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A RAPID HISTOCHEMICAL TEST FOR MAMMALIAN TYROSINASE*

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ABSTRACT

A simple, rapid separation of epidermis from dermis was achieved following incubation of human or guinea-pig skin in one molar sodium bromide at 37° C for 30 min. The resulting epidermal sheets incubated at 37° C in 0.1 M phosphate buffer (pH 6.8) for 15 to 120 minutes with 5×10^{-3} M dopa or for 120 to 240 minutes with a mixture of 5×10^{-3} M tyrosine and 5×10^{-3} M ascorbic acid gave good dopa-tyrosinase and tyrosine-tyrosinase reactions. Melanocytes were readily visualized because new melanin formed within the cytoplasm.

Bruno Bloch scored an important first when he incubated fresh pieces of human skin with dopa† and found that melanocytes became visible [1]. This conversion of dopa to melanin by the action of an enzyme within the pigment cells marked the beginning of modern histochemistry. Credit also must be given to the chemist M. Guggenheim, a relative of Bloch's, who earlier had determined the chemical structure of dopa [2], given some of the amino acid to Block, and pointed out to him the similarities in structure between dopa and adrenaline. Bloch felt that adrenaline or a related substance was somehow responsible for the darkening of skin in patients with Addison's disease because the adrenal glands were involved in that disorder [3]. These positive experiments served to give Bloch a major interest in melanin pigmentation. It turned out that he was wrong on some important points but the dopa test became and continues to be of great significance.

From 1917, when Bloch's detailed paper on the dopa reaction appeared, to the present time several improvements have been made in the test. Becker, who worked with Bloch, introduced a 24-hour preincubation at 5° C in dopa and found that for light-colored human skin a better reaction was obtained when the skin was irradiated in vivo with ultraviolet light for several days before the biopsy was taken for the dopa reaction. In 1950 Fitzpatrick, Becker, Lerner, and Montgomery [5] found that pretreatment of the skin with ultraviolet light for several days permitted tyrosine as well as dopa to be used as substrate for the reaction. It was then clear that human skin possessed both tyrosinase and dopa oxidase activities.

Most of the investigations before 1950 were carried out with vertical sections of mammalian skin. However, for melanocyte counts as well as for fine morphologic studies horizontal sections proved to be better. Three different approaches were used to obtain horizontal viewing of melanocytes. In one, a technique used extensively by Snell [6], fresh pieces of skin are cut horizontally on a freezing microtome and the thick skin sections are incubated with dopa. The other two methods use either enzymic or ionic splitting of the epidermis from the dermis and then the incubation of the small pieces of epidermal sheets, which contain the melanocytes, with the appropriate substrate. In 1941 Medawar [7] found that trypsin could be used to separate the epidermis from the dermis. Later Billingham and Medawar [8] used trypsin-split epidermis for melanocyte viewing. Szabó employed this procedure for numerous studies [9]. A nonenzymic method of separating the epidermis was initiated by Felsher in 1947 when he found from a systematic study that cations and anions varied in their ability to swell dermal collagen—swelling which in turn would bring about a separation of the epidermis [10]. This ionic splitting of epidermis was put to practical use by Hambrick and Blank [11]. Subsequently Staricco and Pinkus selected two molar sodium bromide solutions for the splitting off of epidermis for melanocyte research [12]. Furuya and Ikeda used a combination of these procedures to reduce the time of the dopa reaction [13, 14].

In this paper we wish to report a method for the ionic separation of epidermis from human and guinea-pig skin taking less time than 30 minutes (instead of the usual 2 to 3 hours) and the subsequent incubation of the epidermal sheets with dopa for ¼ to 2 hours or with tyrosine and ascorbic acid for 4 hours to give quick visualization of tyrosinase in melanocytes.

