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The Regulation of Growth Hormone Secretion from the Isolated Rat Anterior Pituitary *in vitro*

THE ROLE OF ADENOSINE 3′:5′-CYCLIC MONOPHOSPHATE

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1. The release of growth hormone from isolated fragments of rat anterior pituitary tissue incubated *in vitro* was studied by employing a double-antibody radioimmunoassay. 2. In the absence of added stimuli, two phases of hormone release could be distinguished, an early phase of 2h duration and a subsequent late phase. In the early phase, hormone release was rapid but could be significantly decreased by calcium depletion and by 2,4-dinitrophenol whereas the rate of release in the late phase was uninfluenced by these incubation conditions. These results have been interpreted as indicating the existence of a secretory component in the early phase of release. 3. In subsequent experiments, the effects of various agents on the rate of hormone output during the late phase of incubation were investigated. Hormone release was increased by theophylline and by dibutyryl cyclic AMP (N^6-2′-*O*-dibutyryl-adenosine 3′:5′-cyclic monophosphate), the response to both of these agents being related to the concentration of the stimulant employed. 4. The stimulation of growth hormone output by theophylline was significantly decreased by calcium deprivation and by 2,4-dinitrophenol. The response to dibutyryl cyclic AMP was diminished by 2,4-dinitrophenol, iodoacetate and 2-deoxyglucose but not by malonate or colchicine. 5. Arginine, β-hydroxybutyrate, albumin-bound palmitate and variation in the glucose concentration of the incubation medium over a wide range were without any statistically significant effect on the rate of hormone release from either control pituitary fragments or those subject to secretory stimulation by dibutyryl cyclic AMP. 6. It is suggested that the regulation of growth hormone secretion is mediated by cyclic AMP (adenosine 3′:5′-cyclic monophosphate). The secretion observed in response to cyclic AMP requires the presence of ionized calcium and a source of metabolic energy but is independent of pituitary protein synthesis *de novo*. The integrity of the glycolytic pathway of glucose metabolism appears to be essential for cyclic AMP-stimulated growth hormone secretion to occur.

Previous studies relating to the control of growth hormone secretion have led to the generally accepted view that, in man, the hormone is secreted in response to stress (Greenwood & Landon, 1966), to hypoglycaemia (Roth, Glick, Yalow & Berson, 1963) and to elevations in the circulating concentrations of amino acids of which arginine appears to be the most effective (Merimee, Lillicrap & Rabinowitz, 1965). Although the precise site of action of these stimuli remains uncertain, the observations that the growth hormone response to insulin-induced hypoglycaemia may be abolished by section of the hypothalamo-pituitary stalk (Roth *et al.* 1963), by certain hypothalamic lesions (Abrams, Parker, Blanco, Reichlin & Daughaday, 1964) and by micro-infusion of glucose directly into the hypothalamus (Blanco, Schalch & Reichlin, 1966), suggest a probable mediation by nerve cells in this part of the central nervous system. This concept has received further support from the finding in mammalian hypothalamic extracts of a substance, the so-called growth hormone releasing factor, which is active in stimulating both the release and the synthesis of growth hormone by the somatotroph cells of the anterior pituitary (Deuben & Meites, 1964; Schally, Steelman & Bowers, 1965). In studies of bovine pituitary slices *in vitro*, however, Schofield (1967*a*,*b*) demonstrated that a number of agents appeared to be capable of influencing growth hormone release by a direct effect on the pituitary gland itself. Amongst these findings was the report that theophylline, a methylxanthine which acts to inhibit the enzyme phosphodiesterase responsible for the inactivation of cyclic

AMP, leads to a significant acceleration of growth hormone release from this preparation. Subsequently, further evidence, obtained both *in vivo* (Müller, Pecile, Kabir Naimzada & Ferrario, 1969) and *in vitro* (Steiner, Peake, Utiger, Karl & Kipnis, 1970), has confirmed the hypothesis, originally advanced by Schofield (1967*b*), that cyclic AMP may be important in the mediation of the effect of growth hormone releasing factor. In addition, Steiner *et al.* (1970) showed that both theophylline and extracts of pituitary-stalk-median-eminence tissue led to an increase in growth hormone release from the isolated rat anterior pituitary which was accompanied by a parallel increase in the pituitary concentration of cyclic AMP.

The purpose of the present study was to devise a simple system *in vitro*, based on measurement of the rate of growth hormone release from isolated fragments of rat anterior pituitary, which would be suitable for the further investigation of the mechanisms by which growth hormone secretion may be controlled. The rat pituitary was chosen for these studies on grounds of its ready accessibility in the fresh state and its relatively small size which allows fragments to be obtained with minimal tissue damage. The investigations described below were made possible by the recent development in this laboratory of a rapid, sensitive and specific double-antibody radioimmunoassay for rat growth hormone which has been shown to be valid when applied to the determination of the hormone released into media in which rat anterior pituitary fragments have previously been incubated.

In the present paper, evidence is presented to show that the rat pituitary fragment preparation provides a reproducible and sensitive system for the investigation *in vitro* of the regulation of growth hormone release and that, by its use, it is possible to distinguish between hormone leakage, occurring presumably from cells damaged during isolation of the gland fragments, and true secretion. The system has been employed to study growth hormone secretion and to investigate the metabolic requirements exhibited by the gland when it is in an actively secreting state. A preliminary account of some of the findings reported here, including the demonstration of a direct effect of the cyclic nucleotide in stimulating growth hormone secretion *in vitro*, has previously been published (Lockhart Ewart & Taylor, 1970).

MATERIALS AND METHODS

Chemicals. Carrier free ^{125}I of specific radioactivity greater than 15Ci/mg was obtained as sodium [^{125}I]iodide in a minimum volume of NaOH solution from The Radiochemical Centre, Amersham, Bucks., U.K. (catalogue No. IMS 3). Purified rat growth hormone for iodination (NIAMD-Rat GH-I-1) and a rat growth hormone reference preparation for radioimmunoassay (NIAMD-Rat GH-RP-1) together with rhesus monkey anti-(rat growth hormone) serum (NIAMD-Anti-Rat GH Serum-1) were the kind gift of Dr A. F. Parlow under the auspices of the Rat Pituitary Hormone Program of the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Md., U.S.A. The biological potencies of the purified rat growth hormone for iodination and of the rat growth hormone reference preparation are 1.5 and 0.6 i.u. (bovine growth hormone)/mg respectively. Rhesus monkey serum was a gift from Professor P. M. Daniel, Institute of Psychiatry, Maudsley Hospital, Denmark Hill, London S.E.5, U.K. Sodium toluene-*p*-sulphonchloroamide (Chloramine-T) and sodium metabisulphite were obtained from Hopkin and Williams Ltd., Chadwell Heath, Essex, U.K. Sephadex G-50 and G-100 were obtained from Pharmacia (G.B.) Ltd., London W.5, U.K.

