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STUDIORUM PROGRESSUS

Production *in vitro* of Melanin-Like Pigmentation by Cells of the Intestinal Mucosa[1]

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The dendritic melanocytes of the epidermis and the cells of the hair matrix contain melanin granules, the amount and/or size of which is increased by the classical dopaoxidase reaction as described by BLOCH and RYHINER[4] and BLOCH[5], or by the tyrosinase reaction (FITZPATRICK and coworkers[6]). In the full series of reactions, beginning with the oxidation of tyrosine and leading to the appearance of cytoplasmic melanin granules, visible with the light microscope, five steps can be considered separately in accordance with current proposals.

The oxidation of tyrosine to dopa, and secondly from dopa to dopachrome is possible through autoxidation of tyrosine, and can be accelerated considerably by small amounts of dopa (LERNER[7]). It is catalyzed by phenoloxidase from mushroom extracts, which is a copper-containing enzyme. Recently the two steps were separated, the first being catalyzed by cresolase, the second by catecholase, both of which are copper-containing proteins (MASON[8]). These two steps require 1 atom of oxygen each. In spite of numerous investigations, the third step (further oxidation of dopachrome to melanin), the structure of melanin, and the fourth step (i.e. the polymerization of melanin) are not completely understood. According to MASON[9] 2·6 more atoms of oxygen are required. MONDER, WILLIAMS, and WAISMAN[10] found that 6 atoms are required. Both groups state that 1 molecule of CO_2 is given off in the course of the oxidation. However, according to CROMARTIE and HARLEY-MASON[11] dopamelanin still contains about $^1/_6$ of its original CO_2. The fifth step, the formation of melanin protein, resulting in microscopically visible granules in the cytoplasm of melanocytes, is also poorly understood. There is no certainty as to whether these five steps are actually catalyzed enzymatically in this exact sequence in animal tissues, or if other reactions lead to their completion.

In the course of experiments on the histochemical detection of aromatic amines, the development of pigmentation in certain cells in the lamina propria of the mucosa of the small intestine of guinea pigs was observed when sections were incubated for short periods in buffered solutions containing 5-hydroxytryptophane. Incubation in 5-hydroxytryptamine solutions resulted in a more pronounced pigmentation. A number of amines and amino acids of the indolalkylamine or the catecholalkylamine series was then investigated. With 3-hydroxytyramine (dopamine) and with dihydroxyphenylalanine (dopa) a very striking reaction was observed. It was then decided to investigate whether this phenomenon was related to the dopaoxidase. It was also hoped to learn more about the phenolic oxidation and the possible role in melanin-formation of these physiologically and pharmacologically active compounds. A number of questions arose in the course of this investigation which cast some doubt on the completeness and the validity of the currently existing theories of the dopaoxidase or tyrosinase reactions as described for skin, which may require reevaluation of the proposed individual steps of these reactions. For this reason it seemed justified to publish these preliminary results at any early stage. Much more must be done to clarify the situation.

Methods.—Most studies were performed with small intestine, generally duodenum and less frequently ileum or jejunum. Other parts of the gastrointestinal tract investigated include stomach, coecum, appendix, ascending and descending colon. The other organs or tissues examined were skin, kidney, liver, brain, heart, spleen, adrenal gland, thyroid gland, and thymus.

Tissues of adult guinea pigs of either sex were used in the majority of the experiments; the remainder were taken from rats, and but a few from man and frog. The animals were sacrificed by stunning and bleeding. Human tissues were obtained shortly after surgery (appendix, normal and inflamed; through the courtesy of Drs. H. T. ENTERLINE and J. J. MORAN, Department of Pathology, Hospital of the University of Pennsylvania).

Frozen sections of fresh or formalin-fixed tissues (10% buffered formalin) were cut usually at 10μ, or occasionally at 20 or 30μ, and placed directly on slides. The incubation medium consisted of 10 ml of 0·1 *M* phosphate buffer containing 0·72% NaCl, or of 0·0067 *M* phosphate buffer (Soerensen), pH = 8·0, plus substrate. Tissues were incubated in covered Coplin jars at 37°C, or at room temperature (25–29°C). The incubation time was usually 30 min, except when noted otherwise.

