Amino Acid Requirement for the Growth-Hormone Stimulation of Incorporation of Precursors into Protein and Nucleic Acids of Liver Slices

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1. Incorporation of [^{14}C]leucine into protein in rat liver slices, incubated *in vitro*, increased as the concentration of unlabelled amino acids in the incubation medium was raised. A plateau of incorporation was reached when the amino acid concentration was 6 times that present in rat plasma. Labelling of RNA by [^3H]orotic acid was not stimulated by increased amino acid concentration in the incubation medium. 2. When amino acids were absent from the medium, or present at the normal plasma concentrations, no effect of added growth hormone on labelling of protein or RNA by precursor was observed. 3. When amino acids were present in the medium at 6 times the normal plasma concentrations addition of growth hormone stimulated incorporation of the appropriate labelled precursor into protein of liver slices from normal rats by 31%, and into RNA by 22%. A significant effect was seen at a hormone concentration as low as 10 ng/ml. 4. Under the same conditions addition of growth hormone also stimulated protein labelling in liver slices from hypophysectomized rats. Tissue from hypophysectomized rats previously treated with growth hormone did not respond to growth hormone *in vitro*. 5. No effect of the hormone on the rate or extent of uptake of radioactive precursors into acid-soluble pools was found. 6. Cycloheximide completely abolished the hormone-induced increment in labelling of both RNA and protein. 7. It was concluded that, in the presence of an abundant amino acid supply, growth hormone can stimulate the synthesis of protein in rat liver slices by a mechanism that is more sensitive to cycloheximide than is the basal protein synthesis. The stimulation of RNA labelling observed in the presence of growth hormone may be a secondary consequence of the hormonal effect on protein synthesis. 8. The mechanism of action of growth hormone on liver protein synthesis *in vitro* was concluded to be similar to its mechanism of action *in vivo*.

It has been established that hypophysectomy of animals lessens, and treatment of animals with growth hormone stimulates, growth and the synthesis of RNA and protein of many tissues including liver (Simpson, Evans & Li, 1949; Talwar, Panda, Sarin & Tolani, 1962; Korner, 1960, 1964). Growth hormone stimulates amino acid incorporation into protein when added to the medium in which diaphragm muscle is incubated (Kostyo & Knobil, 1959; Manchester & Young, 1959) or with which liver is perfused (Jefferson & Korner, 1967) but it has never been shown to be effective on protein or RNA synthesis when added to cell-free systems. Jackson & Sells (1967) were unable to obtain an effect of growth hormone, added *in vitro*, on RNA formation in rat liver slices. There is evidence for an amino acid requirement for stimulation of protein-labelling by growth hormone in perfused

liver (Jefferson & Korner, 1967). We therefore investigated the effects of growth hormone on RNA and protein metabolism in liver slices from intact and hypophysectomized rats in the presence of various concentrations of amino acids in the incubation medium. The readily defined conditions of this system have also permitted experiments designed to test the effect of growth hormone on RNA synthesis in relation to its effect on protein synthesis. A preliminary account of some of these findings has been published (Clemens & Korner, 1969).

MATERIALS AND METHODS

Chemicals. Ox growth hormone (NIH/GH/B13) was kindly supplied by the National Institutes of Health (Bethesda, Md., U.S.A.). The mean potency was given as 0.95 U.S.P. unit/mg. Its thyroid-stimulating-hormone

potency was given as 0.0047 U.S.P. unit/mg and its luteinizing-hormone potency as 0.0017 NIH-LH-51 unit/mg. A solution in 0.9% NaCl (pH8) was prepared immediately before use. An equal volume of 0.9% NaCl was added to the medium used for control liver slices.

Cycloheximide was obtained from Sigma (London) Chemical Co., London S.W.6, U.K. DL-[¹⁴C]Leucine (34mCi/mmol), L-[¹⁴C]leucine (312mCi/mmol) and [³H]-orotic acid (19700mCi/mmol) were obtained from The Radiochemical Centre, Amersham, Bucks, U.K.

Animals. Intact male Sprague-Dawley rats weighing 100–120g were obtained from the Oxford Laboratory Animal Centre, Bicester, U.K. Hypophysectomized male rats of the same strain and in the same weight range were purchased from Charles River Laboratories, Wilmington, Mass., U.S.A. The animals were fed on Diet 86 pellets (Dixons, Ware, Herts., U.K.) and killed by a blow on the head and bled from the neck.

