SUBSTRATE UTILIZATION IN CELL DIFFERENTIATION*

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During embryonic development, the cells of an embryo undergo progressive specialization. This specialization is evident in the morphological appearance and in the functional activity of the cell and, more subtly, in the developmental potentialities of the cell. This report is concerned primarily with the changes occurring in the functional activity of cells during the course of their development, particularly with those changes which determine the ability of the cell to synthesize simple substances. The changes that occur in synthetic ability during development are probably related in a causal way to subsequent developmental capacity—that is, to the prospective potency of a cell. The fact that the prospective potency of a cell is progressively restricted during development may well reflect a reduction or loss in the ability of the cell to carry out various types of syntheses. It seems apparent, however, that in the adult organism, some groups of cells carry on metabolic activities and synthesize various substances which are not detectable in the early embryo. Thus, during development, some cells may lose part of their original endowment, and others may acquire synthetic abilities not manifest in the early embryo. This generalization must be regarded with reservation, however, since present techniques do not permit a critical distinction between a latent and a nonexistent potentiality for a given synthesis.

Although the production of various complex molecules, such as the pituitary hormones of the adult, does not appear to be a detectable function of embryonic cells, it is not yet possible to prove that the production of such hormones represents genuinely new synthetic abilities—that is, those not present, even in a latent sense, in the type of cellular organization found in antecedent cells.

In support of the concept of differentiation by loss of synthetic ability is the observation that many enzymes are normally found in assayable quantities during the earliest stages of development (Moog, 1952), but that they tend to be concentrated preferentially in certain tissues or organs as development proceeds. That is, some enzymes are greatly reduced or lost in some tissues during functional differentiation. The observations of Ebert *et al.* (1955) on the progressive restriction of the areas of the chick blastoderm which synthesize the heart-muscle protein myosin likewise support the concept that biochemical differentiation involves losses of synthetic ability.

However, although antecedent embryonic cells may have the ability to carry out any of the syntheses performed by their adult daughter cells, it is apparent that all of the diverse daughter cells are not characterized by the same metabolic patterns, nor do they synthesize all of the substances manufactured by their sister cells. The cells of the thyroid, parathyroid, and thymus glands, as well as the various types of secretory and nonsecretory epithelial cells found

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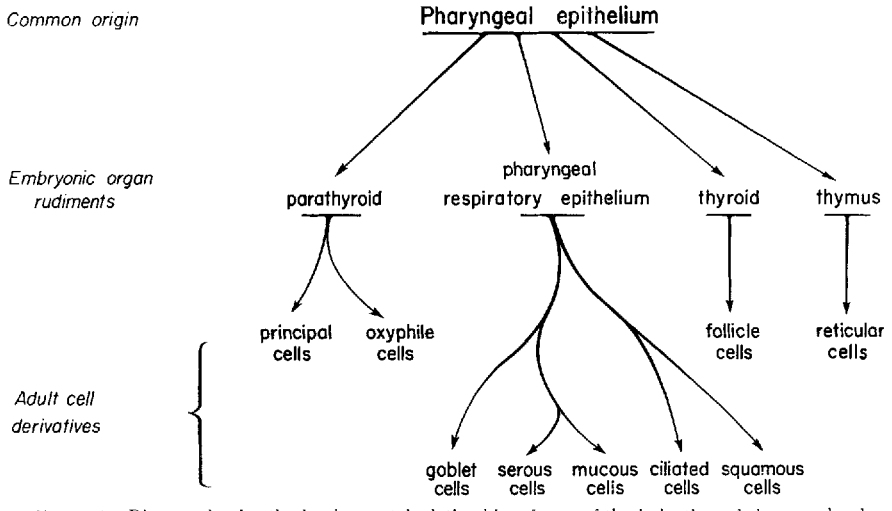

FIGURE 1. Diagram showing the developmental relationships of some of the derivatives of pharyngeal endoderm. The adult cell types possess distinctive synthetic abilities, and may differ also in their ability to synthesize simple metabolites.

in the lining of the pharynx and respiratory system, though all having a common endodermal origin, quite obviously have specialized synthetic abilities which distinguish them from one another (*cf.* FIGURE 1).

