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PHOTOCHEMISTRY OF DL-PHENYLALANINE

By

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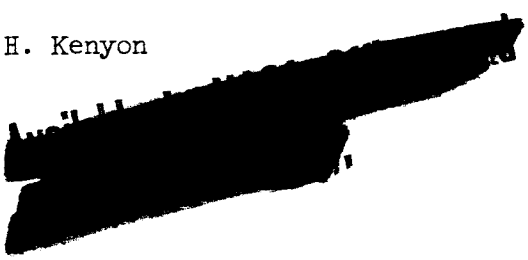

This investigation was supported by a research grant NsG 218-62 from the National Aeronautics and Space Administration, and by training grants 2G-712-C2 and 2G-712-C3 from the National Institutes of Health (Division of General Medical Sciences), United States Public Health Service.

B. L. Report No. 124

December 1964

ACKNOWLEDGMENTS

I wish to thank my thesis advisor, Professor M. S. Blois, for his interest in and support of my investigations during the past two years. His cooperation and encouragement have been invaluable. I am also indebted to Dr. Howard H. Pattee for providing space and facilities, and for many stimulating discussions.

In addition, I am grateful to the following individuals for their special contributions: Professor Victor Burns for helpful suggestions on quantitative paper chromatographic techniques; Dr. John Maling and Mr. Edmund Day for assistance in the electron spin resonance measurements; Mrs. Lina Taskovich for many useful suggestions on various experimental procedures; Messrs. Christopher Platt and Kenneth Nozaki for valuable technical assistance; Mr. Phillip Banda for helpful discussion; and Dr. Kendric Smith for his incisive criticisms.

Finally, I wish to thank Dr. Lubert Stryer and Professor Arthur Giese for serving on my thesis advisory committee.

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I. INTRODUCTION

A. GENERAL

Living organisms respond to ultraviolet light of intermediate wavelengths (2000-3000 Å) in a great variety of ways. Some of the responses of animal cells which have been investigated quantitatively include inhibition of growth (Mayer and Schreiber, 1934), erythema (Coblentz and Stair, 1934), immobilization (Giese and Leighton, 1935), activation of eggs (Hollaender, 1938), inhibition of division (Giese, 1945), sensitization to heat (Giese and Crossman, 1945), retardation of regeneration (Hirschfield and Giese, 1953), and hemolysis (Green, 1956). The mutagenic and killing effects of ultraviolet radiation (UV) have been the subject of much interest and activity, and a large number of quantitative investigations have been carried out on viruses, bacteria, fungi, and higher plants. Representative examples from the vast literature of the subject are the studies of Zelle and Hollaender (1954) on UV inactivation of T 1 bacteriophage, Evelyn Witkin's (1956) work on UV-induced mutations in bacteria, the work of Zetterberg and Giese (1962) on UV-induced back-mutations in the fungus Ophiostoma multiannulatum, and Swanson's (1942) investigations on UV-induced chromatid deficiencies in Tradescantia. Detailed discussions of many of the observed effects of UV radiation on living systems are contained in a comprehensive treatise edited by Hollaender (1955) and in recent reviews by Giese (1964) and Zetterberg (1964).

Underlying a given biological response to ultraviolet radiation are photochemical processes at the macromolecular or monomer level. The absorption of ultraviolet light of intermediate wavelengths by protoplasm is due chiefly to two classes of macromolecules: nucleic acids and proteins (Caspersson, 1950). The ultraviolet absorption maximum for nucleic

acids is at 2600 Å, and that for proteins containing aromatic amino acids occurs at or near 2800 Å (Caspersson, ibid.). Most other quantitatively significant substances in nonpigmented cells absorb only below 2300 Å (Caspersson, ibid.). Therefore, on the basis of absorption spectra alone, it is to be expected that nucleic acids and proteins will be implicated in many of the responses of organisms to UV radiation. This expectation is fully confirmed by action spectral data which show that the relative efficiencies of different wavelengths of ultraviolet light in producing a given biological effect often parallel the UV absorption spectrum of either nucleic acids or proteins containing aromatic amino acids (Giese, 1964).

Since nucleic acids and proteins have been implicated as the primary light absorbing materials in many biological responses to UV radiation, knowledge of the detailed photochemical behavior of these substances is necessary for the interpretation of these responses on a molecular basis. As a result of the efforts of many workers, much qualitative and quantitative information is now available on the in vitro photochemistry of DNA and its constituent purine and pyrimidine bases (see Shugar and Wierzchowski, 1958; Shugar, 1960; Smith, 1964). These studies have added much to our knowledge of the molecular basis of UV-induced mutations and UV inactivation of organisms, although many unsolved problems remain, particularly those concerning the correspondence between the in vitro photochemistry of free purines and pyrimidines and the in vivo photochemistry of these substances when they are part of the structure of DNA (Smith, 1964).

The effects of UV radiation on enzymes include alteration of many physical properties in addition to loss of enzyme activity. Attempts to correlate UV-induced changes in some readily measured physical property, such as optical absorbance, with extent of enzyme inactivation, have shown that in some cases enzyme activity is lost considerably earlier than the appearance of detectable changes in the physical property. For example, Uber and McLaren (1941) have shown that trypsin inactivated by UV radiation to the extent of 60% exhibits no appreciable increase in absorbance. Prolonged irradiation, however, does result in increased absorbance, as evidenced by the appearance of a yellow color (Gates, 1934; Clark, 1936). These results show that in the case of trypsin, the

photochemical events underlying UV inactivation are distinct from those underlying increases in optical density. However, in the case of other enzymes, such as pepsin, chymotrypsin, and soybean trypsin inhibitor, optical density increases do parallel loss of enzymatic activity (Gates, 1934; Finkelstein and McLaren, 1949). Therefore, elucidation of the photochemical events underlying optical absorbance increases would be particularly helpful in determining which amino acid residues are or are not intimately associated with the maintenance of structure necessary for enzyme function.

