A DIFFUSION STUDY
OF
DOPA MELANIN PIGMENT

by

CHARLES ROBISON FRIEDE

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INTRODUCTION

Pigment occurs in many groups of the animal kingdom, and its origin and composition have for years been the subject of much research. The name melanin, given to the black nitrogen containing pigment, has been very loosely used both in chemistry and biology. Practically all black, insoluble deposits found in tissues, or formed during biochemical operations have been called melanin. The term has also been applied to the artificial products obtained by the prolonged action of oxidizing agents, such as hydrogen peroxide on thiophene, benzene, and phenol (40). Many of the so-called melanins do not contain nitrogen and therefore bear no relationship to the tyrosinase melanin, containing about 8.5 percent of nitrogen. Melanin is produced from the amino acid tyrosine by the action of tyrosinase, an oxidizing enzyme found widely distributed in vegetable and animal tissues. The enzyme's specific substrate, tyrosine, is also found in these materials. The enzymatic reaction in vitro in the presence of atmospheric oxygen results in the formation of a black pigment, tyrosinase melanin. The enzyme's function seems to be to introduce a hydroxyl group into tyrosine, forming 1-3-4 dihydroxyphenylalanine, commonly called Dopa. A water solution of the latter substance upon standing in contact with air forms an intense black colored solution of Dopa melanin.

It has been fairly well established that the tyrosine--tyrosinase reaction is responsible for melanin pigment formation in nature. A majority of the investigators have studied the melanin extracted from natural sources such as hair and melanotic

tumors and have reported widely differing elementary composition. This is probably due to the extreme difficulty of purification. Tyrosinase melanin and particularly Dopa melanin should be of a high purity and a study of them should furnish valuable information about the natural melanin.

All of the melanins are characterized by their relative insolubility and therefore most of the work dealing with them has been limited to the determination of their elementary composition, and to spectrophotometric studies (6).

An attempt has been made to further the knowledge of the melanin pigments by determining the diffusion constant of a dopa melanin solution. This constant should be of value in clarifying the structure of melanin. Since the precursor of dopa melanin is known, the diffusion constant might give an indication of the degree of polymerization.

REVIEW OF THE LITERATURE

Melanin

Tyrosinase is an oxidizing enzyme discovered by Bourquelot and Bertrand (12) in 1896 in the fungus Russula nigricans. It is widely distributed in plant and animal tissues and two excellent sources, according to Chodat and Staub (15), are the meal worm, Tenebrio molitor, and potato peelings.

Pugh (42) reported that tyrosinase consisted of only one enzyme while Richter (50) claimed that it consisted of two. The view of the former was upheld by Graubard and Nelson (23) who showed that there is no evidence of a second enzyme in the tyrosinase preparations although it does have activity toward both monohydric (p-cresol) and dihydric (catchecol) substances. Aberhalden (1) also confirmed the work of Pugh, failing to find a carboxylase in tyrosinase as claimed by Richter.

Dalton and Nelson (19) reported in 1938 that they had succeeded in preparing a crystalline copper-protein that possessed tyrosinase activity. The complete preparation of the enzyme in its pure crystalline form however has apparently not been reported.

Bertrand (9), in 1896, found that this enzyme oxidized the amino acid tyrosine to a black pigment and, as the enzyme occurred often with tyrosine, offered this as the explanation of the darkening of the cut surfaces of many plants and vegetables.

Block (10) found that the product of the oxidation of tyrosine by animal tyrosinase was related to certain of the melanins of animal tissues and held that the tyrosine-tyrosinase

reaction is responsible for the production of many normal pigments. Corroborating this hypothesis, was the discovery by Meirowsky (33) of Tyrosinase in the ink-sacs of the squid, which eject an inky fluid containing melanin.

When the enzyme acts upon tyrosine in the presence of air or oxygen a series of color changes (yellow to red to brown to black) is observed before finally the black insoluble melanin separates from the solution. Chodat and Schweizer (14) suggested that an essential step in the action of tyrosinase was a deaminising process. In support of this Folpmers (21) isolated benzaldehyde from the products of the action of tyrosinase on phenylaminoacetic acid. Happold and Raper (25), however, were unable to detect any benzaldehyde from the reaction of tyrosinase on glycine, alanine or phenylaminoacetic acid, unless p-cresol was present along with the amino acid. Robinson and McCance (51) confirmed this in the same year. Chodat and Schweitzer had used the p-cresol supposedly to prevent the reaction between the ammonia and the aldehyde that were formed. Happold and Raper concluded that the deaminization in the presence of p-cresol is not due to direct action of the enzyme, but is caused by the preliminary oxidation of the p-cresol to an orthoquinnone derivative which then attacks the amino group. Von Szent-Györgyi (55) reached the same conclusion independently.