One plausible view of cellular differentiation pictures the cells of the organism as specializing in the synthesis not only of complex substances such as hormones or enzymes, but also in the synthesis of simple substances needed by them all. Thus a division of labor would be effected. Such simple substances as amino acids, purines, pyrimidines, *etc.*, may perhaps be synthesized by some cells and not by others. Since these substances are required by all cells, it is probable that all the cells at some early stage of embryonic development are equivalent in the capacity to synthesize them. As embryonic development progresses, the ability to synthesize these substances may become restricted to only certain types of cells, while the remaining cells are diverted to other synthetic jobs. Such losses of synthetic ability might well account for the apparently irreversible nature of differentiation. In any event, this hypothetical specialization in synthesis would seem to be an efficient way in which to organize the cellular activities of a metazoan. If such losses in synthetic ability do occur, and if they can be chemically defined, then the course of normal cellular differentiation might be controlled, since the chemical environment of the cells undergoing specialization could then be altered in a meaningful way. Efforts to test this hypothesis of the nature of cellular specialization have been concerned, in the investigations to be reported here, with: (1) the ability of differentiated or semidifferentiated cells of various types to synthesize simple substances such as amino acids, purines, or pyrimidines; and (2) the ability of melanoblasts of various genotypes to utilize particular substrates in the synthesis of their individual melanin pigments.

Metabolism of Glucose by Cells in Tissue Culture

In the first of these investigations, cells from organs of seven-day-old chick embryos were isolated and grown in tissue culture. Cells were taken from the liver, heart, skin, spleen, kidney, and the pigmented layer of the retina. These cells were grown in flasks or Petri dishes in the presence of a complex, chemically undefined medium consisting of chick embryo extract, chicken plasma, horse serum, Earle's saline (Earle, 1943), and amino acids or glucose labeled with C^{14}. After varying periods of growth in the presence of this medium, the cells were removed and fractionated, and the protein fraction was hydrolyzed with HCl. Aliquots of the hydrolysate were then chromatographed on filter paper in parallel runs. The distribution of radioactivity on the chromatograms was determined by counting with a Nuclear Scalar equipped with a G. E. open window end counter, and the development of the parallel chromatogram colorimetrically was used as the basis for a tentative identification of the sources of the radioactivity. When glucose was used as the labeled substrate, it was evident that liver and heart cells produced different pattern distributions of radioactivity on the chromatograms and that, therefore, they must have different patterns of synthetic ability. These differences are probably related to the incorporation of part of the glucose carbon skeleton into amino acids, particularly into alanine and aspartic acid, although other amino acids were also involved to a lesser extent.

Differences were also evident in the synthetic patterns of some of the other tissues but, as yet, no positive identification of the chemical differences has been made. These results are preliminary, and any conclusions drawn must be considered tentative pending the outcome of further and more detailed investigations that are now in progress. It may be noted, however, that results are available from other investigations that bear on this general problem. A. Fischer *et al.* (1953) have shown that chick heart fibroblasts grown in the presence of glucose labeled with C^{14} synthesize amino acids from metabolites ultimately derived from the glucose. The largest amount of radioactivity was found in alanine and aspartic acid, signifying a formation of pyruvate and oxaloacetate from glucose, with subsequent transamination to form the two amino acids. Some C^{14} was also found in the tissue serine, glycine, glutamic acid, and proline. In studies of protein synthesis and amino acid turnover in tissue culture, Gerarde *et al.* (1952) demonstrated that, in tissues maintained in Tyrode's medium containing glycine-1-C^{14}, the protein subsequently isolated was found to contain both labeled glycine and serine in the ratios of 3.6 for heart fibroblasts and 2.0 for lung cells. In similar experiments with DL-alanine 1-C^{14} and DL-phenylalanine-3-C^{14}, all of the C^{14} of the proteins was found restricted to those two amino acids (Winnick, 1952). Thus heart fibroblasts and lung cells differ in their ability to convert glycine to serine, but are alike in being unable to convert alanine or phenylalanine to any other amino acid.

Unfortunately, in none of these investigations have clones of cells been used. All have started with populations of cells derived, to be sure, from restricted areas of the embryo, but undoubtedly including cells of diverse synthetic

abilities. Negative results would have had little significance, since populations of cells drawn from different tissues might have the same pattern of synthetic abilities, although composed of a variety of cells of different, perhaps complementary, synthetic abilities. Since, in certain instances, however, these diverse populations of cells were shown to possess different synthetic capacities, certainly clones of cells extracted from the population would have shown similar or greater differences in synthetic ability.

The great difficulty in obtaining clones of cells from single cell isolations, in some instances, may be due to an obligate cross-feeding among closely associated cells. The provision of an adequate medium for such separated cells would be very difficult if the nutritive exchange consisted of labile substances. Much effort has been invested in several laboratories in the attempt to devise a chemically-defined medium which will sustain the indefinite growth of animal cells in tissue culture (*cf.* Waymouth, 1954, for review of the nutrition of animal cells). These efforts, though not yet successful, have made significant progress and, when such media are devised, they will greatly facilitate investigations of reciprocal nutritive relationships among the specialized cells of metazoans.