MATERIALS AND METHODS

All guinea pigs used in these studies had a reddish brown coloring, light-colored plantar surfaces, and dark eyes. Black animals were not used because the large quantities of melanin already in the pigment cells made the detection of new melanin by visual means difficult. Light-colored animals gave weak or variable tyrosinase reactions. Hair was removed from the guinea pigs by the latex method of Freedberg [15]. Two to fourteen days

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† In German dihydroxyphenylalanine is *dioxyphenylalanine*. It is from the German name of the amino acid that the term dopa was derived.

after the epilation procedure multiple 4-mm punch biopsies of back skin were taken. The skin pieces, approximately 0.5 mm deep, were cut off at the base with a scalpel and floated epidermal side up in a solution of 0.1 M sodium phosphate buffer at pH 6.8 containing 1 M sodium bromide at 37° C for 30 minutes. The epidermis with attached melanocytes was gently peeled from the dermis and floated stratum corneum side up in 0.1 M sodium phosphate buffer at pH 6.8 containing either dopa or a tyrosine-ascorbic acid mixture at 37° C for varying periods of time. After incubation the section was fixed in 10% formalin, gradually dehydrated with alcohol, cleared with xylene, and permanently mounted on a slide. Epidermal sheets were obtained in a similar manner from newborn human foreskins after circumcision.

Skin splitting. Several different methods were tested for separating the epidermis with attached melanocytes from the dermis.

We extended the experiments of Felsher [10], Hambrick and Blank [11], and Staricco and Pinkus [12] to find the minimum conditions for effective splitting of the epidermis from the dermis. The small pieces of guinea-pig skin were incubated at 37° C with lithium and sodium salts having iodide, bromide or thiocyanate as anions. Good separation was obtained under the following minimal conditions:

Salt	Concentration	Time
	(Molar)	(min.)
NaBr	0.5	20
LiBr	0.5	15
NaI	0.25	20
LiI	0.25	10
NaCNS	0.25	10
LiCNS	0.15	10

Even though all the above solutions could be used individually to give excellent splitting, we found that the tyrosinase reaction was strongest after sodium bromide splitting. Trypsin and EDTA under appropriate conditions led to separation but offered no advantage. To assure a good separation of the skin layers for all skin samples we decided to use 1 molar sodium bromide in 0.1 molar sodium phosphate buffer at 37°C for 30 minutes.

RESULTS AND DISCUSSION

Dopa as substrate. Fresh epidermal sections from human or guinea-pig skin with the stratum corneum up were incubated in 0.1 molar sodium phosphate buffer, pH 6.8, at 37°C with 1 mg per ml (5×10^{-3} M) of l-dopa for 15 to 120 minutes. Appropriate controls of incubations of skin in solutions without dopa were employed. Dramatic visualization of melanocytes in the sections treated with dopa was possible because dopa was converted to melanin within the melanocytes through the action of tyrosinase (Figs. 1a–c and 3b). The dopa reaction was apparent after 15 minutes and maximum in two hours. For most studies an incubation period of 30 to 60 minutes was adequate.

Tyrosine as substrate. In the formation of melanin, tyrosinase catalyzes the oxidation of tyrosine

