MECHANISM OF ACTION OF THE MAMMALIAN PHENOLASE COMPLEX

by

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A THESIS

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(Chairman, Graduate Council)

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I. INTRODUCTION

The oxidases are the catalysts which are directly concerned with aerobic life. These enzymes are metallo-proteins, among the best-characterized of which are the copper-proteins, uricase, ascorbic acid oxidase, laccase, and the phenolase complex,* which is the subject of the present dissertation. The properties of this enzyme, or enzyme complex, have been the subject of investigation for over 60 years. The phenolase complex is defined as that copper-containing enzyme or enzyme complex which catalyzes the aerobic oxidation of mono- and ortho-dihydric phenols to ortho-quinones. Thus depending upon the reaction conditions, the phenolase complex may catalyze the incorporation of one atom of oxygen into a molecule of monophenol, or the oxidative dehydrogenation of diphenols to ortho-quinones.

1) monophenol + O_2 = ortho-quinone + H_2O

2) 2(ortho-diphenol) + O_2 = 2(ortho-quinone) + $2H_2O$

As indicated in equations 1) and 2), that oxygen which is not incorporated into a monophenol, is reduced directly to water. No hydrogen peroxide is formed.

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*The phenolase complex has been given a variety of names (such as phenol oxidase, polyphenol oxidase, tyrosinase, dopa oxidase, catecholase, catecholase, cresolase, and phenolase) depending in part upon which phenolic substrate was being investigated. The term "phenolase complex" is used throughout this dissertation to emphasize the uncertainty of the identity of the active centers which catalyse reactions 1) and 2).

One of the major questions regarding the phenolase complex has been whether the two activities (equations 1 and 2) are catalysed by separate enzymes, a single enzyme with separate catalytic centers, or a single enzyme with identical catalytic centers. Since mammalian phenolase complex is relatively specific for tyrosine and dopa (β-(3,4-dihydroxyphenyl)-alanine), a study of optical specificity might be expected to elucidate the relation of the two activities.

II. REVIEW OF THE LITERATURE

The literature review has been divided into two sections, the first of which deals with the general problem of the nature and action of the phenolase complex, and the second considers the problems which are more specifically related to mammalian phenolase complex and the melanocyte.

A. Nature and action of the phenolase complex.

Pugh and Raper (1) presented convincing evidence that o-quinones may be formed from mono- and o-dihydric phenols in the presence of phenolase complex (extract from the mealworm, Tenebrio molitor), by isolating the anilino-o-quinones and proving their structure by analysis and synthesis. Identical anilino-o-quinones were formed from the enzymic oxidation of phenol and catechol, and from p-cresol, m-cresol, and homocatechol. The formation of o-quinone by the enzymic oxidation of catechol has been confirmed polarographically by Doskocil (2).

The following criteria establish the phenolase complex as a copper enzyme:

(1) The copper content of purified preparations is proportional to monophenolase and to diphenolase activities (3,4).

(2) Removal of copper results in a loss of activity which is regained when copper is replaced. Other metals are ineffective in reactivating the enzyme (5-8).

Potato phenolase complex (6) takes up carbon monoxide in the presence of diphenol in the molar ratio $CO/Cu \cong 0.5$. The carbon monoxide complex is not dissociated by light, but is stoichiometrically dissociated by HCN. From this it is suggested the enzyme is in the cupric state in the presence of air and absence of reducing agents, is reduced to the cuprous state by o-diphenols (6), and that two copper atoms are associated (9) so that they may combine with one molecule of oxygen.

An interesting phenomenon, which was recognized early, was the autocatalytic nature of monophenol oxidation: the maximum rate of monophenol oxidation is preceded by an induction period (10) which may be eliminated by the addition of small amounts of an o-diphenol (6,10,11). In attempting to explain the autocatalytic nature of monophenol oxidation, Onslow and Robinson (12) and later Richter (13) proposed that diphenols are enzymically oxidised to ortho-quinones and hydrogen peroxide, which then may secondarily oxidize monophenols to the corresponding diphenols. Against this hypothesis were the observations that (a) the catechol-phenolase complex reaction, followed iodometrically, was not affected by added catalase or peroxidase (14), (b) p-cresol or phenol could not be non-enzymically oxidised by means of o-benzoquinone (14), (c) the rate of disappearance of hydrogen peroxide, under nitrogen, was the same in the presence or

absence of p-cresol plus phenolase complex (14), and (d) no trace of hydrogen peroxide could be detected polarographically when catechol was oxidized by phenolase complex and air (2). Recently, O_2^{18} studies have provided convincing evidence that the oxygen atom incorporated into monophenols, in the presence of either purified mushroom phenolase complex (15) or a crude mouse melanoma brei (16), is derived exclusively from molecular oxygen. Adams and Nelson (17) demonstrated that the component which catalyses monophenol oxidation has the characteristics of an enzyme, that is, it is heat labile, is inactivated by dilute acids, it does not pass through a semipermeable membrane, and it can be precipitated from solution by addition of ammonium sulfate. However, in the course of purifying phenolase complex from the common mushroom, Psalliota campestris, they were able to separate fractions which contained a higher ratio of cresolase/catecholase activity (oxidative activity when p-cresol or catechol is used as substrate) than the starting material, as well as fractions which contained a lower cresolase/catecholase ratio. They concluded that separate enzymes were responsible for the oxidation of mono- and diphenols. They were unable to separate the activities starting with extracts from the mushroom, Lactarius piperatus, (cf. 18,19) or the puff ball, Calvatia cyathiformis (17). Keilin and Mann (4) obtained a highly purified enzyme preparation from Psalliota campestris which appeared to become highly specific for o-diphenols and unable to oxidize monophenols. They concluded that some "additional factor" was required for the oxidation of monophenols. Kubowitz (6) commented that o-diphenols, or other substances which shorten the induction period

observed in the oxidation of monophenols, were removed during the purification of the phenolase complex, and that Keilin and Mann simply observed a long induction period. In an extensive study of the factors which affect monophenol oxidation, Bordner and Nelson (14) concluded that the induction period which precedes the maximal rate of oxygen consumption (determined manometrically) was "a) shortened by reducing agents such as potassium ferrocyanide, alanine, hydroquinone and hydrogen peroxide, all of which reduce o-quinones; b) prolonged by oxidizing agents capable of oxidizing homocatechol to homoquinone, such as potassium ferricyanide, a suspension of manganese dioxide and laccase; c) prolonged by the addition of sodium benzene sulfinate; d) shortened as the solution becomes more alkaline." Therefore Bordner and Nelson (14) suggested that diphenols activate the enzyme to permit the oxidation of monophenols, possibly as suggested by Kubowitz (6), by reducing cupric-enzyme to the cuprous form. The reaction mechanism illustrated in Figure 1 was proposed. Nelson, Dawson and co-workers (cf. 20) have amassed considerable data which, in contradiction to earlier work (7), they interpret as supporting the hypothesis of a single enzyme catalyzing the oxidation of both mono- and diphenols.

When catechol is oxidized by the phenolase complex, the enzyme is progressively inactivated. This inactivation is dependent only upon the amount of catechol oxidized, and not upon the rate of oxidation. Monophenols (p-cresol and hydroquinone*) protect the enzyme against this

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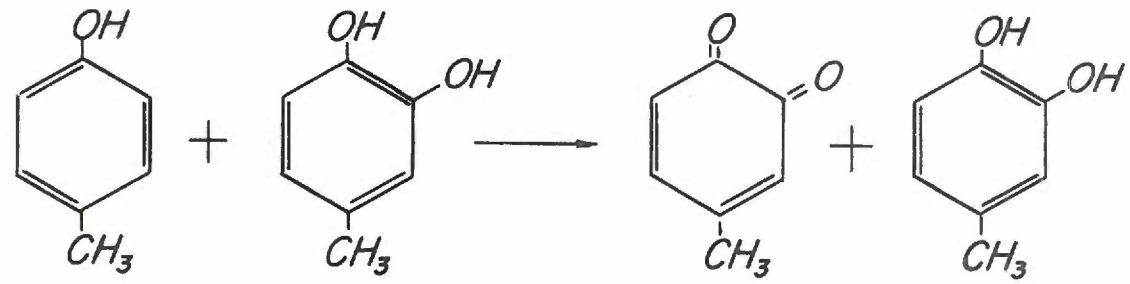
*Hydroquinone was later shown to be oxidized partially by the monophenol mechanism (22) as well as by reducing p-quinone.

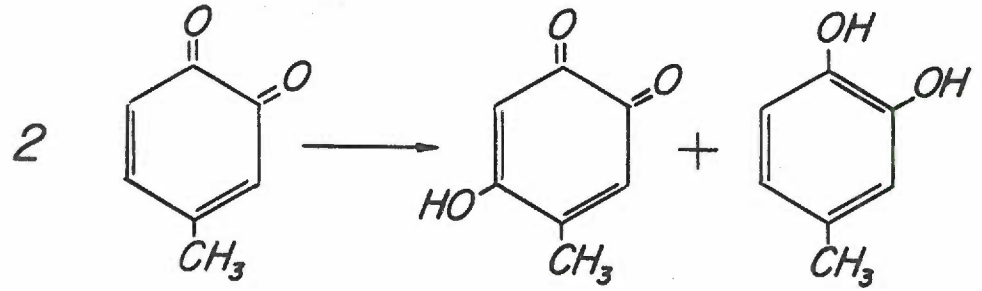
Figure 1

Mechanism of enzymatic oxidation of monophenols, proposed by Bordner and Nelson (14). Reaction 1 indicated the apparent requirement of simultaneous oxidation of diphenols to permit the enzymic oxidation of monophenols. Reaction 2 is the proposed non-enzymic mechanism by means of which diphenols are regenerated to permit monophenol oxidation to become autocatalytic, and to explain the consumption of two atoms of oxygen per mole of diphenol oxidized, and three atoms of oxygen per mole of monophenol oxidized.

