

PHYSICAL STUDIES ON MELANINS

II. X-RAY DIFFRACTION

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ABSTRACT A number of purified natural and synthetic melanins have been examined by X-ray diffraction. A consistent finding with all samples was the lack of structure in the diffraction pattern corresponding to any significant crystallinity in these melanin preparations. A diffuse ring, centered at a Bragg spacing of 3.4 Å was consistently found in samples of melanin from animal sources, and a similar ring at 4.2 Å in all melanins obtained from plants. Models for these two polymer types, based upon the current concept that they primarily involve indole and catechol monomeric units respectively, were then evaluated by a Monte Carlo method. From the comparison of the observed spacings with the calculated ones it was concluded that the 4.2 Å spacing in the catechol melanins is probably related to the average interaction between adjacent monomeric units, with mutually random orientations. The 3.4 Å spacing observed in indole melanins appears to derive from the tendency of indole monomers (probably of adjacent chains) tending to aggregate in near parallel stacks. Some randomness in the form of translations and rotations parallel to the planar groups is consistent with the diffraction patterns. An interesting finding was that the diffraction pattern of synthetic melanin prepared by the alkaline auto-oxidation of catechol gave the 3.4 Å spacing found in the indole melanins of natural origin.

INTRODUCTION

The melanins are a class of polymeric pigments widely distributed throughout the plant and animal kingdoms. Although much is known about the biosynthetic pathways leading to the formation of animal melanins, little is known about the chemical structure of these polymers. Because both analytical and synthetic chemical studies have yielded only partial answers to the problem of melanin structure, we have studied the applicability of physical methods in dealing with this complex material.

In a previous report (Blois et al., 1964) referred to herein as (I) the unusual paramagnetism of melanin was studied by means of electron spin resonance, and some deductions were made on the structure of melanin. It was believed that a thorough study of melanins by means of X-ray diffraction might yield further clues to the melanin problem. This is the report of such a study.

EXPERIMENTAL METHODS

Most of the X-ray diffraction data were recorded photographically using pinhole collimation and flat cassettes. In the case of some samples a closed system was employed and air scattering was eliminated by evacuating the system or filling it with helium. When large enough samples were available a record was also obtained with a Picker X-ray diffractometer (Picker X-Ray Corp., White Plains, N. Y.). K_{α} radiations from copper or molybdenum and occasionally chromium were used. The specimens were generally packed into a 2 mm hole drilled into a 1 mm thick aluminum block. A thin aluminum foil mounted on one side of the block helped prevent spilling of the sample, and provided a check on the film to specimen distances which ordinarily were 50 or 100 mm. Some of the samples were pressed into pellets $\frac{1}{8}$ - $\frac{1}{4}$ inch in diameter and about $\frac{1}{16}$ inch thick, using pressures of approximately 25,000 pounds per square inch. The photographs were scanned either by a Joyce (Joyce, Loebel and Co., Gateshead-on-Tyne II, England) or a Jarrel-Ash (Jarrel-Ash Co., Waltham, Mass.) recording microdensitometer using a magnification of 5:1. For translating film densities into relative intensities a series of graded exposures of a crystal reflection were recorded on a film and were scanned under the same conditions as the diffraction records.

The preparation and purification of melanin samples were similar to the procedures given in (I). A number of purified melanin samples from a variety of sources (chicken feathers, rabbit hair, *Ustilago myadis*, *Luffa cylindrica*, and *Ipomea purpurea*) were kindly provided by Professor R. A. Nicolaus. Squid melanins and mouse melanoma melanins were prepared in our laboratory using previously described procedures followed by room temperature treatment with 6N HCl for three weeks. Synthetic melanins were prepared by both auto-oxidation (under mildly alkaline conditions) of dihydroxyphenylalanine (DOPA) and by the oxidation of DOPA with mushroom tyrosinase (Worthington Biochemical Corporation, Freehold, N. J.). Approximately 200 diffraction records were obtained using some 100 samples of melanin. Thermal transition experiments were carried out with Dupont's Model 900 Differential Thermal Analyser (DTA) and Model 950 Thermo Gravimetric Analyser (TGA), Dupont Co. Instrument Products, Wilmington, Del.

