A Turbidimetric Assay for Tyrosinase Activity in Frozen Sections of Mouse Skin.* (21083)

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(Introduced by J. S. Nicholas.)

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Although the incubation of mammalian skin sections in dopa(1-3) or in tyrosine(4,5) has proved useful in localizing tyrosinase activity, attempts to measure the activity of these preparations have been hampered by the lack of sensitive and objective methods for determining the amount of newly formed dark pigment. Previous estimations of tyrosinase or dopa oxidase activity by visual grading of hair bulbs have served to indicate order of effect as a function of genotype(2,3) or treatment(6). However, a linear scale of visual grades might actually represent much larger changes, involving an exponential relationship, in amounts of pigment(7). Moreover, the visual grading method involves shifting standards for comparing enzyme activities in differently colored genotypes. For these reasons it was considered desirable to develop a sensitive method for assaying tyrosinase activity objectively and on an absolute scale of turbidimetric measurements.

Materials and methods. The slow, partial breakdown of wool keratin in warm, dilute alkaline sulfide(8) and the dispersion of melanin in alkaline media(7,9-12) suggested the possibility that hot concentrated alkaline sulfide might be a suitable dispersion medium for both melanin and keratin. We therefore used a solution molar with respect to both KOH and $Na_2S \cdot 9H_2O$. Hot KOH alone was much less effective in breaking down the skin, although it has been reported to be satisfactory for dissolving human hair keratin(12). Three hundred *frozen sections*, 30 μ thick, from the dorsal skins of two 7-day-old intense brown mouse littermates (genotype bb) were incubated at 38°C for 8 hours in 1½ ml M/10 phosphate buffer, pH 6.8, or in the same volume of buffer containing either L-tyrosine or L-dopa at a concentration of 0.5 mg/ml. Following incubation, the sections were prepared for histological examination as previously described(5). After clearing in toluol, several sections were removed for histological study and visual grading of hair bulbs, while the remaining sections were reserved for turbidimetric tests. Sections used in the latter tests were placed on filter paper to accelerate evaporation of the toluol, and 10 mg samples of dry sections were then transferred to Klett colorimeter tubes. To each of the tubes was added a 5 ml aliquot of dispersion medium. The tubes were placed in a boiling water bath and their contents were stirred occasionally. Within 15 minutes the keratin and pigment underwent dispersion to give brown suspensions. The breakdown of heavily pigmented sections resulted in incomplete dispersion; small, dark, slowly settling, particles were visible. Vigorous agitation of Klett tube contents was therefore necessary before each turbidimetric reading in a Klett-Summerson photoelectric colorimeter (with No. 42 blue filter). The dispersion medium was used for the zero setting of the instrument, and readings for a given suspension were obtained four times in order to determine the variability of the measurements. The turbidimetric stability of the suspensions was checked by storing them overnight in a refrigerator (5°C) and obtaining additional readings on the following day. *Visual gradings* of hair

TABLE I. Comparison of Pigmentary Intensities of Incubated Frozen Skin Sections as Measured by Visual Gradings or Turbidimetric Readings.

Incubation medium	Visual gradings: No. bulbs graded	Visual gradings: Mean grade	Turbidimetric readings (Klett units)
Buffer	602	3.26 ± .03	295 ± 2.5
Tyrosine	381	3.52 ± .03	489 ± 5.8
Dopa	411	3.88 ± .03	773 ± 6.2

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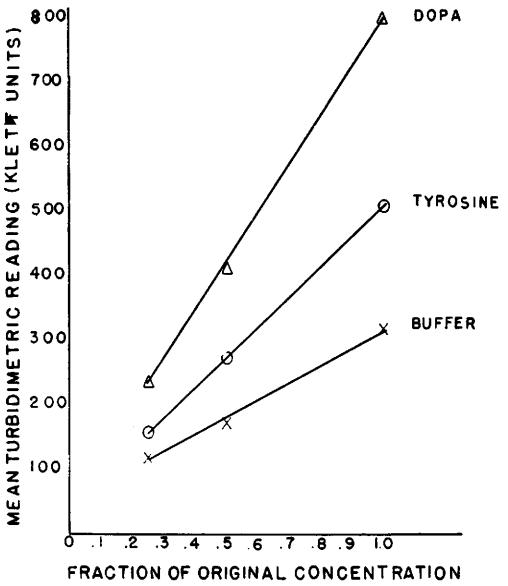

FIG. 1. Adherence of skin and melanin suspensions to Beer's Law as indicated by straight line relationships between turbidimetric readings and degrees of dilution. Only incubation media are indicated.

bulb pigmentation were obtained at low power magnification by one of us (T.A.S.). A scale of 5 grades was adopted to encompass the range from a light brown stippled appearance (grade 1) to a solid black hair bulb (grade 5). Fractional grades were not assigned to any hair bulbs.