Preparation and incubation of liver slices. The livers were rapidly removed into ice-cold Krebs–Ringer bicarbonate buffer (Umbreit, Burris & Stauffer, 1964) which was gassed before use with O₂+CO₂ (95:5). Care was taken to avoid damaging the liver lobes during handling. The livers were rinsed once to remove blood and then rapidly sliced to a thickness of 0.5mm with a Stadie–Riggs tissue slicer (Stadie & Riggs, 1944) which was maintained at 0–4°C throughout. The first and last slices were discarded. The liver slices were rinsed three times in ice-cold Krebs–Ringer buffer, drained, and four slices were placed in the incubation medium in each conical flask, with approx. 1ml of medium/100mg wet wt. of tissue. Equal amounts of tissue were present in all incubation flasks. The incubation medium was Krebs–Ringer bicarbonate buffer containing 3% (w/v) bovine albumin powder (fraction V from bovine plasma; Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex, U.K.), 8mm-glucose and a mixture of 20 L-amino acids in multiples of the concentrations found in rat serum, which were (in $\mu\text{mol}/100\text{ml}$): arginine, 22; asparagine, 3.8; aspartic acid, 3.8; threonine, 29; serine, 29; glutamic acid, 19; glycine, 43; alanine, 48; valine, 20; methionine, 7; isoleucine, 9; leucine, 17; tyrosine, 9; phenylalanine, 8; histidine, 9; lysine, 48; proline, 24; cysteine, 3.8; glutamine, 19; tryptophan, 6.9 (Scharff & Wool, 1964; Schimassek & Gerok, 1965). The labelled leucine or orotic acid was added as described in the legend of each figure and table. The incubation was carried out at 37°C in a shaking water-bath under O₂+CO₂ (95:5). At the end of incubation samples of the medium were taken for determination of radioactivity and each slice in which radioactivity incorporation into protein or RNA was to be measured was rapidly chilled in ice-cold 0.9% NaCl and separately homogenized in the same solution (approx. 1ml/100mg of tissue). Slices in which acid-soluble radioactivity was to be measured were blotted, weighed on a torsion balance and homogenized in 1ml of 5% (w/v) trichloroacetic acid.

Determination of radioactivity incorporated into protein. The homogenized slices were treated with 5ml of 0.5M-HClO₄ and the procedure of Munro, Jackson & Korner (1964) was used to free the resulting precipitate of amino acids, RNA and lipid, and to measure the radioactivity in the sample. The radioactivity of samples was counted in a Nuclear-Chicago gas-flow counter and corrections

were made for background counts and self-absorption. Results are reported as c.p.m. incorporated/mg of protein. The counting efficiency was approx. 18%.

Determinations of radioactivity incorporated into RNA. The homogenized slices were treated with ice-cold 20% (w/v) trichloroacetic acid and RNA was extracted, determined and its radioactivity determined as described by Jefferson & Korner (1967) in a Beckman model LS 100 scintillation counter, with an aqueous scintillation solution as described by Turner (1968). All samples were equally quenched. Results are reported as c.p.m. incorporated/mg of RNA. The counting efficiency was approx. 31%.

Determination of radioactivity in the acid-soluble pool. The tissue homogenates in trichloroacetic acid were centrifuged for 10min at 400g in an MSE Mistral 4L centrifuge and the radioactivities of 0.25ml samples of the supernatants were counted in 3ml of aqueous scintillation fluid in a Packard Tri-Carb scintillation counter. Counting efficiency was determined by the channels-ratio method and was approximately 85% for ¹⁴C and 20% for ³H. Results are expressed as c.p.m./mg wet wt. of tissue.

Determination of radioactivity in the incubation medium. Samples (0.2ml) of the medium were taken at the end of the incubation and their radioactivities counted in 3ml of aqueous scintillation fluid. Counting efficiency was determined by the channels-ratio or external-standard-ratio methods and results are expressed as nmol of labelled precursor/ml of incubation medium.

Statistical treatment of results. Incubations were in quadruplicate. Results are expressed as mean c.p.m. ($\pm$ s.e.m.)/mg of protein or RNA. Where the results of several experiments have been combined, results are expressed as mean percentages of the control incorporation $\pm$ s.e.m. We have claimed a significant difference when the mean values of control and experimental groups showed such a difference that the *P* value was less than 0.05.

RESULTS

Fig. 1 shows that the labelling of liver-slice protein by [¹⁴C]leucine increased linearly as the concentration of unlabelled amino acids in the incubation medium was raised. The radioactivity of the protein reached a plateau at an amino acid concentration about 6 times the normal plasma value. In contrast with this finding, labelling of RNA by [³H]orotic acid was not stimulated by changes in amino acid concentration in the medium. Indeed, incorporation was inhibited at high amino acid concentrations, probably because of changes in rate of uptake of precursor (M. J. Clemens & A. Korner, unpublished work).

