Growth Hormone, Cyclic Nucleotides and the Rapid Control of Translation in Heart Muscle

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Perfused rat heart incorporated L-[¹⁴C]tyrosine into protein at a constant rate for up to 75 min. A purified bovine growth-hormone preparation (1 µg/ml) stimulated the incorporation to a new constant rate that was more than three times the control rate by 10 min after hormone addition to perfusate. The hormone, however, did not alter the intracellular tracer amino acid pool, and the relationship of this to the aminoacyl-tRNA precursor pool is discussed. It is concluded that the increased incorporation largely reflected a rapid increase in protein synthesis at the ribosomes. Measurements of cyclic nucleotide contents during the perfusion showed that these appeared to vary in a systematic way during the perfusion. This stands in contrast with the constant values given by several other parameters measured in this preparation. Further, the cyclic nucleotide variation seems to be independent of external effectors. The steady-state performance of the heart correlates more closely with the [cyclic AMP]/[cyclic GMP] ratio than with the content of the individual cyclic nucleotides. At 10 min after the addition of growth hormone a slight decrease in cyclic AMP content and a large decrease in cyclic GMP were found, suggesting that the hormone's effect in stimulating protein synthesis may be mediated by a decrease in cyclic nucleotide concentrations or an increase in the [cyclic AMP]/[cyclic GMP] ratio. The findings are also consistent with an intracellularly directed role for these nucleotides, and the possibility that the cyclic nucleotide changes are an indirect result of growth-hormone action is discussed.

It is well known that growth hormone stimulates protein synthesis either *in vivo* or *in vitro* in a variety of tissues (Manchester & Young, 1959; Kostyo & Knobil, 1959a; Korner, 1960; Kipnis & Reiss, 1960). This increased ability is not dependent on mRNA synthesis, since it was observed when RNA synthesis had been inhibited by actinomycin (Korner, 1964; Martin & Young, 1965); also the observed stimulation of RNA synthesis (Talwar *et al.*, 1962; Korner, 1963; Wyatt & Tata, 1968; Oravec & Korner, 1971) has been shown in liver to be secondary to increased protein synthesis (Clemens & Korner, 1970). The basis for the effect appears to reside in an enhanced ability of ribosomes from treated animals to incorporate amino acids (Korner, 1960, 1961). Increased activity after the administration of growth hormone is still apparent in liver cell-free systems in the presence of an initiation inhibitor (Korner, 1969), but does not, in rat diaphragm, appear to involve an increased number of nascent peptide chains (Kostyo & Rillema, 1971).

Increased amino acid uptake under the influence of the hormone is also well documented (Noall *et al.*,

1957; Riggs & Walker, 1960; Kostyo & Knobil, 1959b; Snipes & Kostyo, 1962; Kostyo, 1964; Hjalmarson *et al.*, 1969; Rillema *et al.*, 1973; Jefferson *et al.*, 1975). Kostyo (1964) has shown that incubating diaphragm muscle in Na⁺-free (choline) buffer abolished the hormone effect on transport without preventing the stimulation of protein synthesis. Jefferson *et al.* (1975) found that in the perfused liver the effect of growth hormone in stimulating urea production was not seen if this was first increased by using 3-6 times the normal plasma concentrations of amino acids. This suggests that the stimulation is secondary to increased amino acid transport. In contrast, Jefferson & Korner (1967) and Clemens & Korner (1970) found that in perfused liver or liver slices growth-hormone stimulation of amino acid incorporation was observed only at elevated concentrations of amino acids. Further, the enhancement *in vitro* of amino acid uptake by growth hormone appears to be confined to tissues removed from hypophysectomized animals, although tissues from intact animals still show increased protein synthesis under these conditions (Clemens & Korner, 1970; Reeds *et al.*, 1971). These findings suggest that, whereas some effects of the hormone may depend on the increased amino acid transport, the increased

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protein synthesis observed is probably an independent phenomenon.

