 45% in the rate of glucose oxidation. Concentrations higher than these were less effective. When the curve in Fig. 6 is compared with that in Fig. 5, a number of similarities can be seen. Both curves rise sharply at one concentration and remain at a plateau before falling slightly as the concentration is increased even further. It is noticeable that stimulation of glucose oxidation first occurs at $1\mu\text{M}$ -insulin (140 munits/ml) whereas a similar rise occurs in the relative rate of growth hormone synthesis at $2\mu\text{M}$.

Thus, although there is no evidence for a direct relationship between the increase in glucose utilization and the increase in growth hormone synthesis, the fact that both phenomena are caused by approximately the same dose of insulin suggests that there could be some relationship between the two.

It is known that 2-deoxy-D-glucose is absorbed by the intestine (Wilson & Crane, 1958) and is transported in erythrocytes (Hillman *et al.*, 1959) by a

Table 1. *Effect of actinomycin D in vitro on the stimulation of growth hormone synthesis by insulin*

Anterior pituitary lobes were incubated for 5 h at 37°C in the Krebs-Ringer buffer containing [^3H]leucine together with $5\mu\text{M}$ -insulin and $25\mu\text{g}$ of actinomycin D/ml. The effect on incorporation of [^3H]leucine into trichloroacetic acid-insoluble protein and growth hormone was measured as described in the text and compared with the effects of adding $5\mu\text{M}$ -insulin and $25\mu\text{g}$ of actinomycin D/ml to separate incubations. The results are expressed as the means $\pm$ S.E.M. of the numbers of determinations shown in parentheses. Any results significantly different from the control values are indicated as follows: ** $P < 0.01$, *** $P < 0.001$.

Incubation medium ...	Control (8)	$5\mu\text{M}$ -Insulin (6)	$25\mu\text{g}$ of actinomycin D/ml (6)	$5\mu\text{M}$ -Insulin + $25\mu\text{g}$ of actinomycin D/ml (5)
Growth hormone synthesis				
(a) d.p.m./mg	5322 ± 259	$7770 \pm 319^{**}$	4896 ± 639	6386 ± 692
(b) percentage of control value	100 ± 5.1	146.1 ± 5.9	92.0 ± 12.1	120.2 ± 12.9
Total protein synthesis (percentage of control value)	100 ± 6.0	90.2 ± 9.9	108.2 ± 2.9	100.8 ± 4.1
Growth hormone synthesis (percentage of total protein synthesis)	36.7 ± 2.6	$59.4 \pm 2.2^{***}$	31.2 ± 1.5	43.7 ± 4.4

Fig. 6. Effect of various concentrations of insulin in the medium on the rate of glucose oxidation *in vitro*

Anterior pituitary lobes were shaken for 3 h at 37°C in flasks containing the Krebs-Ringer buffer, [^{14}C]glucose and various concentrations of insulin. The amount of radioactive CO_2 released was measured as described in the text and the results are expressed as nmol of glucose oxidized/mg of tissue in 3 h. Each experimental point is the mean $\pm$ S.E.M. (the numbers of observations are shown in parentheses). Results significantly different from control are indicated by an asterisk, ($P < 0.05$).

similar route to glucose. However, it is unable to undergo facilitated diffusion across the intestinal wall (Crane & Crane, 1959) and into human erythrocytes (Lefevre & Marshall, 1958), owing to lack of hydroxyl group on its C_2 carbon atom. Kipnis & Cori (1959) showed that the 2-deoxy-D-glucose, which is transported into rat diaphragm, is phosphorylated by hexokinase in competition with glucose and quickly accumulates as 2-deoxyglucose 6-phosphate. When this reaches a certain intracellular concentration it prevents further penetration of the tissue by glucose. With this in mind, 2-deoxy-D-glucose was added to the incubation medium to determine whether this relatively mild interference with glucose uptake and utilization would affect the action of insulin.