(1)



(2)



"reaction inactivation," but ascorbic acid (a reducing agent) or benzoic acid (an inhibitor) affords no protective action (21). By employing differential adsorption on alumina and kaolin (23), highly purified preparations of phenolase complex have been prepared from the common mushroom (Psalliota campestris) which have greatly different ratios of catecholase activity (oxidative activity when catechol is used as substrate) to cresolase activity (rate of oxygen uptake in the presence of p-cresol). These have been called high catecholase or high cresolase preparations of the phenolase complex. For a series of purified high cresolase preparations (24), there appeared to be an upper limit of cresolase activity when based on the amount of catecholase activity. In these preparations, both activities were proportional to the copper content, and the ratio of activities resembled that in the fresh water extract of the mushroom (24). For a series of high catecholase preparations, phenolase (rate of oxygen uptake when phenol is the substrate) and catecholase, but not cresolase activities were proportional to the copper content (25).

Monophenols and diphenols are mutual inhibitors of oxidation catalysed by phenolase complex as indicated in Table I. This suggests that the catalytic sites for the oxidation of mono- and ortho-dihydric phenols are similar, and may be identical.

Mallette and Dawson (26) studied the electrophoretic behavior of five preparations of phenolase complex from Psalliota campestris which possessed greatly different ratios of catecholase/cresolase activity and various degrees of purity, and concluded that all known properties of the phenolase complex could be explained on the basis of a single enzyme capable of catalysing the oxidation of both mono- and o-dihydric phenols. In order to

TABLE I

Mutual Inhibition of the Phenolase Complex by Mono- and Diphenolic Substrates

Phenolase complex from	Substrate Inhibited	Substrate	Reference
P. campestris	p-cresol	catechol	(78)
(in presence of ascorbic acid)	phenol	catechol	(32)
	p-cresol	homocatechol	(32)
	4,5-dimethylphenol	4,5-dimethyl catechol	(32)
P. campestris or potato	dopa	tyrosine	(31,32)

account for preparations with various catecholase/cresolase ratios, it was suggested that, during purification, portions of the enzyme were successively fragmented from the original molecule, resulting in an increased catecholase activity and a decreased cresolase activity. This explanation was considered to be consistent with the higher copper content of high catecholase preparations compared with that of high cresolase preparations. One high catecholase preparation was studied in the ultracentrifuge. It was calculated to have a molecular weight of about 100,000 and four atoms of copper/mole. The work and interpretations of Mallette and Dawson have been discussed by Mason (9), and by Singer and Kearney (27) who cite the need for sufficient amounts of highly purified phenolase complex to permit electrophoretic, ultracentrifugal, and phase rule studies of the enzyme.

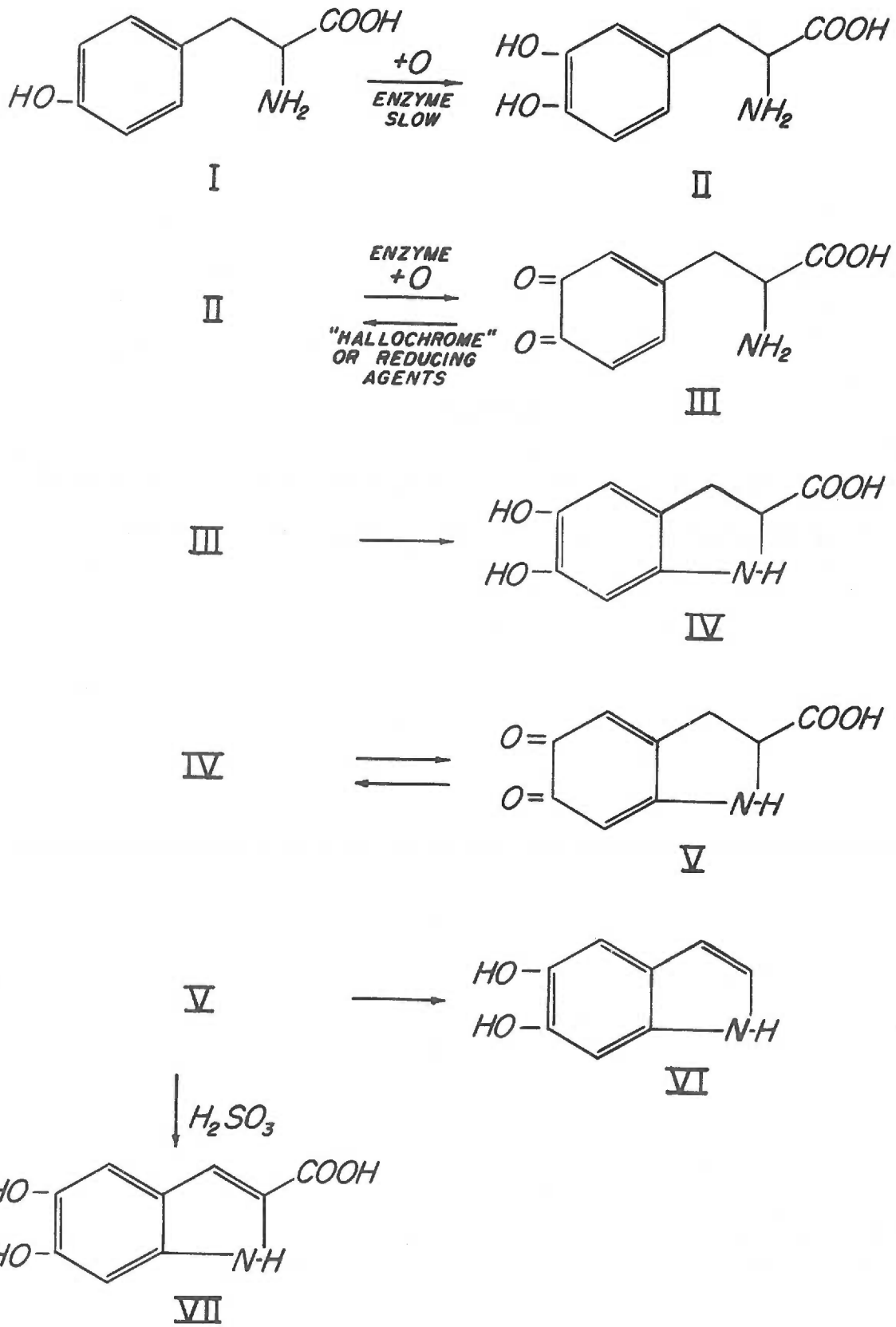
It now appears certain that both monophenolase and diphenolase activities are copper dependent, but the question of the structure of the phenolase complex, that is, whether the catalytic centers are identical or different and whether they reside on the same or different proteins, has not been resolved. Bordner and Nelson's (14) proposal that o-diphenols activate the phenolase complex to catalyse the oxidation of monophenols by reducing cupric-enzyme to cuprous enzyme, appears to satisfy the present data but conclusive evidence remains to be obtained.

B. <u>The melanocyte and mammalian phenolase complex</u>.

The reaction sequence occurring when tyrosine is oxidized to melanin precursors was first proposed by Raper (28), who studied the aerobic oxidation of tyrosine and dopa in the presence of phenolase complex (Figure 2) from the mealworm, <u>Tenebrio molitor</u> (11,29). Raper described the reaction as proceeding in three distinct steps:

Figure 2

Reaction sequence proposed by Raper (28) for the oxidation of tyrosine to melanin precursors. See text for discussion.


COOH
HO-
NH2
+O
ENZYME
SLOW
HO-
HO-
COOH
NH2
I
II
II
ENZYME
+O
"HALLOCHROME"
OR REDUCING
AGENTS
O=
O=
COOH
NH2
III
III
HO-
HO-
COOH
N-H
IV
IV
O=
O=
COOH
N-H
V
V
HO-
HO-
N-H
VI
H2SO3
HO-
HO-
COOH
N-H
VII

1) Tyrosine → red substance (oxidative, enzymic)

2) red substance → colorless substance (non-oxidative, non-enzymic)

3) colorless substance → melanin (oxidative, non-enzymic)

VI and VII (Figure 2) were isolated from the "colorless substance" under neutral conditions and in the presence of acid, respectively, as methoxy derivatives (29). II was isolated from the "colorless substance" (11). The reaction II→III may be reversed by IV (30),(which would be simultaneously oxidized to V) or by other reducing agents such as ascorbic acid (31,32). The presence of V was spectrophotometrically confirmed by Mason (33). The polymerization to melanin has recently been reviewed by Mason (34,35).