Dibutyryl cyclic AMP, L-arginine hydrochloride, malonic acid (disodium salt), 2,4-dinitrophenol, iodoacetic acid (sodium salt), DL-β-hydroxybutyric acid (sodium salt) and 2-deoxyglucose were obtained from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K. Cyclic AMP was obtained from Boehringer Corp. (London) Ltd., London W.5, U.K. L-Leucine, EDTA, theophylline hydrate and colchicine were obtained from British Drug Houses Ltd., Poole, Dorset, U.K. Cycloheximide was obtained from Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K. and bovine plasma albumin from Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex, U.K. Albumin-bound palmitate was prepared from palmitic acid, obtained from Sigma (London) Chemical Co. Ltd., by the method of Garland, Newsholme & Randle (1964).

Assay of radioactivity. Measurements of radioactivity were carried out by using a Beckman LS-233 liquid-scintillation spectrometer (Beckman Instruments Ltd., Glenrothes, Fife, U.K.). The scintillator mixture employed was toluene–Triton X-100 (7:3, v/v) containing 5g of 2,5-diphenyloxazole/l. The counting efficiency for ^{125}I in this system was approx. 50%. All samples were counted for a time sufficient for more than 5000 total counts above background to be recorded.

Preparation of ^{125}I-labelled rat growth hormone. Rat growth hormone (5μg of NIAMD-Rat GH-I-1) was iodinated with 2mCi of ^{125}I by using a modification of the method of Hunter & Greenwood (1962) in which the time of exposure of the hormone to Chloramine-T was decreased to less than 10s. To separate the labelled hormone from free ^{125}I, the reaction mixture was transferred to a column of Sephadex G-50 which had previously been equilibrated with 0.05M-sodium phosphate buffer, pH7.5, containing bovine plasma albumin (0.5mg/ml) and merthiolate (0.6mM), subsequently referred to in this paper as assay diluent buffer. By using the same buffer, the labelled hormone was eluted from the column and samples (100μl) were rapidly frozen and stored for periods of up to 3 months at −20°C. Assuming no losses of growth hormone on the column, the specific radioactivity of the ^{125}I-labelled rat growth hormone prepared in this way was 200–250μCi/μg. As a result of progressive polymerization, which occurred on storage, the labelled hormone was repurified by chromatography on Sephadex G-100 on each day of assay. Elution was carried out by

using assay diluent buffer and 2ml fractions were collected. With a column measuring 40cm×1.2cm internal diam. the peak of the monomeric ^{125}I-labelled rat growth hormone was eluted in a volume of 24ml. For use in the assay, a sample of the peak fraction was diluted in assay diluent buffer so that 100μl of the resulting solution contained approx. 100pg of the ^{125}I-labelled rat growth hormone. After repurification, 92–97% of the radioactivity associated with the labelled hormone was precipitated by 5% (w/v) trichloroacetic acid and, in the presence of an excess of rhesus monkey anti-(rat growth hormone) serum, more than 80% of the protein-bound radioactivity was precipitated under the conditions of the assay.

Antiserum to rat growth hormone. The antiserum supplied by the National Institutes of Health (NIAMD-Anti-Rat GH Serum-1) was diluted 1:1000 with assay diluent buffer and samples were rapidly frozen in a mixture of acetone and solid CO_2 before storage at −20°C. For each assay a fresh sample was thawed and assay diluent buffer added to give an antiserum dilution of 1:40000.

Antiserum to rhesus monkey γ-globulin. This antiserum was prepared in rabbits by the method of Hales & Randle (1963). After the addition of merthiolate to give a final concentration of 0.6mM, the serum could be stored undiluted at 4°C for up to 6 weeks without loss of activity.

Standard solutions of rat growth hormone. Approx. 500μg of the rat growth hormone reference preparation (NIAMD-Rat GH-RP-1) was accurately weighed and dissolved in assay diluent buffer to give a solution containing 100μg/ml. Samples of this stock solution were frozen in a mixture of acetone and solid CO_2 and stored at −20°C for up to 5 weeks before use. For each assay, one such sample was thawed and doubling dilutions of the standard ranging from 100 to 6.25ng/ml were prepared in assay diluent buffer.

Assay of rat growth hormone. A double-antibody procedure (Morgan & Lazarow, 1963) was employed in the immunoassay, which was carried out at room temperature.

Assay diluent buffer (100μl) was added to each assay tube followed by 100μl of anti-(rat growth hormone) serum at a dilution of 1:40000. This dilution of antiserum was sufficient to bind approx. 50% of the radioactivity associated with 100pg of ^{125}I-labelled rat growth hormone in the absence of competing unlabelled hormone under the conditions of the assay. A volume (100μl) of solutions containing standard or unknown concentrations of rat growth hormone was then added and the contents of each tube were mixed and incubated at room temperature for 2h. At the end of this period, ^{125}I-labelled rat growth hormone (approx. 100pg) was added in a volume of 100μl and the incubation allowed to proceed at room temperature for 36h. Rhesus monkey serum (100μl of a 1:500 dilution in assay diluent buffer) was then added to act as a carrier. Finally, 100μl of anti-(rhesus monkey γ-globulin) serum was added at the dilution (1:3 in assay diluent buffer) which had previously been shown to produce optimum precipitation of the ^{125}I-labelled rat growth hormone–antibody complex. Incubation at room temperature was then continued for 4h after which the assay tubes were centrifuged at 1500g for 20min and the supernatants removed by using a Pasteur pipette. The precipitates were taken up in 100μl of water, 1ml of the Triton scintillation mixture was added and, after thorough mixing, the radioactivity associated with the precipitates was assayed. All samples were counted to an error (±2S.D.) of ±3%.

Incubation of rat pituitary fragments

Animals. Male rats of the Sprague–Dawley strain (300–400g) were obtained from Oxfordshire Laboratory Animal Colonies, Oxford, Oxon., U.K. The animals were maintained on Diet 86 (E. Dixon & Sons, Ware, Herts., U.K.) and were allowed free access to food and water to the time of death.