It is already evident, however, that a medium that may be satisfactory for one type of cell may be inadequate for another. A. Fischer (1953) for example, found that cardiac myoblasts were unable to survive on a diet that was entirely satisfactory to liver fibroblasts. He further observed that, in a medium containing a mixed culture of connective tissue cells and macrophages, the connective tissue cells gradually died out, leaving a pure culture of macrophages. The synthetic abilities of the fibroblasts may be more restricted therefore than those of the macrophages.

In studies on morphogenesis and differentiation in explanted chick blastoderms, Spratt (1950a, b) was able to demonstrate quantitative and qualitative differences in the nutritive requirements of different tissues and organs for carbohydrate substrates. Such differences in nutritional requirements are probably reflections of corresponding differences in synthetic abilities. Moreover, the nutritive requirements of the tissues changed, as differentiation proceeded, attesting to the changing patterns of synthetic ability during the course of differentiation.

All of these studies on the nutritive requirements of animal cells involved the use of complex, chemically undefined media. Such investigations would be facilitated and the interpretations made more certain if the media used were chemically defined. The availability of labeled compounds, however, has now, to some degree, circumvented the need for a chemically defined medium in testing the nutritive requirements and, as a corollary, the synthetic ability of cultured cells. With labeled compounds, the pathways of synthesis, interconversion, and degradation of amino acids, purines, pyrimidines, *etc.* may be followed, even though the compound in nonlabeled form is present in the medium, along with numerous unidentified constituents.

Underlying the approach discussed here is one assumption that must be regarded with reservation, namely, that explanted cells retain the pattern of metabolic syntheses which characterized them at the time of explantation.

There is evidence that the pattern of amino acid composition of cells remains constant *in vitro* (Gerarde *et al.* 1952), but the ability to synthesize the constituents of this pattern may not, of course, have remained constant. Nevertheless, this constancy of amino acid composition in cultured cells over an extended period of time is at least reasssuring, though it must be realized that significant changes in synthetic ability may occur immediately on explantation as a consequence of the isolation of the cells from the intact organism. It is also true that the synthetic activities of cells may vary with the type of medium in which they are grown. Fell and Mellanby (1953) have clearly shown that large quantities of vitamin A, added to the culture medium of various epithelial cell types, will induce the cells to assume the functions of a secretory epithelium and will inhibit keratinization.

Quite obviously, only the first steps have been taken in the investigation of the pathways of biochemical differentiation. Once the differences in synthetic ability now evident between related partly-differentiated cell types are clearly specified, then that portion of the study which is particularly significant for embryology may be undertaken: namely, the comparison of the patterns of syntheses found in the early embryonic cell types with those found in their specialized offspring.

Substrates for Melanogenesis

The second part of this general investigation deals with the rather special problem of melanogenesis. This investigation has already been carried far enough to answer specific questions and to raise several others. The problem simply is that of determining the substrates used in melanin synthesis by melanoblasts of different genotype. Melanin is found in all shades from yellow through brown to black. The particular color of melanin pigment synthesized by a melanocyte is governed by the genotype of the cell and the embryological history of the cell. The question which may be posed is: Do the different colors of melanin involve the use of different initial substrates in synthesizing the pigment, or are the colors attributable to different types of polymers of the same monomer or to associated elements or compounds which determine the specific color produced?

This problem was investigated by growing melanoblasts from mice or chicks in the presence of various possible labeled precursors of melanin. Both *in vivo* and *in vitro* techniques were used. Since it has been commonly believed that the amino acid tyrosine is the initial substrate for melanin synthesis, this compound, labeled in the second carbon atom of the side chain, was added to tissue cultures of embryonic chick skin. The skin contained melanoblasts in the process of making melanin. After melanocytes filled with melanin granules had differentiated, the tissue was fixed, sectioned, mounted on glass slides, and stained. Autoradiographs of the tissue were then prepared according to the following procedure. After staining with hematoxylin in water, the tissue on the slide was dipped in 4 per cent polyvinyl alcohol for a few minutes, and then the slide was allowed to dry for 12 hours while lying flat. Thus, the polyvinyl alcohol embedded the tissue and produced an optically smooth surface about