The melanocyte (36) is a melanin-producing dendritic cell (37) from the neural crest (38-40) of vertebrate embryos and, in adult mammals, located adjacent to the basal cell layer of the skin (37), in the hair bulbs (41-43), the leptomeninges (44), the uveal tract (45), and the retinal pigment layer of the eye (46). Malignant tumors of this cell are called melanomas. The cytoplasm of the cells contains (and apparently produces) (47,48) granules pigmented with a brown or black chemically heterogeneous polymer (49-51), melanin. The melanin granules stain similarly to mitochondria (52), but are structurally dissimilar (47, 53, 54). The granules appear to develop in specific pigment-formation centers (48) from cytoplasmic vesicles by the deposition of concentric layers of melanin (47). Melanin granules and melanocytes have been studied extensively by means of the electron microscope (47,53-57). The

ergastoplasm and mitochondria (47,57) appear to be similar to those of other cells. An active phenolase complex has been demonstrated in melanocytes from thorium X (37), or ultraviolet (58) irradiated adult human skin, mammalian (41-43) and avian hair bulbs, embryonic mammalian and avian retinas and uveal tracts and embrionic chicken epidermis (46). (All of these studies, except that using thorium X, have involved the use of Fitzpatrick and Kukita's autoradiographic histochemical technique with tyrosine-2-C^{14} (41,42), as well as other techniques in the case of irradiated adult human skin and embryonic chicken retinas.) The effects of copper chelating agents and added metal salts on vertebrate melanoma suspensions have been interpreted as supporting the hypothesis that vertebrate phenolase complex is copper dependent (7,8,59). Melanin granule suspensions have been reported to contain, in addition to phenolase complex, cytochrome c, cytochrome oxidase, and succinic oxidase (60-62). However, the probable contamination of the granule suspensions with numerous mitochondria (cf.47) and methods of granule preparation) renders this interpretation uncertain. Rigorous studies of the enzyme distribution among the cell particulates of the melanocyte have not been made. In an early work, Hogeboom and Adams (63) demonstrated the presence of the phenolase complex in crude Harding-Passey transplantable mouse melanoma preparations, and reported partial separation of monophenolase from diphenolase activities by means of fractional ammonium sulfate precipitation. However, Lerner, et al. (64) were unable to show any separation of monophenolase from diphenolase activity by Hogeboom and Adams' ammonium sulfate precipitation method, by fractional ethanol precipitation, or by differential centrifugation. During the

fractionation procedures, the induction period found with tyrosine oxidation progressively increased, but could be shortened in a linear manner according to the logarithm of added dopa concentration. The induction period is minimal at about pH 6.8. It is increased by increasing tyrosine concentration. The pH optimum for dopa oxidation is within the range of 6.5-7.5 (64,65). The optimal dopa concentration is about 0.006 M (65). High salt concentration inhibits the enzymic oxidation of dopa (65).

III. STATEMENT OF THESIS

The demonstration of the relative specificity of mammalian phenolase complex suggested the study of optical specificity as a means of gaining information as to whether the complex consists of a single active site on a single enzyme which catalyses the oxidation of both mono- and dihydroxyphenols, or whether these activities reside in separate active centers. Since it has been shown that ortho-diphenols activate the enzymatic o-hydroxylation of monophenols, the activation specificity may be assumed to be the specificity toward o-diphenols of the site which catalyses the oxidation of monophenols. The specificity for the oxidation of o-diphenols (in the absence of monophenols) may be assumed to be the specificity of the site which catalyses the oxidation of o-diphenols. If the specificities are considerably different, separate active sites are most likely involved; but if the specificities are similar, the probability of a single active site which catalyses the oxidation of both mono- and o-dihydroxyphenols will be indicated.

IV. METHODS

A. Preparation of melanin granules.

Suspensions of melanin granules were prepared from Harding-Passey mouse melanomas by the method described by Hogeboom for the preparation of liver mitochondria (68). Balb/C strain mice,* which had been transplanted with tumor material 25-54 days prior to use, were killed by neck dislocation. The tumors (1.2-8.0 grams wet weight/tumor) were immediately dissected, stripped of connective tissue, and chilled in 0.25 M aqueous sucrose solution. The firm, black tumor material was blotted dry, weighed and minced, in a cold room maintained at ± 2°C.

Homogenization: 3 gram aliquots of minced tissue were suspended in cold 0.25 M sucrose (10 ml./gm. of tissue) and homogenized for two minutes in a Potter-Elvehjem homogenizer.** Homogenization was interrupted every half minute to permit rechilling of the brei in a salt water-ice bath.

Fractionation: 10 ml. of homogenate were carefully layered over 10 ml. of 0.34 M sucrose solution and centrifuged for 10 minutes at 700xG, accelerating and decelerating slowly to minimize mixing of the layers. With a little care, the layers remained distinct, with most of the granules remaining in the upper layer, a few in the lower layer, and the nuclear material and cellular debris forming a soft pellet.

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*Purchased from Roscoe B. Jackson Memorial Laboratories, Bar Harbor, Maine.

**Purchased from A. H. Thomas Co., Philadelphia, Pennsylvania.

The entire supernatant was pipetted off, care being taken not to include any pellet material. The pellets were discarded.

The combined supernatants were centrifuged 10 minutes at 5000 g and the resulting supernatant removed. In the initial preparations (1 and 2) this supernatant was removed by decanting, but in the later ones, it was pipetted off with a capillary pipette, care being taken to remove a minimum amount of sedimented material.

The pellet was redispersed by Potter-Elvehjem homogenization in 5 ml. of 0.25 M sucrose per gram of tissue, and centrifuged for 10 minutes at 24,000 g. The supernatant, including the poorly sedimented material, was decanted. The last washing and centrifugation was repeated once. The final pellet, which contained the greater portion of the melanin granules and essentially no nuclei or cellular debris (determined by microscopic examination), was redispersed as before, in a few ml. of 0.25 M sucrose, and stored in the refrigerator until used. Preparations #4 and 5 were in addition, stored at all times in an ice-water bath. All centrifugations were performed with a Servall model "SS1" angle-head centrifuge, or for the high speed centrifugations, with a Spinco model "L" centrifuge in a #40 rotor.

B. Reagents.

All reagents were prepared with distilled, deionized water.

a) Buffer. monopotassium phosphate, reagent grade, Baker and Adamson. Potassium hydroxide, C.P., Baker Analyzed. 0.10 M phosphate, titrated to pH 6.8 with concentrated potassium hydroxide.

b) D- or L-Tyrosine. Dissolved in phosphate buffer. Final concentration when present in reaction vessel = 1.84 M (1.00 mg./3.0 ml. of reaction fluid). Criteria of purity are described in Appendix I.

c) D- or L-Dopa. Dissolved in phosphate buffer. Final concentration when present in reaction vessel = 1.69 M (1.00 mg./3.0 ml.of reaction fluid) or 0.169 M (0.10 mg./3.0 ml. of reaction fluid). Criteria of purity are described in Appendix I.

C. Reaction Conditions.

Oxygen consumption was measured in a Warburg respirometer. The required substrates were carefully pipetted into the reaction vessels and the reaction mixtures brought up to volume with buffer. A filter paper wick in the center well of the reaction vessel was saturated with 0.2 ml. of 10% KOH. The total flask volume was about 25 or 30 ml. The bath temperature was maintained at 38.0°C. with an average deviation of ± 0.005° by means of a Sargent model "S" Thermonitor. Errors due to barometric pressure changes were minimized by reading the thermobarometer between each pair of reaction readings. Zero time was determined by tipping the melanin granule suspension into the main compartment from the side arm of the reaction vessel.

V. EXPERIMENTAL RESULTS

A. Activation studies.

The rates of oxygen consumption in the presence of D- or L-dopa, L-tyrosine, or L-tyrosine plus catalytic amounts of D- or L-dopa, catalysed by the phenolase complex activity of mammalian melanin granules, were measured simultaneously in separate reaction vessels. (All reaction vessels were run in duplicate, and average values are reported here.) L-dopa is oxidized about four times faster than D-dopa, by Harding-Passey melanin granules (twelve complete experiments from six granule preparations) (Figure 3), and L-dopa activates the enzymic oxidation of L-tyrosine to a greater extent than D-dopa (Figures 3 and 5). Thus a relative optical specificity for L-dopa is observed at the site of diphenolase activity and at the site of activation of monophenolase activity. Factors which affect the diphenolase activity, affect the rate of activation as indicated in Figure 4. Table II demonstrates the increase in diphenolase activity which occurs when granule preparations are stored at 0°C. Every change in diphenolase activity is reflected by a change in the rate of activation of monophenolase activity, as indicated in Figure 5. The rate of activation of enzymatic oxidation of L-tyrosine appears to be a function of the rate at which L-dopa may be enzymically oxidized. This suggests that the two functions are closely related.

Figure 3

Representative rates of oxygen consumption illustrating the relation of substrate optical specificity to activation optical specificity. Oxygen consumption was measured in the Warburg respirometer. Each vessel contained 0.18 ml of melanin granule suspension (Preparation 3(1), specific activity = 6.1 dopa units/mg N). Substrates: Curve 1, 1.00 mg L-dopa; curve 2, 1.00 mg D-dopa; curve 3, 1.00 mg L-tyrosine plus 0.10 mg L-dopa; curve 4, 1.00 mg L-tyrosine plus 0.10 mg D-dopa; curve 5, 1.00 mg L-tyrosine. Reaction conditions are described in "Methods."