It is to be expected of a pituitary protein hormone that it would have rapid physiological effects mediated via a plasma-membrane hormone receptor, and there are reports that specific growth-hormone receptors have been found in a variety of tissues (Lesniak *et al.*, 1973, 1974; Kahn *et al.*, 1973; Shiu & Friesen, 1974). Not a great deal of attention has been paid to attempts to establish how quickly growth hormone exerts its effects, although there are several reports that demonstrate quite rapid responses of tissues to growth hormone. For example, stimulation of protein synthesis (Jefferson & Korner, 1967; Kostyo, 1968), of amino acid transport (Kostyo, 1968; Hjalmarson *et al.*, 1969) and of carbohydrate metabolism (Park *et al.*, 1952; Goodman, 1968; Mowbray & Ottaway, 1973*b*) have all been observed within 30 min after injection into an animal or after addition to perfusion or incubation medium. In a systematic study aimed at detecting the earliest significant change in amino acid incorporation into diaphragm muscle, Rillema & Kostyo (1971) found no significant change until 20 min after hormone administration either *in vivo* or *in vitro*, and Kostyo & Nutting (1973) detected no change before 30 min in a range of tissues *in vivo*. However, even if the hormone had had a large immediate effect, it is doubtful whether the method used in these studies would have detected this before 20–30 min. Since it has implications not only for the mode of action of growth hormone but for mechanisms by which protein synthesis can be controlled, we set out initially to study the rapidity with which protein synthesis in a perfused rat heart preparation responds to growth hormone. Several parameters measured in this preparation suggest that for 90 min at least it is in a steady state or in a very reproducible non-steady state (Mowbray & Ottaway, 1973*a*). Further, growth hormone appears to induce a very rapid alteration to a new steady state (Mowbray & Ottaway, 1973*b*). Amino acid incorporation into protein of perfused heart has been shown to be linear for at least 1 h (Sender & Garlick, 1973; Mowbray & Last, 1974), and the effects of growth hormone on protein synthesis are known to persist for much longer than this (Kostyo & Nutting, 1973). Thus, by examining the time-course of amino acid incorporation in a steady state, first in the absence and then after the addition of growth hormone, it might be possible to determine from the incorporation function the earliest time at which the hormone influenced the rate.

Experimental

Methods

Incorporation experiments. Male white rats weighing about 200 g were deprived of food overnight, and

the hearts were removed under ether anaesthesia and perfused as described by Mowbray & Ottaway (1973*a*). The perfusion-medium contents were as described by Mowbray & Last (1974) except that 4 times the concentrations of all amino acids were used. Carrier-free L-[U-¹⁴C]tyrosine was added to give a final concentration of 0.027 μ Ci/ml. Purified growth hormone (see below) was dissolved in a drop of 10 mM-NaOH and made up in perfusion medium to a concentration of 0.5 mg/ml. A 100 μ l volume of this solution was added to the perfusate reservoir at 30 min in some perfusions to give a final concentration of 1 μ g/ml. At the end of the perfusion the heart was freeze-clamped, homogenized and extracted three times with hot 10% trichloroacetic acid containing 2 mM-tyrosine as described by Mowbray & Last (1974). The extracts were collected for counting of radioactivity and determination of raffinose. The protein precipitate was washed further, once with acetone and three times with ether, before being dissolved in 2 ml of 40% (w/v) KOH.

Cyclic nucleotide experiments. These were carried out as above except that the freeze-clamped tissue was homogenized in cold 0.5 M-HClO₄ in 25% (v/v) ethanol, and then centrifuged for 20 min at 10000 rev./min in the 16 $\times$ 15 ml head of the MSE Super-Speed 18 centrifuge at 2°C; the pH of the supernatant was adjusted to 6.6 with KOH solution and the mixture was left for 30 min. After removal of the KClO₄, the supernatant was freeze-dried and stored for up to 2 months at -20°C. The residue was dissolved in 1.0 ml of water before the determination of cyclic nucleotides.

Growth-hormone purification. Bovine growth hormone was purified by applying it to a column (45 cm $\times$ 2 cm) of Sephadex G-75 equilibrated with 0.01 M-glycine-HCl buffer, pH 3.6. The effluent protein was monitored by reading the extinction at 280 nm, and the hormone was recovered from the selected fractions (see the Results and Discussion section) by ethanol precipitation at pH 7.6 (Wilhelmi, 1955) and dried over P₂O₅.

Cyclic AMP assay. Cyclic AMP was determined essentially by the saturation assay procedure of Brown *et al.* (1971). The standard curves, however, were drawn as described by Tovey *et al.* (1974), in which the ratio [radioactivity (c.p.m. - blank) in the absence of unlabelled cyclic AMP]/[radioactivity (c.p.m. - blank) in the presence of unlabelled cyclic AMP], C_0/C_x , was plotted against standard amounts of cyclic AMP. With some batches of crude cyclic AMP-binding protein, prepared from bovine adrenal cortices (Brown *et al.*, 1971), appreciable additional binding activities affected the linearity of the C_0/C_x plot, and so samples were determined only within the primary linear range. All unknowns were assayed at least in duplicate, and samples, selected at random, were cross-checked by using a test kit obtained from

The Radiochemical Centre (Amersham, Bucks., U.K.). Agreement between values obtained by both procedures was excellent. In addition, measurements were made on a number of samples incorporating standard amounts of unlabelled cyclic AMP, as proposed by Weller *et al.* (1972). In these tests good recoveries were obtained for the added internal standards, and so the presence in the extracts of any materials interfering with the assay could be discounted.

