A Direct Effect of Growth Hormone on the Incorporation of Precursors into Proteins and Nucleic Acids of Perfused Rat Liver

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1. The livers of rats were perfused *in situ*. When the amino acid concentration in the perfusing medium was that present in rat plasma, the addition of growth hormone to the medium stimulated the incorporation of labelled amino acids into liver protein only marginally and not to a statistically significant extent. When, however, the amino acid concentration was raised to three times that present in rat plasma, growth hormone significantly and substantially stimulated amino acid incorporation into protein within 30 min. of perfusion of normal rat liver. 2. A significant effect of growth hormone on labelling of normal rat-liver protein was seen with concentrations not much greater than those reported to be present in rat plasma. 3. The labelling of nucleic acids of normal and hypophysectomized rat liver by [³H]orotic acid was enhanced by addition of growth hormone to the perfusing medium when normal concentrations of amino acids were used. 4. At elevated concentrations of amino acids, growth hormone stimulated labelling of nucleic acids of hypophysectomized rat liver at 30 and 60 min. of perfusion. Under these conditions, nucleic acids of normal rats were labelled to about the same extent in control and hormone-treated livers at 30 min. and, because of a fall in the radioactivity of the control livers, there was more labelled nucleic acids in growth-hormone-treated livers at 60 min. than in the control livers. 5. Growth hormone, unlike insulin, had no inhibitory effect on the release of glucose by the perfused liver. 6. It is concluded that growth hormone can stimulate the incorporation of precursor into proteins and nucleic acids of liver directly and without the mediation of other organs or of insulin.

It is well established that hypophysectomy lessens, and that treatment with growth hormone stimulates, the growth of animals and the synthesis of protein and RNA of liver and other tissues (Simpson, Evans & Li, 1949; Talwar, Panda, Sarin & Tolani, 1962; Korner, 1960, 1964). Growth hormone affects the ability of rat-liver ribosomes to incorporate amino acids into protein *in vitro* (Korner, 1961) and to attach to or to translate added template RNA (Korner & Gumbley, 1966; Korner, 1967). The RNA-polymerase activity of rat-liver nuclei is also stimulated by treatment of rats with growth hormone (Pegg & Korner, 1965; Widnell & Tata, 1966).

In all these experiments, the effects of growth hormone on liver were examined after injection of

the hormone into the animal: attempts to demonstrate hormone action *in vitro* with liver cell-free systems have failed. Growth hormone *in vitro* is, however, known to stimulate amino acid incorporation into proteins of isolated diaphragm of the rat (Kostyo & Knobil, 1959; Manchester & Young, 1959). It is therefore possible that growth hormone does not act directly on liver but indirectly through its influence on other tissues or endocrine glands. One suggestion, for example (Young, 1939, 1945, 1964), is that growth hormone acts by stimulating insulin secretion from the β -cells of the islets of Langerhans. The experiments described in the present paper are an attempt to settle this point.

The perfused liver system developed by Mortimore (1961), which has proved useful in studying effects of insulin on liver *in vitro* (Mortimore, 1963; Exton, Jefferson, Butcher & Park, 1966), has been used to study the effects of growth hormone *in vitro*. The results show that growth hormone can stimulate uptake of precursors into protein and

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RNA of liver directly and without the mediation of the other organs or of insulin.

MATERIALS AND METHODS

Growth hormone. Ox growth hormone (NIH-GH-B10 and NIH-GH-B11) was kindly supplied by the National Institutes of Health (Bethesda, Md., U.S.A.). The mean potency was given as 0.81 U.S.P. unit/mg. Its thyroid-stimulating-hormone potency was given as 0.0084 U.S.P. unit/mg. and its luteinizing-hormone potency as 0.0038 NIH-LH-S1 unit/mg. A fresh solution of hormone in 0.9% NaCl was prepared for each experiment and 1 ml. was added to each 50 ml. of perfusion medium. An equal volume of 0.9% NaCl was added to the medium used for control livers.

Radioactive material. DL-[1-¹⁴C]Phenylalanine (44.2 mc/m-mole), DL-[1-¹⁴C]leucine (32 mc/m-mole) and [³H]orotic acid (4720 mc/m-mole) were obtained from The Radiochemical Centre (Amersham, Bucks).

