and epinephrine throughout the body supplies a potent tool for studying the role and interplay of these neurohormones in the brain as well as peripherally.

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22 March 1957

Isolation of a Nutritional Variant from a Culture of Rabbit Fibroblasts

The results of earlier studies dealing with the cultural characteristics of rabbit fibroblasts derived from normal tissue indicated that the over-all nutritional requirements of newly established strains vary as a function of their age *in vitro* (*1*). The quantitative aspects of the data were consistent with the concept that either a limited number of cells in the original population (inoculum) were capable of continuous growth in the medium employed or that such cells appeared in the population as a result of mutation. The present experiments (*2*) were undertaken to obtain information regarding the fundamental question of whether nutritional variants are present in a given culture after prolonged serial cultivation *in vitro*. Rabbit fibroblasts, strain RM3-56, which had been propagated serially *in vitro* for almost 2 years were selected for study (*1, 3*). On the assumption that the proportion of nutritional variants in a particular population might be too small to be detected by examining the progeny of single cells selected at random from the population, it was considered expedient to establish conditions which favor the proliferation of variants by altering the composition of the medium.

Table 1. Isolation of a nutritional variant from a culture of RM3-56 fibroblasts.

No. of passages *in vitro*	No. of cells*	No. of colonies†
79	1.1×10^6	1
87	0.9×10^6	0
91	1.5×10^6	2

* Second passage in medium 73.
† Colonies containing cells capable of continuous proliferation in medium 73.

The following media were employed: medium 56 was 5 percent chick embryo extract, 10 percent normal horse serum, and 85 percent S18 (volume for volume); medium 73 was 2 percent dialyzed horse serum, 98 percent S16. Solutions S16 and S18 are of similar composition and contain amino acids, vitamins, salts, and glucose (*3*). The procedure employed for propagating RM3-56 serially in medium 56 and the methods used in quantitative experiments were described previously (*3*). The experiments were based on the observation that RM3-56 fibroblasts fail to proliferate in medium 73 after the first subculture. Under these conditions, the nonproliferating cells gradually degenerate over a period of from 3 to 4 weeks. The regular schedule of fluid replacements was continued, to permit the detection of any cells capable of proliferating in this medium. The results of three experiments performed with cells of different ages *in vitro* are summarized in Table 1.

In the first and third experiments, three colonies, containing from 50 to 100 healthy fibroblasts, were observed after 9 to 16 days (second passage in medium 73). These cells proliferated rapidly, whereas the remaining fibroblasts degenerated. In the second experiment, none of the cells appeared capable of proliferating in medium 73. The cells comprising one of the colonies were subsequently subcultured, and their progeny continue to proliferate after 40 passages in medium 73. This variant or subline is designated RM3-73, to indicate both the tissue from which the culture was originally derived and the medium in which the strain is propagated. Strain RM3-73 also differs from RM3-56 in that it proliferates at a somewhat slower rate in medium 56. On the other hand, strain RM3-73 is indistinguishable from RM3-56 by such criteria as morphology and susceptibility to infection with vaccinia virus.

Of particular significance is the fact that only a small fraction of the cells in RM3-56 cultures are of the 73-type. For example, the three colonies isolated in experiments (Table 1) that employed 3.5×10^6 cells contained a total of only 225 cells when first observed. The proportion of RM3-73 cells in a population of RM3-56, calculated on this basis, is a maximal value because the 73 cells were proliferating throughout the course of the experiments. It is apparent that, under conditions where the ratio of two nutritional types of cells is less than 1 to 10^4, cloning techniques (*4–6*) are impractical for detecting the minority type. The method of selection applied under conditions where a large proportion of the cells fail to proliferate provides a practical means for studying large populations of cells with respect to nutritional cell types. Furthermore, the presence of nonproliferating cells serves the same purpose, in permitting isolated cells to proliferate, as the addition of irradiated cells ("feeder layer") described by Puck and Marcus (*5*). The method is particularly suitable for studying the genetics of cell lines whose nutritional requirements have been partially defined. In these instances, by combining the selection method and cloning techniques, it becomes possible to study somatic cells with any of the required nutritional factors as genetic markers. It is anticipated that the availability of a broad spectrum of nutritional cell types not only will be of aid in understanding the physiology of cultured cells but will enhance the usefulness of the tissue-culture method in virology and in studies on malignancy.

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25 March 1957

Histochemical Demonstration of Enzymes Separated by Zone Electrophoresis in Starch Gels

Zone electrophoresis in starch gels is a rapid, simple, dependable method of high-resolving power for separating complex mixtures of proteins. We have combined this method with histochemical techniques for locating and identifying enzymes in tissue sections in order to analyze the enzymatic composition of biological material (*1, 2*). The numerous esterases responsible for the high level of esterase activity in the mouse liver

were separated and compared with the esterases found so ubiquitously in other organs. The fact that we have also been able to separate and localize tyrosinase and phosphatase suggests that these analytic methods have general applicability in the study of the enzymatic composition of tissues.

In this investigation, the starch block was prepared according to the method of Smithies (*3*). The tissue was homogenized in an equal volume of water and centrifuged at 1700 *g* for 10 minutes. The supernatant liquid was adsorbed onto a strip of filter paper and inserted into the starch block as described by Smithies (*3*). Satisfactory separation of the proteins was achieved in 5 hours at a *p*H of 8.6 at room temperature using a 6-volt drop per centimeter of travel through the starch block. Migration occurred at about half this rate at 2°C. The starch blocks were sliced into two or more thin slabs, permitting several techniques to be applied to the same sample. When the short-chain esters of α-naphthol and naphthol AS acetate were used as substrates, the techniques described by Gomori (*4*) were employed. We propose the term *zymogram* to refer to strips in which the location of enzymes is demonstrated by histochemical methods.