Cyclic GMP assay. This was carried out by using the radioimmunoassay kit supplied by Collaborative Research, Waltham, Mass., U.S.A. The assay is 10^3 times less sensitive to cyclic AMP, and thus prior fractionation of the nucleotides was not necessary. Added cyclic GMP was recovered in high yield, and incubation of random samples for 30 min with excess of phosphodiesterase showed that no other cross-reactive material was present in the tissue extracts.

Radioactivity assay. The ^{14}C was assayed as described by Mowbray (1975) except that quenching was estimated in the dissolved protein samples by added internal standard hexadecane. The ^{125}I in the cyclic GMP determination was assayed by using a Panax Gamma 160 γ -ray counter.

Raffinose and protein determination. Raffinose was determined by the method of Davis & Gander (1967), and protein by the method of Lowry *et al.* (1951), with bovine serum albumin (Armour Pharmaceutical Co., Eastbourne, Sussex, U.K.) dried to constant weight as standard.

Materials

L-[U- ^{14}C]Tyrosine hydrochloride (403 mCi/mmol) and cyclic [8- ^3H]AMP (27 Ci/mmol) were obtained from The Radiochemical Centre. Bovine growth hormone was purchased from Miles Laboratories (Kankakee, Ill., U.S.A.), theophylline from Sigma Chemical Co. (St. Louis, Mo., U.S.A.), ox heart phosphodiesterase from the Boehringer Corp. (London W.5, U.K.) and DL-raffinose from Fisons (Loughborough, Leics., U.K.). All other materials were of the highest quality commercially available.

Results and Discussion

Growth-hormone purification

One of the difficulties in using growth hormone is that the preparations employed by different workers are somewhat ill-defined. Part of the reason for this is that procedures used to extract pituitaries also extract cathepsins that co-purify with the growth hormone (Bornstein *et al.*, 1973). When a series of commercial preparations of growth hormone were tested on analytical 12% polyacrylamide gels containing sodium dodecyl sulphate, they appeared very heterogeneous. In addition to a major band migrating with an apparent molecular weight of about 20000 (with

one porcine sample there were 11 discrete bands in the 15000–30000-dalton range), there appeared, in all cases, both some fast-migrating material and some which did not penetrate the gel. The preparation with the highest proportion of material migrating as a single species in the 20000–30000-dalton band was a bovine growth-hormone preparation from Miles Laboratories, and this was selected for further purification. This was accomplished by subjecting a solution of the growth hormone to two ethanol precipitation steps (Wilhelmi, 1955), which inactivate (or remove) the cathepsins (Bornstein *et al.*, 1973), and then to chromatography on Sephadex G-75. The elution pattern from a typical sample is shown on Fig. 1. The high-molecular-weight and low-molecular-weight fractions were discarded, and the major peak fractions representing 20000–30000-dalton species were pooled, and the growth hormone was recovered by ethanol precipitation at pH 7.6 (Wilhelmi, 1955) and the protein precipitate was dried over P_2O_5 .

In recent years the existence of a serum factor, variously called sulphation factor, thymidine factor or somatomedin, produced either from, or under the influence of, growth hormone, has been recognized (Salmon & Dauphaday, 1957). This factor appears to be much more potent in stimulating chondriogenesis in cartilage than is growth hormone and, since it has been shown to stimulate amino acid incorporation into muscle, has been proposed as the active principle of growth hormone (Salmon & DuVall, 1970). Somatomedin has been found associated with material of molecular weight between 3900 and 12000 partially purified from human plasma (Van

Fig. 1. Purification of commercial growth hormone

The Figure shows the E_{280} profile of 10mg of bovine growth hormone applied at 4°C to a column (45 cm $\times$ 2 cm) of Sephadex G-75 and eluted with 0.01 M-glycine-HCl buffer, pH 3.6. The fraction size is approx. 3 ml and the flow rate 0.5 ml/min. Further experimental details are given in the text. The bar indicates the fractions used.

