[1] Nass, S., and M. M. K. Nass, *J. Cell Biol.*, **19**, 593 (1963).
[2] *Ibid.*, p. 613.
[3] Schatz, G., E. Haslbrunner, and H. Tuppy, *Biochem. Biophys. Res. Commun.*, **15**, 127 (1964).
[4] Kalf, G. R., *Biochemistry*, **3**, 1702 (1964).
[5] Rabinowitz, J., J. Sinclair, L. DeSalle, R. Haselkorn, and H. Swift, these PROCEEDINGS, **53**, 1126 (1965).
[6] Luck, D. J. L., and E. Reich, these PROCEEDINGS, **52**, 931 (1964).
[7] Wintersberger, E., *Hoppe-Seylers Z. Physiol. Chem.*, **336**, 285 (1964).
[8] Neubert, D., and H. Helge, *Biochem. Biophys. Res. Commun.*, **18**, 600 (1965).
[9] Bolton, E. T., and B. J. McCarthy, these PROCEEDINGS, **48**, 1390 (1962).
[10] Hoyer, B. H., B. J. McCarthy, and E. T. Bolton, *Science*, **144**, 959 (1964).
[11] Burns, K. I., and C. A. Thomas, in *Abstracts*, Biophysical Society Meeting, Chicago (1964).
[12] McCarthy, B. J., and B. H. Hoyer, these PROCEEDINGS, **52**, 915 (1964).
[13] Scherrer, K., and J. E. Darnell, *Biochem. Biophys. Res. Commun.*, **7**, 486 (1962).
[14] Burton, K., *Biochem. J.*, **62**, 315 (1956).
[15] Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
[16] Schneider, W. C., *J. Biol. Chem.*, **176**, 159 (1948).

THE INFLUENCE OF COLLAGEN ON THE DEVELOPMENT OF MUSCLE CLONES

BY STEPHEN D. HAUSCHKA* AND IRWIN R. KONIGSBERG

DEPARTMENT OF EMBRYOLOGY, CARNEGIE INSTITUTION OF WASHINGTON, AND DEPARTMENT OF BIOLOGY, THE JOHNS HOPKINS UNIVERSITY, BALTIMORE, MARYLAND

Communicated by Dietrich H. Bodenstein, November 15, 1965

Previous investigations have demonstrated that single embryonic muscle cells isolated in culture are capable of forming a macroscopic muscle colony within which the differentiation of multinucleated, cross-striated muscle fibers occurs.[1, 2] The development of such muscle clones from single cells, plated in relatively large volumes, is dependent upon the use of culture medium which has been previously exposed, for several days, to the presence of a dense population of cells.[2] Medium prepared in this manner ("conditioned medium") might be altered in many respects by the metabolic activities of the cells during the conditioning period. An almost limitless number of possible alterations to the medium might have been responsible for its ability to support the development of muscle clones. Therefore, it did not seem practical to engage immediately in a detailed biochemical analysis. We chose rather to reduce the complexity by defining more critically the biological parameters of the requirement for conditioned medium.

One approach which has been particularly fruitful was designed initially to examine the possibility that conditioned medium was required only during a particular phase of muscle clone development. In addition to answering this question, the results immediately suggested what the identity of the active component might be. These results[3] may be summarized as follows: (1) Single cells, plated initially in conditioned medium, which was then replaced by unconditioned medium after 3 days of cultivation, ultimately produced differentiated muscle colonies indistinguishable from companion cultures grown in conditioned medium throughout

the entire 2-week period. (2) Petri plates which had been preincubated with conditioned medium for 3 days and subsequently seeded with single cells were able to support growth and differentiation of muscle colonies even when the liquid overlay used was unconditioned medium. (3) Despite repeated rinses with distilled water, the ability of such preincubated Petri plates to support the development of muscle clones was retained.

These results suggested that conditioned medium alters the Petri-plate surface, most probably by the deposition of distilled-water insoluble material upon it.

Two findings lead us to consider the possibility that the material involved might be collagen. First, we found that conditioning could be affected by fibroblasts alone and did not depend upon the presence of muscle cells. We also knew, from previous observations, that medium collected from conditioning "farms" in the log growth phase was ineffective, but that medium collected from crowded, stationary cultures supported the development of muscle clones. It seemed reasonable to assume that the material responsible for altering the Petri-plate surface was a metabolic product of fibroblasts which had reached the stationary phase.