EXPERIMENTAL RESULTS

The X-ray diffraction patterns of the melanins studied fall into three groups:

(a) *Animal Melanins* The main feature of the first group (Fig. 1 a) is the ring at a Bragg spacing of 3.4 Å. Squid melanin, mouse melanoma melanin, and melanin from chicken feathers and rabbit hair belong to this group; so also do the synthetic melanins prepared from catechol or DOPA (*L*, *D*, or the racemic mixture) by auto-oxidation or using mushroom tyrosinase. There was no change in the diffraction whether the crude sample of squid melanin had been extracted with hot or cold HCl.

(b) *Plant Melanins* The characteristic feature of the second group is the ring at a spacing of 4.2 Å. (Fig. 1 b). Melanin isolated from *Ustilago myadis*, *Luffa cylindrica*, and *Ipomea purpurea* seeds (Nicolaus et al., 1964) belong to this group. The last two samples were available only in minute quantities and their X-ray diffraction patterns were very weak.

(c) *Phaeomelanin* Fig. 1 c shows the diffraction pattern of a phaeomelanin sample obtained from red chicken feathers by Nicolaus. This has a halo at the same

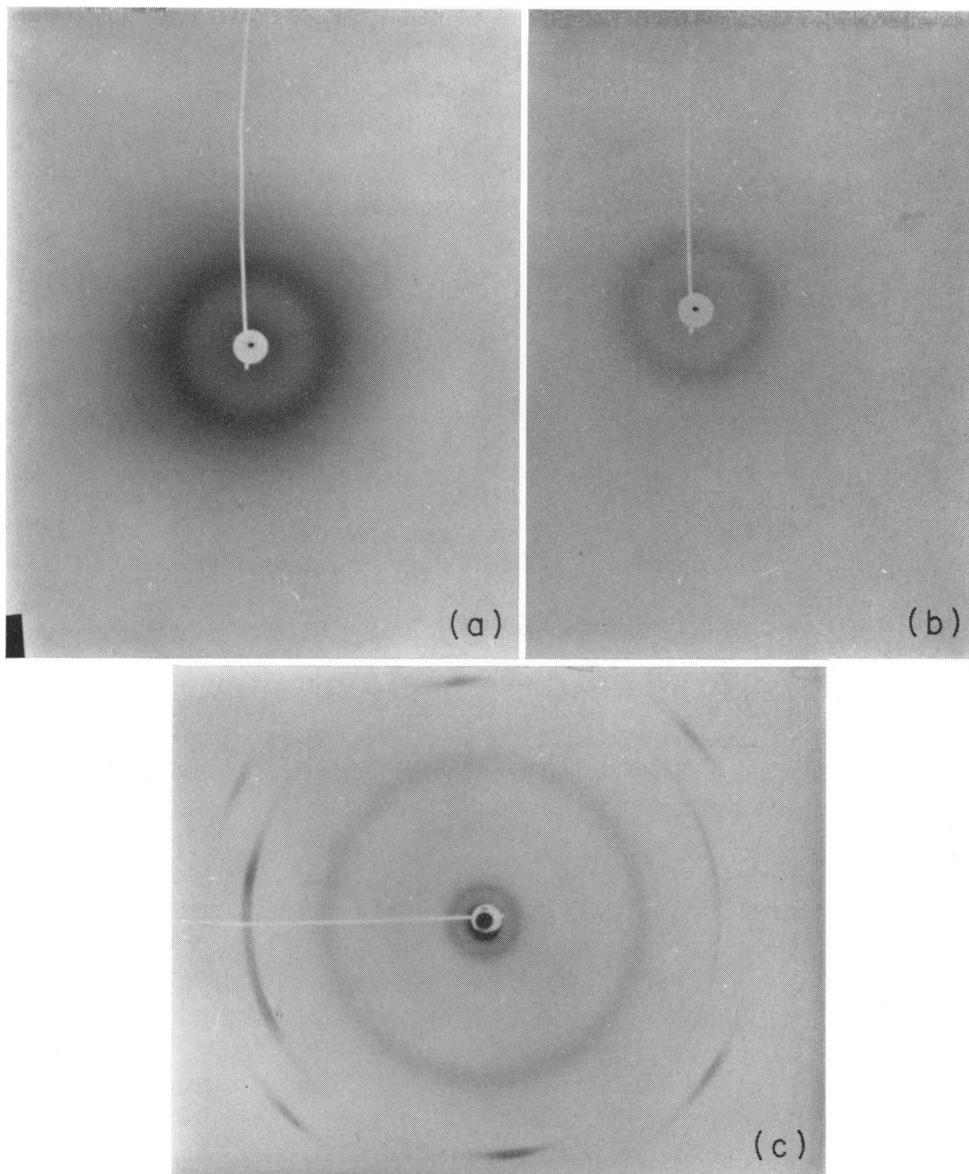


FIGURE 1 X-ray diffraction pattern of (a) squid melanin; (b) *Ustilago* melanin; and (c) phaeomelanin. Radiation used was Mo K_{α} with a film to specimen distance of 50 mm for (a) and (b) and 100 mm for (c). The rings showing preferred orientation in (c) are due to aluminum foil.

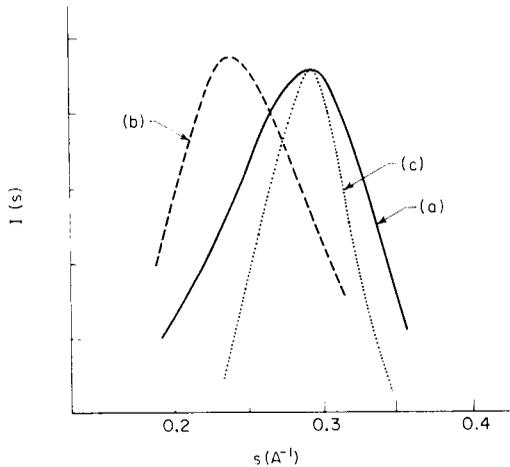


FIGURE 2 Densitometer traces of the diffraction patterns of (a) squid melanin; (b) *Ustilago* melanin; and (c) phaeo-melanin. They have been replotted with $s = 2 \sin \theta / \lambda$ (in \AA^{-1} units) as abscissa and ordinates proportional to the intensity.