Wyk *et al.*, 1972), and, whereas there is a possibility it may aggregate at neutral or alkaline pH, it has been shown to behave as a 6000–8000-dalton species on Sephadex G-75 under acid conditions (Uthne, 1973). Thus our preparation is probably free of somatomedin.

The possibility that somatomedin can be produced from growth hormone during a short heart perfusion seems unlikely, since even *in vivo* no increased thymidine-factor activity was detected at 30 min after an intravenous dose of growth hormone (Kostyo & Nutting, 1973), and the site of somatomedin production is probably liver (McConaghey & Sledge, 1970). Since Kostyo & Nutting (1973) observed an increased amino acid-incorporation rate by 30 min, these data support the view that the rapid effects of growth hormone are not mediated through somatomedin.

Effect of growth hormone on amino acid incorporation into perfused heart

After a 10 min wash-out pre-perfusion, hearts from male rats weighing 220–230 g were perfused in a recirculating system in the presence of glucose, tracer [14 C]tyrosine and a complete amino acid mixture resembling rat plasma (Mowbray & Last, 1974) but at 4 times the normal plasma concentrations. At various times up to 75 min the hearts were freeze-clamped and the radioactivity in the hot-trichloroacetic acid-extractable fraction was counted (see the

Fig. 2. Time-course of incorporation of [14 C]tyrosine into protein in the perfused heart

The Figure shows the radioactivity in the hot-trichloroacetic acid-insoluble fraction of hearts pre-perfused for 10 min and then perfused for the times shown in the presence of [14 C]tyrosine. Each point represents the mean and S.E.M. of four separate hearts, except for that at 45 min which is for a single heart. Growth hormone (1 μ g/ml) was present in the perfusion medium for some hearts (○) from 30 min, but was not added in other experiments (●). Further experimental details are given in the text.

Experimental section). In some experiments sufficient partially purified bovine growth hormone was added to the main perfusate reservoir at 30 min to produce a final concentration of 1 μ g/ml of perfusion medium. The results of this incorporation experiment are shown in Fig. 2. The incorporation rate appears to be linear over the 75 min. At 10 min after the addition of growth hormone there was no detectable change in the incorporation rate, but from that time the rate is linear and is increased to 335% of that shown in the absence of hormone. The fact that the increase is linear from 40 min implies that maximum activation had been achieved by that time. There appears to be a lag of about 10 min before the incorporation rate increases. There is, of course, some delay between addition of hormone to the reservoir and its arrival at the heart receptor sites, although this is not expected to be long. Recent studies with isolated heart cells, which eliminate mixing problems, suggest that the lag may be nearer 3 min than 10 min (D. J. Bates & J. Mowbray, unpublished work).

Precursor specific radioactivity

That this large change in incorporation rate after the administration of growth hormone is not mainly the result of altered specific radioactivity in the tyrosyl-tRNA pool is supported by several lines of evidence. Measurements of the intracellular radioactivity (raffinose extracellular marker) show that this is constant from the earliest time measured and that it is unaffected by growth hormone (Fig. 3). Thus the contribution of the tyrosine pool in the medium to the total intracellular pool is not altered. In these experiments it was not feasible to measure intracellular tyrosine concentration, since unlabelled tyrosine was used in the extraction procedure to obviate adsorption of labelled amino acid. However, growth hormone has been shown not to alter tyrosine pool size in diaphragm (Crowther *et al.*, 1954) and liver (Jefferson *et al.*, 1975). There is, however, some debate as to whether the aminoacyl-tRNA pool equilibrates with either medium or total intracellular amino acid pools (see, e.g., Mowbray & Last, 1974; Fern & Garlick, 1974). It appears that some amino acids show functional heterogeneity in a range of tissues and that the precursor pool used in protein synthesis may be of a higher specific radioactivity than the total intracellular pool (Kipnis *et al.*, 1961; Rosenberg *et al.*, 1963; Manchester & Wool, 1963; Hider *et al.*, 1969, 1971; Mortimore *et al.*, 1972; van Venrooij *et al.*, 1972, 1974; Rannels *et al.*, 1974; Airhart *et al.*, 1974). Two studies in which attempts were made to measure directly the specific radioactivity of a given aminoacyl-tRNA have suggested that in HeLa cells the leucyl-tRNA specific radioactivity is identical with the medium-pool specific radioactivity and higher than total intracellular-pool specific radioactivity (van Venrooij *et al.*, 1974), whereas in