Incubation medium. The basal medium employed for the incubation of anterior pituitary fragments was a bicarbonate-buffered salts solution (Gey & Gey, 1936) containing glucose (5.6mM) as an energy source and bovine plasma albumin (1mg/ml) to decrease adsorptive losses of the released growth hormone. In addition, the quantity of $CaCl_2$ employed in making up the medium was modified to give a final Ca^{2+} concentration of 2mM. The medium was thoroughly gassed with O_2+CO_2 (95:5) immediately before use and its pH, 7.4, checked by means of a Pye 78 pH meter. When additions were made to the medium, as described in the text or in the Tables, the concentration of NaCl was adjusted where necessary to maintain constant osmolality.

Incubation technique. The animals were killed by a sharp blow to the back of the neck. A skin incision to expose the cranium then allowed division of the bones of the skull in the line of the sagittal suture and reflexion laterally of the resulting bone flaps. The brain was displaced posteriorly, the optic nerves severed and the exposed pituitary was then removed intact after anterior displacement of its dural covering. By this means it was possible to obtain pituitary glands from 9 rats within 10min of the death of the animals. The glands were washed in incubation medium at 37°C and the alae were separated from the central portion of the gland which, representing the posterior and intermediate lobes together with a residuum of anterior pituitary tissue, was discarded. The resulting paired anterior pituitary fragments were lightly blotted, rapidly weighed to the nearest 0.1mg and transferred singly to 25ml conical flasks containing 3ml of incubation medium. The weights of the fragments obtained in this way were in the range from 1.5 to 4mg. Incubation was carried out in a water bath at 37°C with gentle shaking and the flasks were gassed continuously with humidified O_2+CO_2 (95:5) throughout the incubation procedure. In initial experiments to determine the time-course of basal growth hormone release, the medium was renewed at intervals of 30min for a total period of 4.5h and samples of each medium were taken for assay of their growth hormone content. In this way it was possible to distinguish two phases of basal hormone release, designated early and late (for details, see the Results section), and to investigate their properties two different techniques of incubation were required. Thus, in the majority of the subsequent experiments, designed to determine the responsiveness of the tissue to alterations in the composition of the incubation medium, preincubation for a period of 2.5h was employed, the medium being replaced on two occasions during this time (late-phase studies). However, in a further series of experiments, designed to investigate the properties of release

in the early phase of incubation, the preincubation period was decreased to 30 min. In both cases, after preincubation, the medium was discarded and the pituitary fragments were briefly washed with 1 ml of medium at 37°C. Freshly prepared incubation medium (3 ml) containing the substances under investigation was then added to alternate flasks, the remaining flasks, which contained the paired fragments from the same gland, receiving an equal volume of control medium. Incubation was then continued for 1 h at 37°C and samples of the medium were taken for assay.

In experiments to determine the effects on release of metabolic inhibitors, the gland fragments were exposed to the inhibitor during the final hour of preincubation (or 30 min in studies of release in the early phase) as well as during the incubation proper. An exception to this is provided by iodoacetate which was added to the medium only during the incubation period itself since prolonged exposure to this agent has been reported to lead to a marked loss of its specificity as an inhibitor of the enzyme, triose phosphate dehydrogenase (Webb, 1966*a*).

In experiments designed to investigate the effect of Ca^{2+} depletion on release, the gland fragments were exposed to Ca^{2+}-free medium throughout the preincubation period and EDTA (0.5 mM) was added to the incubation medium of the experimental fragments during both the final 30 min of the preincubation and the experimental period itself. To exclude the possibility of a direct effect of the chelating agent in these experiments, EDTA (0.5 mM) was also added to the control flasks during the same periods, normal Ca^{2+} concentration, 2 mM, being maintained by the addition of supplementary $CaCl_2$ to the control medium.

At the conclusion of each incubation, samples of the medium were rapidly frozen in a mixture of acetone and solid CO_2 and stored at −20°C for up to 2 weeks until assayed. In each experiment, samples of all media employed were assayed to exclude the possibility of non-specific interference in the assay by any of the added substances.

Application of the immunoassay to unknown solutions

Preparation of samples. For assay of the growth hormone content of the anterior pituitary tissue, gland fragments of approx. 3 mg wet wt. were homogenized singly in 2 ml of 0.01 M-NaOH in an all-glass homogenizer and, after centrifugation at 2000*g* for 5 min to remove particulate material, the supernatant was diluted 1:16000 in assay diluent buffer for assay. Incubation media were assayed at a dilution of 1:150 in the same buffer.

Assay specificity. In the protocol provided with the reagents supplied by the National Institute of Arthritis and Metabolic Diseases, it is reported that there is no interference in the assay from rat follicle-stimulating hormone, luteinizing hormone or thyroid-stimulating hormone but that rat prolactin shows a cross-reactivity of approx. 2%. In the present studies, evidence relating to the specificity of the assay was obtained by determining the growth hormone content of a sample of incubation medium over a range of doubling dilutions from 1:75 to 1:1200. The results indicated that the apparent total growth hormone content of the medium is independent of the dilution employed in the assay. This observation implies parallelism between the binding inhibition curves characteristic of the unknowns and the standard rat growth hormone preparation and provides evidence that there is a protein present in the incubation medium which is immunologically indistinguishable from the standard preparation of rat growth hormone employed in the assay.

Sensitivity and reproducibility of the assay. To assess the sensitivity of the assay when applied to unknown solutions, the results obtained in 105 consecutive assays, each performed in triplicate, of diluted incubation media were analysed by using the method proposed by Brown, Bulbrook & Greenwood (1957). Calculated in this way, the quantitative sensitivity of the assay, defined as the least amount of growth hormone which can be measured with a maximum percentage error ($P = 0.05$) of ±50%, was 400 pg-equiv. of NIAMD-Rat GH-RP-1, or approx. 100 pg of rat growth hormone if allowance is made for the low potency of the standard employed.

The reproducibility of the assay was investigated by assaying a single sample of incubation medium on four separate occasions over a period of 3 weeks, the medium being separately diluted on each occasion. The mean concentration of rat growth hormone determined in the four assays was 31.0±2.9 (S.E.M.) ng/ml indicating a high degree of reproducibility of the method.