spacing as the first group (3.4 \AA), and another prominent one at about 14 \AA . There are two rings between them at spacings of 5.9 \AA and 8.4 \AA . These are relatively weak and are not reproduced well in Fig. 1 c. All the rings are much sharper than those of the previous samples.

(d) *Diffractometer Records of Spacings 7–14 \AA* The diffractometer records of squid melanin and melanins from *L* and *DL* DOPA showed weak and diffuse, but definite, humps centered at Bragg spacing of 13 \AA . Catechol and *Ustilago* melanins showed less certain peaks at about 8 \AA . None of the photographic records revealed these spacings clearly. It is, however, difficult to reject them as spurious for the following reasons: the humps in the diffraction records appeared in the same place when the experiments were repeated a number of times using a freshly mounted sample each time, and blank runs showed no such humps. Furthermore, parasitic scattering from the collimator had interfered with the region from 8 \AA and above in the photographic diffraction records.

Further work using low angle X-ray scattering techniques will be necessary to determine the origin of these large spacings and they will not be further considered here.

Fig. 2 shows the above features of the three diffraction patterns discussed. The densitometric data have been replotted with the reciprocal of the Bragg spacing (s in \AA^{-1} units) as the abscissa. Only the portions of the curves close to the peaks of interest at 3.4 and 4.2 \AA are shown.

