THE JOURNAL OF INVESTIGATIVE DERMATOLOGY
Vol. 56, No. 5
Copyright © 1971 by The Williams & Wilkins Co.
Printed in U.S.A.

REGULATION OF TYROSINASE ACTIVITY IN MOUSE MELANOMA AND SKIN BY CHANGES IN MELANOSOMAL MEMBRANE PERMEABILITY*

MELVIN H. VAN WOERT, M.D., FRANCES KORB, B.S. AND KEDAR N. PRASAD, PH.D.†

ABSTRACT

Compounds which are known to labilize biological membranes have been demonstrated to increase tyrosinase activity in the B-16 melanoma and newborn mouse skin. Detergents, bile acids, chlorpromazine (CPZ), neutral steroids and proteases all increase melanosomal tyrosinase activity. Digitonin (0.5%) produced approximately a five fold increase in tyrosinase activity and released 50% of the enzyme from the melanosome. CPZ (10^{-3}M) produced a 2.7 fold increase in tyrosinase activity without releasing the enzyme from the melanosome. The CPZ-induced activation of tyrosinase was decreased by the membrane stabilizer, chloroquine, and abolished by prior treatment of the melanosomes with digitonin.

Alteration of the outer melanosomal membrane by digitonin and CPZ was confirmed by electron microscopy.

The results suggest that the membrane surrounding the melanosome regulates the accessibility of the substrate tyrosine to the tyrosinase molecule inside the organelle. A change in membrane permeability may be the mechanism by which certain physiological and pharmacological compounds control or alter pigmentation.

Tyrosinase catalyzes the initial steps in melanin formation, i.e. the hydroxylation of tyrosine to dihydroxyphenylalanine (dopa) and the subsequent oxidation of dopa to dopaquinone. In mammalian melanocytes, most of the tyrosinase is confined in a specialized cytoplasmic organelle, the melanosome (1). This particle, which synthesizes and stores the melanin pigment, is bounded by a unit membrane (Fig. 1A).

We have shown that chlorpromazine (CPZ) and other phenothiazine compounds increase melanoma tyrosinase activity *in vivo* and *in vitro* (2, 3). Alteration of membrane permeability is one of the major effects of the phenothiazine tranquilizers (4). The increase in melanoma tyrosinase induced by various phenothiazines is proportional to the surface activity of these compounds suggesting that they might act by labilizing the melanosomal membrane. Menon and Haberman (5) demonstrated that strong detergents also increase melanoma tyrosinase activity.

These findings suggest that regulation of the permeability of the outer melanosomal membrane (Fig. 1A) by physiological and pharmacological compounds could be one mechanism of controlling cutaneous melanin formation (3). This report presents additional experimental evidence in support of this hypothesis. Various agents which labilize or stabilize biological membranes were investigated for their effect on mouse melanoma and skin tyrosinase.

MATERIALS AND METHODS

Chemicals. L-Tyrosine-3,5-H^3 (specific activity, 8.2 c/mmole) was purchased from New England Nuclear Corporation (Boston, Mass.). Digitonin was obtained from Fisher Scientific Company (Pittsburgh, Pa.). L-Tyrosine, L-dihydroxyphenylalanine, sodium desoxycholate, ouabain, and trypsin were purchased from Nutritional Biochemicals Corporation (Cleveland, Ohio). Lithocholic acid, lysolecithin, prednisone, cholesterol, etiocholanolone, progesterone, diethylstilbestrol, chymotrypsin, papain, vitamin E, vitamin A, hydrocortisone and testosterone were purchased from Sigma Chemical Company (St. Louis, Mo.). The nonionic detergent nonylphenoxypoly(ethaneoxyl)-ethanol (Igepal Co-630) was generously supplied by the General Aniline and Film Company (New

Received October 23, 1970; accepted for publication November 19, 1970.

This work was supported by DHEW Grant NB 07542 and Damon Runyon Grant DRG 1041.

* From the Departments of Internal Medicine and Pharmacology, Yale University School of Medicine, New Haven, Connecticut 06510.

† Department of Radiology, University of Colorado Medical Center, Denver, Colorado 80220.

