16851

Inhibitory Action of Extracts of Mammalian Skin on Pigment Formation.

PETER FLESCH.* (Introduced by S. Rothman.)

From the Departments of Pharmacology and Medicine, Section of Dermatology, University of Chicago, Chicago, Ill.

Formation of melanin *in vitro* through oxidation of tyrosine by tyrosinase or by autoxidation of l-dihydroxyphenylalanine (dopa) is inhibited by skin extracts of rabbits[1,2] and of guinea pigs.[3,4] Rothman, *et al.*[5] have observed the inhibitory effect with extracts of isolated human epidermis. These authors advanced the theory that since they were able to counteract the inhibition with iodoacetamide, the inhibitory effect was due to the presence of sulfhydryl compounds in the epidermis. They suggested that pigmentogenic stimuli, such as ultraviolet rays, X rays, heat radiation and inflammatory processes caused pigmentation by oxidizing or destroying these inhibitory sulfhydryl compounds, thus enabling the enzyme to act on the pigment precursor.

The present work was carried out to investigate the nature and possible role of this inhibitory factor. The inhibitory action of aqueous extracts of human epidermis and of skin homogenates from rabbits was studied *in vitro*. In another series, rabbits were subjected to pigmentogenic stimuli and observations were made of the changes *in vivo*.

Methods. Aqueous extracts of human epidermis were prepared according to Rothman *et al.*[5] For preparation of extracts from rabbit skin, the hair on the back of the animals was clipped with scissors and on the bare skin two adjacent areas of equal size were mapped out with ink. One of the areas was covered with cloth, while the other was irradiated with an ultraviolet General Electric, RS, 275 W lamp. The distance varied from 6 to 12 inches and the exposure time from 5 to 15 minutes. Immediately after the irradiation the animal was killed. Skin samples were taken from the irradiated and non-irradiated areas. The subcutaneous fat tissue was removed and skin samples weighing from 0.3 to 0.5 g were frozen in dry ice and thawed rapidly several times in order to break up the cells. The pieces were minced and homogenized by means of glass homogenizers[6] with 2 ml distilled water in an ice bath, diluted with distilled water to a final concentration of 1 ml water for each 50 mg of tissue, placed in the ice box for 24 hours and centrifuged several times in the cold.

The inhibitory power of aqueous extracts of human epidermis and of the supernatant fluid of rabbit skin homogenates was determined by adding these extracts to buffered dopa solutions and by measuring the inhibition of melanin formation colorimetrically, as described elsewhere.[7] Sulfhydryl determinations were made with the ferricyanide method of Anson[8] in ultracentrifuged extracts and supernatant fluids of homogenates.

Results. In agreement with Rothman *et al.*[5] it was found that aqueous extracts of human epidermis inhibited the oxidation of tyrosine by tyrosinase and the autoxidation of dopa *in vitro*. As an index of the potency of the extracts the degree of inhibition of the autoxidation of dopa by 0.1 ml extract was chosen arbitrarily and the inhibition was ex-

* American Cancer Society Fellow, 1948-49.

1 Pugh, E. M., *Bioch. J.*, 1933, **27**, 475.

2 Danneel, R., and Schaumann, K., *Biol. Zbl.*, 1938, **58**, 242.

3 Schaaf, F., *Arch. f. Dermatol. u. Syph.*, 1938, **176**, 646.

4 Ginsburg, B., *Genetics*, 1944, **29**, 176.

5 Rothman, S., Krysa, H. F., and Smiljanic, A. M., PROC. SOC. EXP. BIOL. AND MED., 1946, **62**, 208.

6 Potter, V. R., and Elvehjem, C. A., *J. Biol. Chem.*, 1936, **114**, 495.

7 Flesch, P., PROC. SOC. EXP. BIOL. AND MED., in press.

8 Anson, M. L., *J. Gen. Physiol.*, 1941, **24**, 399.

pressed in percentages according to the formula:

$$\% \text{ Inhibition} = 100 \times \left(1 - \frac{\text{colorimeter reading of tube containing extract}}{\text{colorimeter reading of control tube}}\right)$$