Table 1 shows the results of several incubations in which growth hormone was added to the medium at a concentration of 1 $\mu\text{g}/\text{ml}$, with various concentrations of amino acids present. There is no significant effect of the hormone on labelling of protein or RNA when amino acids are absent or when they are present at normal plasma concentrations. When the concentration of amino acids is

6 times that present in normal rat plasma, growth hormone stimulates the incorporation of [¹⁴C]leucine into protein by 31% and incorporation of [³H]orotic acid into RNA by 22%.

Table 2 shows the effect of various concentrations of growth hormone on labelling of protein in liver slices incubated with 6 times the normal plasma concentration of amino acids. Significant effects of the hormone were obtained at a concentration of 10ng/ml, which is well within the physiological range (Schalch & Reichlin, 1966). Higher doses of

growth hormone did not stimulate incorporation to a greater extent.

Liver slices from hypophysectomized rats also responded to growth hormone *in vitro* in terms of increased incorporation of amino acids into protein (Table 3). Injection of growth hormone into hypophysectomized rats 1h before death had a similar stimulatory effect, but when the hormone was also added *in vitro* to tissue from these animals it did not significantly stimulate any further protein labelling.

The observed increases in incorporation into protein and RNA in the presence of growth hormone could be caused by an increased rate of synthesis of these macromolecules or by increases in the specific radioactivities of the pools of precursors from which they are synthesized, or both.

We have measured the rate of uptake of leucine and orotic acid into the acid-soluble fraction of rat liver slices under the same conditions of incubation that permitted a growth-hormone-induced stimulation of incorporation. There was no effect of the

Fig. 1. Influence of various concentrations of amino acids on the incorporation of [¹⁴C]leucine into protein (●) and of [³H]orotic acid into RNA (○) of liver slices after 60min of incubation. Slices were incubated in Krebs–Ringer bicarbonate buffer containing 3% (w/v) bovine serum albumin, 8mm-glucose and a mixture of 20 L-amino acids in multiples of the concentrations found in rat plasma. The specific radioactivity of leucine was kept constant. The concentration of [³H]orotic acid was 1μCi/ml.

Table 2. Effect of growth hormone added to the incubation medium on the incorporation of [¹⁴C]-leucine into protein of liver slices

Incubation for 60min was carried out in Krebs–Ringer bicarbonate buffer containing 3% (w/v) bovine serum albumin, 8mm-glucose, a mixture of 20 amino acids at 6 times plasma concentration, 0.6μCi of [¹⁴C]leucine/ml and growth hormone at the concentrations indicated. The values are the means ±s.e.m. of three determinations.

Growth hormone added to the incubation medium (μg/ml of medium)	Specific radioactivity of protein (c.p.m./mg)
0	175±10
0.01	288±29
1.0	246±23
10	250±27
100	227±15

Table 1. Effect of growth hormone on the incorporation of [¹⁴C]leucine into protein and of [³H]orotic acid into RNA of liver slices at various amino acid concentrations

Incubation at 37°C was carried out for 60min in Krebs–Ringer bicarbonate buffer containing 3% (w/v) bovine serum albumin, 8mm-glucose, a mixture of 20 amino acids in the proportions in which they occur in rat serum, a constant specific radioactivity of [¹⁴C]leucine, 1μCi of [³H]orotic acid/ml and 1μg of growth hormone/ml. Numbers of observations are given in parentheses. N.S., Not significant.

Amino acid concn. (as multiples of plasma concn.)	Mean percentage of incorporation in the absence of growth hormone (±s.e.m.)	
	RNA	Protein
0	106±8 (9) N.S.	96±6 (10) N.S.
1	112±8 (19) N.S.	95±10 (5) N.S.
6	122±9 (32) P<0.05	131±8 (44) P<0.001

Table 3. *Effect of growth hormone injected in vivo or added to the incubation medium on the incorporation of [14 C]leucine into protein of liver slices from hypophysectomized rats*

The incubation conditions were as given for Table 2. Rats were injected 1 h before death with either 0.2 ml of 0.9% NaCl or 200 μ g of growth hormone in 0.2 ml of 0.9% NaCl. The concentration of growth hormone *in vitro*, where present, was 1 μ g/ml. The values are the means $\pm$ S.E.M. of four determinations. N.S., Not significant.