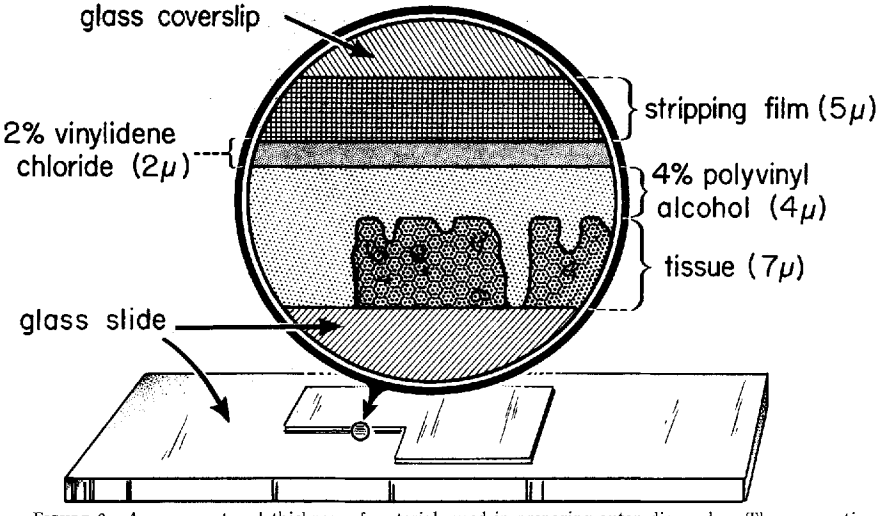

FIGURE 2. Arrangement and thickness of materials used in preparing autoradiographs. The preparation may be made permanent by mounting a coverslip on top of the film after it has been developed.

four μ above the tissue. Next, the slide was dipped in 2 per cent vinylidene chloride (saran), which deposited a chemically impervious layer about two μ thick. Finally, Kodak autoradiographic permeable base stripping film (about five μ thick) was floated onto the slide and allowed to dry in position above the tissue. Depending upon the level of radioactivity of the preparation, the film was exposed from one day to several weeks before being developed. After the film was developed, a coverslip was mounted on top of the film, and the tissue and its autoradiograph could then be viewed as a unit (FIGURE 2).

Autoradiographs prepared of tissues grown in media saturated with labeled tyrosine revealed that the tissue was radioactive, but the tyrosine was not selectively concentrated in the melanin granules (FIGURE 3). It is apparent that the radioactivity is distributed through the sectioned tissue in proportion to the concentration of the protoplasm, and no more radioactivity is lodged in the melanin granules than in the adjacent cytoplasm.

Another contender for the role of initial substrate in melanogenesis is 3-4-dihydroxyphenylalanine, or dopa– the first stable product of the *in vitro* tyrosinase-catalyzed oxidation of tyrosine. Dopa is, in its turn, rapidly oxidized through various intermediates to melanin. Since dopa oxidase is more readily demonstrated in mammalian or avian tissue than is tyrosinase, many investigators have concluded that the initial substrate for melanogenesis, in these tissues, is indeed dopa rather than tyrosine. If dopa is the initial substrate, then of course, it must be formed by some mechanism other than the catalytic oxidation of tyrosine by tyrosinase.

Since labeled dopa was unavailable commercially at the time these experiments were begun, an attempt was made to produce labeled dopa from the labeled tyrosine through catalytic oxidation by tyrosinase in the presence of ascorbic acid. The presence of the ascorbic acid permits the accumulation of

