CLXIV. STUDIES ON MELANINS

. I. PHOTOSYNTHETIC MELANINS

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DARK pigmentations occur in the human and animal body, both under normal and pathological conditions. Nevertheless, there are still discrepancies as to the origin and the chemical nature of the dark pigments, the so-called melanins [Pincussen, 1930]. In order to elucidate some of these problems, the experiments to be reported here were started.

In the course of studies on the influence of short-wave light on proteins, there was ample opportunity to observe a change of colour in the irradiated substances [Spiegel-Adolf, 1927] which has been previously reported by other authors [Stedman & Mendel, 1926]. These colour changes were missed in gelatin, were much more spectacular when the irradiation was made on some aromatic amino-acids (phenylalanine, tyrosine, tryptophan) and were completely absent from aliphatic amino-acids [Spiegel-Adolf, 1934]. Lieben [1927] has described the dark products in irradiated tyrosine and tryptophan solutions as melanoidins, a name used originally by Schmiedeberg [1897] for dark products appearing in albuminoids upon hydrolysis by acids. Their difference from true melanins according to Maillard [1912-13] consists in the fact that only the latter are derivatives of aromatic amino-acids. Nevertheless Fürth & Lieben [1921] have designated hydrolysis products of tryptophan as melanoids. Other authors stress the fact that melanoidins are melanin-like substances of greater solubility.

In order to investigate the formation of melanin-like substances under as simple conditions as possible, solutions of phenylalanine, tyrosine and tryptophan were exposed to ultraviolet light, and the optimum conditions of melanin formation and some of the properties of the dark-coloured products were studied. No attempts have been made in this paper to distinguish between the primary effect of radiation and secondary chemical processes, e.g. the formation of hydrogen peroxide, though Pincussen [1930] discusses the possibility that H_2O_2 formed by irradiation accelerates the formation of melanin.

In preliminary experiments the influence of bright daylight on the three amino-acids was studied. The experiments were done in Miami, Florida, $26\frac{1}{2}^\circ$ north latitude, from the end of December to the beginning of January on a window-sill on a sixth floor having an unobstructed east exposure. The solutions of the three amino-acids (*l*-tyrosine, *l*-tryptophan and *dl*- β -phenylalanine, Eastman Kodak) were equimolar (0.0276 *M*), and in order to overcome the different solubilities, the same amount of NaOH (0.04 *N* final concentration) had been added. 5 ml. of each solution were irradiated in glass dishes of 4 cm. diameter covered with a glass plate. It had been ascertained before that the glass plate cut out the radiation below 290 *m* μ . After 6 days of bright sunshine the tyrosine and the tryptophan were markedly though differently coloured, the former being more brownish, the latter more greenish yellow. The tyrosine took

a shorter time to reach the final colour intensity. The phenylalanine solution was apparently unchanged. These results were not modified in a second series of experiments, in which chlorophyll (final dilution 12,500-fold) and eosin (0.0001 g.) had been added to the original phenylalanine solution, or in which this solution had been diluted ten times. Lieben [1927] had been unable to trace colour changes in alkaline tyrosine and tryptophan solutions exposed to daylight without addition of sensitizers. This discrepancy can be explained by the fact that a longer exposure in a southern latitude was used. The results could be reduplicated in Philadelphia.

The fact that tyrosine and tryptophan react in visible light, whilst phenylalanine does not show any response, could be confirmed by other experiments. Samples of the three aromatic amino-acids covered by the tested glass plate were irradiated for 1 hr. at 15 cm. distance by a quartz mercury arc lamp (Hanovia, 220 V, D.C.). While the tyrosine and tryptophan solutions became dark brown, the neutral and alkaline phenylalanine solutions remained unchanged. These results show that tyrosine and tryptophan are sensitive both to visible and to ultraviolet light, whilst phenylalanine does not show any colour change under the influence of visible light.

Only a few qualitative experiments were made in order to see whether dark heat radiation exerts any influence on the production of melanin-like products in the aromatic amino-acids. An inverted crucible furnace on a tripod served as radiation source; the fluids were placed under the furnace opening, heated to dull red, at 22.5 cm. and at 12 cm. distance for $2\frac{1}{2}$ hr. The results were completely negative as to visible colour formation, nor did the spectrograph show any changes of the absorption spectra of the irradiated solutions. It has been shown previously that heating up to 100° does not markedly affect a tyrosine solution [Lieben, 1927].