The following substrates were used, mostly at a concentration of 0·002 *M*, occasionally at 0·005 *M* or 0·01 *M*: dopamine HCl, L-dopa or DL-dopa, epinephrine bitartrate, levarterenol bitartrate monohydrate, tyramine HCl, DL-tyrosine (not dissolved completely), 5-hydroxytryptamine creatinine sulfate, DL-5-hydroxytryptophane, tryptamine HCl, L-tryptophane. In certain experiments the solutions were bubbled through with N_2 (freed from O_2 by bubbling through pyrogallol solution).

Immediately after the incubation period the sections on the slides were covered with glycerine; alternatively, some were passed through alcohols, xylol and preserved in 'Permount' after counterstaining with hematoxylin-eosin.

Results.—A number of changes in the experimental histochemical conditions were made in the attempts to classify the pigmented cells, and to clarify the underlying biochemical mechanism. Under the experimental conditions described above, the following typical result was obtained consistently when sections of duodenum were incubated at a pH of 8 and room temperature for 30 min in a solution of dopamine that had been allowed to autoxidize for a short period of time (see Figure).

[1] This investigation was supported by research grants [B-282 (C3 and C4)] from the National Institute of Neurological Diseases and Blindness, National Institutes of Health, Public Health Service.

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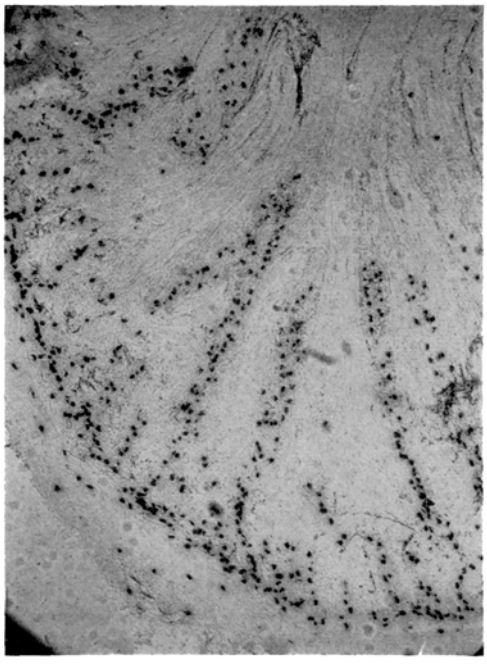
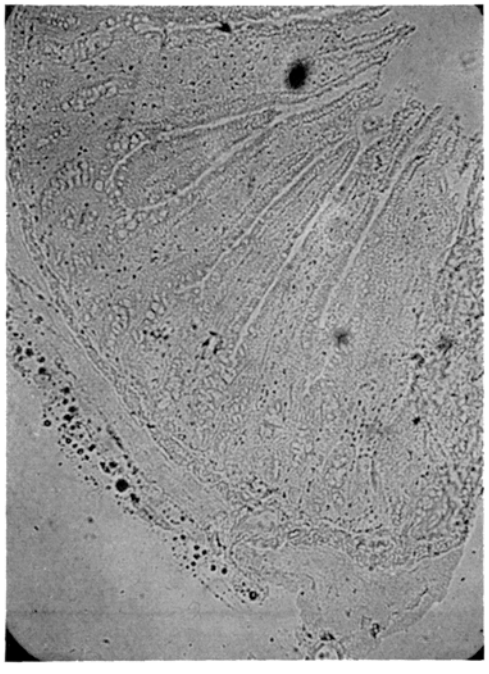
[11] R. I. T. CROMARTIE and J. HARLEY-MASON, Biochem. J. *66*, 713 (1957).



