Tyrosinase in Frozen Sections and Fragments of Mouse Skin.*† (20804)

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The study reported here grew out of efforts to account for differences in results obtained by previous histochemical studies of enzymatic pigment-formation (dopa reactions) in normal mammalian skin(1,2) in contrast to more recent ones in which tyrosinase activity was demonstrated by various methods(3-6). It is important to account for these differences because of conflicting conclusions which could be drawn concerning the physiological genetics of mammalian coat-color pigmentation. For this reason it was considered necessary to attempt new histochemical demonstrations of tyrosinase activity in frozen sections and fragments of normal mammalian skin.

Materials and methods. The mouse genotype used was the triple homozygous recessive for pink eye, brown and maltese dilution (ppbbdd). Genes pp (pink eye) and brown (bb), either separately or in combination, do not cause any marked diminution in dopa oxidase activity although they do cause a drastic reduction in amount of hair pigmentation(7,8). The dilution factor (dd) reduces neither enzyme activity nor amount of pigment, but acts as a pigment clumping factor, resulting in a lighter appearing hair(7,8). Thus low background pigmentation and high enzyme activity should permit ready observation of newly formed pigment. The dorsal skins of 7-day-old mice were prepared by freezing and grinding as previously described (4), or sectioned at 30 μ on the freezing microtome. Incubation of skin samples was carried out at 38°C in 1½ or 2 ml of M/10 phosphate buffer, pH 6.8 (incubated controls), or in the same volume of buffer containing L-tyrosine at a concentration of 0.5 mg/

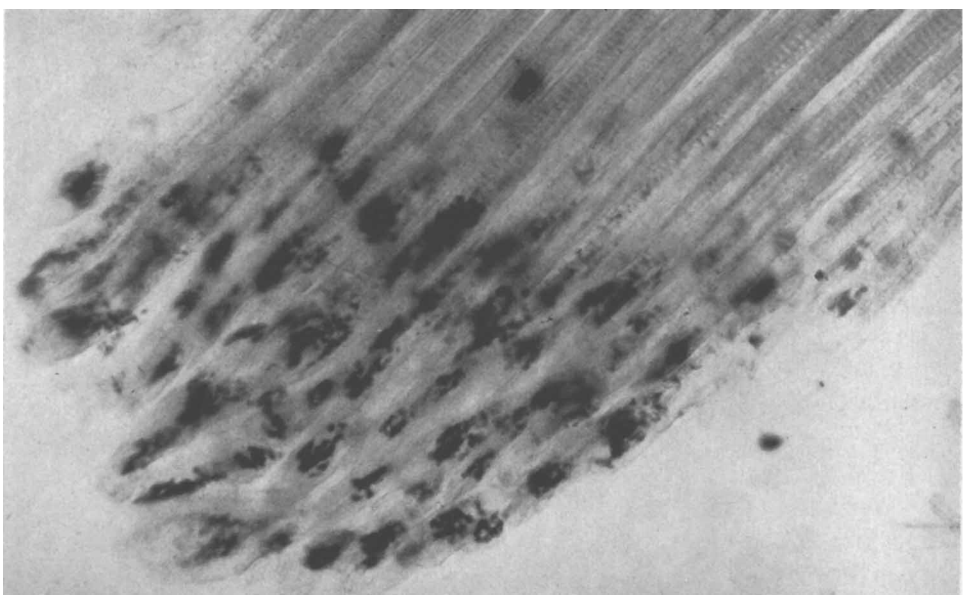

FIG. 1. Relatively discrete pigmentary details in hair bulbs of control skin fragment (incubated in buffer for 20 hr) (× 112).