Since the principal near-UV absorbing residues of proteins are phenylalanine, tyrosine, tryptophan, and cystine (Lerner and Barnum, 1946; Beaven and Holiday, 1952; Wetlaufer, 1962), it is to be expected that photochemical alterations of these substances might be associated with loss of enzyme activity. Action spectral data confirm this view. For example, it has been shown that the action spectrum for UV-induced sensitization to heat closely resembles the absorption spectra of proteins containing aromatic amino acids (Giese and Crossman, 1945). In this connection, it is particularly significant that UV-irradiated solutions of free aromatic amino acids exhibit optical absorbance changes similar to those occurring in UV-irradiated protein solutions (Claesson, 1956). The UV-induced absorbance changes are much more marked in oxygen-saturated than in oxygen-free solutions for both enzymes and free aromatic amino acids, suggesting that photo-oxidation of the aromatic nuclei is occurring in both cases (McLean and Giese, 1950; Claesson, 1956; Kenyon and Blois, 1964). Thus, it is likely that in those instances where optical density increases are correlated with loss of enzymatic activity, aromatic amino acids are involved in the maintenance of aspects of tertiary structure which are essential for enzyme function. However, in the cases in which enzyme inactivation appears to be independent of photo-oxidation of aromatic amino acid residues (i.e., that of trypsin, see above), it is possible that either (1) rupture of a disulfide bridge is required for inactivation and that this process occurs with significantly higher quantum yield than photo-oxidation of aromatic nuclei, or (2) photoexcitation energy initially absorbed by a π-electron in a given aromatic nucleus

migrates down the side chain of the residue into the polypeptide backbone where it either causes rupture of a peptide bond (Mitchell, 1936; Rideal and Mitchell, 1937), or of a number of hydrogen bonds.

In order to determine the extent to which photolysis of aromatic amino acids, cleavage of disulfide bridges, and energy migration participate in UV inactivation of enzymes, it is necessary to know both quantum yields for inactivation and quantum efficiencies for photolysis of the individual chromophoric amino acids. By assuming that the quantum efficiency for photolysis of a given amino acid is the same whether the amino acid is free in solution or incorporated into the structure of a protein, McLaren (1964) has shown that the quantum yields for UV inactivation of chymotrypsin, lysozyme, ribonuclease, and trypsin can be computed with fair accuracy (i.e., to within 60%) from quantum yield data for the free amino acids, without consideration of possible contributions of energy migration. In this connection, it is of considerable interest that in the case of free phenylalanine in aqueous solution, photoexcitation energy initially absorbed by a π-electron in the benzene ring of the molecule migrates to the end of the side chain, resulting in the liberation of CO_2 (see Chapter II). The quantum efficiency for this process ($\varphi = 0.03 - 0.04$) is comparable to the total quantum yield for phenylalanine loss ($\Phi = 0.04 - 0.05$), showing that energy migration occurs in the case of a substantial fraction of those photons whose absorption results in a photochemical reaction.

Although energy migration is necessary for cleavage of a carbon-carbon bond at the end of the side chain, it may not be sufficient. That is, in the case of free phenylalanine, photoexcitation energy may leave the ring with a quantum efficiency considerably higher than 0.04 and be degraded to heat in side chain vibrational modes before photodissociation can take place. However, when phenylalanine is incorporated into the structure of a protein, such transfer of energy to the side chain (and hence to the polypeptide backbone, see Chapter IV) may result in the cleavage of a number of hydrogen bonds near the phenylalanine residue. Evaluation of the extent to which such a process might contribute to UV inactivation of enzymes is a problem for future research.

The possibility that long range energy migration within individual protein molecules might play a fundamental role in certain biophysical processes, such as mitochondrial electron transport, has been discussed in detail by A. Szent-Györgyi (1960). That efficient energy migration does occur in phenylalanine containing model proteins under certain conditions was clearly demonstrated by the elegant experiment of Broser and Lautsch (1956). These workers showed that if CO-mesohemin-IX-poly-DL-(phenylalanine-glutamic acid) was irradiated at 2537 Å, the quantum efficiency for splitting off CO was 0.89, based upon the number of incident photons absorbed by the phenylalanine residues.

Attempts to account for intramolecular energy migration in proteins have been based on either semiconductor models (Evans and Gergely, 1949; Suard, Berthier and Pullman, 1961; Yomosa, 1964), or mechanisms of resonance energy transfer (Förster, 1959; Stryer, 1959, 1960). Although the resonance transfer model does not require a mechanism for transfer of excitation energy from aromatic rings to the polypeptide backbone, such a mechanism seems to be required in the case of the semiconductor model (see Chapter IV).

On the basis of the above discussion, it is apparent that the quantitative photochemistry of aromatic amino acids is of fundamental significance for the interpretation of the effects of UV radiation on proteins, and hence, ultimately, for the interpretation on a molecular basis of those biological effects of UV radiation whose action spectra parallel the absorption spectra of proteins containing aromatic amino acids.

