Reports

Effect of Somatotropin on Cells in Tissue Culture

The growth-promoting effect of somatotropin (pituitary growth hormone) in hypophysectomized and intact animals has been known for many years. However, there has been no evidence indicating its site of action—that is, whether somatotropin acts directly on cells or whether its effects are mediated by a "target organ" (*1*). In earlier studies, Baker and Carrel (*2*) observed that cells derived from a rat sarcoma grew readily in a culture medium containing large amounts of an extract of pepsin-digested pituitary glands. Inasmuch as pituitary growth hormone is destroyed by peptic digestion (*3*), the results of these investigators must be attributed to substances other than somatotropin. Recently, Fell (*4*) has reported that growth hormone has no discernible effect on limb buds of chick embryos in tissue culture. The purpose of this study (*5*) was to determine the direct effect of somatotropin on cells in tissue culture. These experiments indicate that somatotropin acts directly on cells, as manifested by an increased rate of multiplication of cells and an increased rate of protein synthesis.

Pituitary growth hormone, prepared by a method previously reported (*6*), was used. The growth hormone solution was ultrafiltered to insure sterilization and then lyophilized. The same preparation of somatotropin, the activity of which had been determined in hypophysectomized rats, was used in all experiments. The final concentrations of growth hormone in the culture medium were 25, 50, 100 and 200 μg/ml in the various experiments.

Stock cultures of cells (*7*) were grown in media consisting of 40 percent ox serum, 50 percent medium 199 (*8*), and 10 percent chick embryo extract. At the beginning of each experiment, the culture medium was removed from stock cultures, and a solution of 0.25 percent trypsin (*9*) was added to detach the cells from the surface of the glass and to separate the individual cells from one another. At room temperature, this occurred approximately 10 to 15 minutes after the trypsin solution had been added. The cell suspension was centrifuged at 1000 rev/min for 10 minutes. The supernatant was removed; the cells were resuspended in 20 ml of medium 199 and mixed thoroughly by pipetting. A direct count was made using standard WBC pipettes and hemocytometers. A quantity of the suspension sufficient to give a final concentration of 100,000 cells/ml was then added to a medium consisting of 90 percent medium 199 and 10 percent serum.

The basic culture medium used for all experiments consisted of 90 percent medium 199 and 10 percent ox serum. No embryo extract was added to any cultures. The growth hormone was mixed in medium 199 in a concentration 10 times that finally desired. In each instance, 0.5 ml was pipetted into the bottom of a T-30 flask, following which 4.5 ml of cell suspension was rapidly added to each flask; the suspension was stirred during this procedure. The flasks were then stoppered and incubated at 36°C. After 48 hours, 0.5 ml of the hormone solution was again added.

After a total of 96 hours' incubation, the medium was poured off and 5 ml of 0.1*M* citric acid was added. The cultures were incubated at 36°C for 2 to 3 hours. The number of cells was determined by the method reported by Sanford *et al.* (*10*). The figures given in Table 1 represent the average values obtained from three separate determinations on each flask; two to four flasks were used for each group.

The rate of growth of both the experimental and control cultures varied greatly from one experiment to another. However, the results obtained on the individual flasks of any given group were in reasonably close agreement. There was consistently a greater number of cells in the media containing somatotropin than in the respective controls (Table 1). In some instances, the increase was slight and was within the limits of experimental error, whereas in other instances the increase in number of cells was great. A quantitative relationship could not be demonstrated between the concentration of somatotropin and the cellular response in these experiments.

In further studies, the growth response was measured by quantitative determination of the tyrosine content of the cells, as reported by Oyama and Eagle (*11*). The procedures used in these experiments were identical with those used in the previous experiments except for the difference in technique for measuring growth.

As in the experiments in which the cells were enumerated, the rate of growth of cells was consistently greater in the media with growth hormone than it was in the controls (Table 2). The increased rate of growth was slight in some experiments and great in others. A quantitative relationship between the growth response and the concentration of growth hormone could not be established. However, when concentrations of growth hormone ranging from 25 to 200 μg/ml were run simultaneously, the results were suggestive of such a relationship.

There have been numerous studies concerned with the morphologic and metabolic effects of somatotropin (*12*). As a result of these investigations, it has been established that somatotropin promotes an increased rate of growth of intact and hypophysectomized animals with participation in this process by most tissues. It has also been determined that somatotropin exerts a protein anabolic effect and influences carbohydrate and fat metabolism *in vivo*. However, there has been no evidence to suggest that

Table 1. Effect of somatotropin on growth of fibroblasts in tissue culture.

Somatotropin		No. of cells in controls (1000 cells/ml)
Concn. (μg/ml)	No. of cells (1000 cells/ml)	
50	338	327
50	289	258
100	185	148
100	437	375
100	609	496
100	441	410
200	523	430
200	433	418

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Table 2. Effect of somatotropin on protein synthesis in tissue culture.

Somatotropin		Controls tyrosine (μg/ml)
Dose (μg/ml)	Tyrosine (μg/ml)	
50	13.6	13.2
100	11.4	9.6
200	15.0	13.6
200	13.2	11.9
200	9.2	6.5
200	14.8	11.2
25*	14.6	12.6
50*	15.7	12.6
100*	14.9	12.6
200*	17.0	12.6
25†	11.2	10.2
50†	11.0	10.2
100†	12.0	10.2
200†	13.9	10.2

* These experiments were run simultaneously.
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somatotropin acts directly on individual cells. The present observations indicate that somatotropin exerts a significant growth-promoting effect at the cellular level that is manifested by an increase in number of cells and in the rate of protein synthesis.