	$\pm$ Growth hormone added to the incubation medium	Specific radioactivity of protein (c.p.m./mg)
Rats injected with 0.9% NaCl	—	89 $\pm$ 6
	+	164 $\pm$ 26 $P < 0.05$
Rats injected with growth hormone	—	123 $\pm$ 4
	+	146 $\pm$ 24 N.S.

Fig. 2. Uptake of [14 C]leucine into the acid-soluble fraction of incubated liver slices from normal rats. At the start of incubation, the medium contained 8 mM-glucose, 6 times the normal plasma concentrations of amino acids, 1.24 nmol of L-[14 C]leucine/ml and, where indicated, 1 μ g of bovine growth hormone/ml. Each point represents the mean of four determinations. ●, Without growth hormone; ○, with growth hormone.

hormone on uptake of these precursors at any time-point investigated. The time-course of leucine uptake in the presence and absence of growth hormone is shown in Fig. 2. The results for orotic acid uptake are shown in Fig. 3.

These results suggest that the observed increases in incorporation of [14 C]leucine into protein and [3 H]orotic acid into RNA reflect increases in rates of synthesis of the macromolecules. These increases could occur independently of one another or one could be secondary to the other. To test these alternatives, slices were incubated with [3 H]orotic acid and at high amino acid concentrations in the presence of cycloheximide. Table 4 presents results showing that 25 μ g of cycloheximide/ml, which inhibits protein synthesis by 88% (Fig. 4), did not inhibit the labelling of RNA in control slices in 1 h of incubation. It did, however, abolish the growth-

hormone stimulation of incorporation into RNA.

The results in Fig. 4 show that lower doses of cycloheximide, which do not inhibit protein synthesis completely, prevent the stimulation of incorporation of [14 C]leucine into protein by growth hormone.

DISCUSSION

Growth hormone stimulates the incorporation of labelled precursors into protein and RNA of several tissues when it is injected into animals (Talwar *et al.* 1962; Korner, 1960), when it is added to the medium in which diaphragm muscle is incubated (Manchester & Young, 1959; Kostyo & Knobil, 1959) or when it is added to the medium perfusing rat liver (Jefferson & Korner, 1967). It is, however, without effect when added directly to a cell-free protein-synthesizing system from rat liver. The results in the present paper show that, in the presence of an abundant supply of amino acids, physiological concentrations of the hormone added to the medium can stimulate incorporation of radioactive precursors into protein and RNA of incubated liver slices from intact or hypophysectomized rats.

There are difficulties in the interpretation of results from experiments involving incorporation of radioactive precursors into macromolecules in whole cells. The observed rates of incorporation reflect not only rates of synthesis of RNA and protein, but also the specific radioactivities of the functional precursor pools from which these molecules are derived. Growth hormone can stimulate the accumulation of the non-metabolizable amino acid, aminoisobutyrate, into diaphragm (Kostyo, Hotchkiss & Knobil, 1959) and into several tissues in the hypophysectomized rat, including liver (Riggs & Walker, 1960). Kostyo (1964) and Knobil (1966), however, have shown that growth-hormone stimulation of incorporation of amino acids into diaphragm protein can occur in circum-

Table 4. *Effect of growth hormone on the incorporation of [³H]orotic acid into RNA of liver slices incubated in the presence and absence of cycloheximide*

Incubation at 37°C was carried out for 60min in Krebs–Ringer bicarbonate buffer containing 3% (w/v) bovine serum albumin, 8mm-glucose, a mixture of 20 amino acids at 6 times plasma concentration, 1 μ Ci of [³H]orotic acid/ml and, where indicated, 1 μ g of growth hormone/ml and 25 μ g of cycloheximide/ml. Numbers of observations are given in parentheses.

	Mean percentage of control incorporation in the absence of growth hormone and cycloheximide ($\pm$ S.E.M.)	
	Without growth hormone	With growth hormone
Without cycloheximide	100 $\pm$ 6 (15)	127 $\pm$ 7 (15)
With cycloheximide	116 $\pm$ 9 (13)	105 $\pm$ 8 (18)

Fig. 3. Uptake of [³H]orotic acid into the acid-soluble fraction of incubated liver slices from normal rats. The experimental conditions were the same as those given for Fig. 2 except that 14.8pmol of [³H]orotic acid/ml was present instead of [¹⁴C]leucine. Each point represents the mean of four determinations. ●, Without growth hormone; ○, with growth hormone.