Stability of rat growth hormone. To investigate the stability of the hormone under conditions of incubation, 5 μg of the standard preparation of rat growth hormone (NIAMD-Rat GH-RP-1) was added to samples of incubation media in which pituitary fragments had previously been incubated and the growth hormone concentrations of which had already been determined. The media, together with the added growth hormone, were then exposed to a further period of 60 min incubation at 37°C and the total growth hormone content was assayed. In these experiments, the mean recovery of the added hormone was 96.0±3.3 (S.E.M.)%.

The stability of the hormone under the conditions of the assay was assessed by means of a recovery experiment in which 5 ng of standard rat growth hormone was assayed in triplicate in the presence of dilutions of different unknowns. The measurements of recovery of the standard hormone obtained in the presence of dilutions of incubation media and pituitary homogenates were respectively 94.8±3.2% and 99.4±7.0% (mean±S.E.M.).

From these experiments it is concluded that rat growth hormone is stable under the conditions of both incubation and assay and that the results obtained provide an accurate reflexion of the hormonal content both of homogenates and incubation media.

Calculation and expression of results. The rates of release of growth hormone have been calculated and expressed in the Figures and Tables as μg-equiv. of NIAMD-Rat GH-RP-1/unit time per mg wet wt. of pituitary. Except where stated otherwise, the time unit employed is 1 h. The pituitary growth hormone content is expressed as μg-equiv. of the same standard/mg wet wt. of anterior pituitary tissue. Since the NIAMD standard employed is of low biological potency (0.6 i.u./mg) by comparison with the most highly purified known preparations of the hormone (approx. 3.0 i.u./mg), the

results expressed in these terms are subject to a degree of overestimation which may be as great as five-fold. However, since any attempt to correct the results downward would involve assumptions concerning the maximum theoretical purity of rat growth hormone preparations and since essentially the purpose of the results quoted is to provide a comparison between rates of release under different circumstances, the data are presented in their uncorrected form. Rates of release are throughout expressed in terms of the mean±S.E.M. The levels of statistical significance between rates of hormone release from control and experimental fragments were obtained by calculation of t values.

RESULTS

Time-course of growth hormone release in vitro. With the intention of determining the optimum period during incubation *in vitro* for investigation of the effects of various agents on growth hormone secretion, a series of experiments was initially carried out to establish the rate of unstimulated hormone release as a function of time over a prolonged period of incubation. The results obtained are shown in Fig. 1 from which it can be seen that the rate of hormone release into the medium is initially rapid but that it falls progressively during the first 2h of incubation (early phase) to become stable during the succeeding 2.5h (late phase) at a rate equal to approximately one-third of that observed during the first 30min of incubation. To permit expression of these rates of release in percentage terms, the growth hormone content of each fragment was determined at the conclusion of the incubation and corrected for the observed output into the medium. Determined in this way, the growth hormone concentration of the rat anterior pituitary was 173±10μg of NIAMD-Rat GH-RP-1/mg wet weight. On the basis of this estimate, the rate of hormone release, expressed as a percentage of the total growth hormone content of the gland/30min, fell from an initial value of 3.38±0.23% (mean±S.E.M.) during the first 30min to a mean value of 1.22±0.08% during the final 2.5h of the incubation procedure.

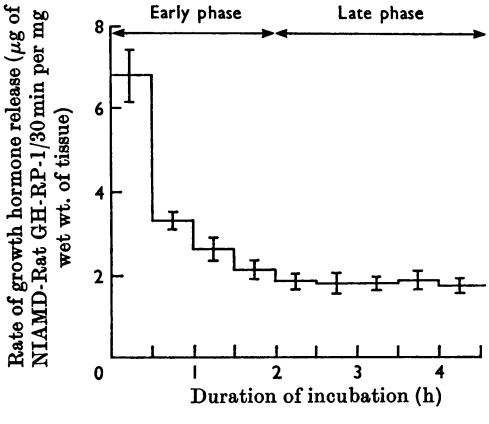

Fig. 1. Effect of the duration of incubation on the observed rates of growth hormone release from isolated rat anterior pituitary fragments *in vitro*. Pituitary fragments, of approx. 3mg wet weight, were incubated singly at 37°C in 3ml of a bicarbonate buffered salt solution containing bovine plasma albumin (1mg/ml) and glucose (5.6mM). At intervals of 30min, samples of the incubation media were taken for the determination of their rat growth hormone content by radioimmunoassay and the tissue was washed and exposed to 3ml of fresh medium. The hormone output during each 30min period is expressed as the mean±S.E.M. for 10 fragments.

Characteristics of growth hormone release in the early and late phases of incubation. In an attempt to determine the nature of the hormone release observed in the early and late phases of incubation, a series of experiments was carried out to investigate the effects of varying the incubation conditions on the rate of hormone output characteristic of the two phases. The results of these experiments are shown in Table 1, from which it can be seen that early phase release was significantly decreased both by 2,4-dinitrophenol (0.2mM) and by calcium depletion to values closely similar to those seen in the late-phase controls, whereas late-phase release itself was unaffected by these conditions of incubation. Conversely, on addition of theophylline (5mM) to the medium, the observed stimulatory effect was greater in the late phase (280% of control) than in the early phase (164% of control) although the absolute rates of release attained in each of the two phases were not significantly different (Table 1). It was concluded from these findings that, during the early phase of incubation, the pituitary fragments *in vitro* were in an active secretory state (see the Discussion section) and, to ensure that the gland fragments were in a truly basal condition, all subsequent experiments were carried out with incubation for 1h after a 2.5h period of preincubation.

Linearity of growth hormone release during incubation in vitro. To determine whether the products released into the medium lead to inhibition of further growth hormone release during the late phase of incubation, an experiment was performed in which the growth hormone content of the medium was determined at 20min intervals during incubation in the presence and absence of 5mM-theophylline. The results of this experiment, shown in Fig. 2, indicate that output from both control and theophylline-stimulated fragments was linear for a period of 1h.

Effect of pituitary fragment size on basal release. The effect on the rate of hormone release of variation

Table 1. *Effects of 2,4-dinitrophenol, calcium depletion and theophylline on growth hormone release from isolated pituitary fragments during the early and late phases of incubation* in vitro

Pituitary fragments were incubated for 1 h at 37°C after preincubation for either 30 min (early phase) or 2.5 h (late phase) in 3 ml of a bicarbonate buffered salt solution containing bovine plasma albumin (1 mg/ml) and glucose (5.6 mM). Other experimental conditions are given in the text. Rates of output are expressed in terms of the mean ± S.E.M. for the numbers of observations shown in parentheses. Details of the statistical calculations are given in the Materials and Methods section.