Green and Goldberg[4, 5] had demonstrated that cell strains derived from embryonic mouse fibroblasts were capable of a high rate of collagen production, but that they synthesized collagen only during the stationary phase. To see whether this correlation was also true of our conditioning farms, we fixed and embedded these cell monolayers *in situ* and examined thin sections by electron microscopy.[3] Numerous fibers exhibiting a periodicity identical to a type described by Green and Goldberg[5] were also found in our conditioning cultures.

All these considerations led us to postulate that collagen was the active material in conditioned medium. If this hypothesis were correct, then purified collagen should replace the requirement for conditioned medium. This corollary has been tested by plating single embryonic chick muscle cells on a substratum of precipitated rat-tail collagen with a liquid overlay of unconditioned medium. The results indicate that this procedure does indeed replace the requirement for conditioned medium.

Materials and Methods.—Medium: The growth medium (unconditioned) has the following composition: 79 parts nutrient mixture F-10,[6] 15 parts horse serum, 5 parts embryo extract, 1 part antibiotic stock (10,000 units penicillin G, sodium, and 0.5 mg streptomycin sulfate per ml), and 0.25 parts fungicide stock (0.8 mg amphotericin B per ml). Embryo extract is prepared by extracting the minced pulp of 12-day chick embryos with an equal volume (per weight) of Pannett-Compton's solution[7] for 1 hr at 5°C. The extract is clarified by centrifugation at 40,000 $\times$ *g* for 3 hr and passed through a Millipore filter of 0.45-μ pore size. Protein concentration of such extracts is approximately 10 mg/ml.

Our protocol for preparing conditioned medium has been modified slightly from that previously published.[2] A secondary cell suspension is prepared from dense primary monolayers grown from trypsin-dissociated embryonic muscle cells. One million secondary cells are inoculated into Pyrex Petri plates (150 mm diam.) containing 10 ml of the modified NCI medium described earlier.[2] The medium is replaced 2 days later with 15 ml of the same type of medium. On the fifth day, the medium is again replaced, this time with 15 ml of the growth medium described above. This medium, after an incubation period of 3 days, is considered "conditioned." It is then collected and sterilized by filtration.

Collagen: Rat-tail tendons were dissected under sterile conditions, as described by Ehrmann and Gey.[8] Collagen was extracted and purified by the technique of Wood and Keech.[9] The collagen used in these studies was the dilute acid-soluble fraction of the neutral salt-insoluble residue described by Wood and Keech. Collagen-coated Petri plates were prepared by mixing 0.05 ml of acid collagen (30 μg Lowry-protein) with 0.01 ml of 6% NaCl. The mixture was then

spread uniformly over the Petri-plate surface with a glass applicator and permitted to gel overnight in a humidified chamber. Coated plates were washed several times with sterile distilled water and once with unconditioned medium prior to use.

Preparation of cultures: Petri plates (Falcon plastic) were inoculated with 400 cells each from a suspension of trypsin-dispersed cells obtained as described previously from the leg musculature of 12-day chick embryos.[10] Each plate contained 2 ml of medium, which was replaced on days 3, 6, and 9 of culture. Cultures were fixed in cacodylate-buffered, 2% glutaraldehyde at day 13, and the colonies were stained with Ehrlich's hematoxylin.

Results.—The major morphological characteristic which distinguishes a muscle clone from a colony of *fibroblast-like* cells is the presence of greatly elongated, multinucleated cells. The cross-striated pattern, typical of skeletal and cardiac muscle, can be demonstrated within these elongated cells.[1, 2] It is these myotubes