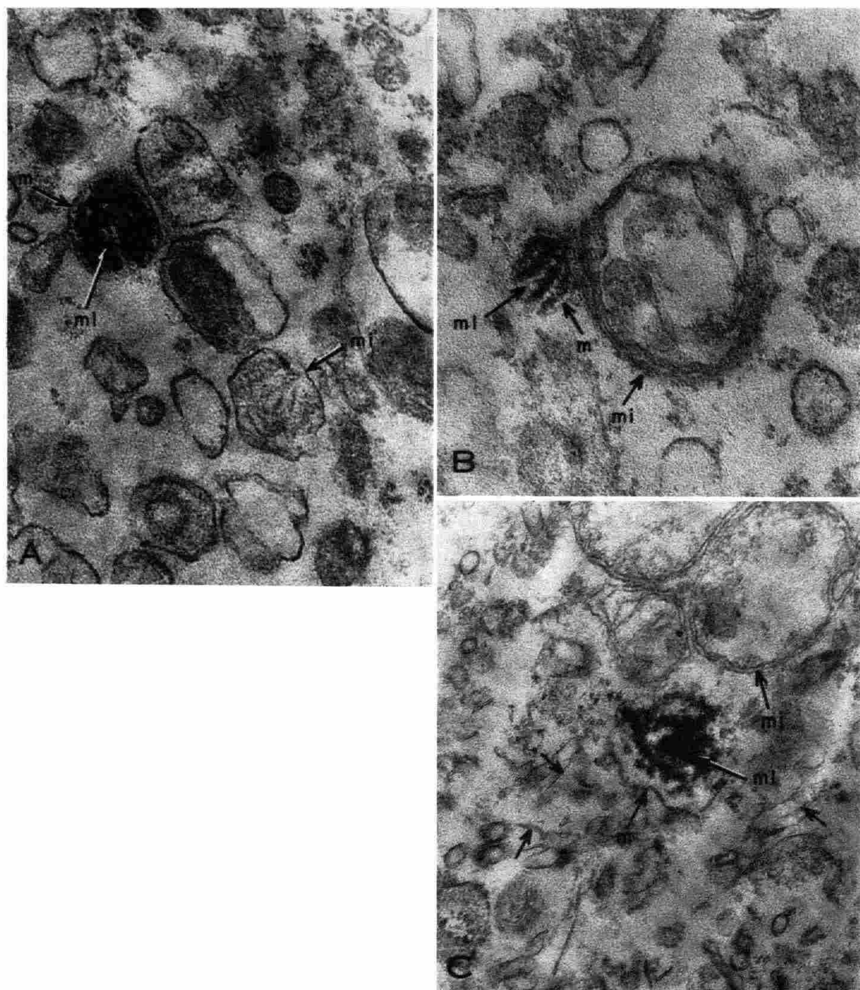

FIG. 1. Electron micrographs of untreated control (A), CPZ treated (B) and digitonin treated (C) melanosome fractions. The melanosome (ml) is surrounded by a unit membrane (m). Mitochondria (mi) are also seen in the sections. Digitonin produces generalized fragmentation of membranes, indicated by the arrows in (C). The pellets were fixed in osmic acid, embedded in epon, sectioned and stained with uranyl acetate followed by lead citrate. × 74,642.

York, N.Y.), chlorpromazine HCl by Smith Kline and French Laboratories (Philadelphia, Pa.), chloroquine diphosphate by Sterling-Winthrop Research Institute (Rensselaer, N.Y.) and chloripheniramine maleate by Schering Corporation (Bloomfield, N.J.).

Preparation of melanoma large granule fraction. B-16 mouse melanoma was obtained from the Jackson Laboratories, Bar Harbor, Maine and serially transplanted subcutaneously in C57Bl/6J mice. The tumors were excised 11–13 days after transplantation and a melanosome-rich fraction was prepared fresh daily for each experiment. The melanomas were weighed and homogenized in a

Potter-Elvehjem homogenizer (Teflon pestle) with 10 volumes of 0.25M sucrose containing 0.08M sodium phosphate buffer, pH 6.8 at 4° C. The nuclear fraction was removed by centrifuging the homogenate for 5 minutes at 700 g. A large granule fraction containing most of the melanosomes was prepared by centrifuging the supernatant at 11,000 g for 10 minutes. The pellet was resuspended in 0.25M sucrose with 0.08M sodium phosphate buffer, pH 6.8 (¼ of the original volume). This large granule fraction also contained mitochondria and lysosomes which do not have tyrosinase activity.