Epidermis samples retained their inhibitory power for a considerable period of time when kept in the ice box before extraction. When the aqueous extracts were stored in the ice box, there was only slight diminution in the inhibitory effect. A fresh extract which caused 71% inhibition, showed at the end of 10 days 70%, and after 3 weeks 65% inhibition. Heating for 10 minutes in boiling water bath reduced the inhibitory effect by 10 to 40%. The inhibitory substance was completely dialyzed against distilled water through collodion membranes.

A few experiments were carried out with light brown extracts of 3 samples of Negro epidermis. No difference could be found between the extracts of epidermis from colored and white persons.

The inhibitory action of the extracts on dopa oxidation could be counteracted with p-chloromercuribenzoic acid, a specific sulfhydryl inhibitor. Cupric ion, a catalyst of sulfhydryl oxidation[9] and of the autoxidation of dopa,[10] also interfered with the inhibitory action of epidermal extracts. (Fig. 1).

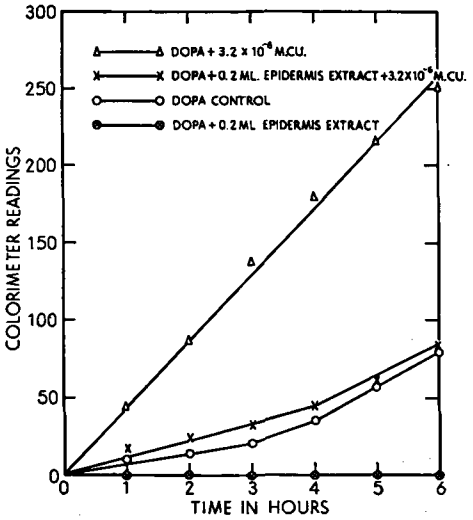

FIG. 1.

Inhibitory effect of human epidermal extract on autoxidation of dopa counteracted by cupric ions.

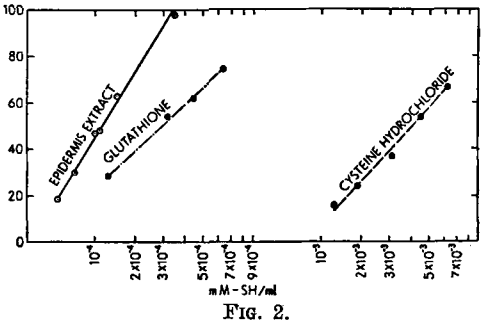

FIG. 2.

Relative inhibitory effect of cysteine, glutathione and extracts of human epidermis on autoxidation of dopa.

Estimation of the sulfhydryl content of the extracts gave values ranging from 4×10^{-5} mM/ml to 4×10^{-6} mM/ml. In most cases a correlation could be found between the sulfhydryl content of the extracts and their inhibitory power. When the -SH content was plotted on a logarithmic scale against the per cent inhibition, a straight line relationship was obtained. Such correlation was also found when glutathione (1.3×10^{-5} to 1.3×10^{-4} M final concentration) or cysteine hydrochloride (2.5×10^{-4} to 1.3×10^{-3} M final concentration) was added to dopa solutions. (Fig. 2). Calculated on the basis of the -SH content and in reference to 50% inhibition, the inhibitory effect of glutathione was about 13 times that of cysteine and the epidermis extracts were about 2.5 times more potent than glutathione.

When skin samples from human autopsy material were irradiated with ultraviolet light, no constant or reproducible differences could be observed between the -SH content of extracts of irradiated and non-irradiated epidermis. In order to eliminate the errors inherent in the preparation of human epidermis extracts (heating at 50°C for varying lengths of time, varying sizes of epidermal strips), living animals were irradiated and changes in the -SH content of the skin were studied in homogenates.