Animals. Male and female Wistar rats from a strain bred in this Laboratory were used. Normal fed males (weighing 100–120 g.) or hypophysectomized females (weighing 120–140 g.) were matched according to age and weight (± 2 g.) for each experiment. Because of the sex and weight differences no comparison is made between normal and hypophysectomized rats in these experiments. Hypophysectomy was performed at least 2 weeks before use and the completeness of the operation was judged by the failure of the rats to gain weight during the post-operative period.

Perfusion of livers. Rats were anaesthetized with sodium pentobarbital (50 mg./kg. body wt.) and the livers perfused *in situ* by the technique of Mortimore (1961). The perfusion medium was Krebs bicarbonate buffer (Krebs & Henseleit, 1932) containing bovine albumin powder (3%, w/v) (fraction V from bovine plasma; Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex), EDTA (0.05 mM) and washed rat erythrocytes to give a haematocrit of 20%. Unless noted otherwise, 10 mM-glucose and an amino acid mixture three times the normal rat plasma concentration was present in the medium at the start of perfusion. The amino acid mixture was made up in 0.9% NaCl (pH adjusted to 7.4 with N-NaOH) and contained the L-amino acids in an amount to make the initial perfusate concentration the following for each amino acid (μ M): arginine, 660; asparagine, 115; aspartic acid, 115; threonine, 870; serine, 870; glutamic acid, 570; glycine, 1290; alanine, 1440; valine, 600; methionine, 210; isoleucine, 270; leucine, 510; tyrosine, 270; phenylalanine, 240; histidine, 270; lysine, 1440; proline, 726; cysteine, 114; glutamine, 570; tryptophan, 207. The labelled amino acid or labelled orotic acid (or both) was added as described in the legend of each Figure and Table. The initial recirculating volume of medium was 50 ml., and 2 ml. samples were taken at various time-intervals and analysed for glucose content by the method of Huggett & Nixon (1957). The flow rate was 7.5 ml./min. At the end of perfusion the liver was rapidly removed into ice-cold 0.9% NaCl, washed twice and homogenized in a total volume of 20 ml. of the same solution.

Determination of radioactivity incorporated into protein. Samples (1 ml.) of the homogenized liver were treated with 5 ml. of 7% (w/v) HClO₄. The procedure of Munro, Jackson & Korner (1964) was used to free the resulting precipitate

of amino acids, RNA and lipid and to estimate the radioactivity in the sample. Corrections were made for background counts and self-absorption. Results are reported as counts/min. incorporated/mg. of protein.

Determination of radioactivity incorporated into nucleic acids. The procedure for isolating nucleic acids was essentially that described by Wool & Moyer (1964). Samples (1 ml.) of the homogenized liver were treated with 5 ml. of ice-cold 20% (w/v) trichloroacetic acid. The precipitate was washed twice with 10% (w/v) trichloroacetic acid and twice with 96% (v/v) ethanol, all in the cold. The residue was then heated with 3 ml. of 10% (w/v) NaCl at 100° for 60 min. The mixture was cooled and centrifuged at 1000g for 10 min., and the supernatant was transferred to volumetric tubes to which were added 2.5 vol. of 96% (v/v) ethanol. The mixture was left overnight at -10°. The precipitated nucleic acids were washed twice with 96% (v/v) ethanol and dissolved in 4 ml. of water. One portion of this solution was transferred to an aqueous scintillation solution (Bray, 1960) and the radioactivity estimated in a Nuclear-Chicago scintillation counter. Corrections were made for background counts. The extent of quenching was determined and found to be small, so no correction was made for it. The efficiency of counting was about 10%. Another portion of the aqueous solution was diluted and the E_{260} value measured to estimate the amount of nucleic acids present. The extinction coefficient used was 32.2 mg./ml./cm. (Jackson, 1966). Results are reported as counts/min. incorporated/mg. of nucleic acid.

Statistical treatment of results. The apparatus used enabled two livers to be perfused simultaneously. One of them served as control and the other as the experimental liver. The summated differences between individual pairs often established a statistically significant difference in experiments where the difference between the mean value and that of controls was not great. Although it is permissible to use summated differences between pairs, we have only claimed a significant difference when the mean values of control and experimental groups showed such a difference that the P value was less than 0.01.