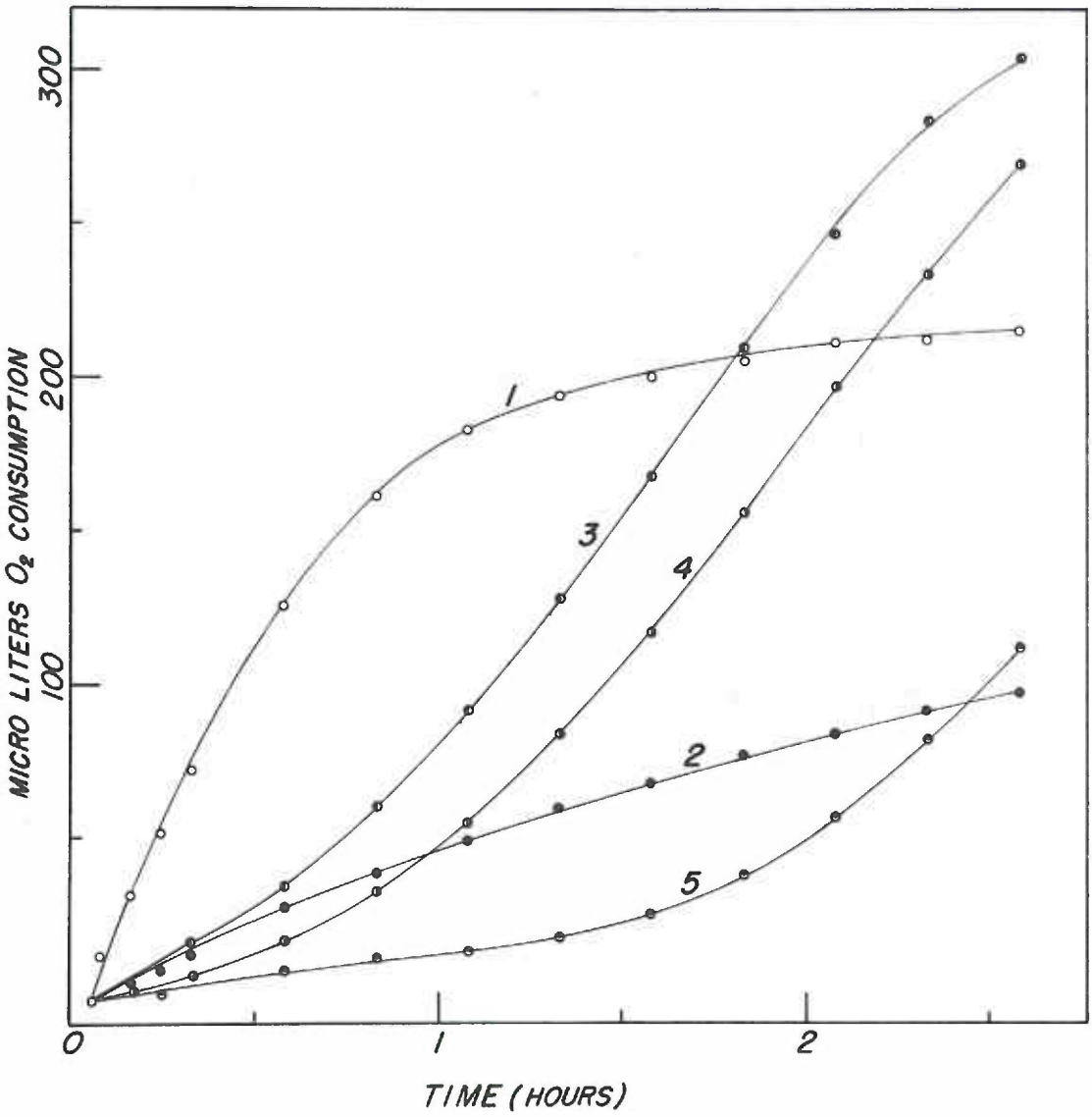

MICRO LITERS O_2 CONSUMPTION
300
200
100
0
1
2
TIME (HOURS)
1
2
3
4
5

FIGURE 3

Figure 4

Increase in enzymic activity of the melanin granules with storage at 0°C. All vessels contained 0.18 ml of melanin granule suspension (Preparation 4) which had been stored continuously at 0°C. Specific activities: curves 3, 5.9 dopa units/mg N; curves 1, 8.1 dopa units/mg N. Equilibration time = 19½ minutes. Zero time was 27 hours (curves 3, closed symbols) and 101 2/3 hours (curves 1, open symbols) after sacrificing the first animal for tumor material. Substrates: curves A, 1.00 mg L-dopa; curves B, 1.00 mg L-tyrosine plus 0.10 mg L-dopa; curves C, 1.00 mg L-tyrosine plus 0.10 mg D-dopa. Reaction conditions are described in "Methods."

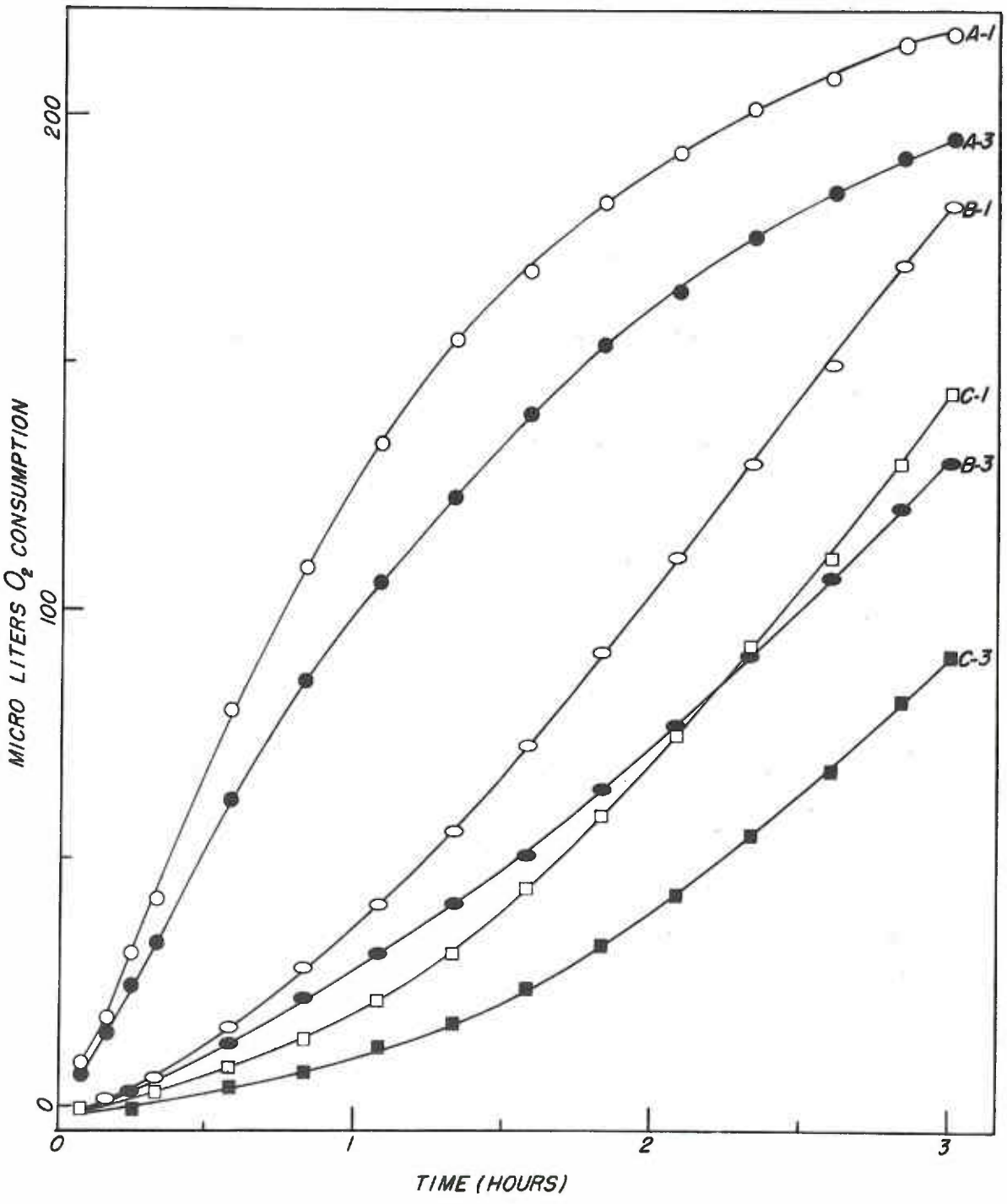

A-1
A-3
B-1
C-1
B-3
C-3
200
100
0
0
1
2
3
MICRO LITERS O_2 CONSUMPTION
TIME (HOURS)

FIGURE 4

Figure 5

Activation dependence upon diphenolase activity: summary of experiments similar to that described in Figure 3, except that various granule preparations were used. The logarithms of the rates of oxygen consumption during the initial 20 minutes of the reactions in the presence of 1.00 mg of L-dopa are plotted against the induction periods obtained in simultaneous experiments when the substrate was 1.00 mg of L-tyrosine, activated by 0.10 mg of D-dopa (open circles) or of L-dopa (closed circles). The reaction time elapsed before 62 microliters (1 gram atom) of oxygen were consumed, is taken as a measure of the induction period.