Medium	Rate of growth hormone release (μg of NIAMD-Rat GH-RP-1/h per mg wet wt. of tissue)	
	Early phase	Late phase
Control	5.58±0.37 (8)	3.84±0.43 (8)†
2,4-Dinitrophenol (0.2 mM)	3.43±0.33 (8)*	3.86±0.28 (8)
Control (2.5 mM-Ca^{2+} + 0.5 mM-EDTA)	6.20±0.42 (15)	3.54±0.37 (8)†
Calcium free (+0.5 mM-EDTA)	3.60±0.32 (15)*	3.30±0.26 (8)
Control	6.48±0.64 (8)	3.68±0.34 (15)†
Theophylline (5 mM)	10.28±1.15 (8)*	10.32±0.98 (15)*

* $P<0.05$ for difference from appropriate control within the early or late phase of incubation.

† $P<0.05$ for difference between the rates of release in the early and late phases under identical conditions of incubation.

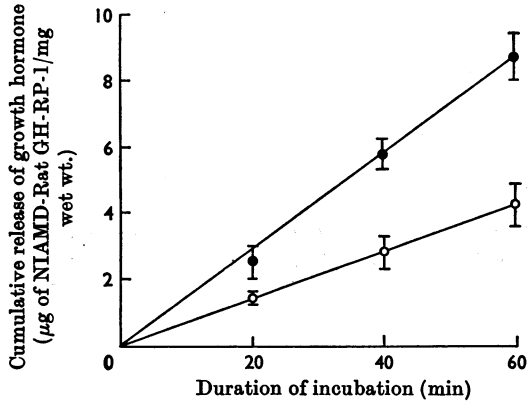

Fig. 2. Time-course of cumulative growth hormone release during the late phase of incubation. After a period of 2.5 h preincubation, pituitary fragments were incubated for 1 h in 3 ml of either control medium (○) or medium containing 5 mM-theophylline (●). At intervals of 20 min during the incubation, 3 samples (50 μl) of each medium were taken for determination of their growth hormone content by radioimmunoassay. Each point represents the mean hormone output (±S.E.M.)/mg wet wt. of pituitary tissue for four fragments.

in the size of the pituitary fragments employed in the incubation system was assessed by analysing the results obtained in six consecutive experiments. The results of this analysis are shown in Table 2 from which it can be seen that the observed rate of release/mg wet weight of tissue was inversely related to fragment size (cf. Schofield, 1967*a*). To avoid possible bias of the results in the subsequent experiments, therefore, it was important to ensure that the control and experimental fragments cut from a single gland were of very closely similar weight. In the studies described below, the weights of the pituitary fragments employed were maintained within the range 2–4 mg.

Effect of 2,4-dinitrophenol, calcium deprivation and cycloheximide on growth hormone release in the presence of theophylline. The results of these experiments are given in Table 3 from which it can be seen that theophylline exerts a significant stimulating effect on growth hormone release at both concentrations tested (0.5 and 5 mM), the response observed being related to the concentration. In an attempt to establish the nature of this stimulation, a number of experiments was carried out to determine whether the response might be modified by alteration in the incubation conditions. Table 3 shows that the rate of growth hormone output in response to 0.5 mM-theophylline was significantly decreased both by 0.2 mM-2,4-dinitrophenol ($P<0.005$) and by calcium deprivation ($P<0.05$) although the observed rates of release from the control fragments were unaltered by these incubation conditions. In the presence of cycloheximide (20 μg/ml), an inhibitor of protein synthesis, the observed rates of growth hormone output from both the control and theophylline-stimulated fragments were increased, only the latter increase, however, attaining statistical significance. Despite these changes in the rates of hormone release, however,

the response to 5mM-theophylline was essentially unchanged by the inhibitor (Table 3). Thus, the stimulation due to theophylline in the control fragments (321%) was not significantly different from that seen in the presence of cycloheximide (293%).

Effect of amino acids, variation in glucose concentration, L-β-hydroxybutyrate, albumin-bound palmitate, cyclic AMP and dibutyryl cyclic AMP on growth hormone release. The results of these experiments are given in Table 4. Both L-arginine hydrochloride and L-leucine, at a concentration of 5mM, regularly appeared to stimulate growth hormone release but the effects were small and did not attain statistical significance. Albumin-bound palmitate (0.6mM) and β-hydroxybutyrate (5mM) were without demonstrable effect on the rate of hormone release and output was likewise unaffected by variations in the glucose concentration of the medium within the range from 2–20mM. Since the observed stimulating effect of theophylline (Tables 1 and 3) seemed most probably to be attributable to the action of this substance in potentiating the effects of endogenous intracellular cyclic AMP by inhibition of the phosphodiesterase normally responsible for its breakdown, we decided to investigate whether the cyclic nucleotide itself might exert a direct effect upon growth hormone release. In initial experiments, cyclic AMP (1mM) was employed and this was found to be without a significant effect on growth hormone release *in vitro* (Table 4). In later experiments, however, employing dibutyryl-cyclic AMP, it was possible to demonstrate a significant stimulation of hormone output in response to both concentrations of the derivative of the cyclic nucleotide tested (0.2 and 1mM) and that, as in the case of theophylline, the response was related to the concentration employed.

Effect of L-arginine hydrochloride, variation in glucose concentration, L-β-hydroxybutyrate and albumin-bound palmitate on dibutyryl cyclic AMP-stimulated growth hormone release. In view of the failure to demonstrate a significant direct effect of arginine, β-hydroxybutyrate, albumin-bound palmitate or variation in the glucose concentration of the medium upon growth hormone release from pituitary fragments in a basal state (Table 4), a further series of experiments was performed to investigate the effects of these incubation conditions on the stimulated release seen in the

Table 2. *Effects of variation of pituitary fragment size on the observed rate of growth hormone release during incubation* in vitro

After preincubation for 2.5h, pituitary fragments of various sizes were incubated for 1h at 37°C in 3ml of a bicarbonate buffered medium containing bovine plasma albumin (1mg/ml) and glucose (5.6mM). At the conclusion of the incubation, samples of the media were taken for assay of their growth hormone content and the rate of hormone release was calculated for each fragment. From the results obtained in this way in six consecutive experiments, the mean rate of release (±S.E.M.) from fragments in the four weight ranges shown was calculated. The numbers of observations within each range are shown in parentheses.