B. PHOTOCHEMISTRY OF PHENYLALANINE - HISTORICAL SURVEY

During the course of a series of investigations on the photochemistry of plasma proteins, D. J. Harris (1926) showed that aqueous solutions of aromatic amino acids take up molecular oxygen when irradiated with ultraviolet light from a mercury arc lamp. In this work, no attempt was made to isolate or identify early photo-oxidation products. In 1934, Spiegel-Adolf reported that aqueous solutions of aromatic amino acids develop a dark brown color during UV irradiation, while UV-irradiated solutions of

aliphatic amino acids remain colorless. Several years later, in the first of a series of papers on melanin pigments, Spiegel-Adolf (1937) referred to the pigments synthesized in UV-irradiated solutions of phenylalanine, tyrosine, and tryptophan as "photosynthetic melanins." Although this conclusion was ultimately proved correct (see Chapter III), it was made on the basis of only a few experimental facts. That is, in 1937 it was known that the UV absorption spectra of "photosynthetic melanins" and of naturally occurring melanins are similar, although in both cases the spectra consist simply of monotonically increasing optical densities with decreasing wavelengths in the entire spectral region from 4000 to 2000 Å (Spiegel-Adolf, 1937). Apart from these optical data, and the known role of tyrosine in the UV-induced, in vivo synthesis of melanin associated with tanning, the only experimental facts then available which suggested that the pigments synthesized photochemically from free aromatic amino acids might be similar to natural melanins, consisted of a few semi-quantitative determinations of the very low solubilities of the two classes of pigments in water and absolute ethanol (ibid.).

That phenols and diphenols occur as a early photoproducts in UV-irradiated aqueous solutions of phenylalanine was first shown by Arnow (1942). This investigator used the Millon (1849) reaction (Arnow, 1937) for detecting phenols and showed that the resulting color closely resembled that obtained with standard tyrosine solutions. A faint orange-red color was obtained with the nitrite-molybdate reagent (Arnow, ibid.), indicating the presence of DOPA. By means of the Schiff (1865; Arnow, 1942), and phenylhydrazine (Arnow, 1942) tests, Arnow was also able to demonstrate the presence of phenylacetaldehyde in UV-irradiated phenylalanine solutions.

Arnow's results were later confirmed by McLean and Giese (1950) during the course of a systematic investigation of changes in the UV absorption spectrum of proteins induced by UV radiation. These workers showed that the optical density increases occurring in UV-irradiated phenylalanine solutions could be reasonably interpreted as being due to the hydroxylation of sites on the benzene ring of the molecule, followed by further oxidation to quinones. Although these conclusions

were based primarily on spectroscopic evidence, McLean and Giese did succeed in demonstrating the presence of phenolic compounds in their reaction mixtures by means of chemical tests using the Folin-Ciocalteu (Snell and Snell, 1937, p 351) and the diazotized sulfanilic acid (ibid., p 357) reagents.

The first detailed, quantitative investigation of the photochemical production of phenolic substances in UV-irradiated phenylalanine solutions was carried out by Schocken (1951). This investigator irradiated aqueous solutions of phenylalanine in quartz vessels with monochromatic radiation at 2700 Å (± 50 Å). The combined concentrations of tyrosine and DOPA were determined as a function of dose of incident radiation by the same color test which Arnow (1937) had used previously. Pigment production was followed by measuring the optical transmission of the solutions at 5400 Å as a function of UV dose. The results of these studies show that tyrosine is photochemically synthesized from phenylalanine and accumulates at a constant rate during the early stages of the reaction (i.e., for incident doses up to 2×10^8 ergs/cm^2). In a separate series of experiments, it was shown that DOPA is a photo-oxidation product of tyrosine and that the concentration of DOPA reaches a low, steady state value (~ 0.3% of the initial concentration of tyrosine) while pigment slowly accumulates. Since the amounts of phenylalanine and tyrosine photochemically decomposed could not be accounted for by the yields of tyrosine and DOPA, Schocken (ibid.) suggested that other types of photochemical reactions, such as "side chain oxidations," decarboxylation, and deamination, were taking place, although he presented no direct evidence in support of these possibilities.

Further support for the hypothesis that other photochemical reactions besides hydroxylations of the benzene ring followed by further oxidation to quinones (McLean and Giese, 1950) and finally to pigmented substances (Spiegel-Adolf, 1937; Schocken, 1951), were taking place in UV-irradiated solutions of phenylalanine, was provided by the work of I. M. Claesson (1956). This investigator conducted a detailed quantitative study of the optical absorbance increases which occur in aqueous phenylalanine solutions during monochromatic UV irradiation at 2537 Å.

By analyzing irradiated phenylalanine solutions on activated charcoal, she found two fractions in addition to unreacted phenylalanine, one of which was tightly bound to the charcoal and accounted for much of the increased light absorption, even though it contained only about 5% of the original mass of phenylalanine. The remaining fraction exhibited low light absorption and represented a small fraction of the starting material. By determining the difference in nitrogen content of the reaction mixture before and after irradiation, Claesson concluded that ammonia had been photochemically liberated. This latter result confirmed the earlier, more detailed studies of Weizmann et al. (1938) on the photochemical hydrolysis of the C-N bond in α-amino acids, including phenylalanine. These workers were able to show that in the case of the aliphatic amino acids, glycine and alanine, photochemical hydrolysis of the C-N bond resulted in the formation of the α-hydroxy derivative of the amino acid with the concommitant liberation of ammonia. In the case of phenylalanine, free ammonia was detected in irradiated solutions but no attempt seems to have been made to detect the corresponding α-hydroxy derivative, i.e., phenyllactic acid. Attempts to determine the quantum yield for the process were unsuccessful since ". . .phenylalanine undergoes a side reaction* leading to the formation of an insoluble [pigmented] coating on the quartz walls" of the reaction vessel (ibid.).