The irradiation experiments were, therefore, limited to the use of ultraviolet light. As source of light, the above-mentioned quartz mercury vapour arc lamp was used. The fluids were placed in dishes of 50 sq. cm.; the thickness of the layer was 0.5 cm. The dishes were covered with a quartz plate. The distance from the source of light was 15 cm. The evaporation, even after an irradiation of several hours, was practically negligible.

The former observations on the formation of melanin-like products in alkaline tyrosine and tryptophan solutions agree with the findings of Lieben. But contrary to our results, Lieben could not detect similar changes in alkaline phenylalanine solutions. Similarly Harris [1926] missed any uptake of oxygen by phenylalanine on irradiation. On the other hand, the requirements for oxidation with colour formation are aromatic compounds of phenolic structure (Pincussen). Experiments were started in order to elucidate these discrepancies. Lieben [1927] reports successful melanin formation in alkaline solutions of tyrosine and tryptophan, and seems to have tested phenylalanine under the same conditions only. For our former experiments on phenylalanine, neutral solutions were used. Experiments were therefore started in which the *pH* of the neutral phenylalanine solution ($cH = 4.37 \cdot 10^{-7}$) was varied either by addition of HCl or NaOH of 0.02–0.04 *N* final concentration.

The results show that, unlike tyrosine and tryptophan, alkaline solutions of which show the most marked black-brown colouring, phenylalanine becomes black-brown only on irradiation in neutral solution. Upon irradiation of phenylalanine, a smell becomes perceptible, which is entirely different from that observed with irradiated proteins. It is similar to that which develops in phenylalanine when heated with sulphuric acid containing potassium dichromate,

and which is attributed to the formation of phenylacetaldehyde [Hoppe-Seyler, 1924]. The alkaline and the acid solutions show a more yellow or reddish hue. This may account for the fact that melanin formation in phenylalanine, upon irradiation, has been missed when working on alkaline solutions. The findings of Lieben can be confirmed in so far that the melanin formation in tyrosine and tryptophan is much weaker in acid and neutral than in alkaline solutions.

In order to isolate the melanin-like substances, the following procedures were used: tyrosine and tryptophan were both irradiated in alkaline solutions (0.04 *N* NaOH) containing 0.5% tyrosine and 0.54% tryptophan respectively. Whilst with proteins the effect of ultraviolet irradiation becomes more marked in solutions of lower concentration, no similar behaviour was noticeable with these two amino-acids. This can be explained by the fact that they are also sensitive to other wave-lengths of light, than those corresponding to their own chief absorption bands, as has been shown above. But the irradiation was interrupted after 1 hr., as the colour of the fluid was then dark brown, and would certainly act effectively as a screen against further irradiation. The dark substances formed through irradiation were precipitated by neutralization with HCl and removed by centrifuging. The supernatant yellow-coloured fluid was made alkaline and again submitted to irradiation. This procedure was repeated twenty times, and all the precipitates were collected, each in one centrifuge tube. The last supernatant fluids still contained traceable amounts of amino-acids. The precipitates were repeatedly suspended in 10 ml. of dilute 0.01 *N* HCl and centrifuged. This was repeated until the last supernatant fluids did not show an appreciable absorption power on the fluorescent screen of the quartz spectrograph, and until the reactions of Millon and Hopkins-Cole, respectively, had completely disappeared. The precipitates were then evaporated to dryness on a water-bath and finally dried in a desiccator. The products were black-brown, practically insoluble in water and weak acids, but easily soluble in weak alkalis. From these solutions, they were precipitated by neutralization.

Isolation of the melanin-like products from irradiated phenylalanine solutions meets with the difficulty that acidification did not cause a satisfactory precipitation. Neither did absorption with aluminium hydroxide in *statu nascendi* [Eisler & Spiegel-Adolf, 1929] prove to be helpful. The only way that could be found was precipitation by neutral salt saturation. Precipitation of the melanin-like product starts at one-third saturation with ammonium sulphate and increases until complete saturation is reached. According to findings with proteins, the presence of neutral salts does not interfere with the effectiveness of irradiation [Spiegel-Adolf, 1927]. The 0.75% phenylalanine solutions were irradiated, therefore, as were the other amino-acids, and ammonium sulphate in substance, enough to cause complete saturation, was added. The precipitate was removed by centrifuging. This procedure was repeated twenty times without exhausting the phenylalanine content of the fluid. The black-brown precipitates were collected in a centrifuge tube. Finally, they were suspended in water and dialysed for 2 days against running tap water. This solution was intensely coloured. Nevertheless, no coloured matter permeated through the parchment membrane, indicating the colloidal state of the phenylalanine melanin in neutral solution. These findings were corroborated by investigations of the Tyndall phenomenon, which was positive in all solutions of the products here described. To rid melanin solutions of all salts, they were electrodialysed, carefully avoiding any heating of the middle cell, until the supernatant fluid showed no appreciable electrical conductivity. Spectrographically, no traces of phenylalanine were detectable. The precipitate was dried on the water-bath and kept