When 2-deoxyglucose is added at a concentration

of 5 mM, the rate of growth hormone synthesis is not significantly different from the control rate (Table 2). However, if 5 mM-2-deoxyglucose is added together with 5 μM -insulin, the 60% stimulation (relative to total incorporation) normally observed, is almost completely abolished. Further, the combination of the two additives leads to a quite drastic inhibition of total protein synthesis including growth hormone. This could be explained by the observation by Kipnis & Cori (1959), that the addition of insulin caused the accumulation of 2-deoxyglucose 6-phosphate in rat diaphragm to reach a higher concentration before further uptake ceased. It is possible that this higher concentration was inhibiting protein synthesis by some means. Despite this effect, expressing the results in terms of growth hormone synthesis as a percentage of total synthesis shows that 2-deoxyglucose is suppressing the effect of insulin. The inference from this is that insulin is acting in some way via glucose.

Effect of a specific inhibitor of glucose transport

If the rate of glucose transport into the cell is the process stimulated by insulin, then it is possible that either the intracellular energy level is limiting under control conditions or there is a specific requirement for glucose for some other reason. To test whether the inhibition of glucose transport is sufficient to block the action of insulin, phloridzin was used. Phloridzin is a glucoside of phloretin. Its effects on glucose transport across intestinal tissue have been well reported, both *in vivo* (Bogdanove & Barker, 1950; Fisher & Parsons, 1950) and *in vitro* (Newey *et al.*, 1959). In addition it has been shown to have a specific effect on glucose uptake into human erythrocytes (Lefevre, 1948), Ehrlich ascites-tumour cells (Crane *et al.*, 1957), and rabbit kidney-cortex slices (Crane & Crane, 1959). The specificity of phloridzin is such that it has been shown not to affect fructose transport in at least one system (Fridhandler & Quastel, 1955). At a concentration of about 0.1 mM, phloridzin does not affect metabolism (Crane *et al.*, 1957) and has been shown to have little or no effect on amino acid transport across the intestinal wall (Ohnell & Höbner, 1939) or into rat kidney-cortex slices (Segal *et al.*, 1963).

When 0.1 mM-phloridzin was added to the incubation medium, there was a slight, but non-significant, inhibition of both growth hormone synthesis and total protein synthesis (Table 3). The presence of phloridzin, however, completely blocked the effect of insulin on growth hormone synthesis, but there was a slight stimulation of total protein synthesis. When growth hormone synthesis, relative to total protein synthesis, was measured, the results were much the same (Table 3). Overall, the effect of phloridzin is to abolish almost completely the effect of insulin on growth hormone synthesis and this increases the

Table 2. *Effect of 2-deoxy-D-glucose on the stimulation of growth hormone synthesis by insulin in vitro*

Anterior pituitary lobes were incubated for 5 h at 37°C in the Krebs–Ringer buffer containing [³H]leucine, 5 μM-insulin and 5 mM-2-deoxy-D-glucose. The effect on incorporation of [³H]leucine into growth hormone and trichloroacetic acid-insoluble protein was measured as described in the text and compared with the effects of adding 5 μM-insulin and 5 mM-2-deoxy-D-glucose to separate incubations. The results are expressed as the means ± s.e.m. of the numbers of observations shown in parentheses. Any results significantly different from the control values are indicated as follows: ** $P < 0.01$, *** $P < 0.001$.

Incubation medium ...	Control (8)	5 μM-Insulin (6)	5 mM-Deoxyglucose (5)	5 μM-Insulin + 5 mM-deoxyglucose (5)
Growth hormone synthesis				
(a) d.p.m./mg	5160 ± 240	7534 ± 309**	4799 ± 361	3148 ± 308**
(b) percentage of control value	100 ± 5.2	145.9 ± 5.5	93.4 ± 7.2	61.1 ± 5.9
Total protein synthesis	100 ± 5.5	90.1 ± 10.1	99.0 ± 5.4	53.0 ± 5.8***
(percentage of control value)				
Growth hormone synthesis	37.3 ± 3.3	60.4 ± 2.2***	35.1 ± 4.8	42.9 ± 3.7
(percentage of total protein synthesis)				

Table 3. *Effect of phloridzin on the stimulation of growth hormone synthesis by insulin in vitro*