Fig. 3. Time-course of the hot-trichloroacetic acid-extractable intracellular radioactivity incorporated from [^{14}C]-tyrosine in the perfused heart

The points depict the means and S.E.M. of the values found for the radioactivity/ml of intracellular water. The data in the absence ($\bullet$) and in the presence from 30 min of growth hormone ($\circ$) are from the same hearts as in the experiment shown in Fig. 2. The perfusate radioactivity was $5.9 \times 10^4 \pm 0.2 \times 10^4$ d.p.m./ml in the absence and $6.0 \times 10^4 \pm 0.5 \times 10^4$ d.p.m./ml in the presence of growth hormone. The extracellular marker was raffinose and the intracellular volume was taken to be 0.506 ± 0.019 ml/g wet wt. of tissue (Mowbray & Ottaway, 1973a).

rat liver, at times up to 15 min after intravenous [^{14}C]valine administration, the valyl-tRNA specific radioactivity is intermediate between that in plasma and total intracellular pools (Airhart *et al.*, 1974).

Infusion *in vivo* to rats of either glycine or serine, which results in the labelling of both pools, led to estimates of the fractional protein-synthesis rate in a number of tissues (including heart) (Fern & Garlick, 1974). The values estimated from each amino acid agreed much more closely if the free intracellular specific radioactivity was used as the precursor, rather than the plasma specific radioactivity. In the perfused heart, estimates of the protein-synthesis rate obtained with different amino acid precursors agree quite well if the tissue specific radioactivity is used in the estimates (Morgan *et al.*, 1971b; Sender & Garlick, 1973; Mowbray & Last, 1974). Together the data suggest that the extracellular and intracellular degradation pools both contribute to the pool used by the tRNA. The unlabelled 'degradation' pool appears to equilibrate, for some amino acids, more slowly than the extracellular pool, although this equilibration can be speeded up by using increased extracellular amino acid concentrations (Mortimore *et al.*, 1972; Rannels *et al.*, 1974).

Thus one way to minimize the uncertainty about the true protein precursor specific radioactivity, and also whether hormone addition might alter intracellular equilibration, is to use an amino acid that

exchanges rapidly across the plasma membrane and has a small pool size, so that internal and external pools rapidly come to an equilibrium at specific-radioactivity values not much different from one another. This means that it is largely unimportant whether the precursor specific radioactivity is nearer the internal or the external values, and that even if the hormone results in a change in precursor specific radioactivity it will only be a small change. Tyrosine appears to have these qualities in perfused heart, and has been shown in the presence of normal amino acid concentrations to reach, within 10 min, a constant intracellular specific radioactivity that was not less than 70% of the perfusate value (Sender & Garlick, 1973). Further, this equilibrium was not altered by insulin, which appears to increase the transport of the same set of amino acids as does growth hormone (Jefferson *et al.*, 1975). Thus all these data lead us to believe that the [^{14}C]tyrosyl-tRNA specific radioactivity in our experiments is constant over the time of the experiment, is close to the perfusate value, and will not be much, if at all, influenced by the addition of growth hormone.

Protein-synthesis rates and protein degradation

From the tyrosine specific-radioactivity values for the medium and data for the amount of tyrosine in heart myosin (Morgan *et al.*, 1971a), the incorporation data in Fig. 2 in the absence of growth hormone give a fractional protein-synthesis rate of 6%/day, which compares with values of 7%/day (Morgan *et al.*, 1971b), 7–9%/day (Mowbray & Last, 1974) and 10–11%/day (Sender & Garlick, 1973) found for similar perfused heart preparations. In the presence of growth hormone the rate has increased to 19%/day. No strictly comparable information has been found in the literature, but, by using the same tracer amino acid, Sender & Garlick (1973) have estimated that a Langendorf heart showed a rate in the presence of insulin of 15–19%/day, the same values as they found for a working heart preparation in the absence of insulin. Thus the rates observed in the present study fall in the expected range.

These rates are strictly net synthesis rates, since some fraction of the total protein is being degraded; but, provided that degradation of total heart protein is random, the dilution of the label in protein ensures that very little of the newly incorporated label is removed. Fulks *et al.* (1975) found that insulin appears to alter degradation and synthesis rates independently in diaphragm muscle. If the main effect of growth hormone were to decrease degradation rates, then these would require to be 2–3 orders of magnitude higher than synthesis rates in order to produce the change in incorporation rate observed. Degradation rates of this order are plainly absurd. Thus, although growth hormone may alter degradation rates, it seems

most unlikely that this could account for any major part of the increase in incorporation rate observed.