in a desiccator. These melanin-like products are black-brown, practically insoluble in water or acids, soluble in weak alkalis. The original water-solubility may have been due to the presence of other substances, or has been lost in the process of purification, indicating that classification according to solubility is only of relative value [Spiegel-Adolf, 1930].

The three melanin-like products were tested as to their solubility in alcohol, since solubility in an organic medium would facilitate some type of investigations. Only the melanin-like product made from tryptophan was soluble to a certain extent in absolute alcohol; according to colorimetric determinations, a saturated solution contains approximately 0.04% tryptophan-melanin. Such a solution is completely clear, but nevertheless gives a strong Tyndall phenomenon, indicating that in alcohol tryptophan-melanin is present in colloidal solution. This finding agrees with a statement of Bloch & Schaaf [1926] according to which the solubility of synthetic melanin in absolute alcohol is 1 : 3000 approximately. The analogous solubility of tyrosine-melanin is lower, approximately 1 : 6000, and the solubility of phenylalanine-melanin in absolute alcohol is practically nil. These findings therefore afford additional evidence that differences exist between the melanin-like products.

Lieben has claimed that differences exist between the irradiation products from tyrosine and tryptophan based on the observation that only tyrosine-melanins are destroyed by hydrogen peroxide and not the analogous products from tryptophan. Experiments made in this direction showed that only quantitative differences exist. All three melanin-like products are entirely bleached by H_2O_2 . But while it required only 5 hr. for 10 drops of Superoxol (Merck) to destroy the colour of 2 ml. of 0.02% tyrosine- or phenylalanine-melanin in 0.032 *N* KOH, 24 hr. were required to reach the same effect in the analogous solution of tryptophan-melanin. But it must be remembered that the tryptophan-melanin solutions were from the beginning darker than the other solutions. The process of bleaching by hydrogen peroxide, which is accompanied by a nearly complete loss of absorptive power in the short-wave spectrum, is not reversible upon renewed irradiation. The colourless solutions nevertheless show a strong Tyndall phenomenon.

In a further series of investigations, evidence was sought as to the identity or difference of these melanin-like substances. Florence *et al.* [1935] made extensive ultraspectrographic studies of genuine and synthetic melanins. They showed that though melanins resemble each other there are nevertheless distinct differences. In order to elucidate this problem quantitatively, ultraspectrographic studies were made on the three melanin-like substances. A small Hilger quartz spectrograph with internal wave-length scale, and a self-made sectorphotometer, with two rotating sectors, one of which was adjustable, were used for this purpose. The sector was driven at a speed permitting 120 rotations in a minute. The source of light was provided by an arc lamp using Eveready Therapeutic C Carbons (containing iron, nickel, aluminium and silicon). Eastman Kodak lantern-plates (slow) were used. The exposure varied from 3 to 10 sec. The adjustable sector was divided into 100 equal parts. As fourteen double exposures could be photographed on one plate, the first and last being used as controls, twelve different positions of the sectorphotometer could be utilized. Always the same positions were used, the logarithms of which were equidistant. As this means that, especially for the higher values, the adjustment of the sector becomes more easily subject to error, a special device was used which automatically guaranteed always the same adjustment for the last five positions of the sector.

The examination of the plates was made on a viewing box, with a low-powered magnifying glass. As the molecular weight of the melanin-like substances is unknown, the percentage extinction coefficient [Weigert, 1927] was computed, $E = \frac{1}{cd} \lg \frac{I_0}{I_s}$ (c = concentration %; d = thickness of fluid layer; I_0 = intensity of light passing through solvent; I_s = intensity of light passing through solution). For the graphic determination $\lg E$ was used. 0.01 % solutions of tyrosin, tryptophan- and phenylalanine-melanins in 0.016 *N* KOH in a tube of 5 mm. thickness were used.