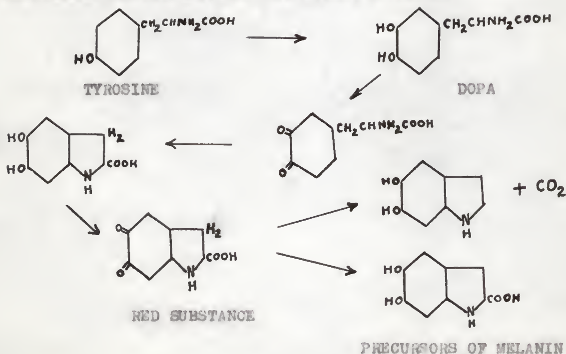
Chodat and Wyss (16) were among the first to call attention to the importance of the hydrogen ion concentration of the reaction. Raper and Wormall (49) gave the pH range of 5 to 10 as the limits in which the enzyme would act on tyrosine and at pH 6.0 tyrosine was quickly changed to the red form while the fur-

ther darkening in color was slow. At pH 8.0 the change from the red form to the black was accelerated.

Raper (45) removed the red form with colloidal ferric hydroxide and found that it lost its color on boiling. The colorless product thus obtained readily underwent oxidation when exposed to air, eventually forming melanin. He therefore concluded that the oxidation of tyrosine to melanin took place in three stages. The enzyme was necessary for the first step only as the second and third took place even after it was destroyed by boiling.

Raper (44)(46) also succeeded in isolating and identifying dopa, 5-6 dihydroxyindole-2-carboxylic acid and 5-6 dihydroxyindole as intermediates in melanin formation. Oxford and Raper (38) prepared by synthetic chemical means the two indole derivatives and found them to be identical with those isolated from the enzymatic reaction.

With this information Raper (45) postulated the following mechanism for the tyrosine-tyrosinase reaction:



While Raper believed that the presence of the enzyme was necessary until the red substance had been formed, Prizbram (41) claimed that very dilute solutions of dopa on exposure to air blackened due to melanin formation. Arnow (4) in fact gave as the preparation of dopa melanin the aerobic oxidation of a water solution of dopa. Raper and Duliere (47) showed that melanin was formed from dopa by the action of silver oxide, a mild oxidizing agent.

Block (10) believed that another enzyme which he called dopa oxidase was necessary for the conversion of dopa to melanin. Block and Schaaf (11) have described the preparation of dopa oxidase. More recently, however, Schmalfuss and Schmalfuss (52) seriously questioned the specificity of dopa oxidase and believe that tyrosinase and laccase are closely related to it.

Sunlight is known to increase the amount of skin pigmentation (freckles and suntan) and this has been explained by Arnow (3) as the result of the ultraviolet light. In the presence of oxygen, tyrosine is converted by ultra violet light to dopa which then forms melanin. Spiegel-Adolf (53) claimed that tyrosine, tryptophane and phenylalanine were all changed to melanin like compounds upon irradiation with ultraviolet light. Recently a patent was issued to Calame (13) for a product which he claimed to be formulated on the basis of these facts so as to hasten the formation of a natural suntan.

The composition of melanin prepared from many different sources has been recorded in the literature. Heinlein (28) gave 7.62 percent nitrogen for tyrosinase melanin, Block and Schaaf (11) reported 8.17 percent, while Raper and Wormall (49) found

8.65 percent nitrogen for the same material by the Dumas method and 8.40 percent by the Kjeldahl method.

The remainder of the analyses (11)(28)(39)(57)(58) were made on melanins from hair, tumors etc. and values given ranged from 6.83 percent to 13.71 percent nitrogen. All of these later melanins contained sulfur in varying amount.

Several investigators, but mainly Waelsch (57) believe that sulfur is not an essential constituent of melanin and that it is probably held in the molecule by physical forces rather than chemical. This is reasonable and offers some explanation of the varied nitrogen contents reported for natural melanins.