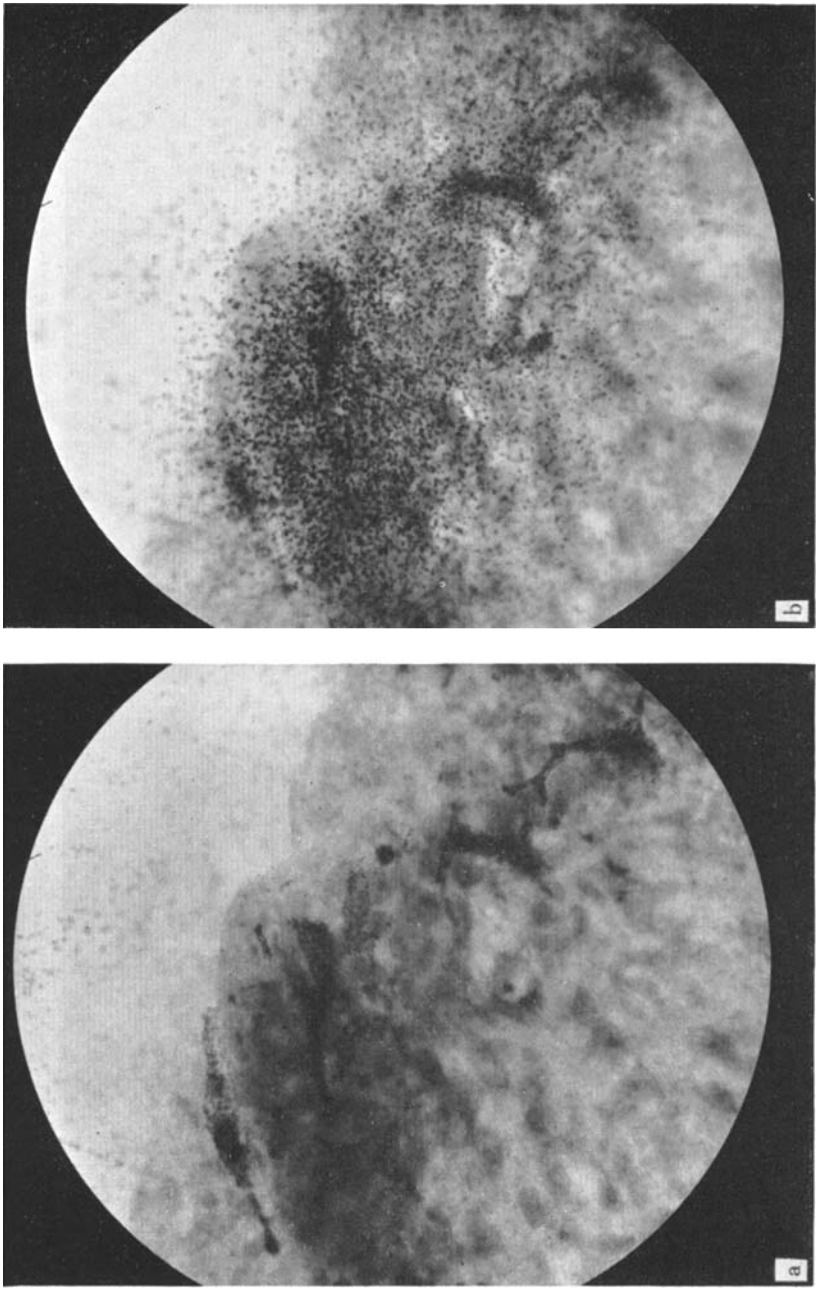

FIGURE 3. Autoradiograph of skin containing melanocytes that develop *in vitro* in the presence of tyrosine labeled in the side chain with C^{14}. The photograph (right) of the autoradiograph is taken in the plane of the film about 10 μ above the tissue. The tissue is therefore out of focus, but the melanocytes can be located by reference to the photograph (left) taken with the tissue in focus.