(e) *Binding of Aromatic Compounds to Melanins* Since melanin is known to have a strong affinity for certain aromatic and polycyclic compounds it was of interest to learn if the finding of the dyes produced structural changes. No change in the diffraction pattern was observed when melanins were treated with various known melanin binding compounds: methylene blue, acridine orange, chloroquine, iodoquine, and chlorpromazine. The retention of these substances in adsorbed form

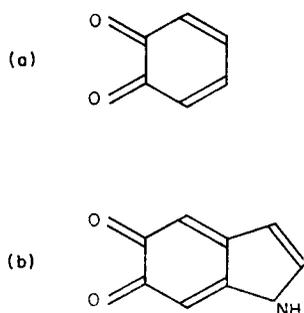


FIGURE 3 Monomeric types expected to be found in (a) catechol melanins and (b) indole melanins.

was insured by dialysis and either radioisotope counting or colorimetry. Evidently the binding of these substances did not involve structural modifications of the kind that would show up in the X-ray diffraction.

(f) *Effect of Heating* A number of melanin samples were heated in an argon atmosphere from room temperature up to 300° C and their X-ray diffraction patterns were recorded before and after the heating. There was no change in the patterns other than minor variations that could be accounted for by the uncertainties of measurement. However, all the samples except *Ustilago* melanin showed a thermal transition at about 130° C. This transition was irreversible and probably represents the removal of water present in the samples in some form.

COMPUTED DIFFRACTION CURVES

Monomer Types

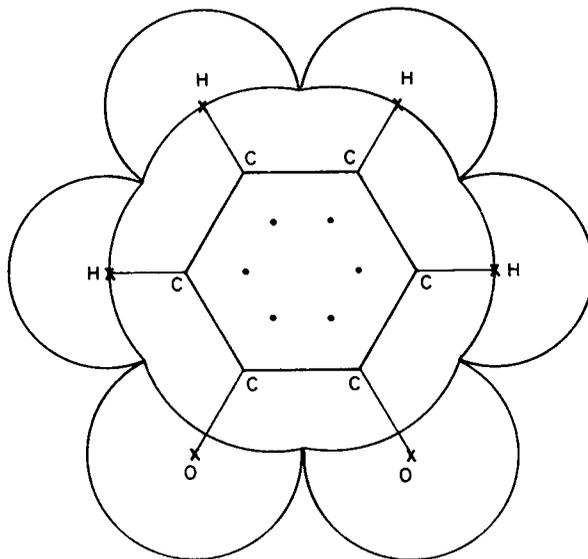
For the computation of the diffraction curves two types of monomers were assumed. They are shown in Fig. 3 and will be referred to as "a" and "b". Type "a" (catechol or *o*-benzoquinone) is expected to occur in *Ustilago* melanin and plant melanins generally, and would probably be expected in synthetic melanins derived from catechol. Type "b" (5-6-indolquinone) is expected to occur in the animal melanins we have studied, and in synthetic melanins derived from DOPA. The C—C and the C—N bonds in the ring structures were all assumed to be of equal length — 1.4 Å. Bond lengths of 1.54 Å for the C—C single bond, of 1.25 Å for the C=O bond and of 1.1 Å for the C—H bond were assumed. These bonds were assumed to be equally inclined to the other two bonds at their C ends. The shape of "a" used in the calculations is shown in Fig. 4. Van der Waals radii of 1.7 Å for carbon, 1.5 Å for oxygen, and 1.2 Å for hydrogen were assumed. These dimensions give "a" a length of 7.5 Å and a thickness of 3.4 Å, and "b" a length of 9 Å, a width of 7.5 Å, and a thickness of 3.4 Å.