Although primary interest has been centered on the photo-oxidative synthesis of phenolic compounds in UV-irradiated solutions of phenylalanine because of the relevance of these processes to the problems of UV inactivation of enzymes and UV-induced in vivo melanin synthesis, two reports of aliphatic photoproducts of phenylalanine have appeared in the Japanese literature. Matsuda et al. (1954) claimed that serine and alanine are synthesized in UV-irradiated alkaline solutions of L-phenylalanine. But Tominaga (1958) using similar experimental conditions was unable to detect either serine or alanine, and instead reported aspartic acid and asparagine as photoproducts. Tyrosine, however, was observed in both cases, thus corroborating the earlier work of Arnow (1942), McLean and Giese (1950), and Schocken (1951). Since it is uncertain whether the Japanese workers used filters to remove short wavelength UV radiation from their illuminating

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* Emphasis mine.

beams, it has been suggested that the aliphatic photoproducts which they report may have resulted from absorption of the 1849 Å photons present in unfiltered mercury resonance radiation (Luse and McLaren, 1963). However, Porter has shown that side-chain photolysis following photoexcitation of ring electrons at 2537 Å does occur in benzene derivatives having linear side chains up to four carbons in length (Porter and Strachan, 1958).

Although Schocken (1951) reported yields of tyrosine in UV-irradiated phenylalanine solutions, he apparently made no attempt to determine the quantum yield for the reaction. Earlier, Weizmann et al. (1938) had attempted to measure the quantum yield for ammonia liberation from phenylalanine, but they were unsuccessful due to the accumulation of an insoluble photoproduct on the walls of their reaction vessels. The first successful determinations of quantum yields for photochemical reactions of phenylalanine were reported by Luse and McLaren (1963). These investigators used quantitative paper chromatography with ninhydrin as color reagent to follow the extent of phenylalanine loss during monochromatic UV irradiation at 2537 Å as a function of the amount of light absorbed by phenylalanine. By this means, they obtained a value of 0.013 for the total quantum efficiency of phenylalanine loss, and a value of 0.002 for ammonia liberation. The yield of ammonia was determined by the Conway microdiffusion technique (Conway and Byrne). Although Luse and McLaren (1963) reported the paper chromatographic identification of tyrosine and DOPA as photoproducts of phenylalanine, they did not determine the corresponding quantum yields.

In a preliminary series of experiments (Blois, 1964) on the long-term photochemistry of DL-phenylalanine in dilute aqueous solution (5×10^{-4} M), it was found that if the solution were saturated with molecular oxygen, the optical absorbance would rapidly increase over the entire wavelength interval from 2200 to 3500 Å, with maximum extent of change occurring just shortward (i.e., 2350 - 2500 Å) of the absorption maximum of phenylalanine (2575 Å), during irradiation with monochromatic light at 2537 Å, as had previously been observed by McLean and Giese (1950) and Claesson (1956). With continued irradiation, the absorbance reached

a maximum and then slowly fell to nearly zero for all wavelengths greater than 2400 Å. However, if the solution contained only small amounts of dissolved oxygen, the absorbance increased more slowly to about the same maximum, and did not undergo further change under prolonged irradiation. In both cases, the solutions were faintly yellow-brown after a UV dose corresponding to maximum absorbance. This color persisted under prolonged irradiation if only traces of oxygen were present, but was bleached if the solution was saturated with oxygen. If higher initial concentrations (~ 0.01 M) of phenylalanine are used, the solutions become intensely pigmented, while the turbidity slowly increases (as previously observed by Spiegel-Adolf, 1937), and if the oxygen concentration is low, a brown precipitate eventually settles out of the solution and accumulates at the bottom of the reaction vessel under prolonged irradiation. In oxygen saturated solutions no such precipitate is formed.

In order to determine the chemical nature of the insoluble pigment, the photochemical processes underlying its synthesis and the extent to which these reactions, as well as other possible, independent reactions, are correlated with the optical density changes occurring in UV-irradiated phenylalanine solutions, a detailed, quantitative investigation of DL-phenylalanine* photochemistry was undertaken using experimental techniques considerably more sensitive than those employed in previous work.

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*DL-phenylalanine, rather than the naturally occurring L-isomer, was used since the present investigation was originally motivated by an interest in the possible relationship of the long-term photochemistry of aromatic amino acids to prebiological chemical evolution and the origin of life (see Blois, 1964).

II. SYNTHESIS OF LOW MOLECULAR WEIGHT PHOTOPRODUCTS

A. SPECTROSCOPY OF DL-PHENYLALANINE

In order for a molecule to undergo a photochemical reaction, it is necessary that the molecule absorb photoexcitation energy, either directly from the electromagnetic field incident upon it or from another molecule of the same, or of a different, species which previously absorbed photoexcitation energy from the radiation field. This requirement is known as the First Law of Photochemistry and was originally formulated in more general terms by Grotthus in 1818 and later extensively confirmed experimentally by Draper (1843). In this study, I shall be concerned exclusively with the direct, non-sensitized photochemical transformations of DL-phenylalanine which occur as a result of absorption of ultraviolet radiation by the benzene ring portion of the molecule.