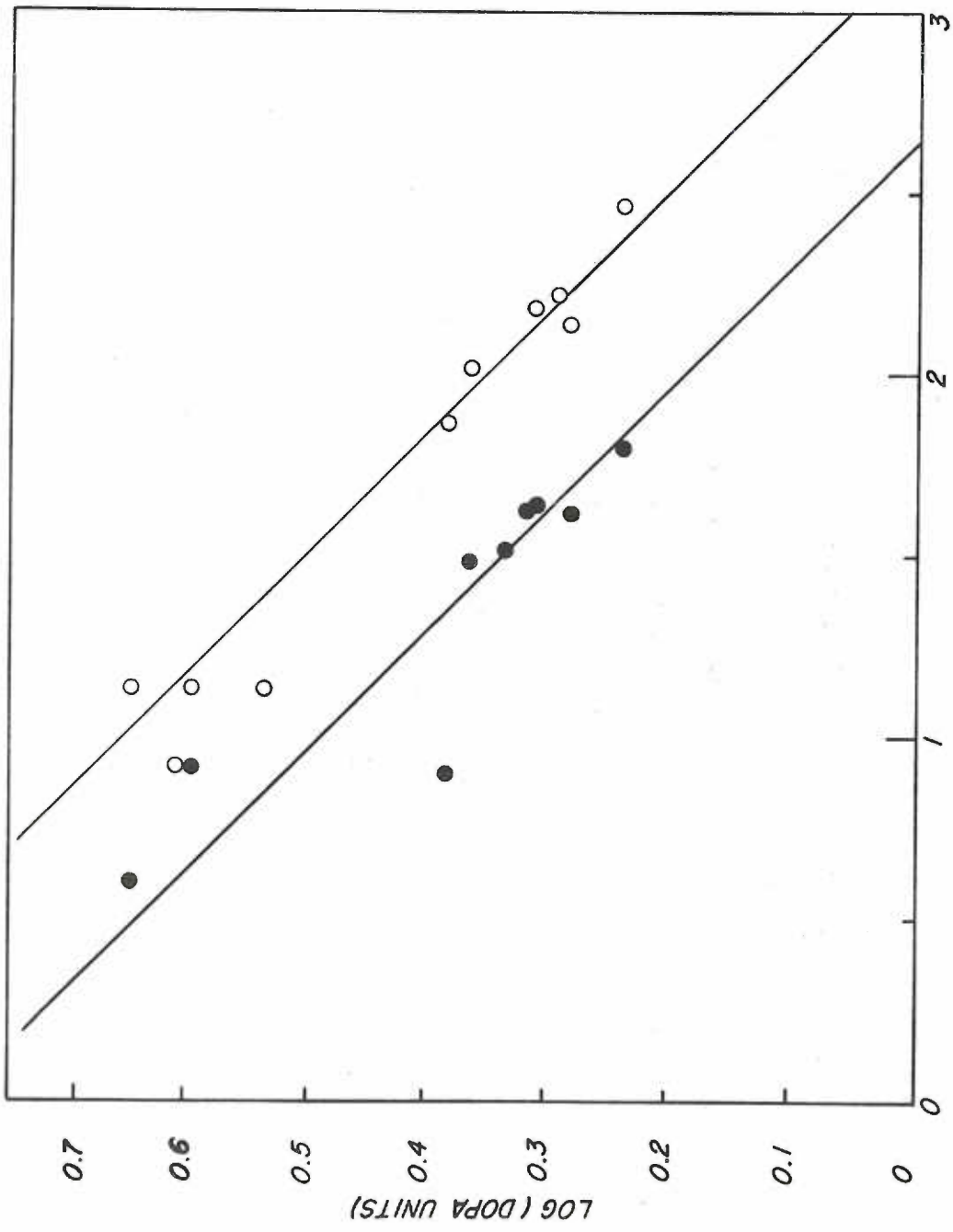

LOG (DOPA UNITS)
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0
1
2
3
TIME OF UPTAKE OF 62 MICRO LITERS (1 ATOM)
(HOURS)

FIGURE 5

TABLE II

Change in Enzymic Activity with Storage
at Zero Degrees, Centigrade

Hours after sacrifice	Diphenolase activity: ul O_2 consumed per minute	
	Preparation 4	Preparation 5
27	1.76	
27.3		2.07
35	1.94	
36.5		2.11
77.5		2.20
101.7	2.45	
101.9		2.35

Increase in diphenolase activity of 0.18 ml aliquots of melanin granule suspension during storage at 0°C. The time elapsed between sacrificing the first animal for tumor material and the "zero time" of each experiment is compared with the rate of oxygen consumed during the first twenty minutes of each experiment when 1.00 mg of L-dopa is the substrate.

B. Preparation of soluble phenolase complex from Harding-Passey melanin granules.

The increase in enzymic activity of the melanin granule suspension (Table II and Figure 4) during storage at 0°C. suggested that the granules may slowly become disrupted under these conditions with possible elution of the phenolase complex. Since solubilization of a protein is a prerequisite to purification, it was desireable to know whether mammalian phenolase complex could be solubilized by a relatively simple technique, such as sonic disintegration of the granules.

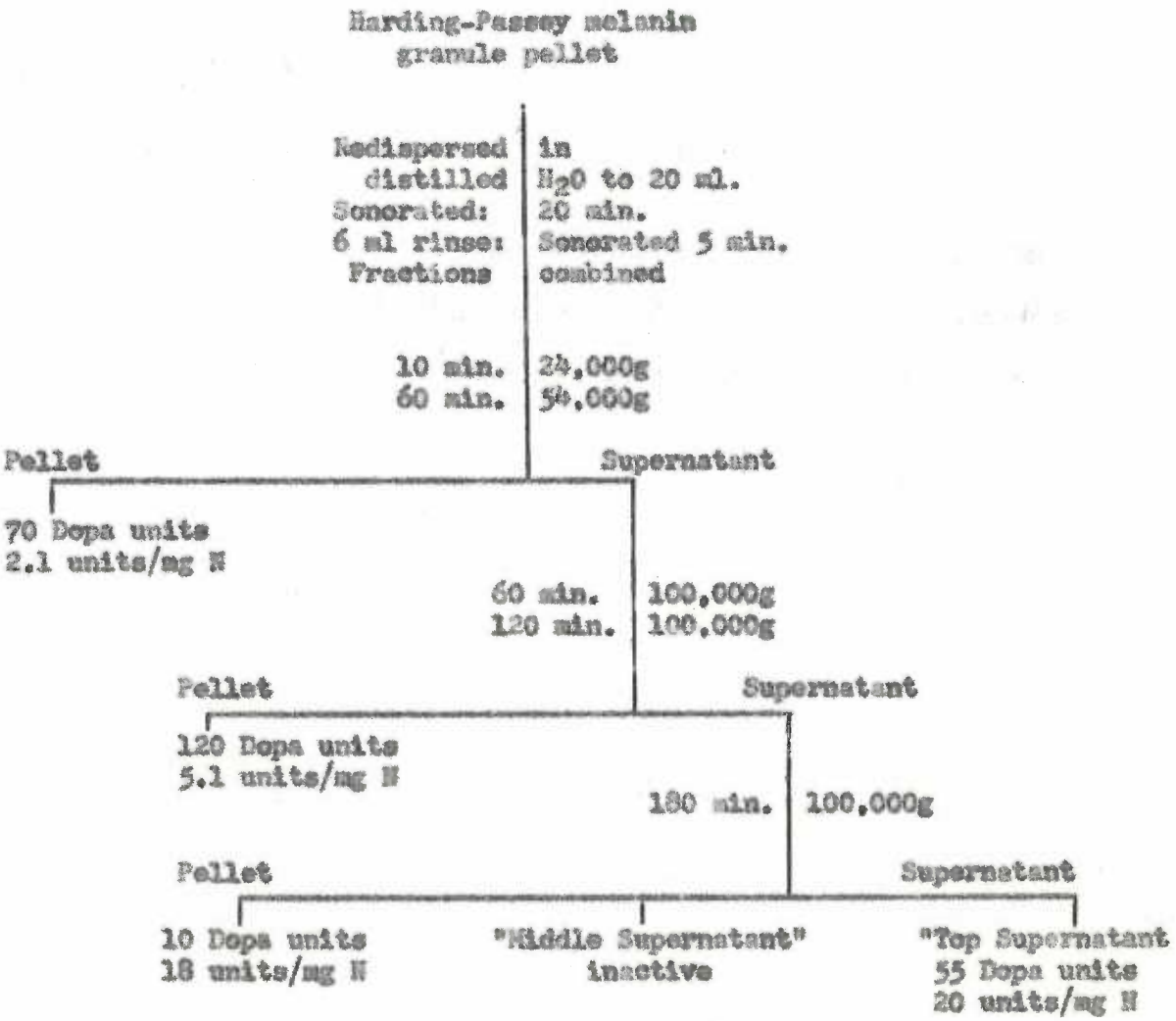
Harding-Passey melanin granules were prepared from 43.9 grams of tumor which had been stored for 2-3 weeks at -40°, as described in "Methods." The final melanin granule pellet was redispersed in sufficient distilled water to make a total volume of 20 ml. The centrifuge tubes were rinsed with 6 ml. of distilled water. The 20 ml. portion was sonorated for 20 minutes in a Raytheon 10kc, 200 watt magnetostriction oscillator. The resulting solution absorbed light intensely, and was optically clear when viewed with the light microscope. The 6 ml. portion was sonorated 5 minutes and added to the 20 ml. portion. The combined solutions were centrifuged as indicated in the flow sheet (Figure 6). The final supernatant was divided into three portions:

1. The upper half of the supernatant, designated "Top Supernatant," was carefully pipetted off with a minimum of mixing, and was a clear, light golden color, except for a small amount of lipid.

2. The "Middle Supernatant" had a color gradient fr m top to bottom of light yellow to reddish black, and was removed as above.

Figure 6

Flow sheet describing the preparation and assay of soluble mammalian phenolase complex. Centrifugation times and relative centrifugal forces are listed.