Range of anterior pituitary fragment weight (mg)	Rate of growth hormone release (μg of NIAMD-Rat GH-RP-1/h per mg wet wt.)
1.00–1.99	4.17±0.32 (30)
2.00–2.99	3.73±0.27 (45)
3.00–3.99	3.43±0.20 (21)
>4.00	2.27±0.23 (12)

Table 3. *Effect of 2,4-dinitrophenol, calcium deprivation and cycloheximide on growth hormone release in response to theophylline*

After preincubation for 2.5h, pituitary fragments were incubated for 1h at 37°C under the conditions indicated. Other experimental details are given in the text. Results are expressed in terms of the mean rates of release (±S.E.M.) for the number of observations shown in parentheses. Details of the statistical calculations are given in the Materials and Methods section.

	Rate of growth hormone release (μg of NIAMD-Rat GH-RP-1/h per mg wet wt.)		
Concentration of theophylline ... / Incubation conditions	0	0.5mM	5mM
Control	3.25±0.40 (6)	5.71±0.50 (6)*	—
2,4-Dinitrophenol (0.2mM)	3.24±0.24 (10)	3.54±0.30 (10)†	—
Control (2.5mM-Ca^{2+}+0.5mM-EDTA)	3.28±0.38 (6)	5.53±0.73 (6)*	—
Calcium-free medium (0.5mM-EDTA)	3.05±0.45 (10)	3.48±0.45 (10)†	—
Control	3.65±0.41 (6)	—	11.68±0.90 (6)*
Cycloheximide (20μg/ml)	4.86±0.44 (10)	—	14.23±0.67 (10)*†

* $P<0.05$ for difference from the result in the absence of theophylline.
† $P<0.05$ for difference from the appropriate control.

Table 4. *Effect of* L-*arginine hydrochloride,* L-*leucine, variations in glucose concentration,* L-β-*hydroxybutyrate, albumin-bound palmitate, cyclic AMP and dibutyryl cyclic AMP on growth hormone release* in vitro

After preincubation for 2.5 h, pituitary fragments were incubated for 1 h in 3 ml of a bicarbonate-buffered medium. All media contained bovine plasma albumin (1 mg/ml) and glucose (5.6 mM) except where otherwise stated and other additions to the medium were as shown. The results are expressed as the mean rates of hormone output (±S.E.M.) calculated for the number of observations in parentheses. Values of P for differences of the results from the appropriate control were calculated as described in the Materials and Methods section.

Medium	Rate of growth hormone release (μg of NIAMD-Rat GH-RP-1/h per mg wet wt.)	P
Control	3.49±0.29 (14)	
L-Arginine hydrochloride (5 mM)	3.96±0.38 (14)	>0.3
Control	2.66±0.25 (9)	
L-Leucine (5 mM)	3.12±0.30 (9)	>0.2
Control	3.12±0.23 (14)	
Glucose (2 mM)	3.02±0.27 (14)	>0.7
Control	2.54±0.24 (9)	
Glucose (20 mM)	2.44±0.22 (9)	>0.7
Control	3.37±0.21 (25)	
L-β-Hydroxybutyrate (5 mM)	3.10±0.17 (25)	>0.3
Albumin (20 mg/ml) (control)	3.26±0.37 (17)	
Albumin (20 mg/ml)+sodium palmitate (0.6 mM)	3.10±0.30 (17)	>0.4
Control	3.56±0.32 (7)	
Cyclic AMP (1 mM)	3.75±0.25 (7)	>0.6
Control	3.40±0.30 (12)	
Dibutyryl cyclic AMP (0.2 mM)	4.57±0.36 (12)	<0.02
Control	3.66±0.30 (19)	
Dibutyryl cyclic AMP (1 mM)	8.13±0.82 (19)	<0.001

presence of dibutyryl cyclic AMP. In these experiments, the dibutyryl derivative of the cyclic nucleotide was added to both the control and experimental flasks at a concentration (0.2 mM) previously shown to produce a significant but submaximal stimulation of hormone release (Table 4). This concentration of the stimulant was chosen so that both inhibition or further stimulation of release by the additions to the experimental flasks would be detectable. The results of these experiments are shown in Table 5 from which it can be seen that growth hormone output in the presence of the cyclic nucleotide and L-arginine hydrochloride was not significantly greater than output in the presence of the cyclic nucleotide alone. Low (2 mM) and high (20 mM) glucose concentrations, L-β-hydroxybutyrate (5 mM) and albumin-bound palmitate (0.6 mM) likewise did not influence the rate of growth hormone output in response to dibutyryl cyclic AMP (Table 5).

Effect of metabolic inhibitors and colchicine on control and cyclic AMP-stimulated growth hormone release. In an attempt to define the metabolic requirements necessary for the stimulating effect of cyclic AMP to occur, a series of experiments was carried out in which the effects of a number of metabolic inhibitors on the response to the cyclic nucleotide were investigated. The results (Table 6) show that, of the inhibitors tested, 2,4-dinitrophenol (0.2 mM), 2-deoxyglucose (11 mM) and iodoacetate (0.4 mM) all led to a significant decrease in the characteristic stimulation of release seen in the presence of 1 mM-dibutyryl cyclic AMP. In contrast, malonate (8 mM) led to a small but significant stimulation of both control release and release in the presence of dibutyryl cyclic AMP but did not significantly alter the magnitude of the secretory response to the cyclic nucleotide (Table 6). The effect of colchicine (0.1 mM) on growth hormone output was also investigated. Table 6 shows that this agent led to small increases in both control and cyclic AMP-stimulated release, the latter effect being greater so that the apparent response to the cyclic nucleotide was also increased. None of these changes, however, was statistically significant.

DISCUSSION

Growth hormone release in the early and late phases of incubation. The finding that growth hormone release from isolated rat pituitary fragments *in vitro* was initially rapid was not unexpected and could have been attributed to non-specific leakage from cells damaged during the isolation or incubation procedures. However, both the duration of this accelerated release and its apparently exponential decay over the first 2 h of incubation did not seem to accord with this explanation. For this reason, it seemed possible that hormone release

Table 5. *Effect of* L-*arginine hydrochloride, variations in glucose concentration,* L-β-*hydroxybutyrate and albumin-bound palmitate on dibutyryl cyclic AMP-stimulated growth hormone release* in vitro

After preincubation for 2.5 h, pituitary fragments were incubated for 1 h in 3 ml of a bicarbonate buffered medium. All media contained bovine plasma albumin (1 mg/ml) and glucose (5.6 mM), except where otherwise stated, together with dibutyryl cyclic AMP (0.2 mM). Other additions to the medium were as shown. The results are expressed as the mean rates of hormone release (±S.E.M.) calculated for the number of observations in parentheses. Values of P for differences of the results from the appropriate control were calculated as described in the Materials and Methods section.