Colored rabbits are suitable for studies of pigmentation, because their hair is always

[9] Bernheim, F., and Bernheim, M. L. C., *Cold Spring Harbor Symp. Quant. Biol.*, 1939, 7, 174.

[10] Flesch, P., *J. Invest. Dermatol.*, 1948, 11, 157.

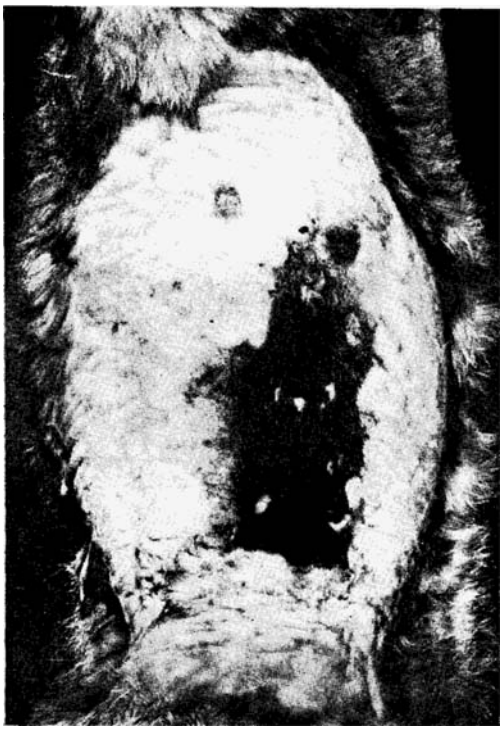

FIG. 3.
Pigmentation in grey rabbit after 12 days of rubbing daily for 1 minute with lanolin.

light colored near the skin. When the hair of colored rabbits is clipped and the bare skin subjected to irritation which leads to inflammation (heat, rubbing, ultraviolet light, etc.), the treated areas turn deeply pigmented after 1 to 2½ weeks and the regrowing hair becomes darker than it was before on the same area and than it is on the surrounding skin. The irritated areas are also characterized by exceedingly rapid regrowth of thick fur.[11-13] Clipping of the hair without irritating treatment leads primarily to an island-like regrowth of hair in pigmented areas[14] whereas hair growth in unpigmented spots is retarded.

In this laboratory one group of animals was rubbed daily for one minute with vaseline or lanolin over a clipped area on the back; the result in a grey rabbit after 12 days' rubbing is shown in Fig. 3. Another group of animals was irradiated daily for 10 minutes at a distance of 15 inches with the G.E. ultraviolet lamp. Pigment formation after ultraviolet irradiation was slightly more delayed than after rubbing. In experiments in which the -SH content of skin was determined, the rabbits were sacrificed immediately after a single irradiation was administered. In other animals one such treatment was found to cause pigmentation after 2 weeks latency period.

In 8 colored rabbits which were treated with ultraviolet light, the water extractable -SH compounds were markedly lowered in the aqueous extracts of homogenates obtained from irradiated skin samples as compared with similar extracts from non-irradiated skin. The average decrease was 53%. The results are summarized in Table I. Each result is the mean of three determinations, carried out in duplicate.

When the inhibitory power of these extracts was tested on the autoxidation of dopa, in 6 experiments the extracts obtained from irradiated skin showed 10 to 73% less inhibition than extracts from non-irradiated skin samples of the same animal. The average inhibition with extracts from irradiated skin was 29% and with extracts from non-irradiated skin 48%. (Fig. 4.)

Similar experiments were carried out on albino rabbits. In 6 experiments the amount of water-extractable -SH compounds did not show any significant change after irradiation. In 2 animals there was a decrease of 62 and 24% respectively.

On the basis of their -SH content, the extracts obtained from rabbit skin had about the same inhibitory power as aqueous extracts of human epidermis. No direct correlation could be found between the inhibitory power of the extracts and their -SH content.