Melanin has been considered by all of the investigators to be an amorphous substance, but recently Spiegel-Adolph and Henny (54) have attempted to distinguish between different melanins by the use of X-ray diffraction patterns. The results were not all that could be hoped for and they plan further work with this technique.

Several theories have been given for the formation of melanin from tyrosine. Nobutani (35) held that the pigment is formed from the reaction between the amino acid and the ortho quinnone that is formed. Angeli (2) maintained that the tyrosine splits to form a pyrrole derivative which then is oxidized to give melanin. The analysis of melanin apparently does not support this mechanism. Block and Schaaf (11) postulated a mechanism in which melanin is represented as the condensation product of one molecule of the 3-4 quinnone of phenylacetaldehyde with two molecules of the 3-4 quinnone of phenylacetaldehyde. Raper (44) believed that melanin is formed by the polymerization of many of the in-

dole nuclei which he has shown to be the precursors of melanin.

Diffusion

Since the first studies on diffusion, made by Graham (22) in 1850, much data have been collected and numerous theories have been brought forth to explain this phenomenon. Northrop and Anson (36) calculated a radius and a molecular weight of particles from the diffusion constants of substances in solution, by the use of the Stoke-Einstein equation assuming a spherical molecule. In many cases this is the only practical means of determining the molecular size or weight, especially for substances of limited solubility. Unfortunately, little use of this has been possible in the past, due to the very low accuracy, many experimental difficulties, and, in the case of slow diffusing substances, excessive time is required.

With the introduction of the sintered glass diffusion cell by Northrop and Anson (36) many of the experimental difficulties were removed. Concerning this technique McBain and Dawson (30) said: "The method of employing constant diffusion columns confined within a porous membrane of sintered glass between two homogeneous bodies of liquid affords one of the simplest, quickest, and yet most precise means of studying diffusion in any field of science." Hartley and Runnicles (26) showed that diffusion takes place only within the diaphragm and a steady state is approximated within the disc. Northrop and Anson (36) depended upon the difference in density of the two solutions to provide ample stirring and later workers, notably Mehl and Schmidt (32), McBain and Lieu (31), and Cole and Gordon (18) have followed his tech-

nique. Hartley and Runnicles (26)(27) and Mouquin and Cathcart (34), however, did not consider this sufficient to prevent the building up of a more or less stagnant layer adjacent to the diaphragm surface so that the diffusion layer was slightly increased as the diffusion progressed. To prevent this, they used a slowly rotating cell with a glass bead on each side of the disc.

Later Runnicles and Hartley (26) found that the thickness of the "stagnant layer" was the same at all concentrations, which indicated that the rotation was not necessary. Mouquin and Cathcart (34) found that diffusion through a given diaphragm was some five percent more rapid when the apparatus was rotated. Hartley and Runnicles (27) believed this indicated that although the effective thickness of the stagnant layer was constant, it could not be considered negligible.

Before the cell can be used to determine the diffusion constant of a substance in solution it must first be calibrated with a liquid whose diffusion constant is known. McBain and Dawson (30) in their experiments used Cohen and Bruins' (17) data for 0.1N KCl for calibration purposes. Although their value is highly accurate, it is only applicable for the particular conditions under which it was obtained. These conditions were quite different from those found in the sintered glass diffusion cell. Using the latter apparatus Mehl and Schmidt (32) have given 1.46 cm^2/day as the value of the apparent or "integral" diffusion constant of 0.1N KCl at 25° C. This constant is related to the true diffusion constant and James, Hollingshead and Gordon (29) have shown that the ratio of the integral to the true constants for 0.1N KCl at 25° C. is 0.924. This gives 1.58 cm^2/day as the value of

the true diffusion constant as determined by Mehl and Schmidt which is the same value as obtained by Gordon (24). This value is apparently the best that is obtainable for the true diffusion constant of 0.1N KCl at 25° C. in the sintered glass membrane.

EXPERIMENTAL PROCEDURE

Preparation of Tyrosinase

A solution of the enzyme was prepared by the method of Bechhold and Erbe (8). Clean potato peelings were moistened with some methyl alcohol and turned through a meat grinder. The mash obtained was pressed through a cloth and it yielded a chocolate brown juice. One volume of the juice was then poured into three volumes of methyl alcohol and the greyish precipitate that formed was filtered off. This precipitate was allowed to stand overnight in an equal volume of water and then filtered, the clear, brown solution serving as a tyrosinase preparation. The entire procedure was carried out in an ice box adjusted to about 5° C. in order to reduce the reaction of the enzyme with the tyrosine present in the potatoes.