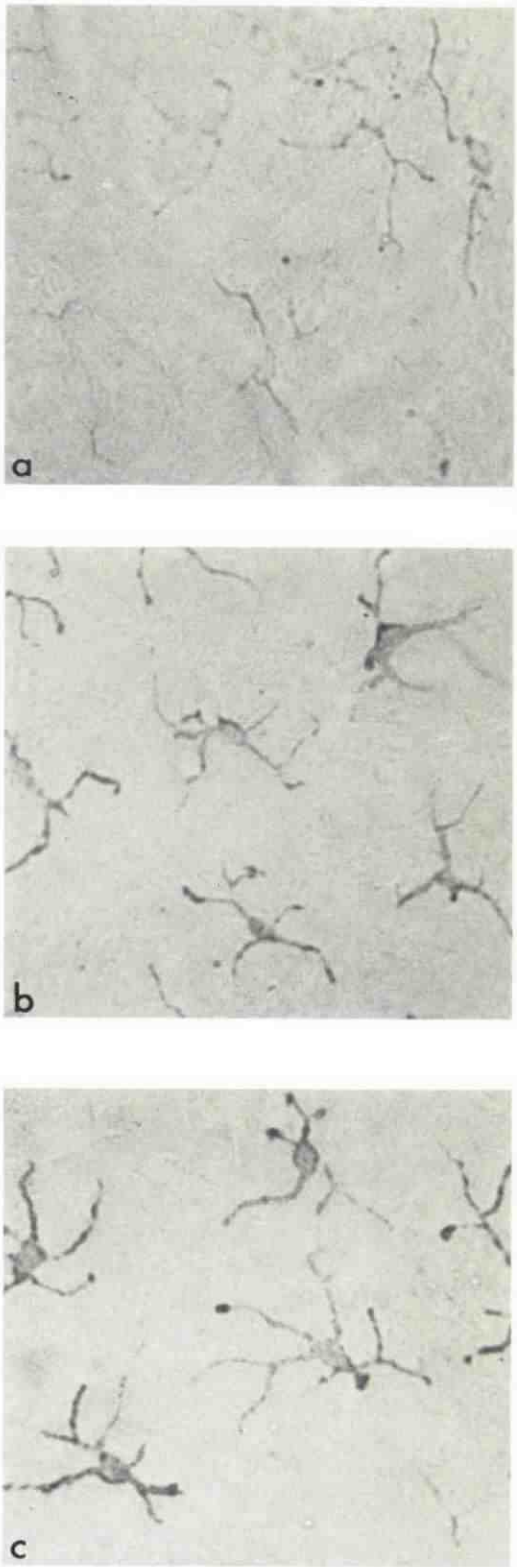
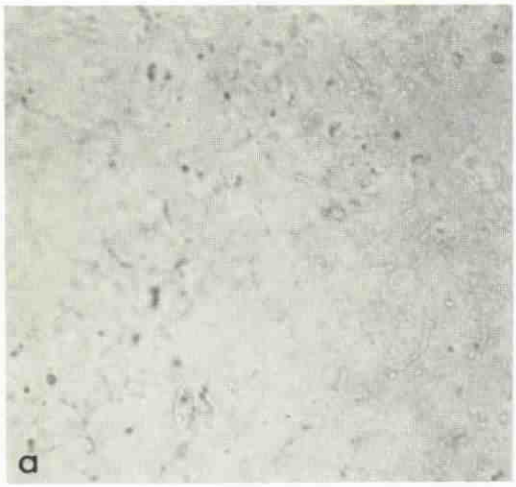
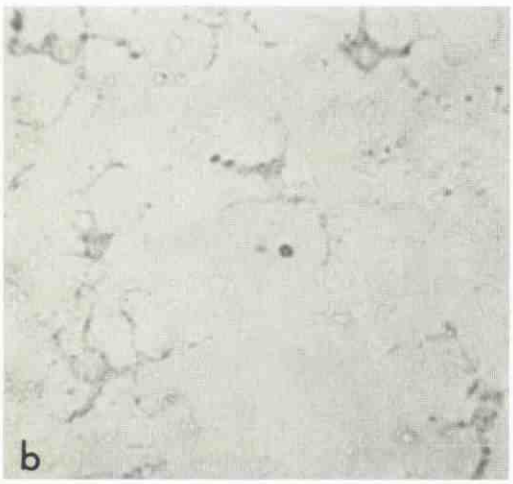


FIG. 1. Pieces of epidermal sheets of guinea-pig skin were incubated with dopa for (a) 0 minutes, (b) 20 minutes, and (c) 40 minutes. The deposition of newly formed melanin within the melanocytes is easily observed. × 540.