Fig. 4. Influence of various concentrations of cycloheximide on the incorporation of [¹⁴C]leucine into protein of liver slices incubated *in vitro* for 90min in the presence and absence of growth hormone. The incubation medium contained 8mm-glucose, a mixture of 20 amino acids at 6 times plasma concentrations, 0.6 μ Ci of [¹⁴C]leucine/ml and, where indicated, 1 μ g of growth hormone/ml and cycloheximide at the concentrations given. The values are the means of four determinations. ●, Without growth hormone; ○, with growth hormone.

stances where no effect on intracellular concentration of label is detectable. Our own results (Figs. 2 and 3) show no effect of the hormone on rate of precursor uptake into the acid-soluble fraction of liver slices, and suggest that the effects on protein and RNA labelling are not caused solely by changes in the specific radioactivities of precursor pools. However, the possible existence of intracellular compartmentation means that sizes and specific radioactivities of acid-soluble pools, as measured, may not be the same as those of the functional pools within cells. Indeed, Hider, Fern & London (1969) have presented evidence in favour of direct incorporation of amino acids into protein from the extracellular pool in the extensor digitorum longus muscle of rats, and Guidotti, Ragnotti & Rossi (1964) have proposed the existence of more than one pool for glycine within the liver cell, based on the kinetics of [¹⁴C]glycine distribution and incorporation into protein in rat liver slices. Hence all measurements of rates of incorporation and specific

radioactivities of precursor pools must be interpreted with care.

An amino acid requirement for an effect of growth hormone on the labelling of protein in perfused liver has been established (Jefferson & Korner, 1967) and is confirmed by the present experiments. We have also shown a similar requirement for a stimulation of RNA labelling by growth hormone. This need could explain the lack of effect of growth hormone on RNA synthesis in liver slices reported by Jackson & Sells (1967), who did not have amino acids in their incubation medium. The concentrations of amino acids in the liver, after incubation in medium containing them in concentrations 6 times those in the plasma, are all raised above the

values for uncubated tissue (M. J. Clemens, unpublished work).

The effect of amino acids in permitting a stimulation of protein labelling by growth hormone may be directly due to a low intracellular concentration of one of them causing it to become rate-limiting during the incubation, unless it is present in abundance. The explanation might be more complex than this, however, since the necessity for amino acids may be connected with the mechanism of action of the hormone. It is known that the ability of liver ribosomes to synthesize protein can be stimulated by growth hormone *in vivo* (Korner, 1968) and amino acids *in vivo* and *in vitro* (Wunner, Bell & Munro, 1966; Staehelin, Verney & Sidransky, 1967; Jefferson & Korner, 1969; Hanking & Roberts, 1965; Baliga, Pronczuk & Munro, 1968; M. J. Clemens, V. M. Pain & A. Korner, unpublished work). Thus growth hormone may stimulate ribosomal activity by increasing the availability of amino acids to the protein-synthesizing machinery of the cell.

Liver slices from hypophysectomized rats also respond to growth hormone *in vitro*, but if the hormone has already been administered 1 h before death the tissue becomes refractory to any further stimulation *in vitro*. This suggests that growth-hormone stimulates protein synthesis in liver *in vitro* by a similar mechanism to its action in the whole animal. It is, of course, possible that some growth hormone remains associated with the liver when the latter is removed from an animal recently injected with the hormone, and that maximal stimulatory effects on liver protein synthesis continue to be exerted *in vitro* just as if the hormone had been added to the incubation medium.

The results of incubations with cycloheximide reported here (Table 4) suggest that the observed stimulation of RNA labelling by growth hormone is secondary to an effect on protein synthesis. The amino acid requirement for a growth-hormone stimulation of RNA labelling also implicates a mechanism involving protein synthesis. Further, the results in Fig. 4 indicate that the growth-hormone-induced increment in labelling of protein is more sensitive to inhibition by low doses of cycloheximide than is basal protein synthesis. Evidence that growth hormone acts at the level of translation rather than transcription is provided by studies on the effects of the hormone on protein synthesis in the absence of RNA synthesis (Korner, 1964; Martin & Young, 1965). Rizzo & Webb (1969) have shown that factors that affect the rate of translation of mRNA and polyribosome size in liver also bring about changes in the rate of ribosome synthesis. Since growth hormone is known to stimulate formation of polyribosomal aggregates, at the expense of free monoribosomes

(Korner, 1964; Staehelin, 1965), such an effect could lead to an increased rate of ribosomal-precursor-RNA synthesis, as a consequence of the increased rate of protein synthesis.

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