Preparation of Melanins

Tyrosinase melanin was prepared by the reaction of the enzyme on a saturated solution of tyrosine. Approximately 50 cc of the enzyme preparation were added to 100 cc of the saturated tyrosine solution. To this were added 100 cc of distilled water and 10 cc of M/5 phosphate buffer, pH 6.8. On standing 12 hours a black solution was produced and the melanin was purified by

precipitating it with HCl, centrifuging, taking it up in NaOH, reprecipitating, etc. Finally it was dialyzed for a week, then evaporated and dried in an oven at 60° C.

The dopa melanin solution had been prepared by dissolving 1 g of 1-3-4 dihydroxyphenylalanine in a liter of distilled water and allowing it to stand for a year in a glass bottle covered with black paper. The black solution was centrifuged at 2,000 r. p. m. and filtered.

Description and Use of the Diffusion Apparatus

The sintered glass cells were made of pyrex glass and were obtained from the Corning Glass Company. They were filled with the 0.1N KCl solution by suction, care being taken to prevent any air bubbles from forming. The lower portion of the cell was a glass jar of suitable size, into which 100 cc of distilled water were pipetted. The upper half was supported by a one hole rubber stopper, which had been cut in half and the membrane placed just below the surface of the water in the lower cell. The stopper served as a support, a leveling device, and also reduced any evaporation of the water in the lower part of the cell.

The entire cell was placed in a thermostat which was kept at 25° C. ± .01. The cell was supported on a level platform that was suspended from the ceiling so as to prevent any vibration caused by the motor in the bath. At the end of the diffusion period the top half of the cell was removed from the solution, emptied by air pressure and washed thoroughly by alternately sucking in and forcing out distilled water. The cell was dried before making another determination with it. The solution in the

lower portion of the cell was placed in flasks and the amount of material that diffused determined.

The same procedure was followed for the dopa melanin diffusions, except that the undiffused solution in the upper half of the cell was saved and combined with that from other runs. When all of original solution had been used, the combined material from the upper part of the cells was again centrifuged, filtered and reused for further measurements. The concentration was of course redetermined. Before using, the cells were all tested for streaming by the method given by Dawson (20).

Physical Determinations and Chemical Analysis

The viscosity of the dopa melanin solutions was determined by the use of an Ostwald viscometer which was kept at 25° C. by immersing it in the thermostat. The density of the solutions was determined by a pycnometer also kept at 25° C. and the volume of the cells was determined by weighing the volume of water that they held. In all cases the value used represents an average of several determinations.

In order to use the Stoke-Einstein equation any diffusion potential across the membrane must be absent. The pH of the dopa melanin solution was measured with a glass electrode and found to be 7.1. This value is near enough to that of the distilled water to make any error due to electro effects negligible.

The amount of KCl that diffused in the standardization of the cells was determined by the Volhard method of analysis for chloride. Nitrobenzene was used to coagulate the AgCl formed with the excess $AgNO_3$ before back titrating with KCNS.

The amount of the dopa melanin that diffused was determined by the micro-Kjeldahl method. The samples were extremely hard to digest and required the use of H_2O_2 in some cases.

The concentrations of the original dopa melanin solutions were determined by evaporating 20 cc samples in platinum crucibles to dryness in an oven at 60° C. The nitrogen content of the solid was then determined by the micro-Kjeldahl method.

As it had previously been thought that dopa melanin solutions did not obey Beer's law a colorimetric method of analysis was not tried until most of the work was completed. It was found, however, that the dopa melanin solution used did obey Beer's law and this permitted a colorimetric analysis of the later diffusions.

Method of Calculation

The fundamental law of diffusion is Fick's law which is written,

$$\frac{ds}{dt} = -AD\left(\frac{dc}{dx}\right) \qquad (I)$$

where ds is the quantity of solute passing through a cross section of area, A, and of thickness, dx, in the time, dt, where dc is the change of the concentration over the distance, dx, and D is the diffusion constant (32). From this the diffusion constant of a solution is defined as the quantity of material that will diffuse across a plane of unit dimensions in unit time under a unit concentration gradient.

