*HORMONAL REQUIREMENTS FOR MYOGENESIS OF STRIATED MUSCLE IN VITRO: INSULIN AND SOMATOTROPIN**

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The development of muscle *in vitro* from embryonic presumptive myoblasts is known to have complex nutritional requirements.[1] One of the necessary ingredients for myogenesis *in vitro*, and indeed for the growth of many cell types, is serum. During the course of studies aimed at understanding the mechanisms involved in the fusion of myoblasts, it became of interest to delete serum from the medium and to determine whether myogenesis could be restored by the addition of known factors. In view of the fact that muscle is dependent upon insulin for its normal function,[2] this hormone was introduced into the culture medium in the absence of serum. It was observed that myotube formation was restored, although not to the extent obtained if serum were present. When somatotropin and insulin were both added to replace serum, an even greater degree of myotube formation took place. This report is therefore concerned with the effects of insulin and somatotropin on myogenesis.

Materials and Methods.—Materials: Crystalline bovine insulin and bovine somatotropin (1 IU/mg) were purchased from the Sigma Chemical Company. Highly purified collagenase, free of noncollagen proteolytic activity, was purchased from the Worthington Biochemical Company. Four-times-recrystallized bovine serum albumin was purchased from the Nutritional Biochemicals Corporation. Stock solutions were prepared by dissolving insulin at a concentration of 0.5 mg/ml in 0.001 *N* HCl, 0.1% NaCl, and somatotropin at the same concentration in 0.02 *M* potassium phosphate buffer, pH 8.0. Each hormone was added to the culture medium at a final concentration of 5 μg/ml. Serum albumin was dissolved in a modified Simm's balanced salt solution (BSS) at a concentration of 7% and the pH adjusted to 7.0; collagenase was dissolved in Ca^{++}-Mg^{++}-free BSS at a concentration of 0.1%. All these protein solutions were sterilized by passage through type GS Millipore filters.

The standard culture medium used consisted of eight parts Eagle's minimum essential medium (MEM), one part horse serum, one part 11-day chick embryo extract, 50 units/ml each of penicillin and streptomycin, and glutamine at a final concentration of 0.002 *M*. When horse serum and/or chick embryo extract were eliminated, they were replaced by one part of 7% serum albumin.

In vitro development of muscle: Breast muscle from 11-day-old chick embryos was dissected free of connective tissue, teased into small pieces, and incubated in collagenase solution at 37°C for 90 min. (This procedure was adopted in order to avoid exposure of the cells to serum which the trypsin method of releasing cells necessitates. The efficiency of this procedure is about 50% that obtained with trypsin.) Tissue removed from the collagenase solution was rinsed once with BSS and resuspended in 2 ml of MEM. The tissue was dispersed by repeated aspirations with a Pasteur pipette, and the cells were filtered through lens paper with the aid of a Swinney hypodermic adaptor. They were then centrifuged at 400 × *g* for 15 min and resuspended in a known volume of MEM; a cell count was made and the density adjusted to 4×10^7 cells per ml. To each milliliter of the medium to be tested, 0.025 ml (10^6 cells) of this suspension was added. One ml of each diluted suspension was added to individual Leighton tubes containing either two 10.5 × 22-mm cover slips or one 10.5 × 50-mm cover slip. The tubes were sealed with rubber stoppers and incubated at 37°C. After 24 hr and every day thereafter, the medium was removed and replaced by an equal volume of fresh medium.

Histological preparations: At 24-hr intervals the small cover slips were removed from the Leighton tubes, rinsed in cold BSS, and fixed in 2% glutaraldehyde in 0.1 *M* sodium cacodylate buffer, pH 7.2, for 90 min and stored in 70% ethanol in the refrigerator overnight. The tissues

subsequently were rinsed in water, stained in Delafield's hematoxylin for 2–3 min, and then dehydrated through graded ethanol and xylene solutions and mounted in Harleco synthetic resin.

Determination of protein and DNA: The developing tissue was thoroughly scraped from the large cover slips into 1 ml of 0.1 *M* NaF solution containing 0.4% glycogen, transferred to small cellulose nitrate tubes, and homogenized by sonication (Branson Instruments, S-75 sonifier with a microprobe). Protein was determined by the method of Lowry *et al.*,[3] and DNA by the method of Burton.[4]

Results.—Myogenesis in the presence and absence of serum: Figures 1 and 2 illustrate the effect of eliminating serum from the culture medium. In the absence of serum (Fig. 2) there is clearly very little fusion of myoblasts to form myotubes. The small but significant amount of myotube formation in this case is due to the presence of embryo extract; in its absence no fusion appears to take place.

Effect of insulin and somatotropin in the absence of serum: Replacement of serum by insulin leads to an increase of myotube formation (Fig. 3) over the basal level seen in Figure 2. No increase in the degree of myotube formation or of their thickness is observed beyond the third day of incubation under these conditions; in fact, upon further incubation, myotubes degenerate. Inspection of the tissue at a higher magnification reveals, as would be expected, that myotubes formed in response to insulin are multinucleated.