Anterior pituitary lobes were incubated for 5 h at 37°C in the Krebs–Ringer buffer containing [³H]leucine, 5 μM-insulin and 0.1 mM-phloridzin. The effect on incorporation of [³H]leucine into growth hormone and trichloroacetic acid-insoluble protein was measured as described in the text and compared with effects of adding 5 μM-insulin and 0.1 mM-phloridzin to separate incubations. The results are expressed as the means ± s.e.m. of the numbers of observations shown in parentheses. Any results significantly different from the control values are indicated as follows: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Incubation medium ...	Control (8)	5 μM-Insulin (6)	0.1 mM-Phloridzin (4)	5 μM-Insulin + 0.1 mM-phloridzin (5)
Growth hormone synthesis				
(a) d.p.m./mg	5510 ± 264	8100 ± 303**	4689 ± 665	6006 ± 595
(b) percentage of control value	100 ± 4.8	147.0 ± 5.5	85.1 ± 15.7	109.0 ± 10.8
Total protein synthesis	100 ± 6.9	90.1 ± 9.4	90.1 ± 12.8	134.5 ± 11.7*
(percentage of control value)				
Growth hormone synthesis	34.6 ± 2.4	56.0 ± 2.0***	32.6 ± 1.6	28.1 ± 3.2
(percentage of total protein synthesis)				

Table 4. *Effect of 2-deoxyglucose and phloridzin on the rate of glucose oxidation in vitro*

Anterior pituitary lobes were shaken for 3 h at 37°C in stoppered flasks containing the Krebs–Ringer buffer, [¹⁴C]glucose and the various additions under test. The amount of radioactive CO₂ released was measured as described in the text. Each value is the mean (±s.e.m.) of nmol of glucose oxidized/mg of tissue during the 3 h period. The numbers of observations are shown in parentheses.

Additions to medium		Rate of glucose oxidation		P (difference from control)
		(nmol/mg)	(% of control)	
None	(8)	7.15 ± 0.49	100	—
5 mM-Deoxyglucose	(6)	5.56 ± 0.41	77.7	<0.05
10 mM-Deoxyglucose	(4)	4.12 ± 0.08	57.5	<0.001
0.1 mM-Phloridzin	(5)	6.57 ± 0.70	91.9	Not significant
0.2 mM-Phloridzin	(5)	5.70 ± 0.45	79.6	<0.05
5 μM-Insulin + 5 mM-deoxyglucose	(5)	5.14 ± 0.47	71.8	<0.02
5 μM-Insulin + 0.1 mM-phloridzin	(5)	6.59 ± 0.60	92.1	Not significant

likelihood that glucose transport is required to enable insulin to stimulate the rate of growth hormone synthesis by the anterior pituitary.

Effect of 2-deoxyglucose and phloridzin on glucose oxidation

At this point it was important to ascertain whether 2-deoxyglucose and phloridzin exhibited similar inhibitory effects on pituitaries *in vitro* as found in other tissues. Therefore the effect of these agents on glucose utilization was measured. The method chosen was to determine the rate of glucose oxidation in the presence of these two substances and also to investigate whether the presence of either modified the stimulation of glucose oxidation observed in the presence of insulin.

The results of these experiments are shown in Table 4. The 5mm-deoxyglucose (the concentration used in the above experiments) inhibited glucose oxidation significantly, by about 22% compared with control values. The 10mm-deoxyglucose was more effective (about 43% inhibition). The combination of 5 μ M-insulin and 5mm-deoxyglucose caused an inhibition not significantly different from that of 5mm-deoxyglucose alone. However, as was shown in Fig. 6, 5 μ M-insulin stimulated glucose oxidation by approximately 25% when included alone in the medium. Therefore one can conclude that 2-deoxyglucose is effective at preventing insulin from stimulating glucose oxidation in the same way as has been shown for growth hormone synthesis in Table 2.

When the results obtained with phloridzin are

examined, a similar picture emerges. Although 0.1mm-phloridzin does not inhibit glucose oxidation significantly, increasing the concentration to 0.2mm causes an inhibition of 30%. The rate of glucose oxidation, when 0.1mm-phloridzin and 5 μ M-insulin are included together in the medium, is not significantly different from that observed for 0.1mm-phloridzin alone. Again, however, the rate is significantly different from that observed with insulin alone. One can conclude that 0.1mm-phloridzin is effective in preventing the stimulation, by 5 μ M-insulin, of the rate of glucose oxidation.