Debye's Formula

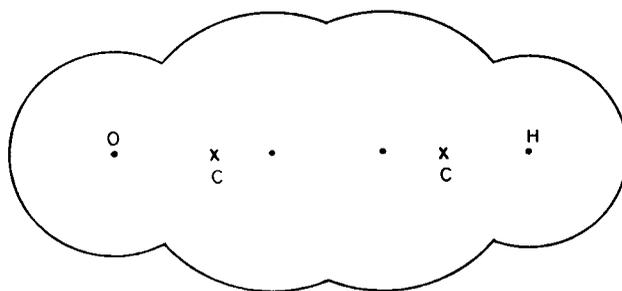
The basis for all the calculations is the Debye expression for the intensity of diffraction in spherically symmetric systems. The diffracting sample is considered to be a

collection of scattering units, the mutual orientations of adjacent units being independent of their separation. If the scattering angle is 2θ , a convenient parameter in the spherically symmetric diffraction space is $s = 2 \sin \theta / \lambda$ where λ is the wavelength of the X-rays used. If we associate a Bragg spacing of d (in A units) with the scattering angle of 2θ , s (expressed in A^{-1} units) is simply the reciprocal of d . The intensity

(a)



(b)



SCALE
0 1A 2A

FIGURE 4 (a) Top and (b) end views of the assumed shape of the monomeric unit in catechol melanin.

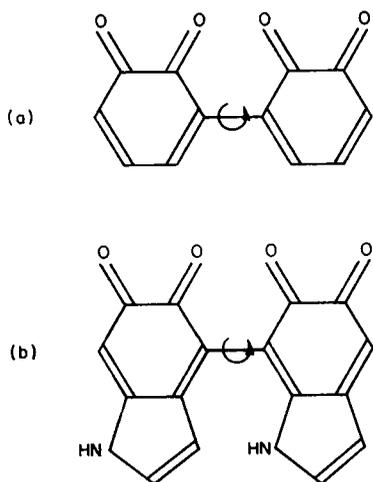


FIGURE 5 A possible dimer expected to occur in (a) catechol melanin and (b) indole melanin.

of diffraction at s is given by

$$I(s) = \sum_i f_i^2 + \sum_i \sum_{j \neq i} f_i f_j \frac{\sin 2\pi r_{ij} s}{2\pi r_{ij} s}$$

where f_i is the scattering factor of the i th atom and r_{ij} is the separation between any pair of atoms in the unit. The first term represents the total independent scattering by all the constituent atoms. The second is the interference term and the summation extends over every pair of atoms present. The derivation of the Debye formula and the conditions under which it is applicable have been described by a number of authors (e.g., Guinier, 1963). All the computations were made with the aid of the IBM/360 (ACME System) of the Stanford University School of Medicine. For each model studied, the distribution of the interatomic distances r_{ij} was calculated, and using the Debye formula the diffraction curves were computed for $s = 0.12 \text{ \AA}^{-1}$ to 0.52 \AA^{-1} in steps of 0.02 \AA^{-1} . The regions of interest are at $s = 0.24 \text{ \AA}^{-1}$ and 0.3 \AA^{-1} corresponding to 4.2 \AA and 3.4 \AA —the observed diffraction maxima.

Adjacent Units with Orientations Independent of their Separation

In an extremely simplified version of the calculations the interatomic distances were taken to be simply those belonging to a single unit. The diffraction curves were computed for the case when the unit was a single monomer and for the case when the unit was a dimer. None of the curves shows any peak in the region of interest. Fig. 5 shows the dimers considered. While other dimers are also involved, the ones considered are known to be present in reasonable numbers in melanins and could be expected to make contributions to the diffraction patterns. The calculations were repeated for various angles of rotation around the C—C single bond. But the diffrac-