Medium	Rate of growth hormone release (μg of NIAMD-Rat GH-RP-1/h per mg wet wt.)	P
Control	4.35±0.33 (15)	
L-Arginine hydrochloride (5 mM)	5.49±0.48 (15)	>0.05
Control	4.70±0.27 (9)	
Glucose (2 mM)	4.56±0.19 (9)	>0.6
Control	4.58±0.26 (15)	
Glucose (20 mM)	4.24±0.19 (15)	>0.25
Control	3.85±0.25 (15)	
L-β-Hydroxybutyrate (5 mM)	4.16±0.31 (15)	>0.4
Albumin (20 mg/ml)	4.51±0.28 (9)	
Albumin (20 mg/ml)+sodium palmitate (0.6 mM)	4.18±0.43 (9)	>0.5

in the early phase of incubation included a secretory component and, to test this hypothesis, use was made of the accepted dependence of true secretory processes on a source of metabolic energy and on the availability of Ca^{2+} (Stormorken, 1969). Thus, the observation that both interference with oxidative phosphorylation by means of 2,4-dinitrophenol and calcium deprivation led to a decrease in the rate of hormone release in the early phase (Table 1), provides strong evidence for the existence of such a secretory component. Conversely, the finding that late-phase release was unchanged by calcium and energy deprivation (Table 1) suggests that the release characteristic of this period of incubation represents a non-specific and presumably unphysiological process which is unlikely to occur *in vivo*. This concept receives indirect support from the results shown in Table 2 which suggest that the rate of hormone release in the late phase may be related more closely to the surface area of the gland fragments than to their mass.

Further interpretation of the present results rests on the observation that, in fragments exposed to 2,4-dinitrophenol or calcium deprivation during the early phase of incubation, the resultant rate of hormone release approximates closely to that characteristic of the late phase (Table 1). This suggests that the non-specific component of hormone release *in vitro* remains relatively constant throughout the incubation period. Thus, it is possible to quantitate the true secretory component of the early phase release in terms of the difference between the rates of growth hormone output seen in the two phases of incubation. Applying this concept to the control rates of release shown in Table 1, the calculated secretory component in the early-phase studies is equal to 2.40±0.33 (mean± S.E.M.) μg-equiv. of NIAMD-Rat GH-RP-1/h per mg wet wt. of tissue. In the absence of any external agent, this secretory component may be attributable to the residual activity of a stimulus *in vivo*, possibly the hypothalamic growth hormone releasing factor, the effects of which decay *in vitro* over a period of 2 h after removal of the gland from the animal.

The demonstration of an endogenous stimulus during the first 2 h of incubation *in vitro* suggested that the early phase of incubation was unsuitable for the further investigation of the regulation of growth hormone secretion. The greater sensitivity of the gland fragments to the stimulating effect of theophylline in the late phase than that seen in the early phase confirmed this view and a 2.5 h-period of preincubation was therefore adopted in the remaining studies. Despite the extended duration of the resultant incubation *in vitro*, the viability of the tissue appeared to be unimpaired. Thus it was possible to demonstrate that both O_2 uptake and the incorporation of labelled amino acids into newly synthesized growth hormone by the gland fragments proceeded at a constant rate over a period of up to 5 h *in vitro* (R. B. Lockhart Ewart, unpublished work). In the context of the present studies, further evidence for the continuing viability of the preparation is provided by the results in Table 3. These indicate that the response of the gland fragments to theophylline during the late phase of incubation shows the properties of calcium and energy dependence characteristic of a true secretory process.

Comparison with other preparations of anterior

Table 6. *Effect of 2,4-dinitrophenol, 2-deoxyglucose, iodoacetate, malonate and colchicine upon control and dibutyryl cyclic AMP-stimulated growth hormone release* in vitro

After preincubation for 2.5 h, pituitary fragments were incubated for 1 h at 37°C in 3 ml of a bicarbonate buffered medium containing bovine plasma albumin (1 mg/ml) and glucose (5.6 mM). Other additions to the medium were as indicated. Further experimental details are given in the text. Results are expressed as the mean rate of release (±S.E.M.) for the numbers of observations in parentheses. Details of the statistical calculations are given in the Materials and Methods section.

	Rate of growth hormone release (μg of NIAMD-Rat GH-RP-1/h per mg wet wt.)		
Concentration of dibutyryl cyclic AMP ...	0	1 mM	Difference
Medium			
Control	3.76±0.43 (4)	7.72±0.36 (4)	3.96±0.22
2,4-Dinitrophenol (0.2 mM)	3.68±0.26 (6)	4.33±0.34 (6)*	0.65±1.03*
Control	3.49±0.40 (8)	7.85±0.69 (8)	4.36±0.56
2-Deoxyglucose (11 mM)	3.97±0.32 (15)	5.99±0.42 (15)*	2.02±0.43*
Control	3.52±0.28 (5)	7.98±0.85 (5)	4.46±0.62
Iodoacetate (0.4 mM)	4.16±0.32 (10)	5.53±0.10 (10)*	1.37±0.39*
Control	3.23±0.32 (12)	6.48±0.38 (12)	3.25±0.36
Malonate (8 mM)	4.58±0.26 (22)*	8.58±0.55 (22)*	4.00±0.45
Control	3.76±0.30 (4)	7.13±0.50 (4)	3.37±0.69
Colchicine (0.1 mM)	4.28±0.78 (6)	8.31±0.75 (6)	4.03±0.48

* $P<0.05$ for difference from appropriate control. For other differences between rates of release from fragments incubated in the presence and absence of inhibitors, $P>0.05$. For simplicity, levels of significance between rates of release in the presence and absence of dibutyryl cyclic AMP are not shown.

pituitary tissue in vitro. Although, in the past, preparations of rat anterior pituitary *in vitro* have been widely employed in the investigation of the control of growth hormone secretion, no systematic study of their properties has been reported. Insofar as comparison is possible, however, the rates of growth hormone release and the response to theophylline observed in the present studies are in good agreement with the values obtained by other workers using the rat pituitary preparation (Birge, Peake, Mariz & Daughaday, 1967; Steiner *et al.* 1970). In more extensive comparisons with the bovine pituitary slice system described in detail by Schofield (1967*a*), the rat pituitary preparation employed in the present studies appears to have the advantages of greater reproducibility and enhanced sensitivity to secretory stimuli. A further apparent advantage of the rat pituitary preparation arises from the observation, shown in Fig. 2, that growth hormone output from both control and theophylline-stimulated fragments was linear, in contrast with the behaviour of the bovine pituitary slice preparation in which the rate of hormone output diminished with continuing incubation in the same medium. Thus, in the present studies, there was no evidence of inhibition of growth hormone secretion by products released into the medium during the incubation period employed.