Harding-Passey melanin granule pellet
Redispersed in distilled
Sonorated: 20 min.
6 ml rinse: Sonorated 5 min.
Fractions combined
H_2O to 20 ml.
10 min. 24,000g
60 min. 54,000g
Pellet
Supernatant
70 Dopa units
2.1 units/mg N
60 min. 100,000g
120 min. 100,000g
Pellet
Supernatant
120 Dopa units
5.1 units/mg N
180 min. 100,000g
Pellet
Supernatant
10 Dopa units
18 units/mg N
"Middle Supernatant"
inactive
"Top Supernatant
55 Dopa units
20 units/mg N

3. The pellet was resuspended in the lower supernatant, which was very black. Specific activities are listed in the flow sheet as dopa units/mg N. One dopa unit is that amount of enzymic activity required to catalyse the consumption of one microliter of oxygen per minute (calculated for the initial 20 minutes) in the presence (initially) of 1.00 mg. of L-dopa in a total reaction volume of 3.0 ml. The maximum specific activity found was that for the "Top Supernatant" (20 dopa units/mg N), which is regarded by the author as being a soluble enzyme.

The optical substrate specificity of the "Top Supernatant" may be compared with that of a previous melanin granule preparation in Figure 7. The time scale for the "Top Supernatant" has been shortened to facilitate comparison. The substrates were D- or L-dopa or D- or L-tyrosine. The relative maximum rates of oxidation of the four substrates are similar for the melanin granules and the "Top Supernatant." It therefore appears that the enzymic activity which catalyses these oxidations is similar for the two preparations.

C. Isoelectric pH of the melanin granule.

Quinones combine readily with basic substances such as primary amino and sulfhydril groups (35,51). Since melanin is a quinonoid polymer, it appeared reasonable that proteins of the melanin granule would be bound to this quinonoid substance through the terminal amino groups. This would result in a more acid isoelectric pH. Melanin granules from the B-16 transplantable mouse melanoma were chosen for this study, since they appear to be more heavily melanized than Harding-Passey granules* and therefore might be expected to have a more acid isoelectric pH than the latter.

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*Footnote page 26.

Figure 7

Optical substrate specificity. Phenolase complex preparations: Open symbols (curves A) = melanin granule Preparation 6 (5.0 dopa units/reaction vessel); closed symbols (curves B) = "Top Supernatant" (3.2 dopa units/reaction vessel). Substrates: curves 1, 1.00 mg L-dopa; curves 2, 1.00 mg D-dopa; curves 5, 1.00 mg L-tyrosine; curves 6, 1.00 mg D-tyrosine. The time scale for the "Top Supernatant" is shortened by one half to facilitate comparison of the enzyme preparations. Reaction conditions are described in "Methods."

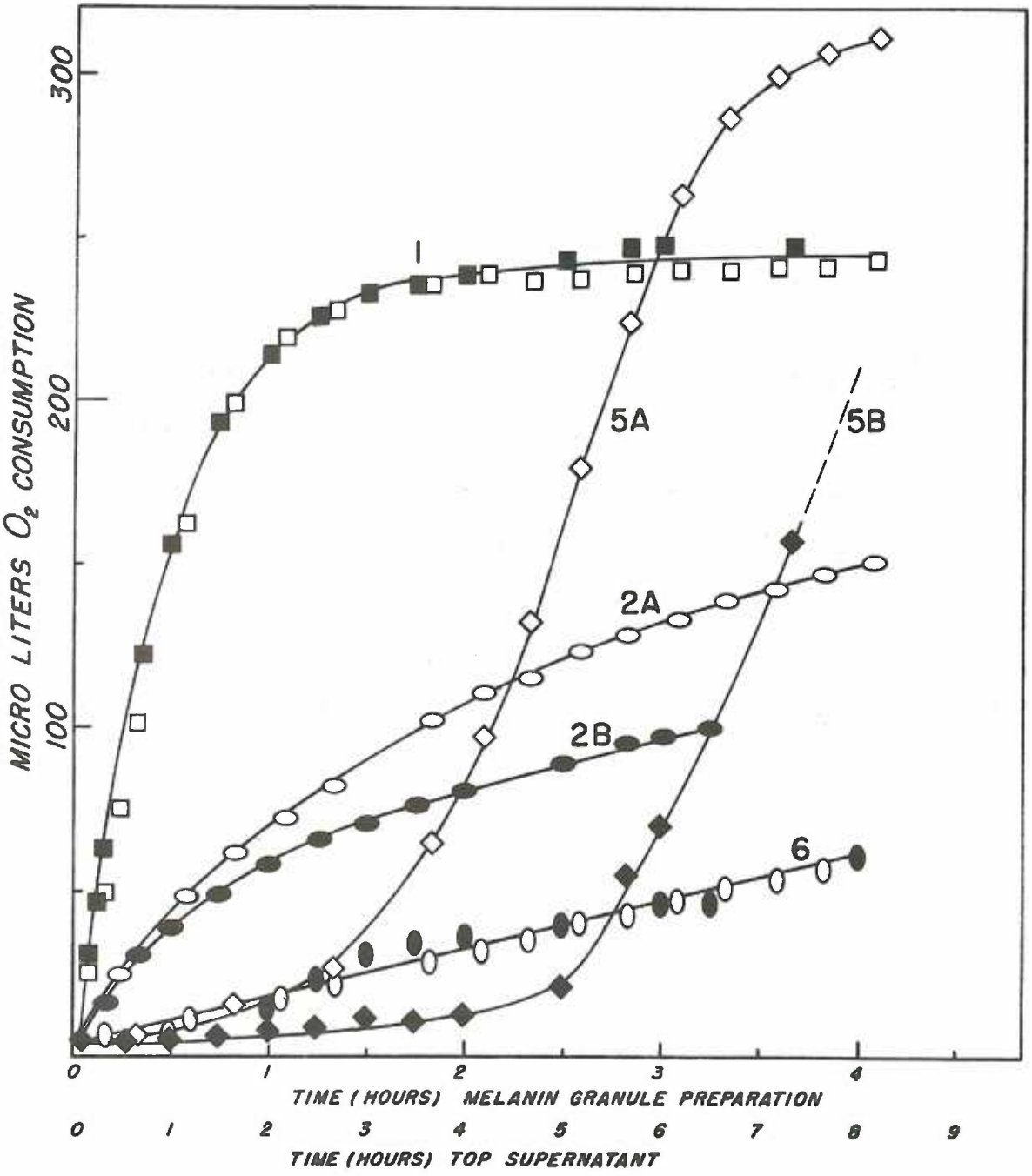
MICRO LITERS O_2 CONSUMPTION
300
200
100
1
5A
5B
2A
2B
6
0
1
2
3
4
TIME (HOURS) MELANIN GRANULE PREPARATION
0
1
2
3
4
5
6
7
8
9
TIME (HOURS) TOP SUPERNATANT

FIGURE 7

Melanin granules from the B-16 transplantable mouse melanoma were prepared in 0.25 M sucrose solution, as described previously for the Harding-Passey melanin granules. The microelectrophoresis cell used was that described by Abramson, et al. (69) (see Appendix II). It consisted of a flat quartz cell of rectangular cross section, with suitable inlet and outlet openings such that the cell could be flushed without disturbing the electrode compartments. The cell was used in conjunction with a microscope so the suspended melanin granules in the cell could be seen. A 25X ocular and a 21X dry objective lens were used for all operations. The direct current power supply was capable of producing 50 milliamperes at 1000 volts. The minimum power necessary to cause migration of the granules was ordinarily used. The polarity was frequently reversed to minimize polarization of the electrodes. Granule migration was observed at the surface of the cell, at the theoretical level of zero endosmosis**, and at the center of the cell, for all determinations.* The cell width was 17.0 mm., the cell depth, 0.438 mm., and a, 91.8 microns from the upper surface. Endosmotic effects were negligable at most pH

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*B-16 mouse melanoma tumors appear much blacker than Harding-Passey tumors, and have a lower specific phenolase complex activity (1-2 dopa units/mg N and 5-11 dopa units/mg N, respectively).

**Komagata equation: $a = \frac{1}{2} \pm \sqrt{\frac{1}{12} - \frac{32}{\Pi^5 k}}$ where a is the theoretical level of zero endosmosis, and k is the ratio of cell width/cell depth.

TABLE III

Electrophoretic Migration of B-16 Melanin Granules

pH	Migration toward positive or negative electrode at: Surface	"a"	Middle	Charge on granules:
4.01	+	+	+	-
3.63	+	+	+	-
3.60	+	+	+	-
3.60	+-	+-	+-	-+
3.59	+-	+-	-	-+
3.55	+-	+-	+-	-+
3.54	+-	+-	-(+)	-+
3.53	-(+)	-(+)	+(-)	-+
3.52	-(0,+)	-(+)	-(+)	+(-)
3.51	-(0,+)	-(+)	-(+)	+(-)
3.49	-(0,+)	-(+)	-(0,+)	+(-)
3.45	-	-(0,+)	-(+)	+(-)
3.44	-(0)	-(+)	-(+)	+(-)
3.33	-	-	-	+

Direction of electrophoretic migration of B-16 melanin granules at various hydrogen ion concentrations. Parenthetic symbols represent migration direction of minor component. See text for conditions.

values. The buffer system was 0.01 M potassium monochloracetate in 0.25 M aqueous sucrose solution, titrated to the desired pH with HCl. A drop of the melanin granule suspension was flushed into the cell with excess buffered sucrose. The direction of migration was determined, the granules were flushed out, and the pH of the suspension determined, with a Cambridge Model R pH meter. The results are listed in Table III.

The major component of the melanin granule suspension was isoelectric at the pH range of 3.53-3.60. The total isoelectric range was about pH 3.4-3.6. The suspended material agglutinated readily in the isoelectric range, but not in unbuffered sucrose solution nor in HCl-sucrose solution of pH 2.08 or 1.77.