Discussion. There are few data in the literature on the effect of ultraviolet light on sulfhydryl compounds of the skin. Keeser[15] found a marked reduction (50%) in the "reduced glutathione" content of rabbit skin after

[11] Lutz, W., *Arch. f. Dermatol. u. Syph.*, 1917, **124**, 233.

[12] Linser, K., *Klin. Wchschr.*, 1926, **5**, 1490.

[13] Linser, K., and Kähler, H., *Klin. Wchschr.*, 1928, **7**, 116.

[14] Königstein, H., *Arch. f. Dermatol. u. Syph.*, 1923, **143**, 314.

[15] Keeser, E., *Arch. f. exp. Path. u. Pharm.*, 1932, **166**, 624.

TABLE I.
Effect of Ultraviolet Light on the –SH Content of Pigmented Rabbit Skin.

Rabbit No.	Hair Color	Exposure time and distance min.	Exposure time and distance in.	–SH expressed in 10^{-5} mM in Non-irradiated skin extracts	–SH expressed in 10^{-5} mM in Irradiated skin extracts	% decrease
1	Grey	10	6	10 ± 0.3*	1.7 ± 0.3	83
2	”	15	8	15 ± 0.4	7.9 ± 0.1	47
3	Red-brown	15	12	39 ± 2.4	30.0 ± 1.3	23
4	”	7	12	19 ± 1.2	12.0 ± 0.7	37
5	”	15	12	18 ± 0.8	8.4 ± 2.1	53
6	Black	15	12	24 ± 2.3	4.6 ± 1.1	81
7	”	15	12	16 ± 1.4	4.8 ± 0.9	70
8	”	15	12	24 ± 1.8	9.2 ± 2.3	62

* Standard deviation of the mean.

TABLE II.
Effect of Ultraviolet Light on the -SH Content of Albino Rabbit Skin.

Rabbit No.	Hair color	Exposure time min.	Exposure time in.	–SH expressed in 10^{-5} mM in Non-irradiated skin extracts	–SH expressed in 10^{-5} mM in Irradiated skin extracts
1	Albino	15	12	11 ± 0.9	12 ± 2.3
2	”	”	”	18 ± 1.2	21 ± 3.2
3	”	”	”	17 ± 0.8	18 ± 2.8
4	”	”	”	28 ± 1.6	30 ± 4.0
5*	”	”	”	41 ± 2.1	43 ± 2.6
6	”	”	”	26 ± 1.8	24 ± 3.1
7	”	”	”	22 ± 1.4	8.5 ± 1.3
8	”	”	”	17 ± 0.6	13 ± 1.1

* Skin homogenate was extracted for 48 hours.

ultraviolet irradiation, while others[16] noted a slight decrease (3%) up to one hour after irradiation. The color of the rabbits used in all these experiments has not been mentioned.

The finding that ultraviolet light decreases the amount of water-extractable -SH compounds in pigmented skin, supports the assumption that pigmentation after irritative stimuli is at least partly due to a decrease in the -SH content of the melanoblast. The observation that a decrease in -SH content does not occur after ultraviolet irradiation of albino skin, can be explained by assuming that most of the radiation passes through the albino epidermis, as it is the case in the mouse,[17] while in the pigmented skin the melanin in the basal layer prevents the radiation from reaching the corium. Absorption of ultraviolet light is therefore much greater in pigmented than in non-pigmented epidermis which is the site of the -SH compounds and of the inhibitory action.[4,18-21]

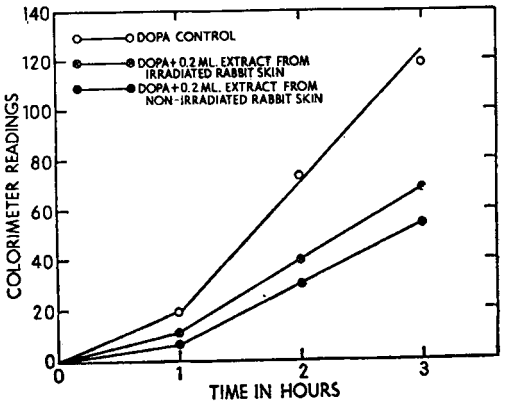

FIG. 4.