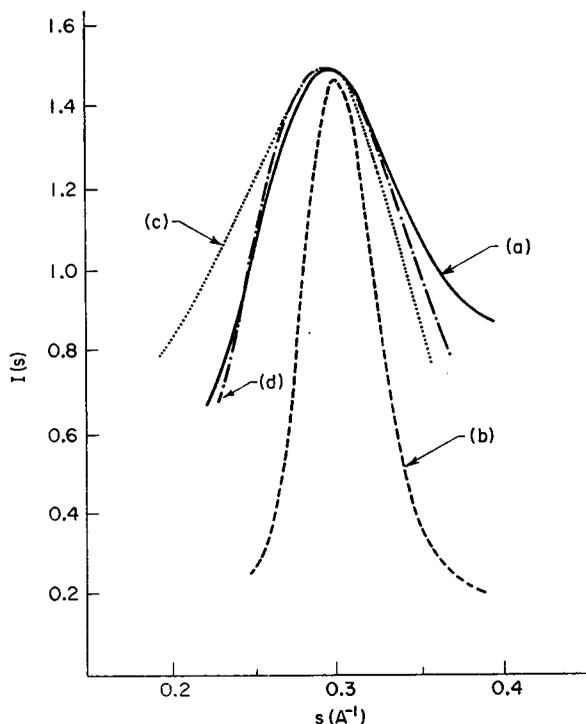


FIGURE 6 Computed diffraction curves of (a) three and (b) five parallel layers of indolequinone, 3.4 Å apart; (c) the experimental diffraction curve for squid melanin; (d) computed diffraction curve for adjacent monomeric units of *o*-benzoquinone with random displacements along the layers and a maximum mutual inclination of 15°. $I(s)$ is in arbitrary units.

tion curves did not differ appreciably from the ones for a rotation angle of 60°. In both cases the calculations imply that the adjacent units could take all possible mutual orientations independent of their separation, i.e., a gas model. In a real situation, however, a large number of these orientations, particularly for small separations, would be excluded for steric reasons.

Parallel Stack of Planar Groups

It has been previously noted that the indole melanins studied and synthetic melanins from catechol show the same spacing in their diffraction patterns though the expected monomeric units are different. While the two monomers vary in their lengths, their "thickness" is the same, 3.4 Å, and is probably related to the observed diffraction spacing of 3.4 Å. One can imagine three or four planar groups belonging to different chains aggregating into a near parallel stack in various sites. This can happen because of folds in the chains, the rotations around single bonds, and Van der Waals interactions. The stacks in different sites would probably be randomly

oriented with respect to one another. Using space filling models it was found that this could occur naturally. In the contributions to the X-ray diffraction pattern, the layer spacings in the stacks would then predominate over the other random interactions involving different portions of one or more polymer chains. The diffraction curves were computed for this model for both monomer types for stacks containing from two to five layers. The calculations were done for the case when all the layers were in register, and for the case in which the layers were displaced parallel to themselves randomly to within the width of the monomer. This arbitrary restriction of the random displacement was used to insure the nearness of the neighbors. The calculations showed that there was little change in the curves when the layers were displaced in parallel fashion. The curves for the two monomer types were also not very different except for the slightly larger breadth of the peak for the type "a". This might be expected in view of its smaller size. As expected, the peak is sharper when there are more layers.

All the curves show the peak at 3.4 Å, the assumed separation between the layers in the stacks. Fig. 6 shows the diffraction curves for three and five layers of the monomer "b" strictly parallel and in register. The experimental curve for squid melanin (Fig. 6 c) is seen to match closely the curve for three layers in regard to the shape and width of the peak. For a given number of layers in a stack the breadth of the peak would be expected to be larger if one allows a slight lack of parallelism between the layers.

Adjacent Units with Random Mutual Orientation

In this model adjacent monomeric units were assumed to take every possible mutual orientation that satisfied sterical requirements. The average distribution of the interatomic distances was then calculated for various values of the maximum separation between the units. The diffraction curves were then computed using Debye's formula.