At the chosen concentration, only three coinciding wave-lengths could be found on the tyrosine-melanin spectrogram. Therefore a 20 mm. tube was used for the determinations of the extinction coefficients at longer wave-lengths, the law of Lambert having been proved to be generally valid for hydrophobic colloids [Lange, 1928]. The wave-lengths at which coincidence was found and $\lg E$ are graphically presented in Fig. 1. The data together with other measurements, are contained in Tables I-III.

Fig. 1. I refers to tryptophan-melanin. II refers to phenylalanine-melanin. III refers to tyrosine-melanin.

The graphic presentation shows that there is a marked similarity in the shape of the absorption curves of the three products. No traces of the spectra of the basic amino-acids are left. The slope of the curves is similar to that observed in melanins by Florence *et al.* [1935]. Nevertheless, there is quantitatively a difference between the absorption values of our products. Tryptophan-melanin shows the highest values, tyrosine-melanin the lowest. The derivative of phenylalanine comes between these two. These findings agree with the above-mentioned observations and show that the derivative of phenylalanine has an individuality of its own. The reasons for these differences can be of different nature, either a chemical difference, or a different degree of polymerization. The similar trend of the extinction curves seems suggestive of the latter. Nevertheless, further experiments are under way to study this problem.

Ultraviolet spectrographic findings can only be used for a further analysis of a substance, when the validity of the law of Beer-Lambert has been proved, i.e. when the extinction coefficients are independent of the concentration of the solution and of the length of its layer (Weigert). Recent investigations of Griebnau *et al.* [1936] have shown that this is also true for hydrophobic colloids. Still, in our case, the presence of strong electrolytes may lead to some complica-

tions. Experiments were therefore started in which the concentration of the fluid and the thickness of the layer were varied. For tyrosine-melanin concentrations of 0.005–0.02 %, for phenylalanine-melanin of 0.0025–0.01 %, for

Table I. *Tyrosine-melanin*

I_s %	$\lg \frac{I_0}{I_s}$	$E = \frac{1}{cd} \lg \frac{I_0}{I_s}$	$\lg E$	λ of coincidence m μ
Plate 5. $c = 0.01$ %; $d = 2$ cm.				
31	0.50000	25.000	1.39794	395
22	0.66667	33.3333	1.52288	385
15	0.83333	41.6667	1.61989	364
10	1	50.0000	1.69897	350
7	1.16667	58.3333	1.76593	329
5	1.33333	66.6667	1.82390	315
3	1.50000	75.0000	1.87506	290
Plate 13. $c = 0.01$ %; $d = 0.5$ cm.				
46	0.33333	66.6667	1.82391	304
31	0.50000	100	2	259
22	0.66667	133.3334	2.12794	240
Plate 15. $c = 0.02$ %; $d = 0.5$ cm.				
31	0.50000	50	1.69897	380
22	0.66667	66.6667	1.82391	345
15	0.83333	83.3333	1.92082	314
10	1	100	2.00000	290
7	1.16667	116.6667	2.06694	265
5	1.33333	133.3333	2.12493	251
Plate 16. $c = 0.005$ %; $d = 2.0$ cm.				
31	0.50000	50	1.69897	380
22	0.66667	66.6667	1.82391	345
15	0.83333	83.3333	1.92082	315
10	1	100	2.00000	290
7	1.16667	116.6667	2.06694	265
5	1.33333	133.3333	2.12493	259

Table II. *Phenylalanine-melanin*

I_s %	$\lg \frac{I_0}{I_s}$	$E = \frac{1}{cd} \lg \frac{I_0}{I_s}$	$\lg E$	λ of coincidence m μ
Plate 17. $c = 0.02$ %; $d = 0.5$ cm.				
22	0.66667	66.6667	1.82391	385
15	0.83333	83.333	1.92082	360
10	1	100	2.00	323
7	1.16667	116.667	2.06694	310
5	1.33333	133.33	2.12493	290
3	1.50000	150.00	2.17609	276
Plate 18. $c = 0.005$ %; $d = 0.5$ cm.				
22	0.66667	66.6667	1.82391	385
15	0.83333	83.337	1.92082	360
10	1	100	2.00	323
7	1.16667	116.667	2.06694	310
5	1.33333	133.33	2.12493	290
3	1.50000	150.00	2.17609	276
Plate 19–21. $c = 0.01$ %; $d = 0.5$ cm.				
46	0.33333	66.6667	1.82391	380
31	0.50000	100	2	324
22	0.66667	133.3334	2.12494	290
15	0.83333	166.6666	2.22183	265
Plate 20. $c = 0.0025$ %; $d = 2.0$ cm.				
46	0.33333	66.6667	1.82391	380
31	0.50000	100	2	324
22	0.66667	133.333	2.12494	289