Dopamine incubation



Control

Duodenum of rat, fresh frozen 10 micron sections, dopamine 5×10^{-3} *M*, 30 min, pH = 8·7, 22°C. Magnification 74 times (Photomicrographs by Dr. P. HUBER, Zürich)

The lamina propria of the mucosa, predominantly in the vicinity of the muscularis mucosae and progressively less to the tips of the villi, showed a large number of round or elongated cells of medium size, having an unindented, rather small nucleus, and cytoplasm filled with brown granules of varying size. Some of the cells were homogeneously brown, the nucleus being hidden by the pigment. Some cells were surrounded by a halo of the same pigment. The epithelial parts of the mucosa did not show any of these cells; this was also true for the whole region of the Brunner glands. The muscular layers contained such cells only occasionally. In the sections which had not been incubated or were incubated in a medium devoid of substrate, and thus served as controls, the same apparent kind of cell showed but a very faintly yellow or pale granulated cytoplasmic structure, provided no counterstain was used. They appeared to be eosinophilic.

A similar reaction, and a similar pattern of the distribution of these cells was found in the ileum, jejunum, coecum and to some extent in the pyloric part of the stomach. In the ascending colon only a few such cells were found, and even fewer were noted in the descending colon. In the fundus of the stomach, which contained a few of such cells, some were scattered outside the lamina propria of the mucosa. The appendix showed numerous pigmented cells in the lamina propria mucosae, and also in the other layers, including the subserosa. A huge number of densely packed pigmented cells was found in a highly inflamed appendix. Here the numerous cells in the inflamed regions (connective tissue and muscularis) were larger and showed much more of a pigmented halo than the cells in the lamina propria of the mucosa.

In the following organs some single, scattered, strongly pigmented cells were found but no typical pattern as to their localization could be established: kidney, brain (various parts), heart, and spleen; none was seen in the liver. In a few cases the thymus and thyroid gland showed a large number of pigmented cells. In skin from the ear of brown-red-pigmented guinea pigs or from albino guinea pigs and rats, the dermis showed a number of strongly pigmented cells. At the same short incubation time (30 min) no increase in granular pigment of the basal epidermal cells or the hair-matrix cells was observed: With an extended time of incubation (overnight), there was some indication of an increase in pigment in the basal epidermal cells as compared to the controls; however accurate quantitation of this was not possible.

In tissues treated with formalin prior to sectioning, leucocytes stained strongly granular, and erythrocytes took up pigment homogeneously though not as much as the leucocytes. This was especially well visible in blood vessels of the inflamed appendix.

Storage of sections on slides in formalin solution for two weeks or for several weeks at room temperature had no apparent influence on the reaction. Subsequent immersion of sections in ethanol or chloroform for 24 h did not affect the pigment formed from dopamine.

The colour which developed in the cells after incubation with dopamine or dopa was brown, dopa yielding a somewhat darker shade. In all cases, a strong pigment reaction was obtained only in the presence of a coloured substrate medium: During the 30 min period of incubation these substrates (at pH 8) turned grey-violet and somewhat turbid, a phenomenon which is also essential in the classical dopaoxidase reaction. After incubation for 13–18 h the solutions turned dark and a marked black precipitate was formed. In these cases, the cells were very strongly, almost black pigmented, the sections were stained overall gray, leucocytes and erythrocytes were stained black.

When other substrates were used, the shade of pigmentation differed: Yellow-brown after adrenaline or noradrenaline, even following prolonged incubation, and light brown, or light red-brown with tyramine, tyrosine, tryptamin, tryptophane, hydroxytryptamine, or hydroxytryptophane. With all these substrates the intensity after short or prolonged incubation at a pH of 8 was much less than in the dopamine or dopa experiments.

After incubation of sections in solutions of dopamine, dopa, or epinephrine which had been oxidized to the corresponding indolequinone by the addition of 4 equivalents of iodine (KOELLE and FRIEDENWALD[12]), the dopachrome from dopamine or dopa resulted in a violet-black stain even after very short incubation, whereas adrenochrome produced a light-yellow pigmentation.

With tryptamine or tyramine, pigmentation was observed to develop even in the absence of visible colour or turbidity of the incubation medium.

Various factors were investigated as to their possible influence on the reaction.

Little or no difference was found for temperatures of 25–29°C (room) or 37°C (incubator). Heating the sections for 30 min at 60°C in 0·9% NaCl solution resulted in little decrease in subsequent pigment-development, whereas heating for 5 min at 85°C abolished the reaction.