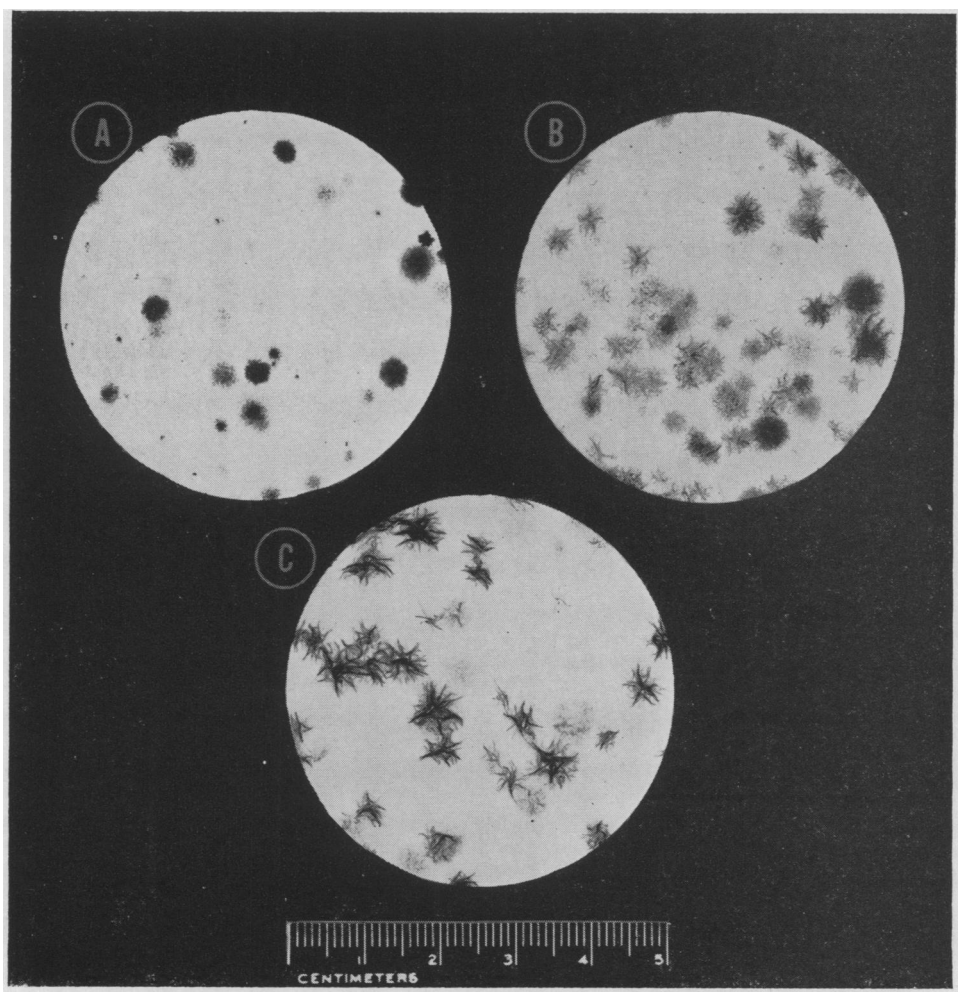

FIG. 1.—The ability of a collagen substratum to replace the requirement for conditioned medium in the development of muscle clones. (*A*) Control culture: untreated surface; unconditioned medium. (*B*) Control culture: untreated surface; conditioned medium. (*C*) Colonies which have developed in unconditioned medium on a surface of reconstituted rat-tail collagen. Each culture was inoculated with an equal aliquot (containing 400 cells) from the same suspension of trypsin-dissociated embryonic (12-day) leg muscle cells. Cultures were fixed and stained on the 13th day of culture.