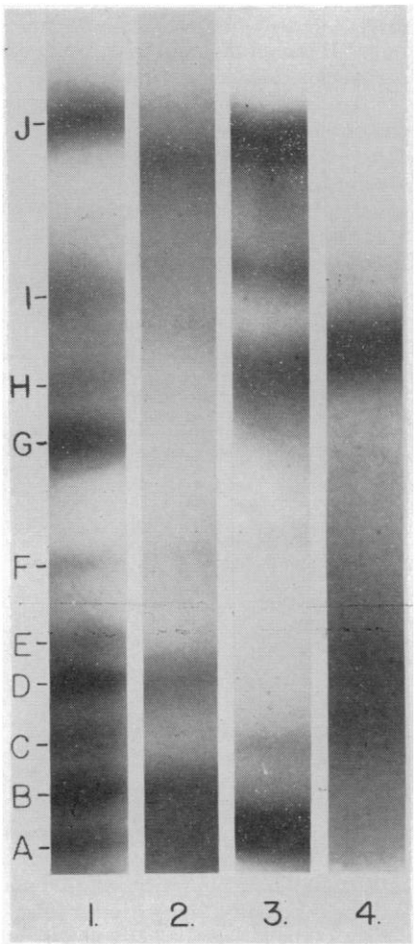

Fig. 1. Zymograms 1 and 2 are from normal adult mouse liver and were prepared using α-naphthyl butyrate as substrate; zymogram 2 was inhibited with eserine at $10^{-4}M$. Zymograms 3 and 4 are from adult mouse lung; these were prepared with different substrates—No. 3 with α-naphthyl butyrate and No. 4 with naphthol AS acetate. Note the inhibition of certain bands in No. 2; the different pattern of bands in lung (No. 3) and liver (No. 1); and the substrate specificity as seen in comparing No. 3 and No. 4.

The distinctive properties of tyrosinases from different species (*5*) were indicated by zymograms of aqueous extracts of mushrooms (*Psalliota*), potatoes, and mouse melanoma. These three tyrosinases were located at different characteristic positions on the starch slabs by their reaction with 3,4-dihydroxyphenylalanine to produce pink bands which later transformed to black melanin.

The separation of esterase-active proteins from mouse liver and other organs yielded a surprisingly intricate and reproducible spectrum of enzymes in the several hundred zymograms so far examined. Characteristically distinct zymograms were obtained from each organ tested (Fig. 1), seemingly reflecting the variety of functions of these organs, and suggesting that these several esterases are metabolically unique and not simply an artifact of the analytic procedures. Some esterases were found in several organs, while others, so far, have been found in only a single organ. However, nearly all the esterases found in any organ also occurred in liver.

Further support for the unique nature of these separated esterases was obtained by reacting the two halves of a starch block with different substrates. Zymograms made using α-naphthyl butyrate as substrate were conspicuously different from those in which naphthol AS acetate was used as substrate (Fig. 1). Certain differences in the distribution of esterases were observed by Gomori (*6*) when he compared α-naphthol esters with naphthol AS esters as substrates. Our results indicate that these differences were due to the substrate specificity of several esterases. To date, procedures using naphthol AS acetate have demonstrated only certain of the esterases that are demonstrated when α-naphthyl butyrate is used as substrate. The esterase in the G band is especially reactive with naphthyl AS acetate. Our results with these substrates demonstrate clearly that the substrate specificity of several of the esterases in the zymograms is not the same.

Finally, the fact that eserine, a known cholinesterase inhibitor, selectively inhibits, for example, the G and H bands (Fig. 1), leads us to suggest that many, if not all, of the bands shown in the α-naphthyl butyrate esterase zymogram are enzymatically distinct.

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References and Notes

1. This investigation was aided by grants from the American Cancer Society and the Michigan Memorial Phoenix Project No. 56.
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14 March 1957

Pituitary Degeneration and Adrenal Tissue Hyperplasia in Spawning Pacific Salmon

In a study of the nature of the death of the Pacific salmons, which occurs regularly following their initial spawning, our attention was drawn to the possible role of the pituitary gland in this process by a series of changes characterizing sexual maturation and subsequent deterioration in these fishes (*1*). The fully mature male and, to a lesser extent, the female salmon exhibit a marked overgrowth of bone and cartilage of the head and vertebral column which produces a hooking of the jaws and a hump back, evidence of greatly increased activity of the pituitary other than the production of gonad-stimulating hormones. At the same time, degenerative changes are occurring—absorption of the scales and focal necrosis of the skin. Following extrusion of sex products, disintegration of the integument increases, and the fish loses its muscular power and balance and dies within a week or two. That such degeneration and death can be hastened by the injection of salmon pituitary glands was observed by R. E. Burrows in the course of experiments on producing accelerated sexual maturation in blueback salmon (*2*). A. P. Rinfret, who, with S. Hane, showed that the salmon pituitary elaborates ACTH (*3*), suggested to us the possibility that the degenerative changes might be caused by intolerable concentrations of a catabolic hormone or hormones secreted by the fish's adrenals under the stimulus of large amounts of adrenocorticotrophic hormones.

Pituitaries and adrenal tissues were secured from several species of the genus *Oncorhynchus*, including king salmon (*O. tschawytscha*), blueback (*O. Nerka*