Effect of various metabolites on growth hormone release. It might be supposed that alterations in the availability of a number of metabolites such as glucose, fatty acids, ketone bodies or amino acids would modify the rate of growth hormone release either by a direct effect on the pituitary or by altering the response to the stimulatory effect of cyclic AMP. From Tables 4 and 5, however, it is clear that these agents are ineffective in modifying growth hormone release both from control and from cyclic AMP-stimulated fragments. Of particular note was the failure of low glucose concentration and of arginine, both of which are powerful stimuli to growth hormone secretion in man, significantly to increase the rate of hormone release from the rat pituitary *in vitro*. This finding might be explained by postulating a hypothalamic site of action for these stimuli in the rat. However, Garcia & Geschwind (1968) have reported that insulin-induced hypoglycaemia in the rat and the administration of arginine to the rabbit lead either to no change or even to a fall in circulating growth hormone concentrations in these animals. Thus, an alternative explanation for the present findings is that they reflect a difference in the responsiveness of the pituitary cells from different species to a number of stimuli.

Role of cyclic AMP in the regulation of growth hormone secretion. In the present studies, the demonstration of a concentration-related stimulation of growth hormone release by both theophylline and dibutyryl-cyclic AMP is in marked contrast with the lack of effect of other metabolites and suggests a major role for cyclic AMP in

the regulation of growth hormone secretion. The failure of cyclic AMP itself to alter the rate of hormone release is not inconsistent with this view since the unsubstituted nucleotide is known both to be more susceptible to hydrolysis by phosphodiesterase and to penetrate cells less rapidly than its dibutyryl derivative (Henion, Sutherland & Posternak, 1967).

The remaining results in the present paper provide further evidence concerning the nature of cyclic AMP-mediated growth hormone secretion. Thus, it is known that the secretion of both luteinizing hormone (Samli & Geschwind, 1968) and thyroid-stimulating hormone (Vale, Burgus & Guillemin, 1968) are independent of protein synthesis *de novo*. In the present studies, with cycloheximide at a concentration previously shown to result in a 90% or greater inhibition of pituitary protein synthesis (R. B. Lockhart Ewart, unpublished work), it was possible to demonstrate that, in the case of growth hormone also, secretion requires neither synthesis of the hormone itself nor of any specific protein concerned in the secretory process over the period of observation.

In the past, there has been some debate as to whether or not energy may be necessary for the secretion of anterior pituitary hormones to occur in response to stimuli. Thus, the secretion of thyroid-stimulating hormone in response to its releasing factor has been reported to depend on a source of metabolic energy (Wilber & Utiger, 1968). Samli & Geschwind (1968), however, were unable to demonstrate an effect of 2,4-dinitrophenol on secretion of luteinizing hormone and a similar lack of effect of cyanide on luteinizing hormone secretion has been reported subsequently by Geschwind (1971). In a recent publication Schofield & Stead (1971) have demonstrated a correlation between the rate of K^+-stimulated growth hormone release from bovine pituitary slices incubated *in vitro* and changes in pituitary concentrations of ATP observed in the presence of uncouplers of oxidative phosphorylation. In the present studies, 2,4-dinitrophenol led to significant inhibition of growth hormone secretion in response to both theophylline and to cyclic AMP in addition to its effect in suppressing the secretion attributed to stimulation by endogenous releasing factor. In this respect, growth hormone secretion resembles that from many other exocrine and endocrine glands in that the process is dependent on a source of metabolic energy. Moreover, the present finding that 2,4-dinitrophenol inhibits the response to cyclic AMP as well as to releasing factor and theophylline, implies that ATP is not required simply as a substrate in the adenyl cyclase-catalysed generation of cyclic AMP within the pituitary cells but that it is necessary at some later stage of the secretory process.

The results in Table 6 further indicate that 2-deoxyglucose and iodoacetate markedly inhibit the pituitary response to cyclic AMP. At the concentration used, these agents would be expected to inhibit glycolysis and we therefore infer that the response to the cyclic nucleotide is dependent on the integrity of the glycolytic pathway, presumably as a first step towards the provision of energy. The failure of 8 mM-malonate to inhibit growth hormone secretion might imply that the operation of the tricarboxylic acid cycle is not essential for secretion to occur. However, it has to be conceded that there are many uncertainties about the role of malonate as a metabolic inhibitor (Webb, 1966*b*).

Up to this point, the results presented are consistent with the currently accepted mechanism by which peptide hormone secretion is thought to occur (for review, see Fawcett, Long & Jones, 1969). This involves the storage of newly synthesized hormone in membrane-limited intracytoplasmic granules, the contents of which are extruded into the pericapillary spaces by exocytosis during the process of secretion. According to this scheme, secretion depends on the migration of the storage granules to the cell periphery for discharge. The present results suggest that this process may be specifically triggered by cyclic AMP, the generation of which *in vivo* is presumably brought about by activation of the adenyl cyclase of the growth hormone-secreting cells by the specific hypothalamic releasing factor. How cyclic AMP may act to cause such granule movement remains obscure. However, Goodman, Rasmussen, DiBella & Guthrow (1970) have proposed a unifying hypothesis to account for cyclic AMP-stimulated secretory processes in which they invoke the cyclic nucleotide as an activator, together with Ca^{2+}, of a contractile microtubular system. That such a process may be involved in endocrine function is suggested by the observations that the secretion of both insulin (Lacy, Howell, Young & Fink, 1968) and thyroid hormones (Williams & Wolff, 1970) are inhibited by colchicine, an agent known to cause disaggregation of microtubules. In the present study, the failure to demonstrate inhibition of cyclic AMP-stimulated secretion by colchicine, under conditions similar to those shown to be effective in blocking thyroid hormone secretion does not support the concept of a role for microtubules in the secretion of growth hormone. Further experiments will clearly be required to elucidate the mechanism by which cyclic AMP may regulate the secretory process in the cells of the anterior pituitary.

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