Preparation of mouse skin homogenate. For mouse skin tyrosinase experiments, C57Bl/6J newborn mice were decapitated 4 to 12 days after littering. The dorsal skin was peeled off, weighed and homogenized with 5 volumes of 0.25M sucrose containing 0.08M sodium phosphate buffer pH 6.8 in a ground glass homogenizer. Two to four skins were pooled for each determination. All of the skins were homogenized under the same conditions but there remained a larger fibrous residue from the older skins. The homogenate was decanted and the residual fibrous tissue discarded.

Protein determination. Protein content of the melanoma melanosome fraction and skin homogenate was measured by the method of Lowry *et al.* (6).

Tyrosinase assay. Tyrosinase was assayed by the method of Pomerantz (7). The tritiated water formed when tyrosine-3,5-^{3}H is hydroxylated by tyrosinase was separated with a Norit A-Celite column and counted in a liquid scintillation spectrometer using Bray's solution (8). Each assay mixture contained L-tyrosine-3,5-^{3}H (2.0 μc, 1.0 μmole); L-Dopa (0.15 μmole); 0.25M sucrose; 0.032M sodium phosphate buffer, pH 6.8 and 0.5 ml of either melanoma melanosome fraction or skin homogenate in a total volume of 1.25 ml. Blanks containing boiled enzyme (100° C for 10 minutes) were run simultaneously. The incubation time was 1 hour, temperature 37° C and each assay was carried out in duplicate. Concentrations of the compounds used in these experiments did not change the pH of the medium. Preincubation experiments with CPZ and etiocholanolone for 45 minutes at 37° C showed no additive effect. Therefore these and other compounds tested were added immediately before the tyrosinase assay. Some of the experiments reported in this paper were repeated, with identical results, using the oxygen electrode method of measuring tyrosinase (3) indicating that the compounds studied were not interfering with the radioactive tyrosinase assay.

RESULTS

Effect of various membrane-active compounds on melanoma (melanosomal fraction) and skin tyrosinase.

The *in vitro* effect on tyrosinase activity of compounds which are known to labilize or stabilize lysosomal (9–12), erythrocyte (13) and mitochondrial membranes (14) are summarized in the Table. Digitonin and another detergent Igepal CO-630 (nonylphenoxypoly [ethyleneoxy] ethanol) produced the greatest increase in B-16 melanosomal tyrosinase. This increase in tyrosinase activity agrees with the previous observations of Menon and Haberman (5) and is thought to be related to the disruption of the membrane surrounding the melanosome. 10^{-3}M CPZ increased skin mouse tyrosinase as well as B-16 melanosomal tyrosinase but preincubation with digitonin or Igepal abolished the ability of CPZ to activate tyrosinase. It is likely that the melanosomal membrane was disrupted maximally by the detergents, therefore CPZ could exert no additional effect. In contrast, chloroquine, which has a stabilizing effect on biological membranes diminished the tyrosinase activation induced by CPZ. The chemical compounds which are known to labilize biological membranes showed the following order of potency for activating tyrosinase: detergents > bile acids > CPZ > neutral steroids. These agents are known to interact directly with the lipid component of membranes. Prednisone and cholesterol are exceptions since they stabilize lysosomal membranes (14) yet increase melanosomal tyrosinase activity. Another membrane stabilizer, hydrocortisone, was inactive in our experiments. Proteolytic enzymes which digest the protein in lipoprotein membranes (15) increased melanosomal tyrosinase activity (see Table).

As shown in the Table, similar qualitative effects were observed in the mouse skin and B-16 melanosomes. However, the smaller percentage increase in skin tyrosinase may be at least partially due to traumatic membrane injury secondary to the vigorous techniques required to homogenize the skin compared to the melanoma tissue.

The concentrations necessary to obtain an effect *in vitro* exceeds the physiological and perhaps the pharmacological tissue levels but are similar to those reported to alter the lysosomal membrane (9, 10). Since many of the compounds used in these experiments are insoluble in water, the experimental procedures do not permit an accurate evaluation of the concentrations in the membrane of the melanosome. In addition, melanin has been observed to absorb non-specifically many polycyclic aromatic compounds (including CPZ, the estrogen mestranol, and the progestin chlormadinone) (16) which

TABLE

Effect of various membrane active compounds on melanoma (melanosomal fraction) and skin tyrosinase

The results are the mean percent ± S.E.M. increase in tyrosinase activity above controls run simultaneously during each experiment. The number of experiments is listed in parenthesis.