RESULTS

At first we attempted to demonstrate an effect of growth hormone at 20 μ g./ml., a dose that stimulates amino acid incorporation into protein in isolated diaphragm (Kostyo & Knobil, 1959), with the arbitrary time of 90 min. of perfusion. Table 1 shows that, under these conditions, growth hormone stimulated the incorporation of [¹⁴C]leucine into liver protein of normal and of hypophysectomized rats, but that the stimulation is only marginal and the differences between means are not statistically significant.

The initial concentration of amino acids in the perfusion medium in these experiments was approximately the same as that reported (Scharff & Wool, 1964; Schimassek & Gerok, 1965) to be present in normal rat plasma (see the Materials and Methods section for details of composition). It was thought that this concentration of amino acids might be limiting for protein synthesis during perfusion, so

Table 1. *Effect of growth hormone on the incorporation of [14 C]leucine into protein of livers from normal and hypophysectomized rats perfused for 90 min.*

At the start of perfusion, the medium contained 10 mM-glucose, the normal rat plasma concentration of amino acids, 0.1 μ C of [3 H]orotic acid/ml., 0.1 μ C of [14 C]leucine/ml. and, where indicated, 20 μ g. of growth hormone/ml. The numbers in parentheses refer to the numbers of observations. The means $\pm$ S.E.M. are given.

Liver donor	Sp. activity of protein (counts/min./mg.)		Increase with growth hormone (%)
	Without growth hormone	With growth hormone	
Normal rats (7)	696 $\pm$ 70	748 $\pm$ 62	14
Hypophysectomized rats (9)	439 $\pm$ 18	495 $\pm$ 22	12

the concentration at the start of perfusion was raised to three times the normal plasma concentration. It was found (Jefferson & Korner, 1967) that the extent of amino acid incorporation into protein of perfused rat liver was greatly influenced by the concentration of amino acids in the perfusing medium.

The results of an experiment with normal rats and with three times the normal concentration of amino acids are shown in Fig. 1. Under these conditions, growth hormone *in vitro* substantially increased the incorporation of [14 C]phenylalanine into liver protein and this effect was apparent within 30 min. of perfusion. The incorporation of labelled phenylalanine into protein was enhanced by 38% after 30 min. and by 41% after 60 min. of perfusion when growth hormone was present in the medium. The growth-hormone effect is statistically significant at 30 and at 60 min.

The results of similar experiments with hypophysectomized rats are shown in Table 2. After 30 min., the differences between control and experimental livers were small (9% with leucine, 16% with phenylalanine), but at 60 min. greater differences are seen (22% and 27%). The results obtained with hypophysectomized rats cannot be compared with those obtained with normal rats for the reason alluded to in the Materials and Methods section.

Table 3 shows the relation between the amount of growth hormone added to the perfusion medium and the stimulation of incorporation of labelled phenylalanine into protein of livers from normal rats. A significant response was detected when 0.1 μ g. of growth hormone/ml. was added to the perfusion medium at the start of perfusion and greater responses (25–30%) were obtained with increasing amounts of growth hormone.

The effect of growth hormone, added to the perfusion medium, on the labelling of liver nucleic acids by [3 H]orotic acid was also studied. The results of the early experiments, with a 90 min. perfusion period and normal plasma concentrations of amino acids, are shown in Table 4. Growth

Fig. 1. Rate of incorporation of [14 C]phenylalanine into protein of perfused livers from normal rats. At the start of perfusion, the medium contained 10 mM-glucose, three times the normal plasma concentration of amino acids, 0.1 μ C of [14 C]phenylalanine/ml. and, where indicated, 20 μ g. of growth hormone/ml. Each point represents the mean of four perfusions. ●, Without growth hormone (control); ○, with growth hormone.

hormone stimulated by over 30% the labelling of nucleic acids in livers of both normal and hypophysectomized rats. This effect is statistically significant for both normal and hypophysectomized rats.

After it had been found that elevated concentrations of amino acids enhanced amino acid incorporation and the stimulation of this incorporation by growth hormone, it was decided to investigate

Table 2. *Effect of growth hormone on the incorporation of labelled amino acids into protein of perfused livers of hypophysectomized rats*

The experimental conditions were the same as those given in Fig. 1 except that 0.1 μ C of [14 C]leucine/ml. was present instead of [14 C]phenylalanine in some of the perfusions. The numbers in parentheses refer to the numbers of observations. The means $\pm$ s.e.m. are given.