Both benzene and phenylalanine exhibit three UV absorption bands between 1800 Å and 2700 Å (Platt and Klevens, 1947; Wetlaufer, 1962). In Fig. 1 the longest wavelength UV absorption band of DL-phenylalanine in aqueous solution is compared with that of benzene. (The absorption spectrum of DL-alanine in this wavelength region is included for comparison.) It is apparent that although the phenylalanine band has somewhat less sharply defined fine structural peaks and is slightly red-shifted with respect to the benzene band, the two bands are very similar in structure, so that the alanyl side chain has very little effect on the absorption spectrum of phenylalanine for wavelengths greater than 2300 Å. Therefore, the absorption of these longer UV wavelengths by phenylalanine is due to the electronic properties of the benzene ring of the molecule, and not to the electronic properties of the side chain.

Several attempts have been made to account for the three characteristic absorption maxima in the ultraviolet absorption spectrum of benzene at wavelengths greater than 1800 Å on the basis of theoretical wave mechanical calculations. One of the most successful of these is the

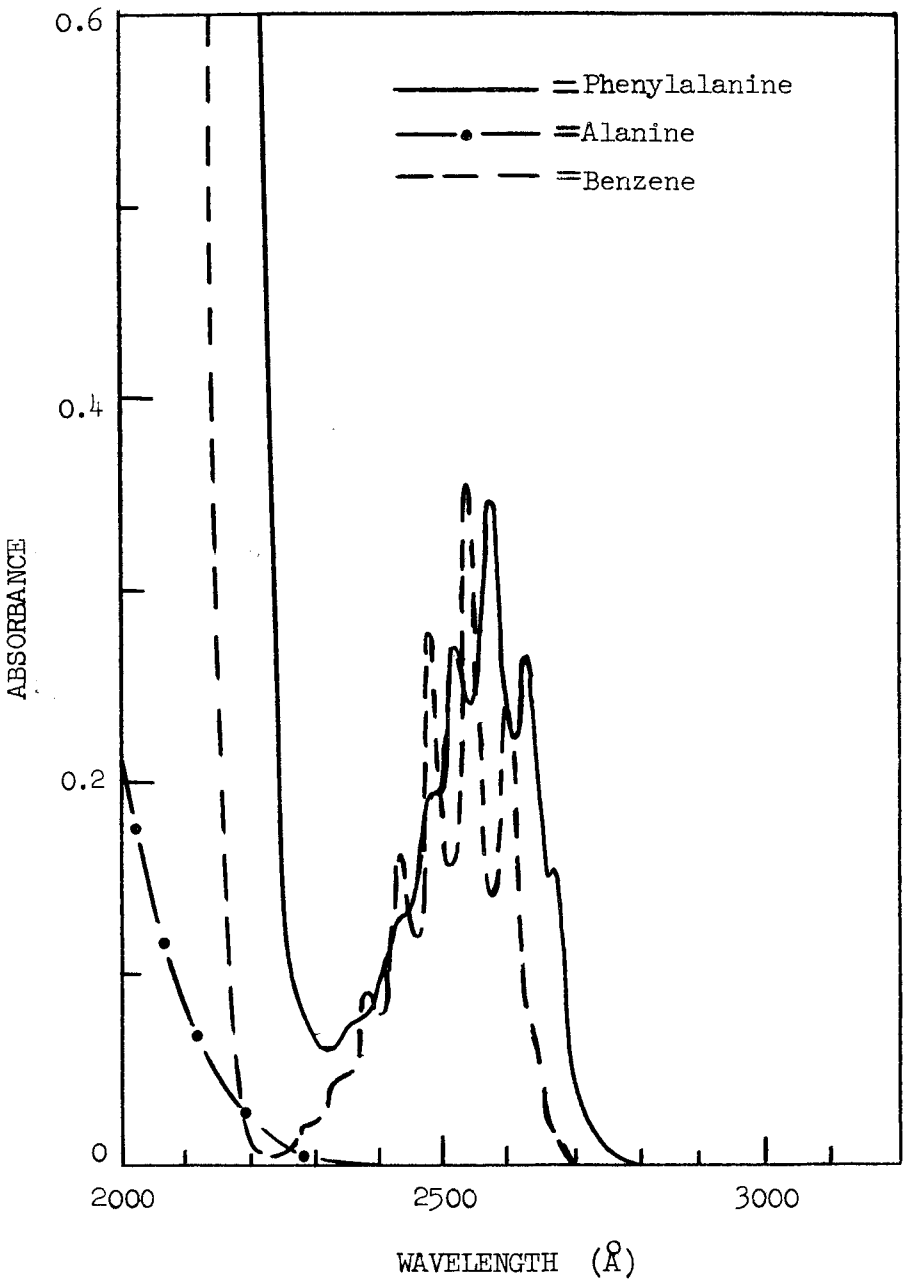

FIG. 1--Structure of longest wavelength UV absorption band of DL-phenylalanine and benzene in dilute aqueous solution (1.75×10^{-3} M). The absorption of an equimolar solution of DL-alanine is included for comparison. Spectra were measured on a Cary model 14M recording spectrophotometer using 1 cm fused silica cuvettes.