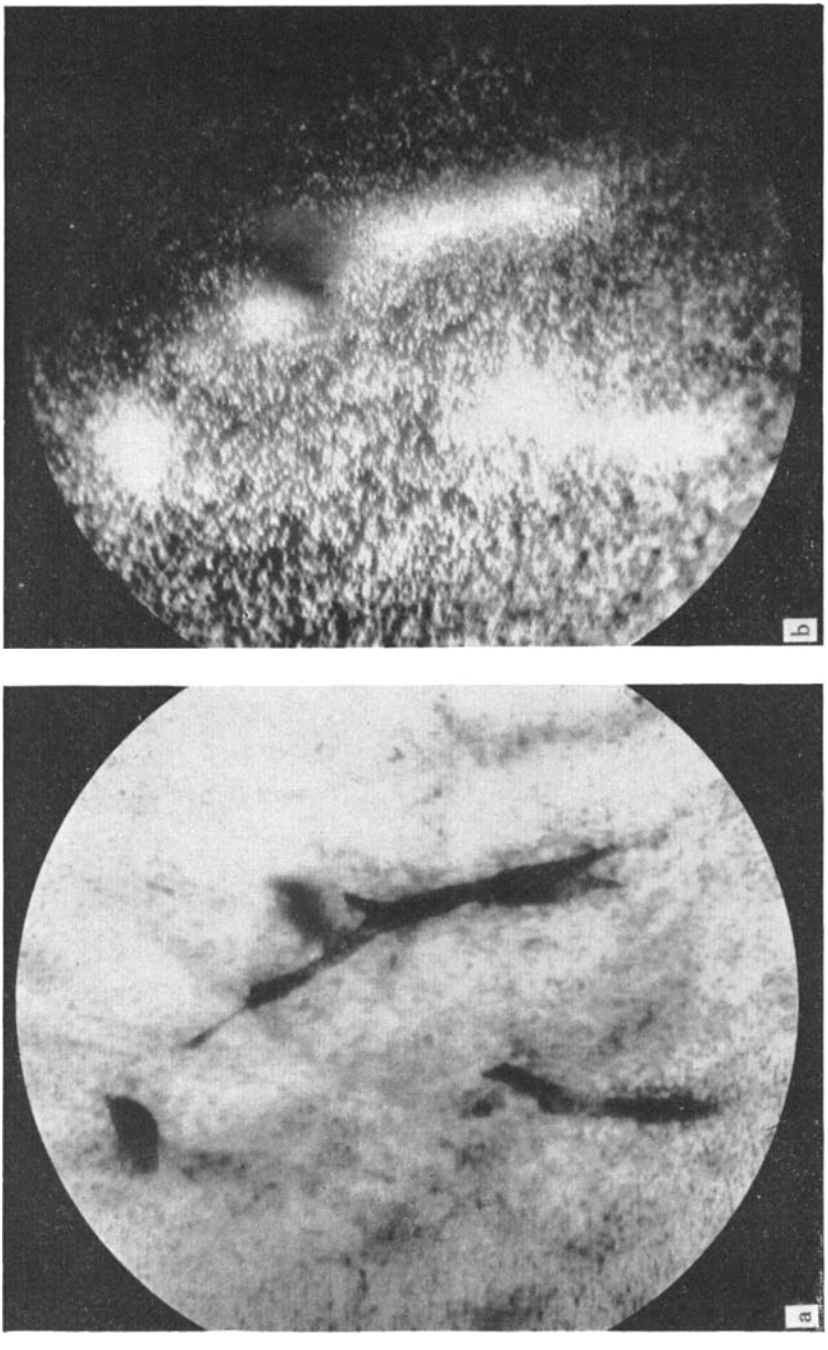

FIGURE 4. Autoradiograph of melanocytes from a tissue culture of embryonic chick skin grown in the presence of radioactive oxidation products of tyrosine. The autoradiograph (right) was photographed by reflected light, thus making the silver grains appear as white specks. That the most intense localization of the radioactivity is in the melanocytes may be determined by comparing the photograph (left) of the melanocytes with the autoradiograph (right).