Compound	Conc.	% Increase in melanosomal tyrosinase activity	
		B-16 Melanoma	Mouse Skin
CPZ	10^{-3}M	172 ± 8 (15)	29 ± 3 (12)
Digitonin	0.5%	412 ± 33 (6)	50 ± 3 (4)
Digitonin and CPZ	0.5%, 10^{-3}M	420 ± 28 (4)	59 ± 4 (4)
Igepal	1.0%	349 ± 30 (4)	57 ± 9 (5)
Igepal and CPZ	1.0%, 10^{-3}M	361 ± 31 (4)	48 ± 4 (5)
Chloroquine‡	10^{-3}M	−14 ± 1 (4)	−3 ± 2 (3)
Chloroquine and CPZ	10^{-3}M, 10^{-3}M	79 ± 5 (4)	18 ± 1 (3)
CPZ	5 × 10^{-4}M	97 ± 6 (4)	
Na desoxycholate	0.1%		72 ± 2 (3)
Na cholate	0.2%		51 ± 4 (3)
Lithocholic Acid*	10^{-3}M	199 ± 19 (4)	
Lysolecithin	0.5%	278 ± 10 (3)	
Prednisone	10^{-3}M	73 ± 4 (3)	
Cholesterol†	10^{-3}M	64 ± 1 (3)	
Etiocholanolone*	10^{-3}M	61 ± 5 (6)	
Progesterone*	10^{-3}M	43 ± 8 (4)	
Diethylstibestrol*	5 × 10^{-4}M	16 ± 2 (6)	
Trypsin§	0.1%	72 ± 7 (4)	
Chymotrypsin§	0.1%	15 ± 2 (3)	
Papain§	0.1%	9 ± 1 (3)	
Others‖	10^{-3}M	0	

‡ The tyrosinase activity was decreased by chloroquine. * These water insoluble compounds were added in 0.015 ml absolute ethanol to 1.5 ml of melanosomal fraction and the same volume of ethanol was added to the control fraction. † Cholesterol was dissolved in 0.015 ml of p-dioxane and added to 1.5 ml of melanosomal fraction; the same volume of p-dioxane was added to the control fraction. § The enzymes were preincubated with the melanosome fraction for 30 minutes at 23° C, pH 6.8, prior to tyrosinase assay. Trypsin, chymotrypsin and papain do not hydroxylate L-tyrosine-3,5-H^3 and when denatured (100° C for 10 minutes) do not increase melanosomal tyrosinase activity. ‖ Other compounds examined were chlorpheniramine, hydrocortisone, testosterone, ouabain, vitamins E and A.

could increase preferentially their concentration in the melanosome.

Effect of CPZ, freezing and digitonin on activity and physical state of tyrosinase in B-16 melanoma melanosomes.

The effect of CPZ, digitonin and freezing and thawing on the physical state and localization of tyrosinase activity was also examined (Fig. 2). Melanosomes prepared as described previously (▨) were incubated either with CPZ 10^{-3}M or digitonin 0.5% for 15 minutes at 4° C or frozen and thawed 5 times (▤). A control melanosome fraction was untreated. The control and treated melanosomes were centrifuged at 30,000 × *g* for 60 minutes; the supernatant saved (□), and the pellet resuspended in the same volume of 0.25M sucrose containing 0.08M sodium phosphate buffer, pH 6.8 (▧). Tyrosinase activity and protein concentration were measured in 0.5 ml samples of each of the four fractions. The increased tyrosinase activity induced by CPZ remained in the sedimented particulate fraction. The tyrosinase was not released from the melanosome; presumably the increase in enzyme activity is due to increased membrane permeability permitting an increased concentration of tyrosine and perhaps dopa to reach the tyrosinase molecule. Removal of the CPZ in solution by repeated washing of the particulate fraction with 0.25M sucrose did not reverse the tyrosinase activation, indicating that the membran

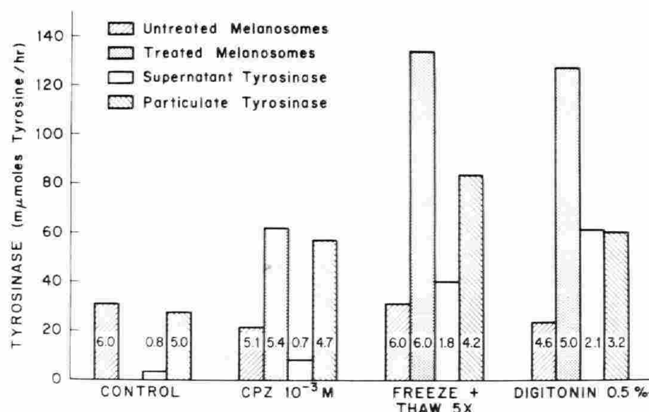

FIG. 2. Effect of CPZ, freezing and digitonin on activity and physical state of tyrosinase in B-16 melanoma melanosomes.