work of Goeppert-Mayer and Sklar (1938). By means of the π-electron approximation (Lykos and Parr, 1956) and the method of antisymmetrized products of molecular orbitals (Mulliken, 1933), Goeppert-Mayer and Sklar were able to approximate the energies of the three excited singlet states of benzene. Electronic transitions between the ground state of the molecule and these three singlet excited states correspond to the three experimentally observed UV absorption bands (i.e., λ_{max} = 2550 , 2030, and 1830 Å , Platt and Klevens, 1947). These transitions, together with the corresponding transitions for phenylalanine, are depicted schematically on the energy level diagram shown in Fig. 2. Sklar (1937) assigned the longest wavelength absorption band of benzene (see Fig. 1) to the symmetry-forbidden $^1B_{2u} \leftarrow {}^1A_{1g}$ transition of the π-electron system. The fact that this transition occurs at all is due to the existence of a non-totally symmetric vibrational mode of the benzene ring (ibid.). The fine structural peaks of the absorption spectra shown in Fig. 1 correspond to a progression of vibrational states consisting of the asymmetric mode plus successive levels of a totally symmetric mode (Sandorfy, 1964). However, the molecular symmetry of the benzene ring is only slightly diminished in these vibrational states, so that the intensity of the absorption band is quite low (molar extinction coefficient = 200 - 220, see Fig. 1 and Platt and Klevens, 1947).

If phenylalanine is illuminated with ultraviolet light of wavelengths less than about 2300 Å (see Fig. 1), several types of electronic transitions may take place, and the photoexcitation energy may not be initially localized in the benzene ring, as is the case with 2475-2675 Å radiation. In particular, if wavelengths near 2000 Å are present in the illuminating beam, the symmetry-forbidden, intermediate energy singlet-singlet transition of the ring π-electron system (λ_{max} = 2030 Å , see Fig. 2) will be excited. If 1849 Å radiation is present in the illuminating beam (as is frequently the case in experiments using unfiltered mercury resonance radiation; Luse and McLaren, 1963; Smith, 1964), then the transition from the ground state to the highest singlet excited state of the benzene ring π-electron system (λ_{max} = 1830 Å ; Jaffé and Orchin, 1962; see Fig. 2) will be induced. Since the π-electron system of the carboxyl group absorbs maximally near 2000 Å (ibid.), it is

FIG. 2--Energy level diagram showing the π-electron transitions of phenylalanine and benzene. The thick arrow represents the transition of primary interest in the present investigation. References: (1) Wavelengths and molar extinction coefficients for far UV absorption bands of phenylalanine - Wetlaufer (1962). (2) Wavelengths and molar extinction coefficients for far UV benzene absorption bands – Platt and Klevens (1947). (3) Selection rules for benzene transitions – Sandorfy (1964). (4) Group theoretical assignments for benzene energy levels – Goeppert-Mayer and Sklar (1938).

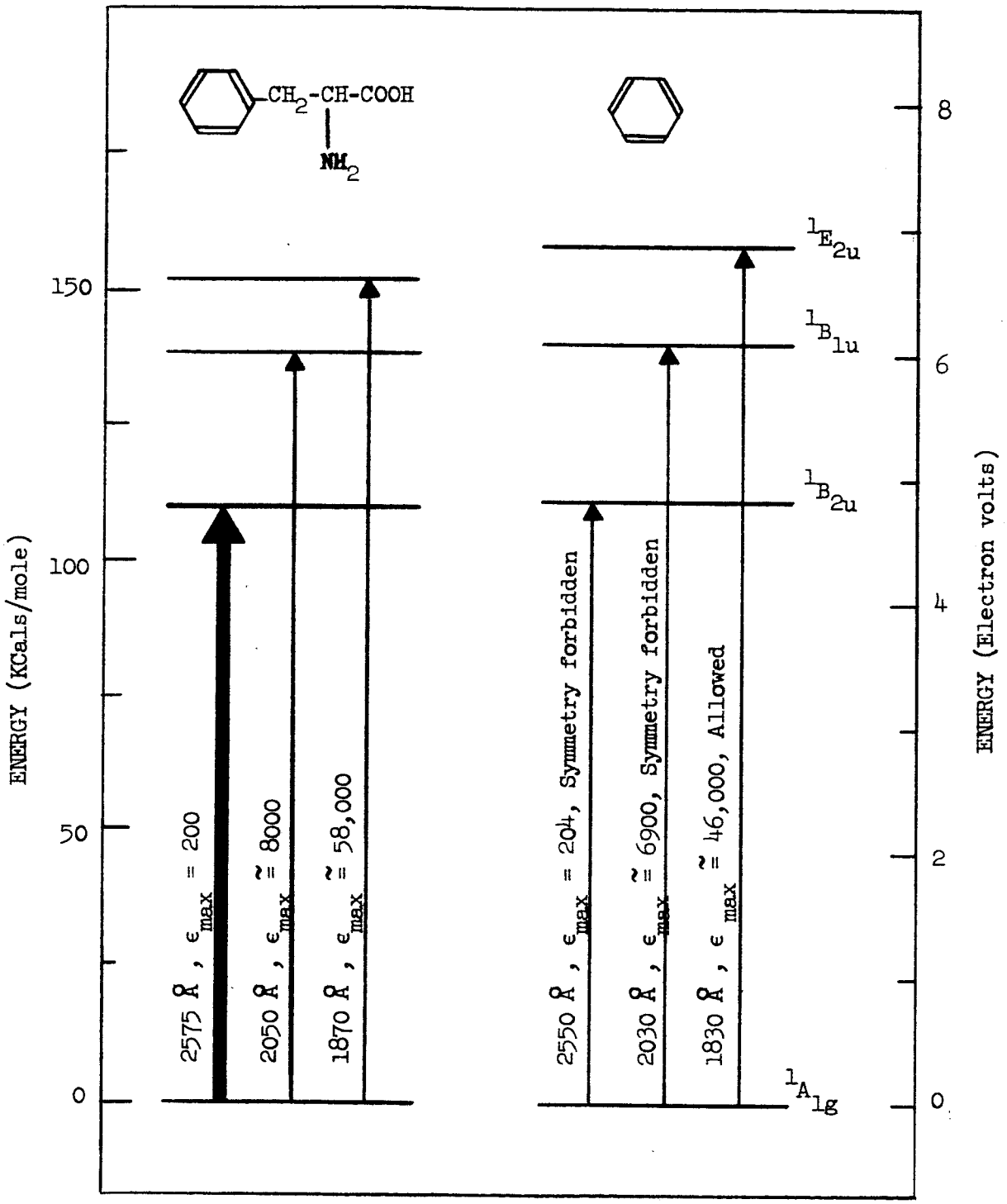
CH2-CH-COOH
NH2
ENERGY (KCals/mole)
ENERGY (Electron volts)
150
100
50
0
8
6
4
2
0
1E2u
1B1u
1B2u
1A1g
2575 Å , εmax = 200
2050 Å , εmax ≈ 8000
1870 Å , εmax ≈ 58,000
2550 Å , εmax = 204, Symmetry forbidden
2030 Å , εmax ≈ 6900, Symmetry forbidden
1830 Å , εmax ≈ 46,000, Allowed