Thus it seems fair to conclude that the experiment described above shows that growth hormone can elicit a very pronounced increase in protein-synthesis rate within 10 min in the perfused heart; in all probability this is an effect on the translation mechanism.

Use of hypernormal amino acid concentrations

In the present study 4 times the normal plasma concentrations of amino acids have been used because it has been reported that these increased concentrations are required to demonstrate effects of growth hormone *in vitro* on liver (Jefferson & Korner, 1967; Clemens & Korner, 1970); also, higher amino acid concentrations would tend to aid the equilibration of radioactive tyrosine between the cell and the medium (see above). Jefferson & Korner (1969) found that increased amino acid concentrations could prevent the decrease in polyribosome content observed during the course of perfusion, and this may be connected with the report that 3 times the normal amino acid concentrations are required for liver perfusion to maintain intracellular amino acid concentrations at the values found *in vivo* in that tissue (Jefferson *et al.*, 1975). No similar requirement appears to be necessary to maintain amino acid concentrations in heart (Scharff & Wool, 1965; Morgan *et al.*, 1971b), and whether the increased concentrations used contributed to the large increase in the synthesis rate produced by growth hormone is unknown. Two effects of raised amino acid concentrations in muscle have been reported that may have had some influence. In perfused heart 5 times the normal amino acid concentrations stimulated protein synthesis and decreased the numbers of free ribosomal subunits (Morgan *et al.*, 1971b). In incubated diaphragm they similarly stimulated protein synthesis and diminished both the rate of disaggregation of polyribosomes and the decrease in the number of nascent chains (Manchester, 1974).

Relation of growth-hormone concentration used to concentrations in vivo

The concentration of growth hormone in rat plasma *in vivo* has been estimated, by radioimmunoassay against pure rat growth hormone as standard, to be 50–100 ng/ml (Schalch & Reichlin, 1966). Although bovine growth hormone has been claimed to have a potency equal to that of the native form in rat (see Schalch & Reichlin, 1966), bioassay of rat plasma has suggested that the equivalent concentration of bovine growth hormone lies between 1 and 2.5 µg/ml (Contopoulos & Simpson, 1957). This latter estimate, however, probably includes sulphation-factor activity. The effects *in vitro* of bovine growth hormone on protein synthesis appear to be constant over a wide range (10 ng/ml–10 µg/ml) of

hormone concentration (Clemens & Korner, 1970), and, since both the biological potency of our preparation and the life-time of native hormone in the perfusion are unknown, a hormone concentration which was probably about an order of magnitude higher than that *in vivo* was added in the present study.

Effect of growth hormone on cyclic nucleotide concentrations

The results presented above demonstrate that it is possible to produce a very pronounced increase in translation rate with attainment of the maximum stimulation within 10 min of the addition of growth hormone. The rapidity of this stimulation and the reported existence of plasma-membrane receptors for growth hormone in several tissues suggests that a second-messenger type of mechanism may be involved, and one of the questions that this poses is the method by which the message is transferred from the hormone receptor to the ribosomal sites.

There is quite a body of data suggesting that cyclic AMP and cyclic GMP concentrations are related to growth. Cyclic AMP concentrations have been observed to rise in conditions unfavourable to growth in bacteria (Bernlohr *et al.*, 1974), in protozoans (Voichick *et al.*, 1973), in fungi (Van Wijk & Konijn, 1971; Malkinson & Ashworth, 1973; Silverman & Epstein, 1975) and in a range of cultured animal cells (Otten *et al.*, 1972; Burger *et al.*, 1972; Korinek *et al.*, 1973; Oey *et al.*, 1974; Rudland *et al.*, 1974); cyclic AMP has also been reported to inhibit cell-free protein synthesis in a postmitochondrial supernatant from rat liver (Sellers *et al.*, 1974). By contrast, cyclic GMP concentrations have been reported to rise in conditions that favour growth in bacteria (Bernlohr *et al.*, 1974), in a fungus (Silverman & Epstein, 1975) and in mammalian cells (Hadden *et al.*, 1972; Illiano *et al.*, 1973; Rudland *et al.*, 1974) and to stimulate polypeptide synthesis in cell-free systems (Varrone *et al.*, 1973; Lanzani *et al.*, 1974). These opposing actions of the two nucleotides have given rise to the suggestion that there are bidirectional systems in the cell that respond in opposite senses to the two effectors so that the resultant cell action is determined by the ratio of the nucleotides (Goldberg *et al.*, 1973; Estensen *et al.*, 1973). Although a number of findings is in accord with this suggestion (Watson *et al.*, 1973; Bernlohr *et al.*, 1974; Rudland *et al.*, 1974; Sapag-Hagar & Greenbaum, 1974), there are also reports of situations in which only one of the nucleotides responded to a stimulus (George *et al.*, 1970; Hadden *et al.*, 1972; Lee *et al.*, 1972; Silverman & Epstein, 1975), suggesting on the contrary that the nucleotides are altered selectively.