Low temperatures, e.g. 4°C, slowed the reaction considerably, with respect to both the development of visible colour in the solution and the appearance of pigmentation in the cells (even after 16 h there was no strong reaction found). However, quite marked pigmentation was observed after 5 min of incubation at 3°C with iodine-oxidized substrates (dopamine, at 0·01 *M* concentration).

Regardless of whether the sections were incubated in freshly prepared dopamine or dopa solutions, or in solutions where autoxidation had been allowed to take place for 30 min, there was a definite latency in the onset of the pigmentation; incubation for 10 min resulted in the development of much less intense pigmentation than the standard 30 min incubation. However, further increase in incubation time beyond 30 min did not seem to produce a proportionate increase in the amount of pigmentation. With lower concentrations than 0·002 *M* (e.g., 10^{-4} or 10^{-5} *M*) the pigmentation which developed was negligible even after prolonged incubation. No attempt was made to determine the optimal concentration. The pH of the incubation medium was of great importance; at a pH range of 7 to 6, little autoxidation and little pigmentation occurred with dopamine or dopa. However, pigment uptake took place even at pH 5, and very markedly at pH 9, when previously autoxidized solution of dopa had been adjusted to the corresponding pH-figures.

When N_2 was bubbled through the incubation medium in the Coplin jars containing slides, no visible color change in the medium was observed and no pigmentation occurred. When an incubation medium was allowed to autoxidize first, then slides were added and N_2 was bubbled through, no further visible color development was noticed in the solution, but pigmentation developed in the cells which could not be distinguished in intensity from the corresponding reaction under aerobic conditions. This was observed with incubation media containing autoxidized dopamine, dopa, or adrenaline.

In 0·002 *M* solutions of dopa or dopamine, to which 0·004 *M* cysteine HCl or sodium diethyldithiocarbamate had been added before autoxidation had taken place, no visible color change occurred in the solution during the following 30 min, and no pigmentation of the cells was found. When the solutions had been allowed to autoxidize prior to addition of the thiol compounds, then a positive cell-pigmentation reaction was observed. Exposure of sections to cysteine or diethyldithiocarbamate solutions for 30 min prior to incubation in the dopamine medium did not have any noticeable influence on the pigmentation.

Incubation of sections in a suspension of precipitated dopa-melanin, washed by repeated centrifugation, did not lead to pigmentation of the cells. Likewise, India ink suspensions were not taken up by the cells.

A few exploratory experiments gave evidence for a strongly positive ferric-ferricyanide reaction, according to the technique of SCHMORL (PEARSE[13]), in the cells that had taken up pigment, but not in the controls.

There was evidence also for a strongly positive benzidine-peroxidase reaction, according to the techniques by VILLAMIL and MANCINI (LILLIE[14]) and DEROBERTIS and GRASSO (PEARSE[13]), in the pigment-developing cells in untreated and in dopamin-pigmented sections, formalin-fixed or untreated.

Discussion.—The foregoing observations indicate that certain cells, found mainly in the lamina propria of the mucosa of the small intestine, develop intense melanin-like pigmentation after a short period of incubation in partially autoxidized, visibly colored solutions of a number of catechol alkylamines and indol alkylamines or their corresponding amino acids. These cells resemble macrophages or histiocytes, cells belonging to the originally mesodermal reticulo-endothelial system, which are known to be capable, under proper conditions, of phagocytosis or pinocytosis. The phenomenon observed here does not, however, seem to be of the intravitally occurring type of phagocytosis. India ink particles or washed (and fully oxidized) melanin-particles prepared from dopa were not taken up. Furthermore, the pigmentation of the cells occurred at the same rate in sections subjected to prolonged drying or treatment with formalin. They did not stain with cresylviolet or toluidine blue, and did not take up trypan blue after its injection into the living animal. In comparison with the known types of cells which have been reported to contain melanin-like pigments, the cells under consideration do not resemble particularly the typical melanocytes of epidermis, which are derived from the neural crest, of the pigmented tissues of the eye, or certain nervous tissues. However, they may be related to the cells found in the somewhat vaguely defined clinical state of pseudomelanosis of the appendix (PEARSE[13]) or in melanosis coli of man; or to the tissue-eosinophils.