Specific requirement for glucose

Further support for the idea that insulin stimulation is mediated by a specific effect on glucose utilization would be the observation that insulin was unable to act if an alternative energy source was used. To test this, a medium was used that contained 11.2mm-D-fructose instead of the 11.2mm-glucose normally used in the Krebs-Ringer buffer.

Replacing glucose by fructose causes no significant difference in the amount of incorporation into growth hormone, but protein synthesis as a whole is increased by about 35% in the fructose medium (Table 5). The net result of this is to lower the relative proportion of growth hormone compared with total newly synthesized protein.

When insulin was added to the fructose-containing medium there was a stimulation of incorporation into growth hormone of about 25% compared with the 50% stimulation observed in glucose medium (Table

Table 5. *Effect of replacing glucose in the medium by fructose on the stimulation of growth hormone synthesis by insulin in vitro*

Anterior pituitary lobes were incubated for 5h at 37°C in either Krebs-Ringer buffer with glucose or Krebs-Ringer buffer with 11.2mm-D-fructose, containing [3 H]leucine and 5 μ M-insulin. The effects on incorporation of [3 H]leucine into growth hormone and trichloroacetic acid-insoluble protein were measured as described in the text and compared with the effects of the two media without added insulin. The results are expressed as the means $\pm$ S.E.M. of the numbers of observations shown in parentheses. Any results significantly different from the control values are indicated as follows: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Incubation medium	Control (Krebs-Ringer buffer) (8)	Krebs-Ringer buffer + 5 μ M-insulin (6)	Fructose medium (4)	Fructose medium + 5 μ M-insulin (5)
Growth hormone synthesis				
(a) d.p.m./mg	5261 $\pm$ 263	7679 $\pm$ 351**	4940 $\pm$ 472	6103 $\pm$ 355
(b) percentage of control value	100 $\pm$ 5.0	145.9 $\pm$ 6.6	93.9 $\pm$ 8.9	116.0 $\pm$ 6.7
Total protein synthesis (percentage of control value)	100 $\pm$ 7.2	90.1 $\pm$ 8.9	134.3 $\pm$ 9.2*	139.3 $\pm$ 7.1**
Growth hormone (percentage of total protein synthesis)	37.6 $\pm$ 2.6	61.9 $\pm$ 2.3***	26.3 $\pm$ 3.7*	31.6 $\pm$ 3.6

5). When the relative rate of growth hormone synthesis is considered (Table 5) the addition of insulin causes a 20% stimulation in the fructose medium compared with a 60% stimulation in the Krebs-Ringer buffer. Insulin may still have an effect on growth hormone synthesis when fructose replaces glucose, but the magnitude is much decreased. Since there is no reason to suppose that fructose is an inferior energy source compared with glucose (in fact the stimulation of protein synthesis might suggest the opposite, if anything), it must be concluded from these results that there is a specific requirement for glucose in the medium to enable insulin to act.

Discussion

The stimulation of incorporation into growth hormone caused by insulin is not a surprising result when it is considered that growth hormone is known to have diabetogenic properties *in vivo* and plasma concentrations of growth hormone are known to increase after administration of insulin (Roth *et al.*, 1963). The effect of insulin on incorporation of amino acids into rat diaphragm protein is well documented (see Wool *et al.*, 1968). The stimulation of amino acid incorporation has been shown to occur when glucose transport cannot take place (Manchester & Young, 1958) and Wool (1965) has shown that incorporation is not dependent on amino acid transport into the tissue. These observations only relate to total muscle protein synthesis and probably reflect the anabolic role of insulin. Any effect on protein synthesis in the pituitary might be expected to relate to the interrelationship of insulin and the pituitary hormones which affect metabolism. Goodner & Krouse (1972) were able to show a 20–30% stimulation of incorporation into total pituitary protein in the presence of insulin at a concentration of $4\mu\text{g/ml}$ (i.e. $0.7\mu\text{M}$) but they were unable to detect any effect on the synthesis of specific proteins such as growth hormone and prolactin. We were able to obtain more specific results concerning growth hormone synthesis by using an insulin concentration of between 1 and $5\mu\text{M}$ (140–700 munits/ml), whereas at insulin concentrations greater than $10\mu\text{M}$ (1.4 units/ml) there was a non-specific stimulation of total protein synthesis. It may be that these relatively high concentrations are required because insulin does not easily penetrate a piece of tissue the size of an anterior pituitary lobe under the conditions used. The results with the radioimmunoassay showed that the concentration of insulin in the medium decreased to a certain extent, presumably owing to proteolytic activity, but not sufficiently to explain the need to add insulin at concentrations a 100-fold in excess of those found in plasma. The successful production of a dispersed-cell preparation may show whether the stimulation also occurs at more physiological insulin concentrations.