FIGURE 2

likely that it will absorb some 1849 Å radiation. Also, excitation of σ-electrons will occur both in the ring and in the side chain (giving rise to the typical "end absorption" of saturated hydrocarbons; see Jaffé and Orchin (1962), p. 173), with the result that carbon-carbon bonds may be cleaved with high probability at the sites of photon absorption since the energy of an 1849 Å photon (i.e., 153.6 Kcals/mole) far exceeds the dissociation energies of these bonds in phenylalanine (30 - 85 Kcals/mole; see Table 4).

Of even greater significance than high energy electronic transitions in phenylalanine is the photolysis of water molecules to H atoms and OH. radicals at 1849 Å . The quantum efficiency for this process has been shown to be as high as 0.6 (Barrett and Baxendale, 1960), so that the reactions which phenylalanine undergoes and the products which accumulate in dilute aqueous solutions illuminated with 1849 Å mercury resonance radiation may be largely determined by secondary processes involving free radical attacks on phenylalanine. If molecular oxygen is present in the system, the number of possible secondary reactions is very large, since several types of complex free radical chain processes can take place involving H. , OH. , and HO_2. radicals, H^+ , OH^- , and HO_2^- ions and the O_2^- radical ion, as well as H_2 , O_2 , H_2O_2 and H_2O (for a general review of these processes, see Uri, 1952). Therefore, if short wavelength UV radiation is present in the illuminating beam, determination of photochemical reaction mechanisms is exceedingly difficult, since such radiation interacts with the system in a non-specific way (i.e., electronic transitions are promoted in nearly all the chemical bonds in the system, including those of the solvent molecules). Therefore, in order to be reasonably assured of at least some success in the elucidation of photochemical reaction mechanisms, it is necessary to limit the interaction of the radiation field with phenylalanine to one electronic transition. This requirement can be met by irradiating with wavelengths from 2475 - 2675 Å such that only the $^1B_{2u} \leftarrow {}^1A_{1g}$ transition of the benzene ring is excited (see Fig. 1), special precautions being taken to filter out all stray radiation of $\lambda < 2300$ Å . The irradiation apparatus designed and constructed for this purpose will be described below in detail.

B. GENERAL EXPERIMENTAL PROCEDURES

The apparatus which was used routinely for monochromatic UV irradiation of DL-phenylalanine solutions is shown schematically in Fig. 3. The light source is a 900 W, high pressure, dc xenon arc (Hanovia No. 538 C 9, ⑤ in Fig. 3). This source, together with a Bausch and Lomb grating monochromator, model No. 33-86-45 (500 mm grating; ⑪ in Fig. 3) provided intensities of monochromatic radiation at 2575 ± 100 Å up to 7000 ergs/cm^2/sec at a distance of several millimeters from the exit slit. Stray light of wavelengths less than 2400 Å is filtered out of the illuminating system by several Vycor filters (⑩) mounted on the window of the arc housing (See Appendix A).

Interference filters calibrated by Optics Technology, Inc. were used in conjunction with a 1P28 photomultiplier tube to calibrate the wavelength dial of the monochromator. This was achieved by mounting the interference filters in front of, and the photomultiplier tube behind, a small aperture centered on the rear wall of the light tight irradiation chamber, the sample mounting block having been moved out of the beam (see Fig. 3, ⑲ and ⑳).

Solutions are irradiated in Cary fused silica spectrophotometer cells (⑭) mounted in a specially machined slot in a copper cooling block (⑮) as shown in Fig. 4. The copper block is mounted inside a light tight chamber (⑱) and aligned such that the sample cell is directly behind a circular aperture on the front wall of the chamber and centered on the exit slit of the monochromator. Thermal contact between the sample cell and the copper block is provided by a thin layer of steel wool. Rapid circulation of cold water through the copper block by means of an external reservoir and pump system (⑰) provides temperature control of the sample cell to within ± 1°C during irradiation. The temperature of the sample cell is followed continuously during an irradiation experiment by means of a copper-constantin thermocouple, the test junction of which is inserted between the cell and the layer of steel wool (Fig. 4).