Computational Techniques

The computations were carried out only for the monomer "a" although they could be applied equally well to "b". One unit was taken as reference. The z-axis was taken to be perpendicular to the plane of the aromatic ring, the center of which was chosen as origin. The orientation of the second unit was specified by the three Euler angles γ , ϕ , ψ , with the range $0 - \pi$, $0 - 2\pi$, $0 - 2\pi$ respectively. γ represented the angle between the adjacent planar groups. The Euler angles have been described by a number of authors (e.g., Condon, 1958). The position of the second unit was specified by the spherical coordinates D , α , β . D represented the distance between the origins, α the inclination of the line joining the origins with the z-axis of the reference frame, and β its azimuth. α and β had the range $-\pi/2 - \pi/2$, $0 - 2\pi$ respectively. Random values of the five angular parameters were generated.

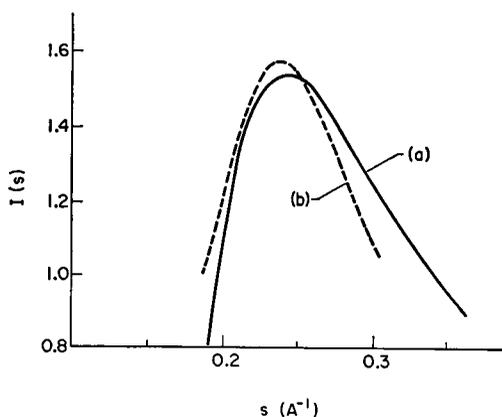


FIGURE 7 (a) Computed diffraction curve for adjacent monomeric units of *o*-benzoquinone assuming all possible mutual orientations that are sterically satisfactory; (b) experimental diffraction curve of *Ustilago melanin*. $I(s)$ is in arbitrary units.

D was chosen in a variety of ways for different sets of computations. The distance between the centers of the 12 Van der Waals spheres of the two units were then calculated and if any distance was less than the sum of the corresponding Van der Waals radii the trial was abandoned and a new trial was begun with a different set of random values for the parameters. If all the Van der Waals distances were satisfactory, the interatomic distances were computed and stored in "boxes" along with their weights. Distances in the range $R \pm \Delta R$ were stored in the box labeled R . A "size" of the boxes $2\Delta R = 0.5 \text{ \AA}^{-1}$ was used for the inspection of the distribution of the distances and a size 0.05 \AA for the calculation of the intensity using the Debye expression. Distances between the centers of the adjacent units were also stored in boxes of size 0.5 \AA . All the angular parameters were stored in boxes of size 0.2 radian.

In each run up to 3000 trials were made and the progress of the calculations was checked periodically. It was found that a good convergence was obtained even at 500 trials. When the range of D was chosen for a set of computations, the program estimated the reduced range of α and γ . When it was required that for a given set of values of the five angular parameters the two units should be as close together as possible, the corresponding value of D was estimated by the program. In this way wasteful searches were considerably reduced. In a typical run 40–65% of the trials were found successful, i.e., sterically satisfactory.

4.2 \AA ring

When the two planar groups were in the same plane, the line joining their centers was nearly along their lengths, and the two groups were as close together as possible the distance between the centers was found to be about 7.5 \AA . When the two units were parallel, the line joining their centers was along the z -axis, and the two groups were as close together as possible the distance between the centers was found to be 3.4 \AA . We could assume D to vary between these two values. If D was allowed

to take all values in this range and the calculations described were carried out the computed diffraction maximum was at a spacing larger than 5 Å. However due to Van der Waals interactions we could expect the adjacent groups to get as close together as possible for any mutual orientation and for any orientation of the line joining their centers. Therefore D was chosen for each set of values of the five angular parameters so that this requirement was fulfilled and the computations carried out. Fig. 7 shows the computed diffraction curve (a) and the experimental diffraction curve for *Ustilago* melanin (b). The two are seen to agree very closely. Their maxima are at 0.24 \AA^{-1} corresponding to a Bragg spacing of 4.2 Å. The distribution of the angular parameters was found to be uniform. In about 15% of the accepted trials the mutual inclination between adjacent planar groups was less than 15° .