Table III. *Tryptophan-melanin*

I_s %	$\lg \frac{I_0}{I_s}$	$E = \frac{1}{cd} \lg \frac{I_0}{I_s}$	$\lg E$	λ of coincidence m μ
Plate 6. $c=0.01$ %; $d=20$ cm.				
31	0.50000	100	2.000	355
22	0.66667	133.33334	2.12494	314
15	0.83333	166.66667	2.22183	293
10	1	200	2.30103	275
7	1.16667	233.33333	2.36799	265
5	1.33333	266.66667	2.42596	257
3	1.50000	300.00000	2.47712	250
Plate 8. $c=0.0025$ %; $d=2$ cm.				
31	0.5000	100	2	360
22	0.66667	133.33334	2.12494	320
15	0.83333	166.66667	2.22183	300
10	1	200	2.30103	282
7	1.16667	233.3333	2.36799	273
5	1.33333	266.66667	2.42596	257
3	1.500	300	2.47712	251

tryptophan-melanin of 0.0025–0.01 % were used. The dilutions were made with water. The KOH-melanin ratio was the same in all samples. The two pairs of tubes available had lengths of 5 and 20 mm. respectively. The results are summarized in Tables I–III.

From the figures of Tables I–III graphs were made by plotting the logarithms of the extinction coefficients against the wave-lengths at which coincidences were observed. By interpolation $\lg E$ was determined at three different wave-lengths from the single graphs. These values are recorded in Table IV.

Table IV

	Plate no.	$\lambda=250$ $\lg E$	$\lambda=300$ $\lg E$	$\lambda=350$ $\lg E$
Tyrosine-melanin	5	—	1.845	1.70
	13	2.045	1.845	1.70
	15	2.12	1.96	1.79
	16	2.12	1.96	1.79
Phenylalanine-melanin	17	—	2.08	1.91
	18	—	2.08	1.91
	20	—	2.065	1.90
	21	—	2.065	1.90
Tryptophan-melanin	6	2.48	2.18	2.01
	8	2.48	2.21	2.02

The figures show that, within the investigated concentrations, the extinction coefficients are practically independent of the concentration and of the thickness of the layer, indicating the validity of the law of Beer-Lambert. The only exception seems to be the behaviour of tyrosine-melanin. The extinction coefficients of Pls. 5 and 13 and the values from Pls. 15 and 16 agree with each other, but the values determined from these two sets are not identical. Whilst in all other samples the concentration has been determined by gravimetric methods, the concentration used in Pls. 15 and 16 has been reached by concentrating a more diluted solution, using volumetric determinations only. The validity of the law of Beer within a certain range of concentrations suggests that the solution under investigation is, under these conditions, stable from a colloid-chemical point of view [Lange, 1928]. This may be helpful for a later evaluation of the

extinction graphs, in comparing the influence of physical conditions and chemical structure.

Only a few conclusions can be drawn as to the chemical import of these findings. Evidence has been given that, without the action of a ferment, by action of light alone on watery solutions, melanin-like substances can be formed from aromatic amino-acids. The fact that phenylalanine too can be a source of melanins, points to the importance of the phenol ring. Though decarboxylation probably occurs in all cases, and the aliphatic group is the same, some of the original differences are still manifest in the final products. The solutions of the melanin-like substances are colloids, indicating polymerization. The name of photosynthetic melanins is suggested for the irradiation products here described.

SUMMARY

1. Conditions necessary for the formation of melanin-like derivatives of aromatic amino-acids by irradiation with short-wave light are described. These products are called photosynthetic melanins.

2. Not only tyrosine and tryptophan, but phenylalanine as well can be the source of photosynthetic melanins.

3. The products were isolated and purified. They show slight differences in solubility, in their reactions with hydrogen peroxide and in their optical absorption.

4. The extinction coefficients fall in the sequence tryptophan-, phenylalanine-, tyrosine-melanin.

5. It is shown that for the absorptive power of melanin solutions, within a certain range of concentrations, the law of Beer-Lambert is valid.

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