In integrating equation (I) the method of McBain and Lieu (31) as revised by Mehl and Schmidt (32) has been followed. In the cell the two solutions are separated by the porous glass

disc with the more dense solution above to keep the solutions stirred and uniform. Diffusion will therefore take place only within the sintered glass disk. Barnes (7) has justified the assumption that the concentration varies linearly within the disc, or that

$$\frac{dc}{dx} = \frac{c_0'' - c'}{x}$$

where c_0'' is the original concentration of the upper solution, c' the concentration of the lower solution at any time, and x is the thickness of the disc. The original concentration of the lower solution is zero in all the determinations and therefore the expression for the concentration gradient at any time is,

$$\frac{dc}{dx} = \frac{c_0'' - \left(1 + \frac{V'}{V''}\right) c'}{x}$$

where v' is the volume of the lower solution and v'' that of the upper. It is also true that

$$ds = -V' \, dc'.$$

Making the necessary substitutions in equation (I), and integrating the following equation was derived.

$$D_1 Kt = \frac{V''V'}{V'' + V'} \left\{ \ln c_0'' - \ln\left[c_0'' - \left(1 + \frac{V'}{V''}\right) c' \right] \right\} \quad \text{(II)}$$

D_1 represents a mean value over a range of concentrations and is the integral diffusion coefficient. K is a cell constant and is the mean effective area of the disc divided by the thickness, or A/x.

Mehl and Schmidt (32) have shown that the integral diffusion constant D_1 is related to the true diffusion constant D in the following way,

$$D = D_1 + c\left(\frac{dD_1}{dc}\right) \quad \text{(III)}$$

Thus it is possible to solve for D by determining D_1 over a

range of concentrations. This was not done, but the value 0.924 (29) for the ratio of D_1/D was used for 0.1N KCl at 25° C. Using $D = 1.58\ cm^2/day$, the best value obtainable, in the place of D_1 in equation (II) and substituting in the other values, the cell constants K were determined.

Knowing the value of K_1 and substituting the volumes and concentrations found into equation (II) it is possible to obtain D_1 for the dopa melanin solution. Since a wide range of concentrations was not available it was not possible to determine D for the dopa melanin by the use of equation (III).

The relation between the diffusion constant at infinite dilution and the radius of large, uncharged, spherical molecules is given by the Sutherland-Einstein equation

$$D_o = \frac{RT}{6\pi\eta rN} \qquad \text{(IV)}$$

where D_o is the diffusion constant at infinite dilution in cm^2 per second, R is the gas constant in ergs per degree mole, T is the absolute temperature, is the viscosity of the medium and N is Avogadro's number. The radius of the molecule r, in cm, was calculated from equation (IV).

It is then possible to use the relation

$$M = 4/3\,\pi\, r^3 g\, N \qquad \text{(V)}$$

to calculate an approximate molecular weight if the specific gravity in grams per cubic centimeter is known.

RESULTS

The Nitrogen Content of Dopa and Tyrosinase Melanins

The results of the nitrogen analyses are given in Table 1.

Table 1. The nitrogen content of various melanins.

Source of Melanin		Percent N
Dopa Solution	No. 1	7.38
		7.50
Dopa Solution	No. 2	7.41
		7.28
Dopa Solution	No. 3	7.29
		7.48
Diffused Dopa Solution	No. 3	7.01
		7.28
Tyrosinase		12.1
		11.7

The poor agreement in the values is probably caused by the severe digestion methods necessary. The average, 7.41 percent, is somewhat lower than the 8.40 percent reported by Raper and Wormall (49) and the 8.17 percent reported by Block and Schaaf (11). The high value for the Tyrosinase melanin is in accordance with the results of other investigators. No doubt this was caused by the fact that the tyrosinase preparation used contained foreign protein whose nitrogen contents are higher than that of the dopa melanin.

Values for the Cell Constants

The values obtained for the diffusion cell constants are shown in Table 2. The results were consistent for a given cell over the wide time ranges used and the average values were used

in the calculation of the diffusion constants. The value used for the D of 0.1N KCl at 25° C. was 1.58 cm^2/day.

Table 2. Determination of the cell constants.