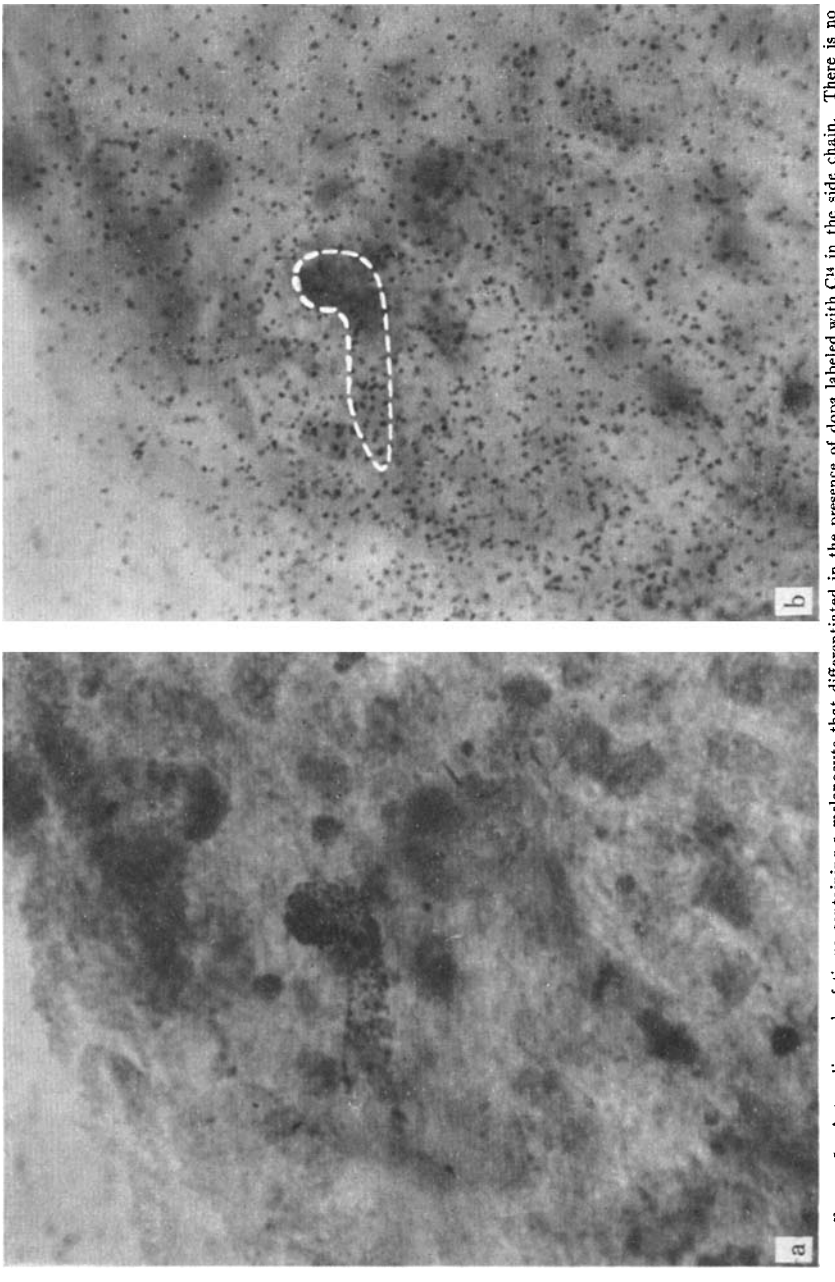

FIGURE 5. Autoradiograph of tissue containing a melanocyte that differentiated in the presence of dopa labeled with C^{14} in the side chain. There is no selective accumulation of radioactivity in the melanin granules. The outline of the melanocyte has been drawn on the autoradiograph (right) to facilitate comparison with the tissue photograph (left).

dopa in the reaction mixture (Krueger, 1950). The reaction mixture containing 2 ml. ascorbate at .67 mg./ml., 0.5 ml. DL tyrosine at 1 mg /ml , and 0.1 ml. of a weak solution of a commercial tyrosinase was allowed to react until a distinct pink color became evident, indicating that some of the tyrosine had been converted to dopachrome. The reaction was then stopped by boiling. Chromatographic analysis revealed the presence of a small amount of dopa in the mixture. This mixture, containing a variety of melanin intermediates including dopa, was then added to cultures of chick melanoblasts, and autoradiographs taken of the culture after extensive melanization occurred. These autoradiographs revealed a distinct selective accumulation of radioactivity in the melanin granules, and these positive results were attributed initially to the small amount of dopa in the medium (FIGURE 4). After commercially produced dopa, labeled in the side chain, became available, these experiments were repeated with large concentrations of the dopa. Surprisingly, the autoradiographs prepared were completely negative so far as any selective incorporation of the dopa into melanin granules was concerned (FIGURE 5). Apparently, in the mixture of oxidation products of tyrosine, some melanin intermediate other than dopa was responsible for the positive autoradiographs obtained.

Since the tissue culture medium represents an artificial environment for the development of melanoblasts, it was desirable to check the results of the tissue culture experiments by corresponding experiments carried out on living animals. Consequently, labeled dopa and labeled tyrosine were injected subcutaneously into new-born C57 black mice and also yellow mice. Injections were made three times a day for four days, at which time the skin and hair of the mice appeared deeply pigmented. A total of about two microcuries was injected into each mouse. The skin was then removed, fixed, sectioned, and stained, and autoradiographs of the sections were prepared. Again, no selec-

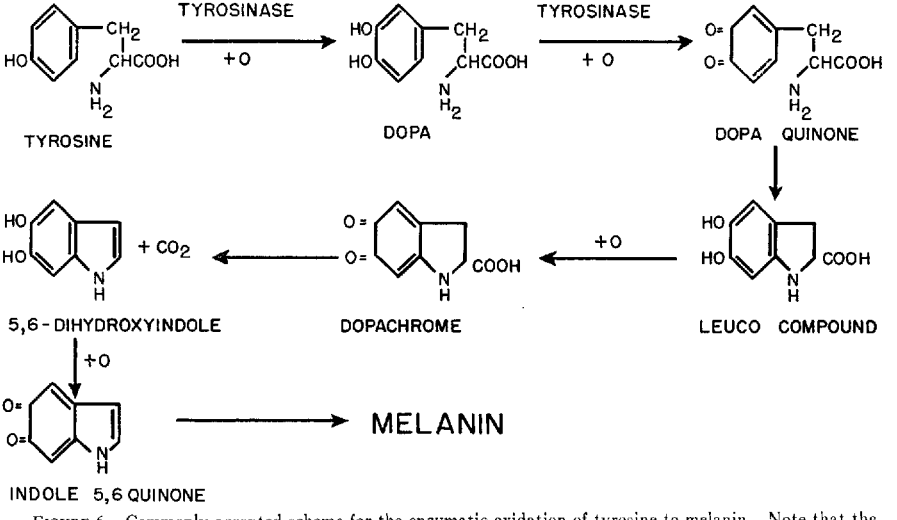

FIGURE 6. Commonly accepted scheme for the enzymatic oxidation of tyrosine to melanin. Note that the side chain of tyrosine is incorporated into the monomer that polymerizes to melanin.