VI. DISCUSSION

A. Mechanism of monophenol oxidation

It has been seen in the introduction that monophenol oxidation most likely occurs by means of direct enzymic catalysis, and that molecular oxygen is incorporated into the ortho-dihydric structure. Several possible mechanisms have been proposed, which depend on separate, separate but associated, or a single active center for the catalysis of mono- and dihydric phenol oxidation. Against the hypothesis of separate enzymes for the two activities is the fact that no enzyme has been found which will catalyse the oxidation of monophenols but not diphenols. The copper content of purified high cresolase preparations is proportional to both cresolase and catecholase activities (24), and the copper content of high catecholase preparations is proportional to both phenolase and catecholase

activities (25). Thus a single enzyme with monophenolase and diphenolase activities seems most probable. Kendal proposed that the phenolase complex consists of a single enzyme which has separate catalytic centers for the two activities. In attempting to explain the apparent requirement of diphenol oxidation concomitant with monophenol oxidation, Mallette (70) proposed that a ternary complex of enzyme, monophenol and diphenol be formed. Actually this scheme would require the formation of a quarternary complex, since oxygen is also involved. This system appears possible, but unlikely because of the increased time necessary to form a quarternary complex, and the necessity for resonance over a considerable portion of the protein backbone to permit activation of the monophenolase site. The mutual inhibition of monophenols and diphenols (see Table II) suggests that the protein configuration at the two active sites is similar or perhaps, identical. The data presented in this paper are entirely consistant with the hypothesis of a single active site which may catalyse the oxidation of either monophenols or diphenols. Mammalian melanin granules are relatively specific for the L-configuration, in the oxidation of dopa and in the activation by dopa of tyrosine oxidation. Factors which alter the rate of enzymic dopa oxidation alter the rate of activation (induction period) and the maximum rate of oxygen consumption in the presence of L-tyrosine. The rate of activation appears to be a function of the rate at which dopa may be enzymically oxidized.

If it is assumed that the phenolase complex consists of a single enzyme in which the catalytic centers of oxidation of mono- and o-dihydroxy phenols are identical, then, as suggested by Mason (9), a single step oxidation of monophenol to ortho-quinone appears most probable. It

would appear very unlikely that a molecule of monophenol would complex with an active center on the enzyme, dissociate as an ortho-diphenol, recomplex with the same active center, and finally dissociate as ortho-quinone. Mason (9) proposed the following sequence of events for the enzymic oxidation of monophenols:

1) Activation: Protein-Cu^{++}_2 + o-diphenol = Protein-Cu^{+}_2 + o-quinone + H_2O

2) O_2 complex formation: Protein-Cu^{+}_2 + O_2 = Protein-Cu_2-O_2

3) Monophenol oxidation: Protein-Cu_2-O_2 + Monophenol = Protein-Cu^{+}_2 + o-quinone + H_2O

Recently, Ingraham (71) presented kinetic studies of the phenolase complex, o-diphenol, and oxygen, which are consistent with the hypothesis that the enzyme combines with oxygen before it does with the hydrogen donor. Mason (9) proposed that the active center of the phenolase complex consists of two neighboring copper atoms which are attached to the protein, and may complex with an oxygen molecule in such a manner that one of the oxygen atoms is co-ordinated with both copper atoms. This proposed structure for the active center suggested a possible detailed mechanism for reaction 3 (see Figure 8). The following structural considerations are suggested:

1) The bond angles of the couprous copper are tetrahedral, similar to simple cuprous complexes (72). Two tetrahedral bonds of each copper atom are bound to basic groups on the protein. The remaining tetrahedral bonds may complex with oxygen, substrate, or water.

2) The data of Kubowitz (6) suggests that the enzyme may complex with carbon monoxide to form a protein-Cu_2CO structure analogous to formal-

Figure 8

Proposed detailed mechanism by which tyrosine may be directly oxidised to dopa quinone.

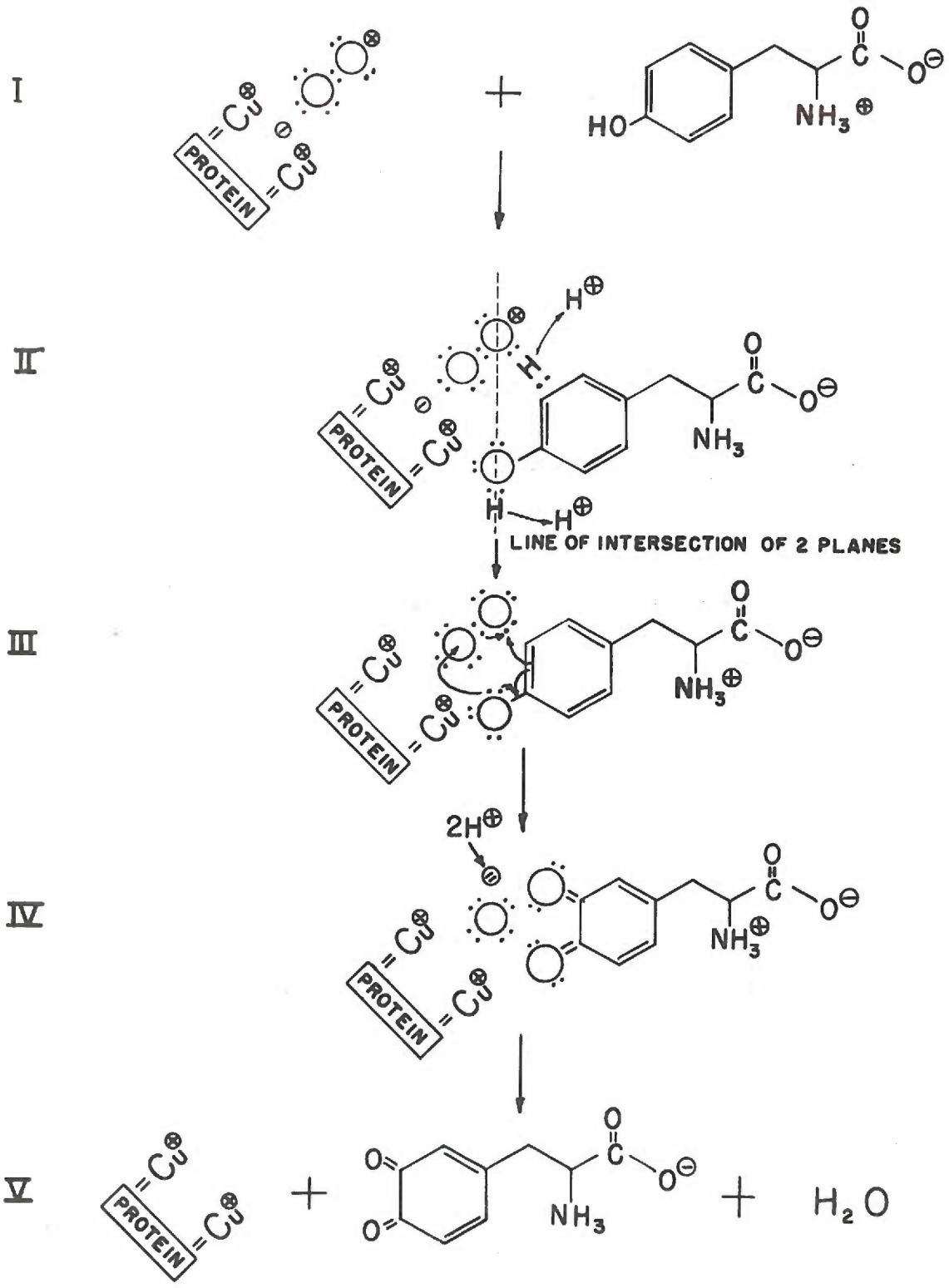

FIGURE 8

OXIDATION OF L-TYROSINE TO L-DOPA QUINONE

dehyde, in which the hydrogens are replaced by copper. Such a structure would be planar with respect to Cu_2CO, with the Cu-C-Cu bond angle greater than the H-C-H bond angle of formaldehyde ($118^\circ \pm 2^\circ$) (73) due to the relatively large size of copper. Since Kubowitz demonstrated that the enzyme-carbonyl complex formation is reversible in the presence of inert gases and that Cu is not displaced from the enzyme (6), it is assumed that the complex formation does not significantly alter the enzyme, and that the protein-Cu_2O_2 complex is structurally similar to the enzyme-carbonyl complex. Thus the Cu_2O_2 complex is assumed to be planar, and the Cu-O-Cu bond angle is assumed to be greater than 118°. If the Cu-O bonds are assumed to be equal, the Cu-O-O bond angle would be less than 121°.

3) The ortho-diphenol structure is planar, and intersects with the plane of the Cu_2O_2 complex at the line of centers of the ortho-oxygen atoms. The intersecting planes are required to satisfy the bond angle, Cu-O-C. The resulting C-O-O bond angle disparity would tend to dissociate the C-O bond and thus increase the velocity of the reaction. The assumed approximate bond angles are: C-O-Cu, 109° (water); O-Cu-O, 108° (tetrahedron); C-C-O, 120° (regular hexagon).

4) The intersecting plane configuration would permit the copper atoms and the alanyl side chain of tyrosine or dopa to complex with the same protein backbone.

5) Inhibitor and substrate specificity (66,74) suggest that L-tyrosine complexes with the enzyme at (at least) the following points: a) alpha-amino group, b) carboxyl group, c) p-hydroxy group. The binding of the alpha-amino and carboxyl groups is required for optical specificity. Inhibitor studies indicate the importance of the p-hydroxy group (74).