Results. Measurements of tyrosinase and dopa oxidase activities by both methods described above are shown in Table I. Mean values of visual grades or turbidimetric readings are followed by their standard errors. The following points are noteworthy: 1) Both methods for determining pigmentary intensity agree as to order of effect. In both cases the differences between mean values are significant at the 1% level. 2) A fractional change in average visual grade corresponds to a turbidimetric change of several hundred Klett units. Thus the latter method is far more sensitive than the former.

The suspensions follow Beer's Law; the relationships between Klett readings (proportional to optical densities) and degrees of dilution appear to be linear. These relationships are shown in Fig. 1. Both medium and suspensions had been stored overnight at 5°C prior to diluting the suspensions and obtaining Klett readings.

Despite the sensitivity of the turbidimetric method it still suffers from 2 shortcomings: 1) The dispersion medium appears to be unstable; a small number of dark particles appear after overnight storage at 5°C. 2) The skin and pigment suspensions showed slight darkening, approximately 20 Klett units, after overnight storage at 5°C, but with readings taken after the suspensions had been warmed to room temperature.

Conclusion. The new turbidimetric method for assaying tyrosinase activity reported here represents a marked improvement over the previously used visual grading method, not only in objectivity and in greatly increased sensitivity, but also in making possible the use of an absolute scale. While immediately useful for determining order of effect, this method might also be sufficiently improved to permit comparison of amounts of naturally occurring pigment in various skin and hair samples.

Summary. A new sensitive and objective method for the turbidimetric assay of tyrosinase activity in frozen skin sections is described. The procedure involves the dispersion of both melanin and skin and hair keratin, from previously incubated sections, in hot molar Na_2S and KOH. Turbidimetric readings are then obtained on the absolute scale of the Klett-Summerson photoelectric colorimeter. This method is immediately useful for determining order of effect, and, if sufficiently improved, might also be useful for comparing amounts of naturally occurring pigment in different skin and hair samples.

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Anticoagulant and Other Pharmacologic Effects of Sulfated Chitin in Animals. (21084)

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The sulfation of various polysaccharides to produce compounds having anticoagulation properties was first described by Bergstrom (1). He reported on the characteristics of the sulfated products of cellulose, starch, glycogen, pectin, chitin and chondroitin sulfuric acid. Most of his investigations were conducted *in vitro,* but later workers, including Piper(2) and others, extended much of the study to *in vivo* testing. A side reaction frequently observed was the agglutination of the thrombocytes. This, coupled with other toxic symptoms, led to the conclusion that the compounds were not suitable for clinical application. Sorenson and Wright(3) described a polysulfuric acid ester of polyanhydromannuronic acid which was tried clinically. Field *et al.* (4) tested the sodium salt or sulfated polygalacturonic acid methyl ester methyl glycoside, and Walton(5) reported on the biological behavior of sulfated dextran. In all instances there was evidence of potential side reactions or toxic phenomena.

Preliminary tests with the sulfuric acid ester of chitin were sufficiently promising to justify extensive studies on the biological behavior of this material. The properties and toxicity of various preparations are herein reported.

Materials and methods. Samples with various average molecular weights were produced by Cushing *et al.*(6), who also described the material as follows: "The product can be defined as the sodium salt of the sulfuric acid ester of poly (1-4) N acetylglucosamine. The sulfur assays (13.5 to 14.5%) showed the degree of sulfation to be somewhat below the maximum theoretical sulfur content of 15.7% for a product having two sulfate groups on each acetylglucosamine residue. The nitrogen assays ranged from 2.3 to 3.2%, the variation being due chiefly to small amounts of residual triethylamine (as a salt on sulfate) used for neutralization of the sulfation mixture. Curiously, the elimination of the residual triethylamine did not significantly decrease the toxicity of the product. All molecular sizes are represented according to the usual frequency distribution curve except that molecules below 5000 mol wt are mostly eliminated during dialysis or repeated alcohol precipitation. In a product having an average molecular weight of 14000, the amount of high molecular weight material (above 17000) is less than 0.5% of the total. In the selected material, relative viscosities (R.V.) in 1% aqueous solution were remarkably uniform, with 1.3 R.V. as the preferred product, and a maximal permissible range of 1.25 to 1.35. The *in vitro* potency varied between 27 and 35 I.U./mg(7)."

Injections were almost exclusively intravenous. Unless high doses were required, sulfated chitin and heparin were used as 1% aqueous solutions. Blood samples for all tests were withdrawn through silicone-treated needles, either into siliconed syringes for transfer to siliconed, chilled glassware or directly into capillary tubes thrust into the needle hub. Coagulation times were determined by the capillary tube method(8). Prothrombin determinations were made by the Quick one-step method. Platelets were counted directly from samples diluted with 3.8% sodium citrate, as described by Quick *et al.*(9). Acute intravenous toxicity was determined in mice. Subacute intravenous toxicity was observed