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FIG. 2. Obliteration of discrete pigmentary details by newly formed pigment in hair bulbs of skin fragments incubated in tyrosine (20 hr) (× 112).

ml, in vessels of 5 ml capacity. The amount of skin incubated varied from a few fragments or sections to approximately ½ dorsal skin prepared as fragments or sections. As a check against the possibility that incubated control samples might undergo some darkening due to utilization of endogenous substrate, an additional control in the form of frozen, but unincubated, skin was prepared. After incubation, 3 hours in the case of the sections, 20 hours in the case of the fragments; the skin samples were fixed in 1:10 formol-alcohol (20 minutes), dehydrated in absolute alcohol (20 minutes), cleared in toluol (at least 20 minutes) and mounted in Permount (Fisher Scientific Co.).

Results. The darkening of both sections and fragments in tyrosine was apparent on gross examination. The tyrosine incubation media containing large amounts of skin developed a brown color. Even the skin samples incubated in buffer underwent a slight darkening as compared with the unincubated controls. Microscopic examination of hair bulbs under low power magnification revealed that the discrete pigmentary details, so readily apparent in the case of the controls (Fig. 1 and 3), tended to become obliterated when the skin was incubated in tysosine (Fig. 2 and 4). In these experimental cases many hair bulbs showed a dense oval mass of dark brown-black pigment, and when large amounts of skin were incubated, the peripheral portions of the hair bulbs, not normally

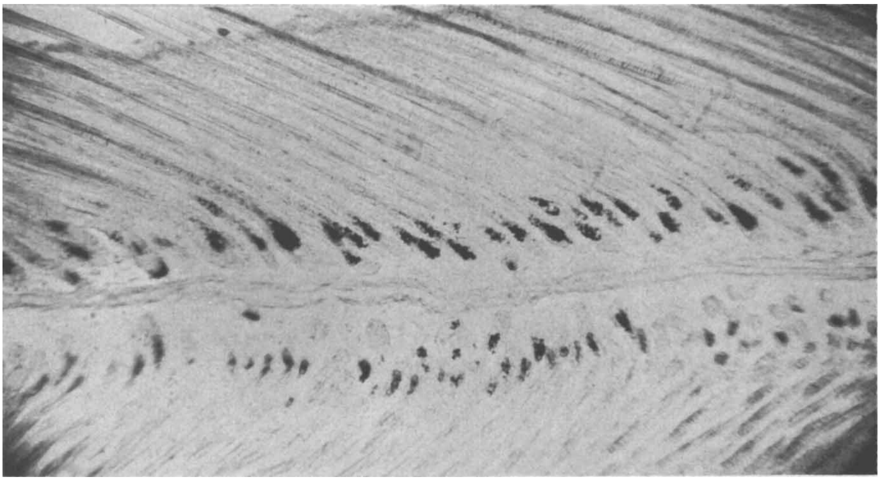

FIG. 3. Hair bulb pigmentary details in unincubated frozen skin sections. The skin was folded before sectioning so that 2 attached sections are shown (× 52).

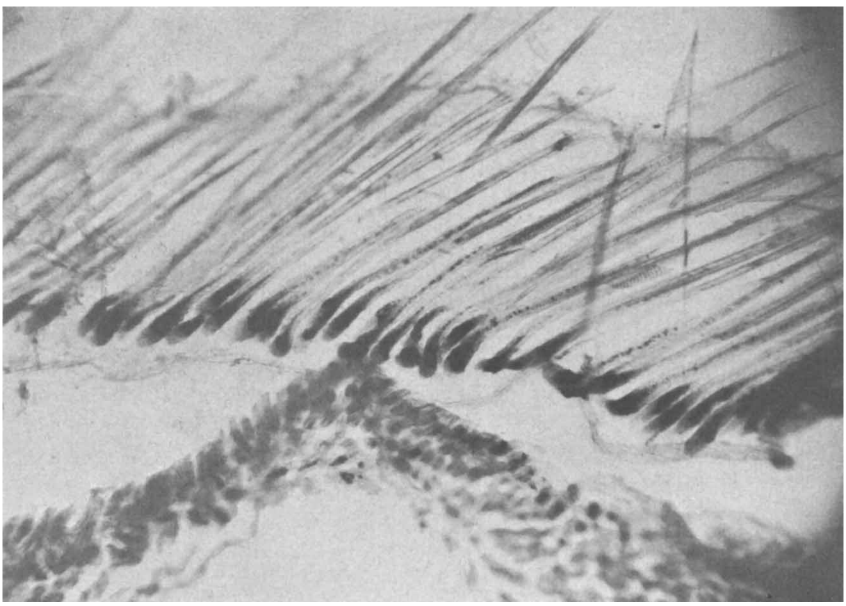

FIG. 4. Obliteration of pigmentary details by newly formed pigment in hair bulbs of frozen skin section (incubated in tyrosine for 3 hr) (× 52).