In addition to the observed effects of the cyclic nucleotides on cell-free protein synthesis, cyclic AMP-dependent protein kinases have been observed to

phosphorylate ribosomes at from one to 16 sites, although no functional differences have yet been reported for these phosphorylated particles (Eil & Wool, 1973a,b; Cawthon *et al.*, 1974; Gressner & Wool, 1974). Crude preparations of elongation factors from rat liver have been found to bind cyclic GMP (Lanzani *et al.*, 1974) and to possess guanylate cyclase activity (Varrone *et al.*, 1973).

To test whether these nucleotides could be involved in mediating growth-hormone action in heart, the protocol of the incorporation experiment was repeated, the freeze-clamped hearts homogenized in cold 0.5M-HClO₄ and the cyclic nucleotides measured in the extract (see the Experimental section).

The behaviour of the cyclic AMP concentrations in heart during 1 h of perfusion is shown in Fig. 4. As before, addition of growth hormone was made to some hearts at 30 min. It was not surprising to see that the zero-time values (after a 10 min wash-out pre-perfusion) were higher than at 30 min, since this might have been expected as a result of stress *in vivo* before removal of the heart. However, the very significant rise in tissue cyclic AMP concentrations in control hearts between 30 and 40 min was most unexpected, since the 10 min wash-out pre-perfusion appears to remove the effects of the endogenous polypeptide hormones insulin and growth hormone (Mowbray & Ottaway, 1973a) and throughout this period the O₂ consumption, glucose uptake, glycogen turnover, nicotinamide nucleotide ratio and amino acid incor-

poration of hearts perfused by this Langendorf technique have been shown to be constant (Mowbray & Ottaway, 1973a; Mowbray & Last, 1974). Although it may be that the rise is caused by the decay of some influence *in vivo* that was removed when the heart was isolated but whose effect persisted for 40 min of perfusion, the suddenness of the rise and the subsequent fall suggest an immediate response to a trigger. If this latter were the case, then it would imply that heart adenylate cyclase was responding, not to an external effector, but to an internal one. Such a response would parallel that of bacteria and fungi under poor nutrient conditions (see above). The observation that the sharp rise in cyclic AMP concentrations is succeeded by a fall towards the 30 min value need not be inconsistent with this mode of action, because it appears that the slime mould *Dictyostelium discoideum*, in poor medium, may produce short pulses of increased cyclic AMP concentration with a frequency of about 0.2 min⁻¹ over several hours before aggregation (Gerisch & Hess, 1974).

One other possibility arising from this variation in cyclic AMP concentration is that it may be fundamentally wrong to expect a constant cyclic AMP concentration in a physiological steady-state condition, since cross-coupling of chemical reactions in a steady state can lead to oscillations (Bonhoeffer, 1947). Indeed, studies of stable glycolytic oscillations in yeast have caused Hess *et al.* (1969) to conclude that 'oscillations are a general property of metabolic systems and an implicit function of their feedback (control) structure'. The data in Fig. 4 would be consistent with oscillations in tissue cyclic AMP concentrations, though clearly further experimental evidence is required.

The addition of growth hormone to the perfusion medium appears to have lowered ($0.1 > P > 0.05$) the 40 min tissue cyclic AMP concentration by about 20%, without, however, preventing the rise and fall in the tissue content seen in the absence of hormone between 30 and 60 min.