Inclusion of somatotropin in addition to insulin leads to an even greater degree of myotube formation, as seen in Figure 4. Somatotropin alone, however, does not have the effect of insulin. It should be mentioned here that the addition of these two hormones to the complete medium containing serum does not lead to any greater degree of myogenesis. It has been reported that oxytocin has an insulin-like activity in stimulating the metabolism of glucose by isolated fat cells.[5] However, it was observed that the addition of 10 μg/ml of oxytocin had no effect on myogenesis in the absence of serum.

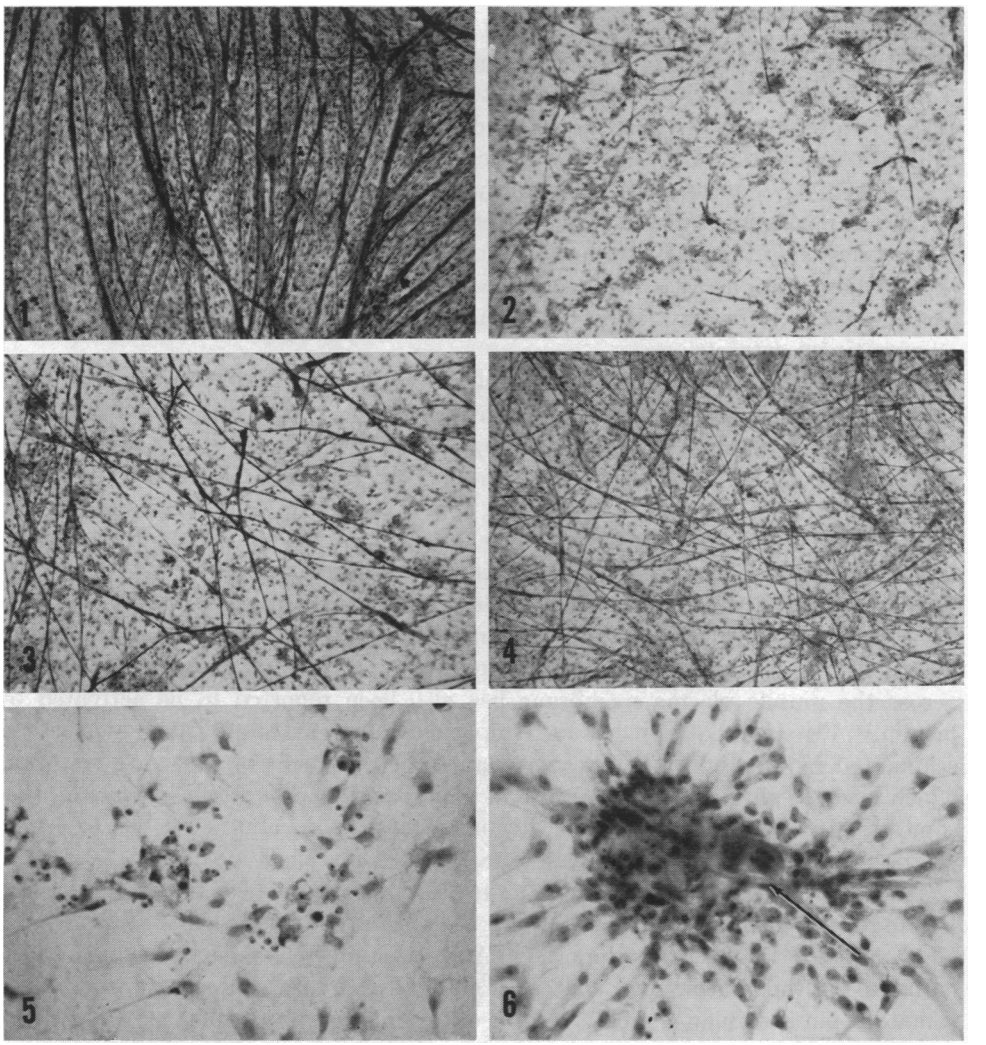
One effect of insulin may be the stimulation of the fusion of myoblasts. This is suggested by the following experiment: If both serum and embryo extract are eliminated from the culture medium, only a few cells survive (Fig. 5). If, however, insulin is added to MEM, greater numbers survive, division figures are frequent, and fusion of cells appears to take place. The fused cells appear to form globular, syncytial masses which are always seen to lie over a cluster of mononuclear cells (Fig. 6). In a few cases, these masses appear to have elongated into a myotube configuration. In addition, insulin also may stimulate cell division. As seen in Table 1, in the absence of serum and in the presence of insulin there is an almost twofold increase in the amount of DNA over the basal level (culture no. 2). Somatotropin does not have this effect nor does it increase that caused by insulin. It may well be that in addition to its possible function in the fusion of myoblasts, insulin is also required to activate cell division prior to fusion. This appears to be a role of this hormone in the histodifferentiation of mammary tissue in organ culture.[6]

TABLE 1

ACCUMULATION OF PROTEIN AND DNA UNDER VARIOUS CONDITIONS OF CULTURE

Culture no.	Condition	Protein (μg)	DNA (μg)
1	Control (8:1:1 medium)	900	17.9
2	−Serum	200	4.3
3	−Serum, + insulin	390	7.7
4	−Serum, + somatotropin	200	4.9
5	−Serum, + insulin and somatotropin	420	7.1

Each value represents the total quantity from two Leighton tube cultures after 3 days *in vitro*.