In the present investigation, no evidence was obtained that any enzymatic process occurred in the tissue sections prior to the formation of colored compounds such as dopachrome or adrenochrome. Controls and additional modifications must be done to rule out the possible dependence on autoxidation for the initial steps in performing the histochemical dopaoxidase experiments with skin as they are described in the literature. It is unlikely that the small amount of tissue per slide incubated in the 10 ml of substrate solution (approximately 0·1 mg of wet weight of tissue, or 0·2 micro mol of nitrogen) contributed to the first two steps of oxidation, from tyrosine, dopa, or dopamine, in the surrounding medium. Survey of the literature on the histochemical reaction for dopa oxidase skin reveals the general assumption of an enzymatic reaction for these first two steps in epidermal cells; on the other hand it has not been ruled out that cells elsewhere than in the epidermis might produce enzymatically such soluble compounds as dopachrome. Since dopa-melanin is insoluble, whereas dopachrome and adrenochrome are readily soluble, the appearance of the former pigment in cells might have resulted from entrance of a soluble precursor or by phagocytosis of an insoluble stage. In the case of 10 micron-frozen sections, practically all of the cells are transsected. It has therefore to be kept in mind

[12] G. B. KOELLE and J. S. FRIEDENWALD, Arch. Biochem. Biophys. *32*, 370 (1951).

[13] A. G. E. PEARSE, *Histochemistry, Theoretical and Applied* (Little Brown, Boston 1953).

[14] R. D. LILLIE, *Histopathologic Technic and Practical Histochemistry*, 2nd ed. (Blakiston, New York 1954).

that mere adsorption of insoluble compounds could take place, but probably not their active uptake into cytoplasmic granules. Phagocytosis in these experiments seems ruled out by the previous treatment by freezing, followed in some experiments by formalin-fixation or drying.

The fourth and fifth steps, melanin polymerization and binding to proteins, are so little understood that speculation about these reactions in the cells may be permitted. The fact that the pigment-uptake did not take place after subjecting to heat-inactivation which occurs with most proteins (5 min, 85°C), suggests that some cytoplasmic protein is involved in the phenomenon observed. Whether this protein would act enzymatically in the polymerization of melanin or by some other type of catalysis is not known. The same must be said about the fixation of polymerized melanin to proteins of the cytoplasmic granules.

Which stage of oxidation of dopa was taken up by the cells, dopachrome or a later step on the way to the subsequent formation of melanin, is obscure. Under the experimental conditions in an atmosphere of nitrogen, no direct aerobic oxidation could have taken place; nevertheless, active pigment-uptake from previously partially autoxidized substrate solutions was obtained. This was seen with autoxidized solutions of dopa, dopamine, and epinephrine. With dopa, dark brown or black melanin-like pigments were observed in the cells, whereas after incubation with epinephrine, yellow or yellow-brown pigmentation was obtained. This was typical also for the iodine-oxidized solutions, where rapid and complete oxidation of the substrates to dopachrome or adrenochrome had been produced. Two explanations for this difference in color are possible. The final melanins produced from dopa and epinephrine may be significantly different in this respect. Alternatively, soluble compounds, like adrenochrome, may be taken up by some cytoplasmic proteins and then not altered, stay yellow, whereas in the case of dopa further oxidation occurs to black dopa melanin.

Present findings indicate that the pigment-production in the intestinal cells, or in skin or in leucocytes, is not highly specific, as has been claimed to be the case for the histochemical dopaoxidase reaction in the skin.

The pigmentation developed in previously autoxidized solutions over a pH range of 5 to 9 or higher. It has also been shown recently in a critical study of the dopa-factor by VAN DUIJN[15] that mushroom-phenoloxidase-oxidized dopa solutions give a similar positive reaction at a low pH (4·65).

The epidermis reaction was claimed to be specific for L-dopa and negative for D-dopa (BLOCH and SCHAAF[16]); however dopamine, which has no asymmetric carbon atom was reported to give the same reaction (MULZER and SCHMALFUSS[17]). In the present study it was found that intestinal cells give the same strong reaction with pre-oxidized epinephrine or its dextro-isomer, suggesting that no such specific phenolase was involved in this step.