The cyclic GMP content of the tissue extract was measured in the presence of cyclic AMP by using a radioimmunoassay technique that is capable of measuring between 0.1 and 10 pmol of cyclic GMP (Fig. 5, inset). The presence of cyclic AMP had no significant effect on the determination, and no phosphodiesterase-insensitive material cross-reacted in the assay. At zero time the cyclic GMP content was not different from the 30 min value, in contrast with cyclic AMP (Fig. 5). This would be in line with the reciprocal behaviour suggested for the cyclic nucleotides and the expected result of stress *in vivo*. In the absence of hormone, cyclic GMP responded just as did cyclic AMP, with a very significant rise in the concentration between 30 and 40 min. The fact that no significant change was observed in the amino acid-incorporation rate would cast doubt on the role

Fig. 4. Tissue content of cyclic AMP in the perfused heart

Hearts were perfused in the absence (●) or in the presence from 30 min of growth hormone (1 μg/ml) (○), and the points each represent the mean and S.E.M. of four separate hearts. Further experimental details are given in the text. The probability that growth hormone has not decreased the cyclic AMP concentration at 40 min is less than 1 in 10 (t test $0.1 > P > 0.05$).

Fig. 5. Tissue content of cyclic GMP in the perfused heart

The hearts and symbols are those in the experiment shown in Fig. 4. Inset: response of the cyclic GMP assay to standard quantities of cyclic GMP.

of cyclic GMP in stimulating protein synthesis. Conversely, the addition of growth hormone at 30 min completely abolished the rise in cyclic GMP concentration seen in the control perfusions. This response to growth hormone is consistent with a report by Rillema *et al.* (1973) who showed that growth-hormone action on glucose and amino acid uptake and on protein synthesis in diaphragm preparations *in vitro* could be abolished by phosphodiesterase inhibitors. However, it is clear that the protein-synthesis rate does not correlate with the total content of either cyclic nucleotide. Rather, since in the control set of hearts the nucleotides vary in similar fashion, there is the possibility that it is the ratio that reflects the true response. Fig. 6 depicts the time-course of the [cyclic AMP]/[cyclic GMP] ratio. It is noticeable that, although the ratio varies much less than do the concentrations of the individual nucleotides in the absence of hormone, it still changed by half between 30 and 40 min. The addition of growth hormone resulted in a significantly higher ratio at 40 min than in controls. This high 40 min ratio had, however, decreased significantly by 60 min. Thus the total cyclic nucleotide concentration ratio hardly appears to correlate well with the protein-synthesis rate either.

This being so, it seems reasonable to wonder whether the observed cyclic nucleotide variations are indirectly related to the mechanisms of growth-hormone action. Since the stimulation of protein synthesis would lead to an increased demand for GTP, the competition for GTP might have deprived the guanylate cyclase of substrate. Although it might seem unlikely that the cell would permit such a large random variation in an important effector, this

Fig. 6. Time-course of the cyclic nucleotide concentration ratio in the perfused heart

The data are derived from the values shown in Figs. 4 and 5 (which see for definition of symbols) and represent the mean ratio and its standard error.

decreased cyclic GMP content could be a component of the signal that more energy is required to finance the increased activity. Whether or not competition for nucleotide triphosphate does affect the cyclic nucleotide concentrations, the data do appear to suggest that the cyclic nucleotide concentration ratio reflects more closely the steady-state condition observed in several parameters than do the concentrations of the individual cyclic nucleotides, and that the response of the cell to a sudden increased energy demand may be mediated by an increased [cyclic AMP]/[cyclic GMP] ratio. How altering this concentration ratio might increase energy production in heart is not clear; it is possible that cyclic GMP might diminish the ability of cyclic AMP to stimulate protein kinases, or it might activate the phosphoprotein dephosphatases. With such a mechanism large variations in the concentrations of individual nucleotides, such as appear to occur in perfused heart, would be less important than changes in the ratio.

The emphasis on the role of cyclic nucleotides in cells of higher organisms has been almost entirely related to their response to extracellular influences. The cyclic AMP 'second-messenger' response has been assumed to be the result of a direct effect of hormone receptor on adenylate cyclase. In the present work some of the findings have implied that, although growth hormone produced a change in cyclic nucleotide concentrations in heart, this may have been an indirect effect. Some of the effects 'produced' by the hormone are probably mediated by the change in cyclic nucleotide ratio and in this sense these effectors are acting as second messengers of the hormone. If

this is true of growth hormone it seems likely it will be true of other hormones also. Such a mechanism does not constrain all the physiological sites of adenylate cyclase and guanylate cyclase to the plasma membrane. Thus reports that adenylate cyclases can be found on sarcoplasmic reticulum (Entman *et al.*, 1969) and in rat liver nuclei (Soifer & Hechter, 1971), and the soluble nature of guanylate cyclase in rat liver (Thompson *et al.*, 1973), need not be dismissed as preparation artifacts.

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