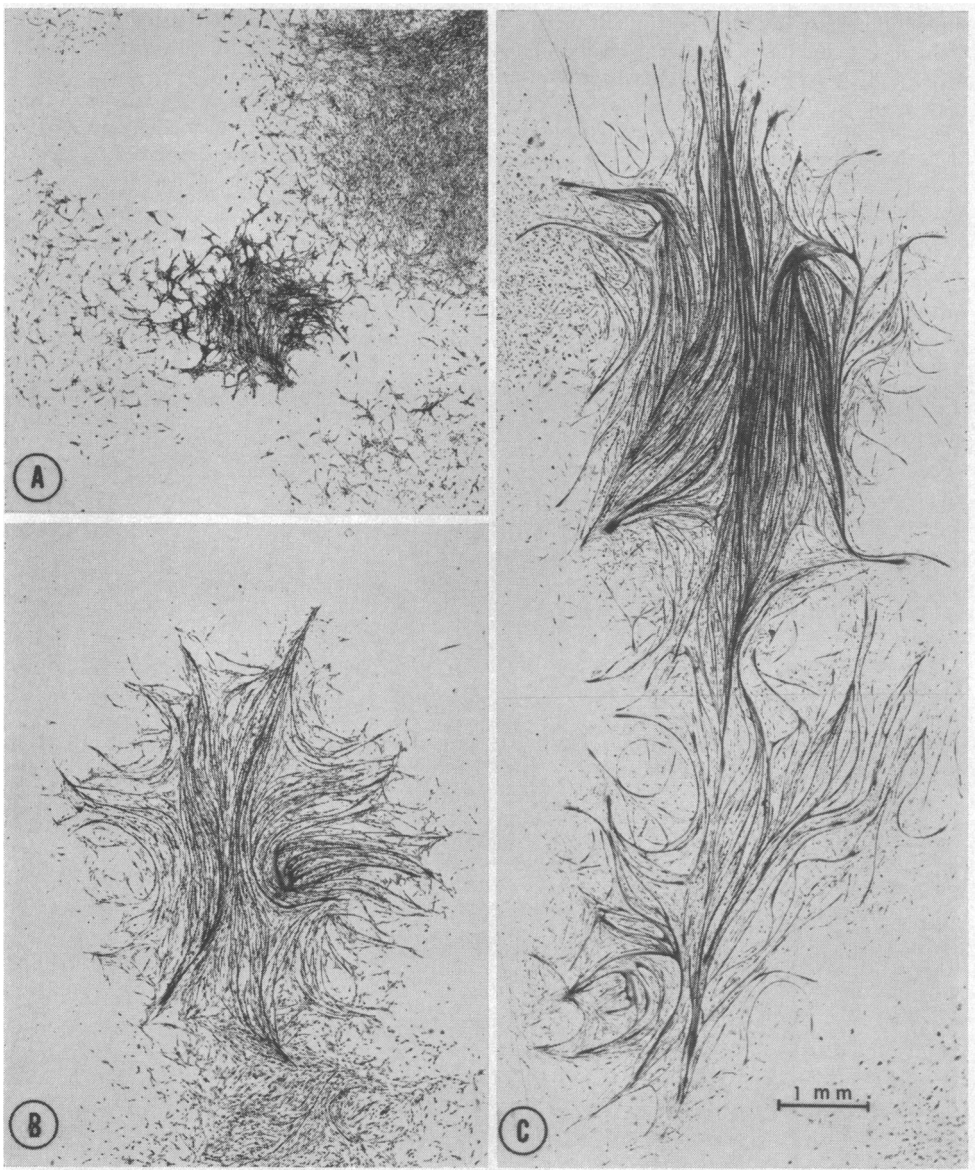

FIG. 2.—Comparison of representative muscle colonies which develop under each of the three sets of conditions tested. (*A*) Small colony containing a few multinucleated muscle fibers, typical of muscle clones which develop in unconditioned medium. Those few muscle colonies which develop in unconditioned medium are most frequently observed in close proximity to colonies of fibroblast-like cells. (*B*) Muscle colony of a culture grown in conditioned medium; muscle fibers are longer and more numerous than in (*A*). (*C*) Two muscle colonies typical of those which develop on a collagen-coated surface in unconditioned medium.

which give the muscle colony developed in conditioned medium its typical fibrous morphology (see Fig. 1*B*). By macroscopic inspection alone, such colonies are not evident in cultures reared in unconditioned medium (Fig. 1*A*), although a small number of minute, poorly developed muscle colonies can be observed by microscopic examination (Fig. 2*A*). However, when cells are plated on a collagen

substratum, despite the fact that the growth medium is unconditioned, large numbers of macroscopic muscle colonies develop. When such cultures are compared with the appropriate controls, it is evident that in the presence of a collagen substratum (Fig. 1*C*), the colonial morphology of the muscle clones is comparable to that observed in conditioned medium. In fact, most of the muscle colonies which develop on collagen with unconditioned medium appear larger and better developed than those usually obtained in conditioned medium (see Fig. 2).

Judged by these qualitative criteria, the collagen substratum appears to replace the requirement for conditioned medium. The same conclusion can be drawn as well from a quantitative evaluation of the yields of muscle clones obtained under the various culture conditions. Table 1 summarizes the results of seven consecutive trials. In every instance, the percentage of muscle [(muscle colonies ÷ total number of colonies) × 100] was dramatically increased by the use of a collagen substratum and differed significantly ($p < 0.001$) from the value for cultures grown in unconditioned medium without collagen. Possibly of greater interest is the finding that the percentage of muscle clones in cultures grown on collagen in unconditioned medium is approximately equivalent to the value for cultures on conditioned medium alone. Of the seven experiments, only two indicate a higher percentage of muscle colonies in the conditioned medium group (expts. 4 and 5). In neither of these two cases, however, is the difference statistically significant. The only significant differences were found in experiments 1 and 6, where the arithmetic mean percentage of muscle colonies was higher in the group in which a collagen substratum was used.