Cell No.	Vol. (cc)	T (days)	C" (Normality)	C' (Normality)	K (cm)	Av. K(cm)
20	23.42	0.963	0.100	0.00589	4.52	
	"	1.487	"	0.00786	4.52	
	"	1.175	"	0.00536	4.44	
	"	1.812	"	0.00958	4.74	
	"	0.980	"	0.00577	4.66	
	"	0.373	"	0.00150	4.40	
						4.54
42	107.59	1.188	"	0.0203	14.82	
	"	1.280	"	0.0212	14.69	
	"	1.268	"	0.0205	14.78	
	"	1.025	"	0.0175	14.30	
	"	1.113	"	0.0190	14.69	
	"	1.815	"	0.0274	14.69	
	"	0.980	"	0.0180	14.43	
	"	0.159	"	0.00437	14.70	
						14.73
48	24.75	1.260	"	0.00646	4.32	
	"	1.330	"	0.00693	4.42	
	"	1.039	"	0.00535	4.26	
	"	1.100	"	0.00600	4.39	
	"	1.812	"	0.00904	4.54	
	"	0.977	"	0.00526	4.36	
	"	0.378	"	0.00236	4.42	
	"	0.249	"	0.00149	4.32	
						4.38

Values for the Diffusion Constants of Dopa Melanin

The integral diffusion constant D_1 for the dopa melanin solutions are given in Table 3. The values are of the same order, but are not in good agreement.

The last values for nitrogen for each cell in Table 3 were checked by the colorimeter and found to be slightly low. The values shown however are those obtained by the micro-Kjeldahl so as to keep the results consistent. The average value was used for

the calculation of the molecular weight.

Sample Calculation of the Molecular Weight

The calculation is based on the data taken from the top line of Tables 2 and 3, substituting into equation (II).

$$K = \frac{23.42 \times 100}{123.42 \times 0.963 \times 1.58} \left\{ \ln 0.1 - \ln \left[0.1 - \left(1 + \frac{100}{23.42} \right) .00589 \right] \right\}$$

$$= 12.49 \times .370 \quad \frac{cc \times cc}{cc \times days \times cm^2/day}$$

$$= 4.52 \text{ cm.}$$

$$D_1 = \frac{107.59 \times 100}{207.59 \times 2.87 \times 14.73} \left\{ \ln 6.25 - \ln \left[6.25 - \left(1 + \frac{100}{107.59} \right) 1.08 \right] \right\}$$

$$= 1.225 \times .406$$

$$= 0.498 \text{ cm}^2/\text{day.}$$

Use of the average value for D_1 for the dopa melanin solution in equation (IV) gives.

$$D_1 = \frac{8.3 \times 10^7 \times 298}{6 \times 3.1416 \times 6.06 \times 10^{23} \times .0089 \times r}$$

Table 3. Determination of the integral diffusion constant D_1 for the dopa melanin solution

Cell No.	Vol. (cc)	K (cm)	C" (mgN/100cc)	C' (mgN/100cc)	T (days)	D_1 (cm^2/day)
42	107.59	14.73	6.25	1.08	2.87	0.498
	"	"	6.25	1.81	4.97	0.628
	"	"	6.25	0.664	0.956	0.848
	"	"	5.26	0.410	1.57	0.364
	"	"	5.26	0.379	1.12	0.466
	"	"	5.26	0.798	2.09	0.578
	"	"	4.30	0.528	1.84	0.517
20	23.42	4.54	6.25	0.587	5.47	0.517
	"	"	6.25	0.393	3.38	0.498
	"	"	5.26	0.441	3.42	0.775
	"	"	5.26	0.314	3.07	0.510
	"	"	4.30	0.209	2.55	0.471
48	24.75	4.38	6.25	0.724	6.91	0.568
	"	"	6.25	1.02	10.87	0.687
	"	"	6.25	0.746	5.50	0.807
	"	"	6.25	0.608	3.85	0.791
	"	"	5.26	0.386	3.42	0.601
	"	"	5.26	0.302	3.06	0.496
					Average =	0.590

$$D_1 = \frac{2.43 \times 10^{-13}}{r} \quad \frac{\text{erg·deg·mole·cm}^3}{\text{erg·deg·mole·cm·sec}}$$

$$= \frac{2.43 \times 10^{-13}}{r} \quad \text{cm}^2/\text{sec}$$

$$= \frac{2.43 \times 10^{-13}}{r} \times 0.864 \times 10^5$$

$$= \frac{2.10 \times 10^{-8}}{r} \quad \text{cm}^2/\text{day}$$

$$= \frac{2.10 \times 10^{-8}}{0.590} = 3.66 \times 10^{-8} \text{ cm}$$

Substitution of r and the value for the specific gravity, g, into equation (V) gives an absurdly low value for the molecular weight.

$$M = 4/3 \times 3.1416 \times 0.9998 \times 49.0 \times 10^{-24} \times 6.06 \times 10^{23}$$

$$M = 125$$

The specific gravity value 0.9998 was an average of several determinations on each of the dopa melanin solutions.