6) It is difficult to visualize how the adjacent cupric ions could be specifically reduced by dopa (equation 1), if dopa must complex with the enzyme only at a specified angle, unless the copper atoms are bound to the protein in such a manner that resonance is possible between them.

In presenting this detailed mechanism, it is hoped that it will be directly tested, and the understanding of the structure and action of the phenolase complex thereby furthered.

B. Relation of mammalian phenolase complex to the melanocyte.

The current views concerning the structure of the melanin granule and its relation to the melanocyte have been discussed in the literature review. The relation of the phenolase complex to the melanocyte is less clear. The phenolase complex appears to be rather loosely bound to a cytoplasmic particulate (61,64) (presumably to the melanin granule). It has recently been prepared, in soluble form, from melanin granule suspensions (independently) by Ward and Brown (75), and by the author. It appears likely that the increase in enzymic activity of granules stored at 0°C. (see Table II) was due to partial breakdown of the granules and elution of the enzyme. Robertson et al. (76) report phenolase activity in all of their centrifugally isolated cellular fractions from Harding-Passey mouse melanoma. Again, partial elution of the enzyme appears to be the most probable explanation.

The isoelectric pH of the B-16 melanin granule (3.4-3.6) is considerably lower than that reported for liver mitochondria (4.2-4.5) (77). This may be due to a relative absence of free amino groups on the surface of the granule, or to the acid properties of melanin.

VII. SUMMARY

1. The optical substrate and activation specificity of mammalian phenolase complex has been determined, and interpreted as favoring the hypothesis of a single type of catalytic site on a single enzyme, capable of catalysing the oxidation of both mono- and o-dihydric phenols.

2. A soluble mammalian phenolase complex has been prepared, and its optical substrate specificity determined.

3. The approximate isoelectric pH of the B-16 melanin granule has been determined.

4. The mechanism of enzymic oxidation of monophenols has been discussed, and an hypothesis presented.

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Appendix I

PURITY OF AMINO ACID SUBSTRATES

A. Amino acids used in experiments with melanin granule preparations 1-5.

L-Tyrosine. Purchased from Eastman Kodak Company Research Laboratory, Rochester, N. Y.

L-Dopa. Purchased from California Foundation for Biochemical Research, Los Angeles, Calif. CfP grade. Specific rotation = -12.2°, c = 1, in N HCl.

D- Dopa. Purchased from California Foundation for Biochemical Research, Los Angeles, Calif. Purified grade. Specific rotation = +11.7°.

B. Amino acids used in experiments with melanin granule preparation 6 and "Top Supernatant" Purchased from Mann Research Laboratories, New York, N. Y.

Tyrosine.

Mann Assay of Tyrosine L-isomer	D-isomer	Theoretical	Specific rotation in 1 N HCl	Isomer
C = 59.7%	59.7%	59.657%	-10.2° to -10.7°	L(-)
H = 6.10%	6.10%	6.120%	+10.2° to +10.7°	D(+)
N = 7.74%	7.74%	7.731%		

Dopa.

Mann Assay of Dopa L-isomer	D-isomer	Theoretical	Specific rotation in 4% HCl	Isomer
C = 54.8%	54.8%	54.820%	-12.7°	L(-)
H = 5.62%	5.62%	5.623%	+12.7°	D(+)
N = 7.10%	7.10%	7.104%		

Paper Chromatography of Mann Research Amino Acids.
Solvents: organic phase of Butanol:acetic acid:water (5:1:4).
Forty micrograms of amino acid applied per spot.

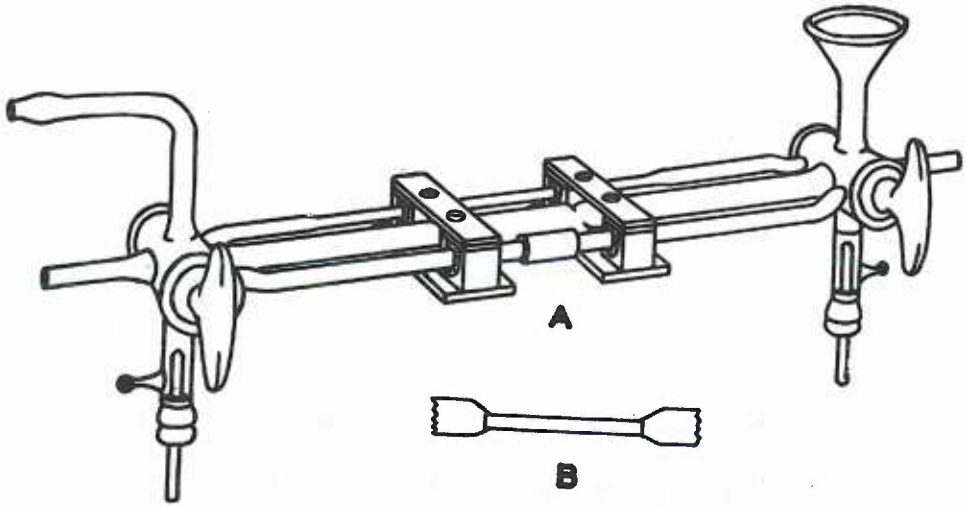
Amino Acid	R_f determined by means of: Ninhydrin	Ultra-violet light
L-Tyrosine	0.363	none
D-Tyrosine	0.356	none
L-Dopa	0.215	0.416? (very faint); 0.0608 (white fluorescent spot)
D-Dopa	0.299	0.408? (very faint); 0.0666 (white fluorescent spot)

The impurities found in D- and L-dopa appear to be similar in R_f, color of fluorescence, and quantity present. The amounts of impurity were assumed to be small, and the amino acids were used without further purification. No difference in experimental results due to the use of Mann Research Laboratories' amino acids, was observed.

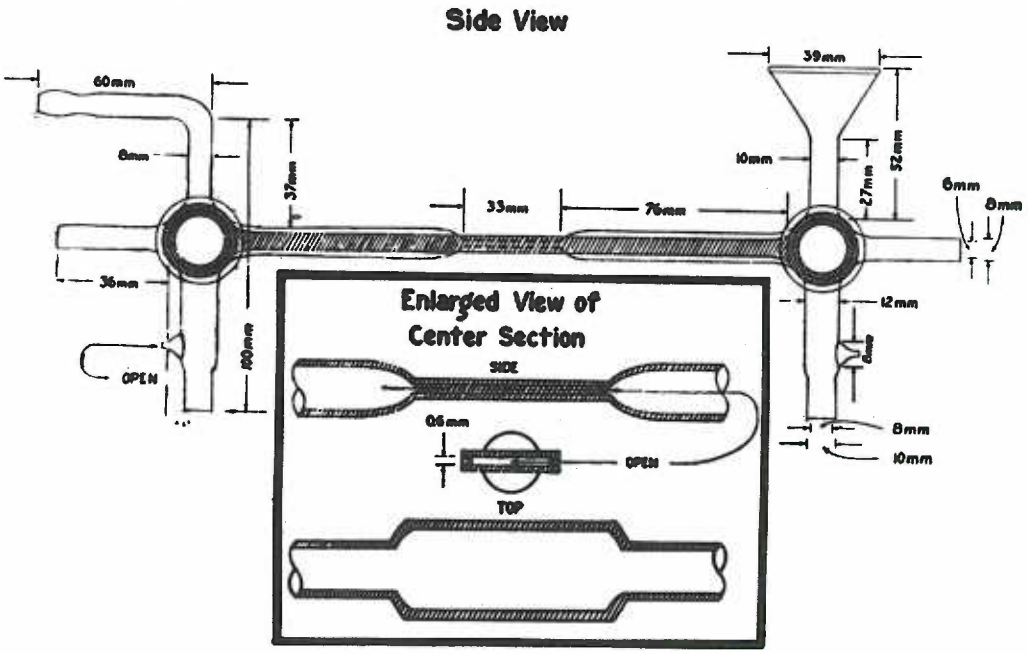
Appendix II

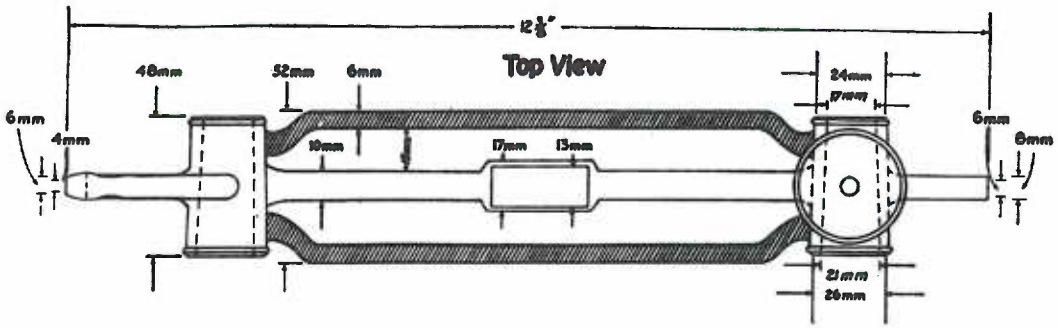
MICROELECTROPHORESIS CELL

Graphic description of the micro-electrophoresis cell which was used to determine the isoelectric pH range of B-16 mouse melanoma granules. Reporduced from Abramson et al. (69), p. 45.



The flat, horizontal electrophoresis cell made of one piece of glass and a supporting frame





Working drawing of the flat horizontal electrophoresis cell made of one piece of glass