By gross observation alone it is obvious that the muscle colonies on a collagen substratum are larger. Undoubtedly, multiplication of myoblasts is affected. Consideration of the quantitative data suggests,

TABLE 1

THE EFFECT OF A COLLAGEN SUBSTRATUM ON THE DEVELOPMENT OF MUSCLE CLONES

		Conditioned Medium			Unconditioned Medium — Untreated Surface			Unconditioned Medium — Collagen Surface		
Expt. no.	No. of Petri plates of each type	Mean no. of muscle clones	Mean no. of fibro. clones	Mean % muscle*	Mean no. of muscle clones	Mean no. of fibro. clones	Mean % muscle*	Mean no. of muscle clones	Mean no. of fibro. clones	Mean % muscle*
1	6	38.5 ± 1.1	32.2 ± 2.3	54.8 ± 1.6	1.2 ± 0.4	42.2 ± 3.3	2.6 ± 0.9	31.0 ± 1.1	15.2 ± 0.8	67.3 ± 2.5
2	8	20.8 ± 1.3	28.5 ± 1.4	42.0 ± 0.7	0.13 ± 0.05	34.4 ± 1.2	0.4 ± 0.4	21.9 ± 1.4	14.8 ± 1.4	59.9 ± 3.9
3	6	28.8 ± 2.3	32.7 ± 2.0	48.4 ± 4.0	0.2 ± 0.2	31.5 ± 2.4	0.6 ± 1.8	19.0 ± 0.9	17.0 ± 1.9	53.6 ± 0.9
4	6	20.8 ± 1.9	9.2 ± 0.8	69.4 ± 2.5	0.0 ± 0.0	35.3 ± 2.3	0.0 ± 0.0	20.8 ± 1.3	12.7 ± 0.8	62.2 ± 2.3
5	5	21.6 ± 2.0	10.2 ± 0.4	67.1 ± 2.0	0.0 ± 0.0	27.2 ± 6.7	0.0 ± 0.0	16.6 ± 1.1	17.6 ± 1.5	47.8 ± 5.5
6	6	29.0 ± 2.2	26.2 ± 1.1	52.5 ± 2.0	1.5 ± 0.5	24.2 ± 1.5	6.4 ± 1.9	21.7 ± 0.7	10.3 ± 0.9	67.7 ± 2.3
7	10	26.9 ± 0.8	32.6 ± 1.7	44.6 ± 1.4	0.2 ± 0.4	41.1 ± 1.9	0.5 ± 0.3	31.9 ± 1.6	25.4 ± 1.3	55.7 ± 1.9
Cumulative means		26.5	25.7	50.8	0.8	34.5	2.3	24.1	16.8	58.9

Values are expressed as mean number per Petri plate ± standard error of the mean.
* Mean per cent muscle value [(muscle colonies)/(total colonies) × 100] are presented since they normalize the data with respect to fluctuations in plating efficiency from experiment to experiment. Tests of the significance of differences between means for per cent muscle indicate that in every experiment the values for cultures maintained in conditioned medium or unconditioned medium plus collagen were significantly different from the values for cultures maintained in unconditioned medium alone ($p < 0.001$ in all cases).

at least, that the initiation of myotube formation may be effected as well. When a collagen substratum is used, roughly 60 per cent of the colonies which form are muscle colonies as compared to only 2 per cent in the control group (unconditioned medium; without collagen). If the low yield of muscle colonies in the control group were due simply to failure of the majority of the myoblasts either to attach or to divide in the absence of collagen, then these control plates should contain less than half the total number of colonies in the collagen-treated plates. This, however, is not the case.