3.4 A ring

The calculations were repeated for various upper limits of D starting from 7.5 Å. The diffraction maximum was found to decrease from 4.2 Å and when the maximum value of D was 3.7 Å the diffraction maximum was 3.4 Å. The adjacent planar groups had a maximum inclination of about 15° and the line joining their centers had a maximum inclination of about 30° to the z -axis. When the maximum value of D was reduced further the diffraction maximum stayed at 3.4 Å while the deviation of the planar groups from parallelism and the deviation of the line joining their centers from the z -axis became less. All these are in conformity with what has been previously said in describing the model "parallel stack of planar groups". Fig. 6d shows the diffraction curve thus derived.

DISCUSSION

Analytical studies of Nicolaus (1962) and in vivo radioactive tracer studies of Hempel (1965) lead to the following concepts of melanin structure. Melanin is not a homopolymer. In animal melanins indoles appear to predominate but may be present in oxidized or reduced form, and with or without a carboxyl unit. In addition, uncyclised DOPA units may be present. All potential bonding sites appear to be involved to some extent. The monomers are apparently joined by several bond types, such as C—C, —CO—, —O—, —O—O—.

The polyfunctionality of the monomers would favor branching and formation of three dimensional networks. However these studies do not answer the question of whether the polymer structure is an ordered one or a random one.

The electron spin resonance results (I) suggest that melanin is not a highly conjugated polymer with extensive electron delocalization. The absence of hyperfine structure in the e.s.r. spectrum is consistent with, but does not prove that, melanin is a disordered polymer, since this absence could be due to a tight, cross-linked structure which prevented molecular tumbling.

In the present computation of diffraction curves, effectively homopolymeric

models have been assumed. However, the presence of other monomer types is not likely to alter the diffraction pattern very much since they are closely related and would have the effect only of altering the bond lengths and angles somewhat in a few cases and in the addition of sidechains like carboxyl groups in a few others. Also, the covalently bonded neighbors were not considered. These could be expected to be a little bit closer together than those associating due to Van der Waals attractions. None of these, however, is expected to alter significantly the shape and height of the computed diffraction curves and hence the conclusions based on them.

The spherical symmetry observed in the diffraction patterns could be due to a random structure. It could also be expected even if the materials examined consisted of particles possessing some regularity of structure (fibrous or lamellar domains for example) but with random mutual orientations. So far no melanin sample has been found to have a preferred orientation of any sort.

Melanins from other sources have been reported to have diffraction rings with spacings — 3.9–4.2 Å for native and digested sepia melanin, for tyrosine and tryptophan pigments, and melanin from human tumors; 4.5–4.7 Å for melanins from human tumors and mouse melanoma (Spiegel Adolf and Henny, 1939). All the indole melanins studied by us, however, showed a spacing significantly smaller, i.e., 3.4 Å. Since there is little information available on the nature of the materials used by these authors it is difficult to comment on the observed difference in the diffraction spacings. Larger spacings in the region 7–10 Å have also been reported for these melanins.

It is possible to deduce a few positive conclusions from the work described in this paper.

1. The diffraction pattern of *Ustilago* melanin (and the plant melanins) is consistent with a random structure in which adjacent monomeric units of *o*-benzoquinone or closely related monomer types are in random mutual orientations that satisfy the steric requirements. The small size of the monomer, and the availability of a number of bond types probably favor this random structure.

2. Somewhat greater regularity seems to occur in the structure of the animal melanins and the synthetic catechol melanins. They seem to aggregate with their adjacent planar groups nearly parallel and as close together as possible (3.4 Å). Random displacements in the plane of the groups may occur as these have little influence on the diffraction curves. However, we do not have sufficient information to rule out the presence of greater regularity that would inhibit these random displacements.

One can hope to get some more clues to the structure of melanin when the low angle diffraction studies, studies on model compounds, and more detailed studies on model building including covalently bonded and hydrogen bonded neighbors become available.

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