The bars are the mean value of two experiments; each assay was run in duplicate. The ordinate is the tyrosinase activity expressed as mμmoles of tyrosine hydroxylated per hour in 0.5 ml of each fraction. The number over each bar is the protein concentration of that fraction in mg/ml.

damage was irreversible or the drug-membrane bond was strong. Freezing and thawing also increased the melanosomal tyrosinase presumably by disrupting membrane structure as has been reported for mitochondria (17) and lysosomes (18); 30% of the tyrosinase was released from the melanosomes. Digitonin produced a similar increase in total tyrosinase activity but 50% of the tyrosinase was found in the supernatant after treatment with this detergent. The protein content of the fraction in each experiment was proportional to their tyrosinase activity. The particulate fractions of the control, CPZ-treated, and digitonin-treated melanosomes were examined by electron microscopy (Fig. 1). Intact outer melanosomal membranes were frequently seen in the untreated control melanosomal pellet (Fig. 1A). The outer membranes of the CPZ-treated melanosomes appeared thickened and swollen (Fig. 1B). Membrane swelling with more generalized fragmentation was also evident in the digitonin-treated melanosomes. CPZ and digitonin also produced swelling of the mitochondrial membrane (Fig. 1B and C).

DISCUSSION

The observations above demonstrate that compounds which labilize lysosomal, erythrocyte, and mitochondrial membranes increase melanosomal tyrosinase activity. Chloroquine, which stabilizes biological membranes, partially protects against this increase in tyrosinase activity. The experimental data suggest that the outer membrane surrounding the melanosome impedes the accessibility of the substrate tyrosine to the inner tyrosinase molecule with the result that under physiological conditions the melanosomal tyrosinase is only partially active. Therefore the rate of synthesis of melanin from tyrosine can be regulated by agents which labilize or stabilize the melanosomal membrane. A variety of physiological and pharmacological compounds can control permeability of this membrane.

These biochemical observations may have clinical implications if compounds which labilize the melanosomal membrane increase cutaneous pigmentation whereas those that stabilize the membrane reduce or prevent pigmentation. There is clinical experience to support further investigation of this hypothesis. Contraceptive medication containing progestational and estrogenic compounds (19), pregnancy (20), liver disease associated with elevated bile acids and estrogens (21) and long term CPZ therapy (22) can induce cutaneous hyperpigmentation; whereas chloroquine produces hypopigmentation of the hair (23). Posttraumatic and postinflam-

matory skin hyperpigmentation (20) could result from damage to melanosomal membranes either directly, or secondarily, by the release of lysosomal proteases. CPZ has been demonstrated to increase melanoma tyrosinase *in vivo* (2). Similar experiments are in progress with other compounds which have been shown to activate tyrosinase *in vitro*.

From the experimental results obtained here, it is evident that similarities exist between melanosomes and lysosomes. Each of the subcellular organelles is surrounded by a single membrane, the permeability of which can be altered by some of the same agents. In both melanosomes and lysosomes slight injury may alter the availability of enzyme to substrate in the organelles while disruptive membrane damage releases them into solution. Seiji *et al.* (24) have suggested another similarity between lysosomes and melanosomes. They found that melanosomes contain acid phosphatase, the enzyme which has been thought to be specifically localized in lysosomes (25). Previous studies in this laboratory have demonstrated that lipofuscin which is synthesized in lysosomes, contains a melanin component (26). The structural and developmental relationships of melanosomes to lysosomes requires further exploration.