The possibility was considered that this reaction could be related to some peroxidase-activity. If in some of the oxidation-steps before or after the formation of dopachrome a peroxide should be formed (MASON[9]), and if these cells should contain peroxidase activity, it is conceivable that an amount of melanin could be formed out of the partly oxidized substrate even in an atmosphere of nitrogen to a sufficient extent to lead to cytoplasmic pigmentation. In a few experiments a strong benzidine peroxidase reaction was found with pigmented cells, and also in similar cells of sections not previously treated. This observation needs further confirmation. Peroxidases are known to be HCN-sensitive, whereas the pigment-uptake is very little affected by HCN according to VAN DUIJN[18].

A number of the present observations obviously require further study for their elucidation. It is so far not clear why with incubation media containing tryptamine, which remained clear and visibly colorless for a number of days, similar pigment-uptake was found as with tryptophane, hydroxytryptamine, etc. Reaction media which have been allowed to stand at room temperature for over 2–3 h or overnight have not been checked for bacterial growth, which could possibly account for some of the 'spontaneous' oxidation taking place in them.

Nothing is known regarding the further fate of these pigmented cells, and whether they are capable of eliminating the pigment. It was reported that in cases of melanosis coli of man, ascribed to the prolonged use of cascara sagrada medication (anthraquinone or emodin), the pigmentation was reversible (BOCKUS, WILLARD, and BANK[19]).

A re-evaluation of the histochemical dopaoxidase reaction in skin is desirable, according to these results and to those which have been obtained by VAN DUIJN[15]. In spite of these differences between the classical melanocytes in the epidermal part of the skin and the cells in the intestine or in the dermis described here, the possibility that some stages of the pigment development and uptake by these cells may be related or identical cannot be ruled out. It would not seem unlikely, that the reaction found in epidermal melanocytes is but a cytologically specialized or adapted form of a more general phenomenon of the possibly non-enzymatic oxidation of dopa, epinephrine, or tryptamines. If this reaction, which is quite rapid, occurs *in vivo*, and if quantitation could be achieved, it might provide information about the oxidative metabolic pathways of the physiologically and pharmacologically important compounds mentioned above.

Zusammenfassung

Gefrierschnitte von frischem oder formalin-fixiertem Dünndarm verschiedener Tierarten werden in teilweise autoxydierten, 2×10^{-3}-molaren Lösungen von Dopamin, Dopa, Adrenalin, Hydroxytryptamin und verwandten Verbindungen bei pH = 8 inkubiert. Gewisse Zellen der tunica propria der Schleimhaut zeigen dann nach relativ kurzer Zeit eine zum Teil sehr starke granuläre Pigmentierung ihres Zytoplasmas.

[15] P. VAN DUIJN, J. Histochem. Cytochem. *1*, 143 (1953); Acta physiol. pharmacol. Neerland *5*, 413, 428 (1957).

[16] B. BLOCH and F. SCHAAF, Klin. Wschr. *11*, 11 (1932).

[17] P. MULZER and H. SCHMALFUSS, Med. Klinik *27*, 1099 (1931).

[18] P. VAN DUIJN, Acta physiol. pharmacol. Neerland *5*, 413, 428 (1957).

[19] H. L. BOCKUS, J. H. WILLARD, and J. BANK, J. Amer. med. Ass. *101*, 1 (1933).

Corrigendum

G. ZBINDEN und A. STUDER: *Histochemische Untersuchungen über den Einfluss von Ipronazid (Marsilid) auf die durch Reserpin erzeugte Freisetzung von Adrenalin und Noradrenalin aus dem Nebennierenmark.* Exper. *14*, fasc. 6, 201 (1958).

Die beiden letzten Sätze der englischen Zusammenfassung müssen richtigerweise wie folgt lauten:

In animals pretreated with equimolar doses of isoniazid, however, histochemical catecholamine reactions show a marked decrease in all cells of the adrenal medulla. These results suggest that monoamine oxidase plays a part in the reserpine-induced release of catecholamine.