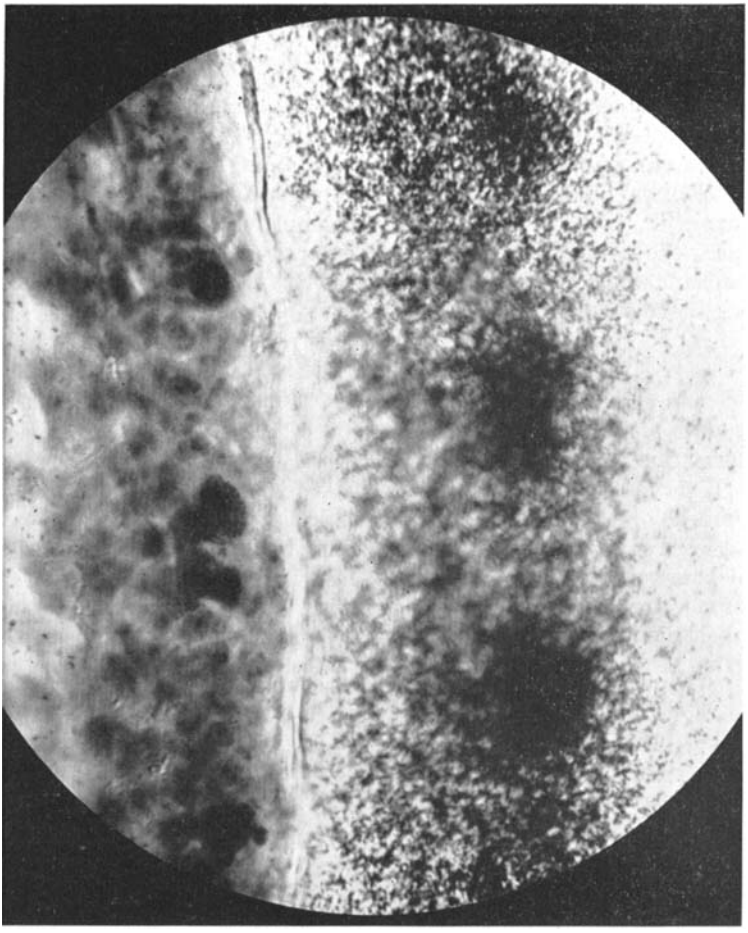

FIGURE 7. Autoradiograph of tissue and melanocytes grown in the presence of uniformly labeled tyrosine. The autoradiograph has been displaced obliquely to the right of the tissue. The melanocytes have rounded up, but are obviously the sources of intense radioactivity.

tive accumulation of the tyrosine or the dopa was evident in the melanin granules, even under these conditions of *in vivo* synthesis of the melanin. These results are difficult to reconcile with the commonly accepted scheme for melanogenesis starting with tyrosine or dopa (FIGURE 6).

The picture was further complicated by the results of experiments using uniformly labeled tyrosine, rather than tyrosine labeled only in the side chain. When the uniformly labeled tyrosine was added to tissue cultures of developing chick melanoblasts, the labeled material was selectively concentrated in the melanin granules, as shown by autoradiographs (FIGURE 7). This result implies that the side chain of tyrosine is not incorporated in the melanin, whereas the ring portion of the molecule does become a part of the melanin (*cf.* FIGURE 6). There is the difficulty, however, of explaining the previous results, in which some oxidation product of tyrosine labeled only in the side chain was

incorporated selectively into melanin granules. At present, no satisfactory explanation is at hand, and further experiments will be needed to clarify these seemingly contradictory results.

Since tryptophane had previously been implicated in the synthesis of a yellow pigment by mouse skin extracts (Foster, 1951), this amino acid labeled in the side chain was added to cultures containing developing chick melanoblasts. Not only did the tryptophane fail to yield selective autoradiographs of any part of the tissue but, in addition, it completely inhibited the synthesis of melanin without adversely affecting the general viability of the tissue cultures. Tryptophane would thus appear to be excluded as a substrate for melanogenesis in chick-tissue cultures.

Summary and Conclusions

(1) Semidifferentiated cells derived from different organs of the embryonic chick, when grown in tissue culture, exhibit different patterns in the metabolic transformation of glucose.

(2) Of several possible substrates which might be used by melanoblasts in the synthesis of melanin pigment, only uniformly labeled tyrosine and oxidation products of tyrosine were found to be differentially incorporated into melanin granules as shown by autoradiographs. The fact that the labeled side chain of neither tyrosine nor dopa served as melanin precursors in these tissue cultures, casts doubt upon the generally accepted scheme for melanogenesis based upon the action of tyrosinase upon tyrosine or dopa.

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