Amino acid	Perfusion	Sp. activity of protein (counts/min./mg.)	
		At 30 min.	At 60 min.
[14 C]Leucine	Without growth hormone (2)	96 $\pm$ 24	197 $\pm$ 13
	With growth hormone (2)	105 $\pm$ 12	241 $\pm$ 2
[14 C]Phenylalanine	Without growth hormone (2)	341 $\pm$ 8	448 $\pm$ 189
	With growth hormone (2)	395 $\pm$ 4	567 $\pm$ 56

Table 3. *Effect of growth hormone added to the perfusion medium on the incorporation of [14 C]-phenylalanine into protein of livers from normal rats perfused for 60 min.*

The experimental conditions were the same as those given in Fig. 1.

Growth hormone added to perfusing medium (μ g./ml. of medium)	Radioactivity of protein (% of control)
0.01	101
0.10	115
1.00	124
10.00	128

the effect of the presence of three times the normal plasma concentration of amino acids on the labelling of nucleic acids by [3 H]orotic acid and on the stimulation of the incorporation by growth hormone. We noticed that the incorporation of precursors into nucleic acids was enhanced by the high concentration of amino acids (Jefferson & Korner, 1967). At high amino acid concentrations, the nucleic acids of the growth-hormone-treated normal livers were more radioactive than those of the control after 60 min. of perfusion (Fig. 2). No difference between them was apparent at 30 min. and the difference at 60 min. appears to have resulted from a fall in the radioactivity of the nucleic acids of the control livers during the 30–60 min. period of perfusion (see the Discussion section).

Fig. 3 depicts the time-course relationship of the incorporation of [3 H]orotic acid into nucleic acids of perfused livers from hypophysectomized rats at elevated amino acid concentrations. In this case a significant effect of growth hormone is evident within 30 min. and the amount of labelled nucleic acid is increased by 47% after 60 min. perfusion with growth hormone.

We have observed (unpublished work) that insulin can produce stimulation of incorporation of

labelled precursors into nucleic acids and protein of perfused liver of normal rats. The possibility existed that the action of growth hormone in stimulating orotic acid and amino acid incorporation might have been mediated by release of insulin bound to the liver (see Ottaway, 1953). It has been shown that insulin suppresses by nearly 50% the net production of glucose by the liver (Exton *et al.* 1966; Mortimore, 1963), so the effect of growth hormone on glucose output of perfused liver was studied under the same conditions as those used by Exton *et al.* (1966) and under the conditions used in our experiments. As shown in Table 5, no inhibition of the glucose output of the perfused liver could be detected when preparations were perfused with growth hormone, yet addition of insulin caused substantial inhibition of glucose output.

DISCUSSION

Young (1939, 1945, 1964) has argued that growth hormone stimulates the release of insulin from the β -cells of the islets of Langerhans of the pancreas, and that some of the effects that follow injection of growth hormone could be caused by insulin. A modification of this view, based on the ideas of Randle, Garland, Hales & Newsholme (1963) on the role of tissue interaction in controlling metabolism, has also been advanced. Randle *et al.* (1963) suggest that any rise in the concentration of plasma non-esterified fatty acids is followed by preferential use of them by muscle and other tissues and a concurrent inhibition of glucose utilization. The consequent rise in blood glucose concentration stimulates insulin secretion, which normally restores metabolic balance. Situations that accelerate the release of fatty acids from adipose tissue would be accompanied by high plasma concentrations of glucose and insulin. It is well known (Raben, 1959; Raben & Hollenberg, 1959) that administration of growth hormone evokes a rapid rise in the concentration of plasma free fatty acids, and this would

Table 4. *Effect of growth hormone on the incorporation of [^3H]orotic acid into nucleic acids of livers of normal and of hypophysectomized rats perfused for 90 min.*

The experimental conditions were the same as those given in Table 1. $P < 0.01$ for both normal and hypophysectomized groups.

Liver donor	Sp. activity of nucleic acids (counts/min./mg.)		Increase with growth hormone (%)
	Without growth hormone	With growth hormone	
Normal rats (7)	834 $\pm$ 52	1095 $\pm$ 63	31
Hypophysectomized rats (9)	1091 $\pm$ 70	1442 $\pm$ 72	32

Fig. 2. Rate of incorporation of [^3H]orotic acid into nucleic acids of perfused livers from normal rats. At the start of perfusion, the medium contained 10 mM-glucose, three times the normal plasma concentration of amino acids, 1.0 μC of [^3H]orotic acid/ml. and, where indicated, 20 μg . of growth hormone/ml. Each point represents the mean of at least five perfusions. ●, Without growth hormone (control); ○, with growth hormone.