After it was shown that insulin was not having any significant effect on leucine uptake and incorporation into total pituitary protein over the range from $1\text{--}2\mu\text{M}$ (140–280 munits/ml), it became almost certain that the effect of insulin, at these concentrations, was a true stimulation of synthesis of a specific protein, i.e. growth hormone (or a precursor). From the experiments with actinomycin D, it is clear that insulin exerts some of its effect via a process that probably involves RNA synthesis. On the other hand, Labrie *et al.* (1971) have shown that the stimulation of incorporation into total pituitary protein (and release of this protein into the medium), which is caused by dibutyl adenosine 3':5'-cyclic monophosphate (dibutyl cyclic AMP), is completely insensitive to actinomycin D. The inference is that the two stimulations occur by different mechanisms and it seems probable that cyclic AMP is not a mediator of the effect of insulin on growth hormone synthesis. Similar studies on the stimulation of insulin biosynthesis in isolated pancreatic islets, or pancreatic slices, caused by raising the glucose concentration in the medium (Howell *et al.*, 1965; Parry & Taylor, 1966; Lin *et al.*, 1972; Morris & Korner, 1970; Permutt & Kipnis, 1972a,b) suggest that this process may be partially susceptible to actinomycin D. Indeed, Permutt & Kipnis (1972a) showed that the effect of glucose is biphasic and the second phase can be completely inhibited by preincubation with actinomycin D and then removing the stimulus.

Since actinomycin D could not completely block the stimulation of growth hormone synthesis by insulin, further experiments were carried out to determine what other processes were involved.

From the results it is concluded that the rate of utilization of glucose, by the anterior pituitary, is stimulated by insulin in a similar way to growth hormone synthesis. The results with 2-deoxyglucose and phloridzin imply that these inhibitors can prevent the effects of insulin on both growth hormone synthesis and glucose utilization. The fact that all treatments tested showed similar effects on the two phenomena suggests that they could be linked in some way.

When fructose replaces glucose, the rate of total protein synthesis in the pituitary is slightly increased, suggesting that it is at least as good an energy source as glucose. However, the stimulation of growth hormone synthesis by insulin is very much decreased in the fructose-containing medium. As insulin is unable to act if glucose oxidation is inhibited, utilization of glucose by the anterior pituitary is an important factor controlling the rate of growth hormone synthesis. Insulin could therefore act by stimulating the rate of glucose utilization in the rat pituitary, causing growth hormone synthesis to increase by some mechanism. The fact that insulin causes a smaller stimulation of growth hormone synthesis, when fructose replaces glucose in the medium, can be explained in two ways.

Either the intracellular requirement for glucose utilization is not simply as a supply of energy, i.e. some specific metabolite of glucose is important, or insulin is unable to stimulate fructose oxidation in the same way as it does glucose oxidation. If the latter is the case, the decreased ability of insulin to stimulate growth hormone synthesis in fructose-containing medium could be due to a limiting supply of energy.

The results presented in the present paper strongly suggest that insulin stimulates the biosynthesis of growth hormone. Roth *et al.* (1963) showed that the stimulation of growth hormone released by insulin-induced hypoglycaemia could be prevented by section of the pituitary stalk. This would suggest that insulin may only affect growth hormone release, as distinct from synthesis, via a pathway involving the hypothalamus. The fact that we were unable to measure any effect on the release of growth hormone with our assay system, substantiates this view.

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