REFERENCES

1. Seiji, M., Fitzpatrick, T. B., Simpson, R. T. and Birbeck, M. S. C.: The melanosome: a distinctive subcellular particle of mammalian melanocytes and the site of melanogenesis. J. Invest. Derm., *36:* 243, 1961.
2. Van Woert, M. H. and Palmer, S. H.: Inhibition of the growth of mouse melanoma by chlorpromazine. Cancer Res., *29:* 1952, 1969.
3. Van Woert, M. H.: Effect of phenothiazines on melanoma tyrosinase activity. J. Pharmacol. Exp. Therap., *173:* 256, 1970.
4. Guth, P. S. and Spirites, M. A.: The phenothiazine tranquilizers: biochemical and biophysical actions. Int. Rev. Neurobiol., *7:* 231, 1964.
5. Menon, I. A. and Haberman, H. F.: State of tyrosinase in melanoma melanosomes. Life Sci., *8:* 207, 1969.
6. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J.: Protein measurement with the Folin phenol reagent. J. Biol. Chem., *193:* 265, 1951.
7. Pomerantz, S. H.: The tyrosine hydroxylase activity of mammalian tyrosinase. J. Biol Chem., *241:* 161, 1966.
8. Bray, G. A.: A simple efficient liquid scintillator for counting aqueous solutions in a liquid scintillation counter. Anal. Biochem., *1:* 279, 1960.
9. de Duve, C., Wattiaux, R. and Wilbo, M.: Effects of fat soluble compounds on lysosomes *in vitro*. Biochem. Pharmacol., *9:* 97, 1962.
10. Weissmann, G.: Lysosomes. New Eng. J. Med., *273:* 1143, 1965.
11. Koenig, H. and Jibril, A.: Acidic glycolipids and the role of ionic bonds in the structure linked latency of lysosomal hydrolases. Biochem. Biophys. Acta, *65:* 543, 1962.
12. Weissmann, G.: The effect of neutral steroids and bile acids on lysosomes *in vitro*. Biochem. Pharmacol., *14:* 525, 1965.
13. Weissmann, G. and Keiser, H.: Hemolysis and augmentation of hemolysis by neutral steroids and bile acids. Biochem. Pharmacol., *14:* 537, 1965.
14. Gallagher, C. H.: The mechanism of action of hydrocortisone on mitochondrial metabolism. Biochem. J., *74:* 38, 1960.
15. Beaufay, H. and de Duve, C.: Enzyme release of bound hydrolases. Biochem. J., *73:* 604, 1959.
16. Blois, M. S. and Taskovich, L.: The reversible binding of some aromatic and cyclic compounds to biopolymers in vitro. J. Invest. Derm., *53:* 344, 1969.
17. Witter, R. F., Morrison, A. and Shepardson, G. R.: Effect of lysolecithin on oxidative phosphorylation. Biochem. Biophys. Acta, *26:* 120, 1957.
18. Sellinger, O. Z., Beaufay, H., Jacques, P., Doyer, A. and de Duve, C.: Intracellular distribution and properties of β-acetylglucoaminidase and β-galactosidase in rat liver. Biochem. J., *74:* 450, 1960.
19. Paschkis, K. E., Rakoff, A. E., Contarow, A. and Rupp, J. J.: P. 707, *Clinical Endocrinology*. Harper and Row, New York, 1967.
20. Fitzpatrick, T. B., Seiji, M. and McGugan, A. D.: Melanin pigmentation. New Eng. J. Med., *265:* 374, 1961.
21. Sherlock, S.: P. 283, *Diseases of the Liver and Biliary System*. Blackwell Scientific Publications, Oxford and Edinburgh, 1968.
22. Greiner, A. C. and Berry, K.: Skin pigmentation and corneal and lens opacities with prolonged chlorpromazine therapy. Canad. Med. Assoc. J., *90:* 663, 1964.
23. Saunders, T. S., Fitzpatrick, T. B., Seiji, M., Brunet, P. and Rosenbaum, E. E.: Decrease in human hair color and feather pigmentation of fowl following chloroquine diphosphate. J. Invest. Derm., *33:* 87, 1959.
24. Seiji, M. and Iwashita, S.: Intracellular localization of tyrosinase and site of melanin formation in melanocyte. J. Invest. Derm., *45:* 305, 1965.
25. Novikoff, A. B.: Pp. 36–74, *Ciba Foundation on Lysosomes*. Eds., de Reuck, A. V. S. and Cameron, M. P., Little Brown, Boston, 1963.
26. Van Woert, M. H., Prasad, K. N. and Borg, D. C.: Spectroscopic studies of substantia nigra pigment in human subjects. J. Neurochem., *14:* 707, 1967.