Fig. 3. Rate of incorporation of [^3H]orotic acid into nucleic acids of perfused livers of hypophysectomized rats. The experimental conditions were the same as those described in Fig. 2. Each point represents the mean of four perfusions. ●, Without growth hormone (control); ○, with growth hormone.

be expected to cause an increased secretion of insulin.

The results of the present experiments indicate that, under some conditions, growth hormone added to the perfusing medium stimulated the incorporation of labelled precursors into nucleic acids and protein of perfused liver of both hypophysectomized and normal rats. This finding shows that these effects of growth hormone are not a secondary consequence of the increased release of fatty acids by adipose tissue, nor are they a consequence of growth-hormone-stimulated release of insulin from the β -cells of the pancreas.

Ottaway (1953) has suggested that the effects of growth hormone on the isolated rat diaphragm

incubated *in vitro* might actually be caused by the release of insulin bound in an inactive form to the tissue. Although insulin can stimulate the incorporation of amino acids into protein and of orotic acid into nucleic acids in perfused liver (L. S. Jefferson, T. E. Martin & A. Korner, unpublished work) the results presented above demonstrate a simultaneous difference in the effects of the two hormones on glucose metabolism. No inhibition of glucose output of the liver could be detected when liver preparations were perfused with growth hormone, yet the results in Table 5 show that small amounts of insulin suppress the net production of glucose by nearly 50% during liver perfusion, confirming the observations of Exton *et al.* (1966) and of Mortimore (1963). It therefore seems unlikely that growth

Table 5. *Effect of growth hormone and of insulin on net glucose production by perfused liver of normal rats*

The conditions were the same as those given in Fig. 1. Insulin, where present, was added at 0.001 unit/ml.

Perfusion	Net glucose production (μ moles/100 g. body wt.)		
	At 30 min.	At 60 min.	At 90 min.
With growth hormone (20 μ g./ml.) (5)	198 $\pm$ 12	351 $\pm$ 17	461 $\pm$ 17
Without hormone (5)	180 $\pm$ 17	391 $\pm$ 27	499 $\pm$ 27
With insulin (0.001 unit/ml.) (6)	123 $\pm$ 4	229 $\pm$ 7	309 $\pm$ 8
Without hormone (6)	188 $\pm$ 9	320 $\pm$ 12	401 $\pm$ 15

hormone exerts its effect on liver through stimulating the release of tissue-bound insulin.

An effect on the incorporation of labelled amino acid into protein was detectable with 100 μ g. of growth hormone/ml. and a somewhat greater effect was observed with larger doses of the hormone. The plasma concentration of growth hormone in the rat is reported (Schalch & Reichlin, 1966) to be 94.2 μ g./ml. in female rats and 54.9 μ g./ml. in males. The lowest concentration at which an effect on amino acid incorporation was observed in our experiments (100 μ g./ml.) is near the physiological concentration of growth hormone in rat plasma. It is not known whether growth hormone is metabolized or degraded by the liver, as is insulin, but growth hormone does disappear rapidly from the circulation (Glick, Roth, Yallow & Berson, 1965), so a requirement for a somewhat higher than physiological dose to maintain the effect in the isolated liver system might be expected.

We have, as yet, no evidence on whether the increased incorporation of precursors into nucleic acids and protein brought about by growth hormone results from stimulation of the rates of synthesis of the macromolecules or from other effects such as stimulation of the uptake of precursors from the medium, or both. The results of Talwar *et al.* (1962), which show that growth hormone injected *in vivo* does stimulate the labelling of the nucleotide pool of rat liver, would lead us to expect a similar occurrence in our experiments with perfused liver.

The result (Fig. 2) that showed that the radioactivity in nucleic acids of liver of normal rats declines during the 30–60 min. period of perfusion is puzzling: we can offer no explanation of this observation, but we suspect that it is connected with the stimulation of precursor incorporation brought about by the presence of elevated concentrations of amino acids.

The fact that a higher concentration of amino acids than that found in rat plasma enhanced the growth-hormone effect and stimulated amino acid

incorporation into protein is also noteworthy. It suggests that one of the amino acids quickly becomes limiting during perfusion.

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