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References and Notes

1. We gratefully acknowledge the advice and assistance of C. Henry Kempe of the department of pediatrics, University of California School of Medicine, San Francisco, and of Hao Li of the Hormone Research Laboratory, University of California, Berkeley.
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5. This investigation was aided by research grant C-2155 from the National Cancer Institute of the National Institutes of Health, U.S. Public Health Service; American Cancer Society Institutional Grant 43; a grant from the E. C. Fleischner Memorial Fund and from the Edwards Fund by the Committee of Research, University of California, San Francisco.
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Oxygen Consumption in Ovulating Fragments of Ovaries of Rana pipiens

It has been reported previously (*1*) that two known inhibitors of the cytochrome oxidase system inhibit *in vitro* ovulation of fragments of ovaries of *Rana pipiens*. Since the specificity of these inhibitors (KCN and sodium azide) is neither perfectly established nor invariable, the following experiments were undertaken to study further the dependency of ovulation on oxygen by (i) observing the effect of anaerobic incubation on ovulation in isolated ovarian fragments, (ii) comparing the oxygen consumption of ovulating and nonovulating ovaries, and (iii) attempting to associate the inhibitory effect of cyanide on ovulation with concomitant inhibition of oxygen consumption.

Standard techniques of Warburg respirometry were used (*2*). A fragment of ovary containing 20 eggs was placed in each manometer flask. The total volume of fluid and tissue was kept at 4 ml throughout the respirometry. In all runs the system was allowed to equilibrate for ½ hour before readings were begun. In view of the potential danger of damage to the follicles from continuous shaking over the necessarily long incubation period of 24 hours, the manometers were shaken (80 cycles per minute) only during the 20-minute period immediately preceding a reading. For experiments involving anaerobic incubation, manometer flasks were gassed with either carbon dioxide (in which case the KOH was omitted) or nitrogen and allowed to equilibrate for ½ hour. Ovulation was stimulated by the addition of pituitary homogenate in a volume representing one-sixteenth of a triturated homoplastic anterior lobe.

Under anaerobic conditions, ovulation was not observed in 18 ovarian fragments taken from six frogs. In 36 comparable control fragments, taken from the same frogs but incubated aerobically, 27.3 percent of the available follicles extruded eggs.

In Fig. 1 the data from an experiment measuring the oxygen consumption of a number of ovulating and nonovulating ovarian fragments are compared. Respiration was minimal during the first 12 hours of incubation, for ovulation did not begin until 10 to 12 hours after the introduction of pituitary extract. In four experiments, utilizing 27 fragments from ovaries of four frogs, the average rate of oxygen consumption over the entire incubation was found to be 1.64 μlit/hr in nonovulating fragments and 2.46 μlit/hr in ovulating fragments. In two experiments, in which ovarian fragments that were exposed to pituitary extract failed to ovulate, the average oxygen consumption was 1.44 μlit/hr. Analysis of the distribution of differences of 24 pairs of oxygen-consumption values from four experiments indicates a statistical confidence level of $p < 0.01$.

Control studies were carried out on (i) oxygen consumption of pituitary extract in the absence of fragments of ovary, (ii) oxygen consumption of freshly ovulated eggs in the absence of ovarian tissue, (iii) the effect on respiration of inhibiting maturation of ovulated eggs by colchicine, and (iv) the contribution to over-all oxygen consumption made by contaminant bacteria. The increased oxygen consumption observed in ovulating ovaries could not be shown to be owing to any of these factors.

The addition of potassium cyanide ($10^{-4}M$) to flasks containing fragments of ovary completely inhibited oxygen consumption in all flasks and completely inhibited ovulation in the flasks that contained pituitary extract. Upon introduction of methylene blue from a second side arm, which brought the concentration in the medium bathing the fragments to $10^{-5}M$, the rate of oxygen consumption returned to approximately normal and the fragments subsequently ovulated. Although ovulation in fragments so treated was not completely normal, it did reach an average of 62.5 percent of that seen in control fragments exposed to methylene blue but not to potassium cyanide.

It appears that the follicular mechanisms which respond to pituitary stimulation producing the discharge of ova are aerobic and utilize molecular oxygen through the cytochrome oxidase system; this constitutes presumptive evidence of energy expenditure in ovulation. These data are consistent with the hypothesis

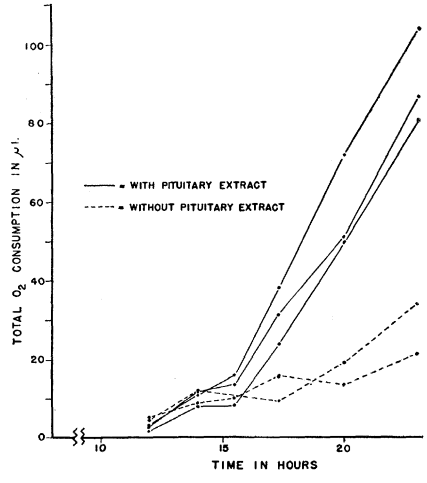

Fig. 1. Oxygen consumption in ovulating and nonovulating fragments. Each curve represents the course of oxygen consumption of a separate 20-egg fragment. Final ovulation of the fragments represented by the top three curves was 83 percent; by the bottom two curves, 0 percent.