colored, took on the appearance of a brown region surrounding the brown-black central mass.

Although, on gross examination, skin incubated in tyrosine appears to undergo uniform darkening, microscopic examination reveals a good deal of variability. Thus an apparently uniform darkening is better described as a shift of the hair bulb population average in the direction of heavier pigmentation.

We also have observed that frozen sections of skin take up oxygen as well as do frozen fragments on incubation in tyrosine.

Discussion. The new demonstrations of tyrosinase activity reported here provide a basis for reconciling conflicting results and interpretations reported in the very recent and earlier studies cited above. Use can now be made of a variety of attacks for determining enzyme activity. Frozen sections are especially favorable for histological observation, while oxygen consumption measurements are independent of the amount of pigment already present in the hair bulbs at the beginning of an experiment. Independent checks on the enzymatic behavior of a given type of skin are therefore possible.

Summary. New histochemical demonstrations of tyrosinase activity in frozen sections and fragments of normal pigmented mouse skin are described. These demonstrations provide a basis for reconciling apparently conflicting results and interpretations arising from earlier studies, in which tyrosinase activity was not demonstrated, and later studies, in which this enzyme was readily demonstrated.

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Interference Phenomenon of Browning and Gulbransen in Experimental Infection of Mice with *Trichomonas vaginalis*. (20805)

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The interference phenomenon of Browning and Gulbransen(1), *i.e.*, the antagonism of the trypanocidal dye stuff p-rosaniline hydrochloride toward 3,6-diaminoacridinium-10-methochloride (Acriflavine) has been primarily studied in trypanosomal infections with p-rosaniline resistant(1) and normally sensitive trypanosomes(2,3). According to Wright and Hirschfelder(4) the antagonism can also be demonstrated *in vitro* using the growth inhibiting activity of the dye stuffs toward yeast cells (*S. cerevisiae*). It could be expected that other organisms as long as they were sensitive to both the triphenylmethane and the acridine dye stuff would also show the interference. The pathogenicity of *Trichomonas vaginalis* for mice(5) and the response of this infection to local administration of p-rosaniline HCl and Acriflavine suggested an attempt to demonstrate the therapeutic antagonism of the 2 dye stuffs in the trichomonad infection.

Materials and methods. The strain of *T. vaginalis* was the same as that used in earlier experiments(5-7). Overnight growth in CPLM medium(8) was used in all instances. White mice from one colony were infected subcutaneously on the ventral side with 0.5 ml of a culture diluted to contain 500000 parasites as ascertained by actual count in a hemocytometer chamber. This dose represented at least 10 minimal infective doses. After 2 hours the marked site of the infection was injected locally with 1 ml of different dilutions of aqueous solutions of p-rosaniline hydrochloride and this was followed by a local injection of 1 ml of Acriflavine solutions at the same site after another interval of 2 hours. Control series with p-rosaniline alone and Acriflavine alone as well as groups of untreated mice were included in every experiment. Four days after the infection the mice were sacrificed, autopsied, and examined microscopically for presence or absence of active trichomonads.

Experimental. It had been found in preliminary experiments that p-rosaniline HCl and Acriflavine exerted a marked activity if administered locally under the experimental conditions described above. Acriflavine is one of the most active anti-trichomonad agents which is evident from the low CD_{50} of approximately 12 μg/ml. The corresponding CD_{50} of p-rosaniline HCl was 162 μg/ml.