Applicability of Beer's Law

If Beer's law holds for a solution, the ratio of the depths of the solutions in the two cups of the colorimeter at the match point will vary inversely as their concentrations or

$$\frac{D_1}{D_2} = \frac{C_2}{C_1}$$

$$\text{So, } D_1C_1 = D_2C_2$$

It can be seen from Table 4 that this was true for the particular dilutions used by comparing column three with column six. These dilutions were such that they were approximately the same concentration as that in the lower half of the diffusion cells at the end of the diffusion period.

Table 4. Colorimeter readings of dopa melanin solution Number 3 at various dilutions.

Left cup			Right cup		
Av. Depth	Conc.	D_1xC_1	Depth	Conc.	D_2xC_2
4.95	10	49.5	10	5	50
9.9	10	99	20	5	100
14.8	10	148	30	5	150
19.8	10	198	40	5	200
19.9	2.5	49.7	5.0	10	50
39.9	2.5	99	10	10	100
10.1	2.5	25.2	2.5	10	25
6.7	7.5	50.2	5.0	10	50
13.5	7.5	101	10	10	100
26.7	7.5	200	20	10	200
40.1	4.5	301	30	10	300

DISCUSSION

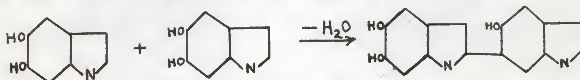
The excellent checks that were obtained for the individual cell constants indicate that this method of diffusion is probably limited in accuracy mainly by the analytical procedure used to determine the concentrations. The cells were recalibrated after they had been used for the determination of the diffusion constant of the dopa melanin solution and it was found that their calibration values had not changed. The last three determinations for each cell in Table 2 are recalibration values.

The values obtained for the diffusion constant of dopa melanin however, varied considerably, but they were all of the same order of magnitude. This range of values can undoubtedly be explained as being due to errors in the nitrogen determination, principally in the digestion stage. Errors here would lead to a loss of ammonia, giving correspondingly low values for the percent of nitrogen in the melanin and also for the amount that diffused. This latter amount is so small that a very slight loss of nitrogen would affect the value of D_1 appreciably.

An average value of the integral diffusion constant of the dopa melanin solution was used rather than the true diffusion constant at infinite dilution to determine the radius. The error caused by this method is certainly less than that introduced in the nitrogen determination by the Kjeldahl method. The value for the molecular weight is apparently meaningless. However, it does show that the assumption that the molecule was spherical was not justified. If the value of D_1 for the dopa melanin were decreased by a factor of ten, the calculated molecular weight

would be in the neighborhood of 50,000.

Raper (44) has shown that one of the precursors of melanin is 5-6 dihydroxyindole. It had been hoped when this problem was started that from the molecular weight determination it would be possible to determine the approximate number of these molecules that were condensed together. The alpha carbon of indole is extremely active and therefore the alpha carbon of 3-4 dihydroxyindole would also be expected to be active. A possible mechanism for the condensation is the splitting out the alpha hydrogen with one of the OH groups forming the dimer and eliminating water. This could continue to give a long chain molecule.



The results indicate that the molecule must be much longer than it is thick and such a mechanism might possibly be correct.

SUMMARY

1. Melanin from tyrosinase and from dopa have been prepared and their nitrogen content determined.

2. The sintered glass diffusion cells were calibrated and used to obtain the integral diffusion constant of the dopa melanin solution.

3. Apparently Beer's law holds for the concentrations of the dopa melanin solutions used and it appears that due to digestion difficulties the colorimetric method gives an easier and more accurate determination of the quantity of dopa that diffused than does the micro-Kjeldahl.

4. An attempt was made to relate the diffusion constant to molecular weight. The results seem to indicate that for the dopa melanin solution the diffusion constant is related to the shape of the molecule rather than to its molecular weight.

5. A possible mechanism for the formation of dopa melanin from its precursor, 5-6 dihydroxyindole has been suggested.

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