A comparison of the plating efficiency [(colonies ÷ cells inoculated) × 100] of aliquots of the same cell suspension in unconditioned medium inoculated into Petri plates with or without a collagen substratum indicates that there is no significant difference in the total number of colonies which form from identical inocula on the two types of surfaces (see Table 2). The most plausible explanation is that in the

TABLE 2

THE EFFECT OF A COLLAGEN SUBSTRATUM ON PLATING EFFICIENCY

	Mean Plating Efficiency*			Significance of the Difference between Means†		
	Conditioned medium	Unconditioned medium	Unconditioned medium plus collagen	Cond. med. with uncond. med.	Cond. med. with uncond. med. + coll.	Uncond. med. with uncond. med. + coll.
1	17.7 ± 0.4	11.0 ± 1.0	11.5 ± 0.5	<0.01	<0.001	>0.8
2	12.9 ± 0.7	8.6 ± 0.4	9.1 ± 0.4	>0.1	>0.2	>0.6
3	14.9 ± 0.6	7.9 ± 0.6	9.0 ± 0.6	<0.01	<0.01	>0.4
4	7.5 ± 0.6	8.9 ± 0.4	8.4 ± 0.2	>0.2	>0.3	>0.5
5	8.0 ± 0.7	6.7 ± 1.0	8.3 ± 1.0	>0.4	>0.8	>0.4
6	13.8 ± 0.6	6.4 ± 0.5	7.7 ± 1.0	>0.001	<0.01	<0.5
7	15.1 ± 0.4	10.3 ± 0.6	14.3 ± 0.5	>0.01	>0.6	>0.02

* Values are expressed as mean plating efficiency [(total colonies per plate)/(no. of cells inoculated per plate) × 100] per Petri plate ± standard error of the mean.

† Values listed are probabilities (p) as determined by student's t test. In none of the experiments were the differences in plating efficiency between unconditioned medium groups and unconditioned medium plus collagen groups (last column) statistically significant. (See text.)

control cultures a considerable number of potential muscle colonies fail to differentiate and are scored as clones of fibroblastic cells. The only alternative would require the dual assumptions that the collagen substratum enhances the attachment or growth of muscle cells to about the same extent that it suppresses attachment or growth of colonies of fibroblastic cells.

Discussion.—The results of these experiments clearly demonstrate that the presence of a collagen substratum replaces the requirement for conditioned medium in the development of clones of differentiated muscle. This finding adds further weight to the observations (see introduction) which prompted us to postulate that collagen might be the material responsible for the activity of conditioned medium. It appears less likely, then, that the role of conditioning in the muscle-cloning situation is simply a matter of relieving nutritional deficiencies in the conventional sense.

The implication, rather, is that muscle differentiation is somehow dependent upon the presence of a specific macromolecule produced by another type of cell—the fibroblast—with which the myoblast is intimately associated *in vivo*. Since collagen is deposited extracellularly, the protein would normally be directly available to the myoblast. We know of no evidence that such a dependency does, in fact, play a role in the normal development of muscle tissue. However, the cellular basis for inferring that such a relationship may exist would have escaped detection

without recourse to cloning. In those instances in which we recognize that the differentiation of a particular cell type is directed by the presence of a dissimilar type of cell, the two component tissues have always been histologically segregated, and interacting at the tissue interfaces. No such simple topographic relationship exists in muscle, the two major cell types being intermingled in this tissue.

Although we have no evidence of cellular interaction between these two classes of cells *in vivo*, one of the two, the fibroblast, is known to participate in a variety of developmentally significant cellular interactions. These so-called "epithelio-mesenchymal inductions" occur between various epithelial organ primordia and their subjacent connective tissue elements.[11] In these instances it has been demonstrated that cytodifferentiation of the epithelial component requires the presence of the mesenchyme or connective-tissue precursor cells. Some of these systems show a strict requirement only for the homologous mesenchyme; in others, the response can be elicited by mesenchymal cells from a variety of sources. The commonest activity of such cells, irrespective of their normal location, is the synthesis of collagen. Thus, it seems reasonable to suggest that collagen plays some common role in many of these different specific cytodifferentiations. Although the suggestion that extracellular materials may exert a directive role in cellular differentiation has been widely aired for many years, there is remarkably little evidence to support this notion.

One line of evidence which has been developed recently, although still only suggestive, seems nevertheless particularly relevant. Differentiation of the pancreatic epithelium into the exocrine component of the gland depends upon the presence of either the mesenchyme which normally subtends the epithelium or that from any of a number of other sources.[12] With a culture technique in which an ultrathin Millipore filter is interposed between the two components, it was first established that the presence of the mesenchyme is required only during the first 48 hr of culture. After this time, the epithelium differentiates on schedule even though the mesenchyme is removed.[12] An examination of fine structure in such cultures has demonstrated that during the 48-hr "critical period," fibers which exhibit the periodicity of collagen are deposited in association with the epithelial cells.[13] A more recent radioautographic study further suggests that material having the same enzymatic sensitivity as collagen is synthesized by the mesenchymal cells; this diffuses across the Millipore filter and accumulates on the side of the filter to which the epithelium is attached.[14] Whether the collagen deposited during the critical period mediates the directive influence of mesenchyme upon the pancreatic epithelium is still a matter for speculation.