Inhibition of autoxidation of dopa with extracts of irradiated and non-irradiated rabbit skin.

[16] Matusis, I. I., and Grechanovskii, V. P., *Bull. biol. med. exp. U.R.S.S.*, 1937, **3**, 489.

[17] Kirby-Smith, J. S., Blum, H. F., and Grady, H. G., *J. Nat. Cancer Inst.*, 1942, **2**, 403.

[18] Percival, G. H., and Stewart, C. P., *Brit. J. Dermatol. Syph.*, 1930, **42**, 215.

[19] Giroud, A., and Bulliard, H., *La kératinisation de l'épiderme et des phanéres.* G. Doin, Paris, 1930.

[20] Kaye, M., *Bioch. J.*, 1924, **18**, 1289.

[21] Walker, E., *Bioch. J.*, 1925, **19**, 1085.

Summary. The inhibitory effect on melanin formation of aqueous extracts of isolated human epidermis and of homogenates of rabbit skin was found to be due to heat-stable, dialyzable, non-protein-like sulfhydryl compounds which were counteracted by cupric ions and p-chloromercuribenzoic acid. A direct relationship was found between the -SH concentration and the inhibitory power of extracts of human epidermis. Ultraviolet irradiation caused an immediate decrease in the amount of water-extractable -SH compounds of the skin of colored rabbits. No such decrease could be observed in albino animals. These findings support the previously advanced theory that pigment forming stimuli cause pigmentation by oxidizing or destroying the sulfhydryl compounds of the epidermis, whereupon the enzyme can freely act on the melanin precursor.

The author wishes to express his appreciation to Dr. Stephen Rothman for his valuable advice and interest in this work.

16852

Method for Determination of Sucrose and Sorbose in Blood and Urine.

CLARK D. WEST AND S. RAPOPORT.

From the Children's Hospital Research Foundation and the Department of Pediatrics, University of Cincinnati College of Medicine.

In this note is described a modification of the method of Hubbard and Loomis[1] for the determination of inulin which has proved satisfactory for the determination of sucrose and sorbose.

The method deviates from that described by these authors in the following ways.

The hydrochloric acid reagent is made by adding 5 volumes of concentrated HCl (sp. gr. 1.19) to one volume of distilled water. The resorcinol reagent is the same.

Plasma or serum filtrates are usually made in 1:20 dilution by precipitation with $Ba(OH)_2$ and $ZnSO_4$ according to the method of Somogyi.[2] Urine dilutions should be 1:100 or more. The one ml sample should contain between 30 and 160 μg of sucrose or between 20 and 150 μg of sorbose.

The procedure of Hubbard and Loomis is followed except for the following details. The test tubes are calibrated at 7.0 ml. After heating the mixture for 45 minutes in a water bath maintained at 70°C, 95% alcohol is added to the 7.0 ml mark, the solutions are mixed and are read in the Coleman spectrophotometer at a wave length of 530 mμ. In the case of sorbose, the density diminishes on standing; hence readings are made exactly 20 minutes after the end of heating.

Over the concentration ranges specified, the density was proportional to concentration for both sucrose and sorbose, sorbose being approximately 8% more chromogenic. Instead of running a blank we prefer to calculate a blank value by extrapolating the density of standards to zero content. In our experience the calculated blank density was usually less than 0.010.

Summary. The method of Hubbard and Loomis for the determination of inulin has been modified and adapted for the determination of sucrose and sorbose.

1 Hubbard, R. S., and Loomis, T. A., *J. Biol. Chem.*, 1942, **145**, 641.

2 Somogyi, M., *J. Biol. Chem.*, 1945, **160**, 69.