These photomicrographs illustrate the differentiation of 11-day-old chick embryo muscle cells after 3 days *in vitro* under varying conditions of culture.

FIG. 1.—Culture in control medium consisting of Eagle's MEM 11-day chick embryo extract, horse serum, 8:1:1; ×71 magnification.

FIG. 2.—Culture with horse serum deleted, supplemented with one part of 7% bovine serum albumin; ×71.

FIG. 3.—As in Fig. 2 with addition of 5 μg/ml of bovine insulin; ×71.

FIG. 4.—As in Fig. 3 with the further addition of 5 μg/ml of bovine somatotropin; ×71.

FIG. 5.—Culture in 9 parts Eagle's MEM supplemented with 1 part of 7% bovine serum albumin; ×318.

FIG. 6.—As in Fig. 5 with the addition of 5 μg/ml of bovine insulin; ×318. The arrow indicates syncytial mass overlying a layer of spread mononuclear cells.

Discussion.—The results reported here pose the question as to whether insulin, or an insulin-like activity, is required for myogenesis *in ovo*. According to Grillo,[7] the pancreas of the chick embryo does not begin to synthesize insulin before the twelfth day of development; yet the development of muscle begins as early as the third to

the fourth day. If myogenesis is indeed dependent upon insulin or an insulin-like activity, one must assume an extrapancreatic source for such a factor.

An insulin-like activity was reported to be present in egg yolk by Shikinami in 1928.[8] However, this report has been disputed by Grillo,[7] although a different method of extraction of the yolk was used by this investigator. It remains a distinct possibility that insulin is present in yolk, although in a bound form, inactive in the rat diaphragm assay used in the latter study. Antoniades[9] has reported that a major fraction of the insulin in human blood is present in a bound state, inactive in the rat diaphragm assay, which can be activated either by acid precipitation of the carrier protein(s) or by incubation of the insulin complex(es) with human or rat adipose-tissue extracts.

With regard to the differentiation of muscle in the chick embryo, one would have to postulate that during development an inactive insulin in yolk is mobilized and activated by some tissue of the developing embryo, such as the yolk sac epithelium. Alternatively, some tissue other than that of the pancreas may elaborate an insulin-like activity in early development. In this regard, it has recently been reported that the isolated perfused rat liver forms an insulin-like substance[10] and that the thymus in various species produces an insulin-like activity.[11]

It is now well established that muscle tissue requires insulin for a variety of its biochemical activities. The most-documented action of this hormone on muscle metabolism is its stimulation of the uptake of sugars;[12] the transport of amino acids across the muscle plasma membrane has also been reported to be activated by insulin.[13] More recently, it has been reported that the rate of protein synthesis is stimulated by this hormone by a mechanism independent of substrate transport which leads to an increase in the rate of amino acid incorporation by muscle ribosomes.[14] In addition, it has been demonstrated that insulin also leads to an activation of the UDPG-α-glucan transglucosylase in muscle.[15]

The action of insulin may be to promote cell division as well as to cause an alteration in the cell membrane prior to fusion. It is clear from Table 1 that somatotropin alone does not significantly promote DNA synthesis beyond the basal value in these cultures. Although the DNA content of cultures with insulin alone and insulin plus somatotropin is similar, a greater degree of fusion occurs in the latter case. These experiments do not provide any insight into the role of somatotropin in increasing cell fusion. In connection with a postulated role of insulin in the alteration of the cell membrane prior to cell fusion, it is of interest that the presumptive myoblasts with which we start our experiments are insensitive to insulin in their uptake of galactose-C^{14}; only after the third day *in vitro*, when myotubes are well developed and contain cross-striated myofibrils, is insulin stimulation of the transport of galactose detectable.[16]

The exact chemical requirements for myogenesis *in vitro* are clearly yet to be defined. The evidence presented in this report implicates insulin in the differentiation of muscle *in vitro*, and somatotropin in an enhancement of this effect. One is led to speculate on other factors that serum and embryo extract contribute to myogenesis. It seems plausible, indeed, that the whole retinue of biochemicals that are involved in the normal function of muscle may in fact be involved in its differentiation. Significant in this regard is a recent report on the differentiation of mammary tissue in organ culture using a defined medium. It was observed that the

hormones prolactin, insulin, and hydrocortisone stimulate the synthesis of a specific group of phosphoproteins as well as the histodifferentiation of this tissue to an alveolar secretory appearance.[17]

Summary.—It has been observed that fusion of presumptive chick embryonic myoblasts to form myotubes *in vitro* is almost completely reduced in the absence of animal serum. Addition of insulin greatly stimulates the formation of myotubes, and the further inclusion of somatotropin in the culture medium further enhances, but does not replace, the effect of insulin.

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