Summary.—Single embryonic chick muscle cells in culture are capable of forming macroscopic colonies containing differentiated muscle fibers. The development of such muscle clones depends upon the use of medium harvested from dense culture (conditioned medium). Several new experimental findings suggested that collagen, in some form, might be present in conditioned medium and might be responsible for its effectiveness in supporting the development of muscle clones.

As a test of this hypothesis, we have examined the ability of a substratum of precipitated collagen from rat-tail tendons to substitute for conditioned medium. The results demonstrate that single cells plated in unconditioned medium on a collagen-coated surface give rise to muscle colonies whose morphology is comparable

to those which develop in conditioned medium. By quantitative criteria also (percentage of muscle colonies which develop) the use of collagen substratum replaces the requirement for conditioned medium.

The implication of these results is that the development of muscle cells requires the presence of a metabolic product of an associated cell type—the fibroblast. The development of a number of different cell types has been shown to be dependent upon the close proximity of connective tissue elements. It is not unlikely that collagen plays a common role in a variety of differentiative events.

We gratefully acknowledge the invaluable assistance of Mr. Francis J. Kupres during the course of these studies. We are also indebted to Dr. and Mrs. David W. Bishop and Dr. Malcolm S. Steinberg for their helpful criticism during the preparation of the manuscript.

* Predoctoral fellow of the U.S. Public Health Service.

[1] Konigsberg, I. R., these PROCEEDINGS, **47**, 1868 (1961).

[2] Konigsberg, I. R., *Science*, **140**, 1273 (1963).

[3] Konigsberg, I. R., and S. D. Hauschka, in *Reproduction: Molecular, Subcellular and Cellular*, ed. M. Locke (New York: Academic Press, Inc., in press).

[4] Green, H., and B. Goldberg, *Nature*, **204**, 347 (1964).

[5] Green, H., and B. Goldberg, *J. Cell Biol.*, **22**, 227 (1964).

[6] Ham, R. G., *Exptl. Cell Res.*, **28**, 489 (1962).

[7] Pannett, C. A., and A. Compton, *Lancet*, **24**, 381 (1934).

[8] Ehrmann, R. L., and G. O. Gey, *J. Natl. Cancer Inst.*, **16**, 1375 (1956).

[9] Wood, G. C., and M. K. Keech, *Biochem. J.*, **75**, 588 (1960).

[10] Konigsberg, I. R., N. McElvain, M. Tootle, and H. Herrmann, *J. Biophys. Biochem. Cytol.*, **8**, 333 (1960).

[11] Grobstein, C., in *Aspects of Synthesis and Order in Growth*, ed. D. Rudnick (Princeton, N. J.: Princeton Univ. Press, 1954).

[12] Goslow, N., and C. Grobstein, *Develop. Biol.*, **4**, 242 (1962).

[13] Kallman, F., and C. Grobstein, *J. Cell Biol.*, **20**, 399 (1964).

[14] Kallman, F., and C. Grobstein, *Develop. Biol.*, **11**, 169 (1965).

---

*INDIVIDUALITY IN NUTRITION: EFFECTS OF VITAMIN A-DEFICIENT AND OTHER DEFICIENT DIETS ON EXPERIMENTAL ANIMALS**

BY ROGER J. WILLIAMS AND RICHARD B. PELTON

CLAYTON FOUNDATION BIOCHEMICAL INSTITUTE AND DEPARTMENT OF CHEMISTRY, UNIVERSITY OF TEXAS, AUSTIN

Communicated November 24, 1965

The genetotrophic principle can be stated in a few words, namely, *the nutritional needs of an organism are determined by the characteristics of the metabolic machinery it has inherited.* This is so obviously valid when applied to different species (e.g., rats, guinea pigs, fruit flies, maize) that it requires no argument. That differences in nutritional needs based upon inheritance also carry over to individuals *within* animal and the human species was postulated by our group in the journal *Lancet* in 1950.[1]

It was further postulated that because of substantial inborn differences in individual nutritional needs, many human disorders of obscure etiology, which character-