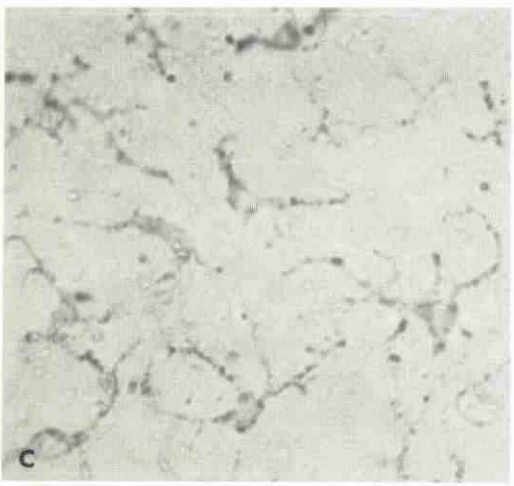
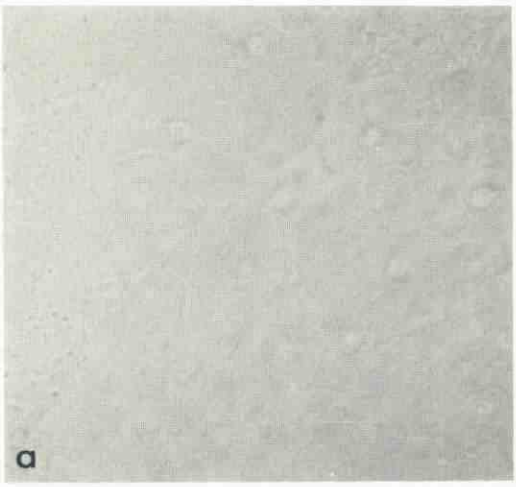
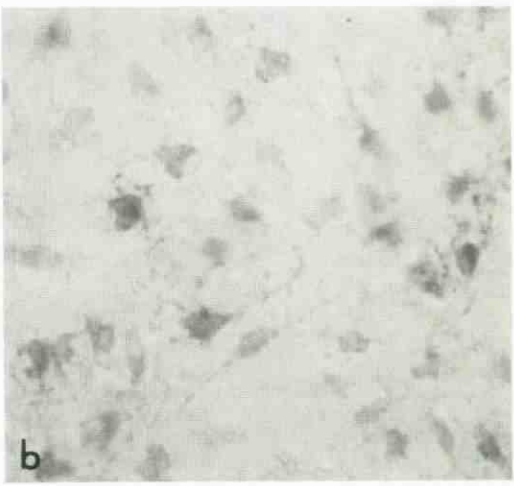


FIG. 2. Epidermal bits of guinea-pig skin were incubated with tyrosine–ascorbic acid mixtures for (a) 0 minutes, (b) 90 minutes, and (c) 180 minutes. As in the case when dopa is the substrate, melanin formation increased with time. Without ascorbic acid very little melanin formed from tyrosine. × 540.





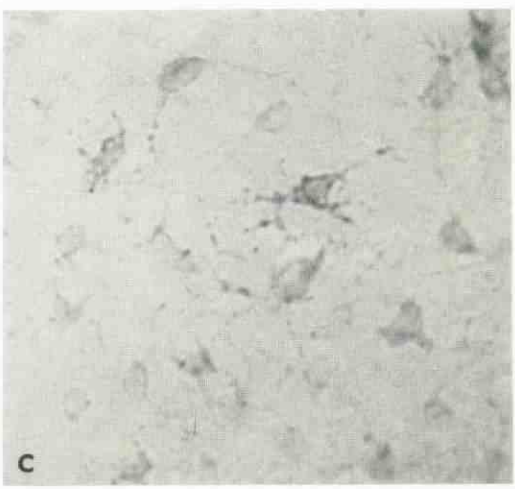


FIG. 3. Fresh epidermal sections from human foreskin were incubated with (a) buffer alone for 4 hours, (b) dopa for 2 hours, and (c) tyrosine–ascorbic acid mixture for 4 hours. Ascorbic acid was required for tyrosine to be used as substrate. × 540.

to dopa and then the oxidation of dopa to dopa quinone. However, the tyrosine–tyrosinase reaction is normally slow and must first be primed with dopa. No priming is required for the faster dopa–tyrosinase reaction. Ordinarily, when fresh sections of skin are incubated with tyrosine nothing happens. However, if the skin is irradiated with ultraviolet light for several days before biopsy, a positive tyrosine reaction will result. The ultraviolet light permits the tyrosine–tyrosinase reaction to occur and enhances the dopa–tyrosinase reaction.

Ascorbic acid can substitute for dopa in the priming process because this reducing agent leads to the accumulation of dopa. When trace amounts of dopa are formed, the dopa is rapidly oxidized to dopa quinone. Ascorbic acid will reduce the dopa quinone back to dopa. This reduction will continue until all the ascorbic acid is oxidized. By allowing an increase in the dopa concentration, ascorbic acid behaves as a tyrosine–tyrosinase priming agent.

Epidermal sections were incubated in phosphate buffer with tyrosine 5×10^{-3} M and ascorbic acid 5×10^{-3} M and ascorbic acid 5×10^{-3} M for 120 to 240 minutes. Positive reactions for melanin formation within melanocytes was observed (Figs. 2 a–c and 3 c). Weak or negative reactions occurred when tyrosine without ascorbic acid was used. Ascorbic acid alone gave no reaction.

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