Prior to exposure of the sample, the absolute intensity distribution within the illuminating beam at the exit slit of the monochromator was

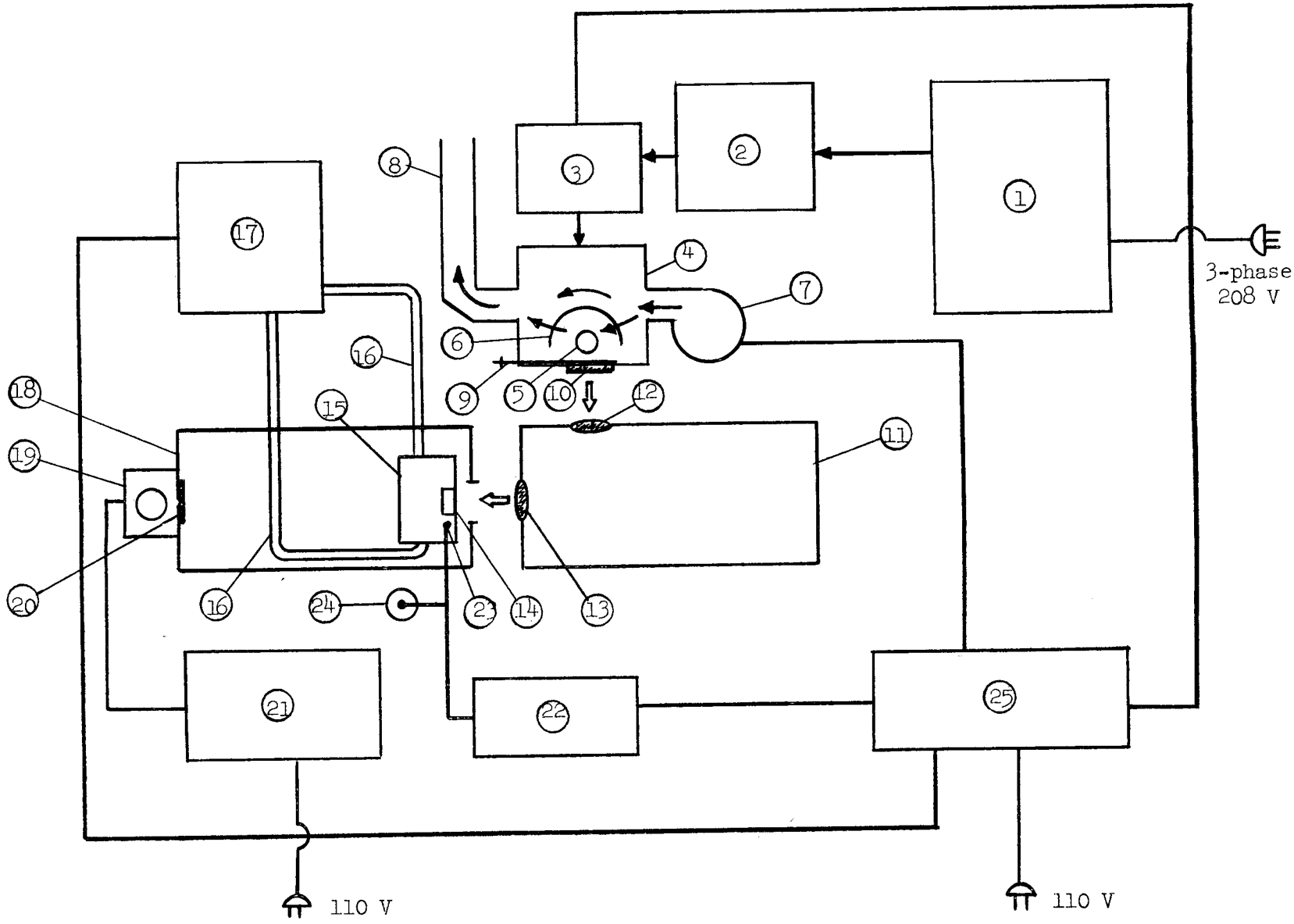

FIG. 3--Schematic diagram of xenon arc irradiation apparatus. See following page for key to components. The major functional components are discussed in detail in the text.

Components of Xenon Arc Irradiation Apparatus (Figure 3)

1. DC power supply, 3-phase 208 volt input, 3 kilovolt-amperes maximum output.
2. DC ballast, 0-3 Ω , Hanovia No. 29143.
3. Radiofrequency starter, Hanovia No. 29009.
4. Arc housing, material: 1/4 inch aluminum plate.
5. DC xenon arc, 900 watts, Hanovia No. 538 C 9.
6. Stainless steel hemispherical reflector.
7. Blower, Dayton No. 2C781.
8. Ozone exhaust system.
9. Shutter assembly.
10. Vycor plates, O.D. > 1.5 at 2300 Å (see Appendix A).
11. Grating monochromater, Bausch and Lomb No. 33-86-45.
12. Monochromator entrance slit and quartz collimating lens.
13. Monochromator exit slit and quartz collimating lens.
14. Fused silica sample cuvette, 1 cm. path length, Applied Physics Corp.
15. Copper temperature control block (see Fig. 4).
16. Water cooling lines.
17. Water cooling and circulating system.
18. Irradiation chamber, material: 1/4 - inch aluminum plate.
19. Photomultiplier tube, RCA No. 1P28.
20. Interference filters, Optics Technology, Inc. No. 1063.
21. Amplifier, Photovolt Corp., No. 520M.
22. Millivolt-microvoltmeter, Keithly Instruments, No. 149.
23. Copper-constantin thermocouple, test junction.
24. Thermocouple reference junction, T = 0°C.
25. Switch box.

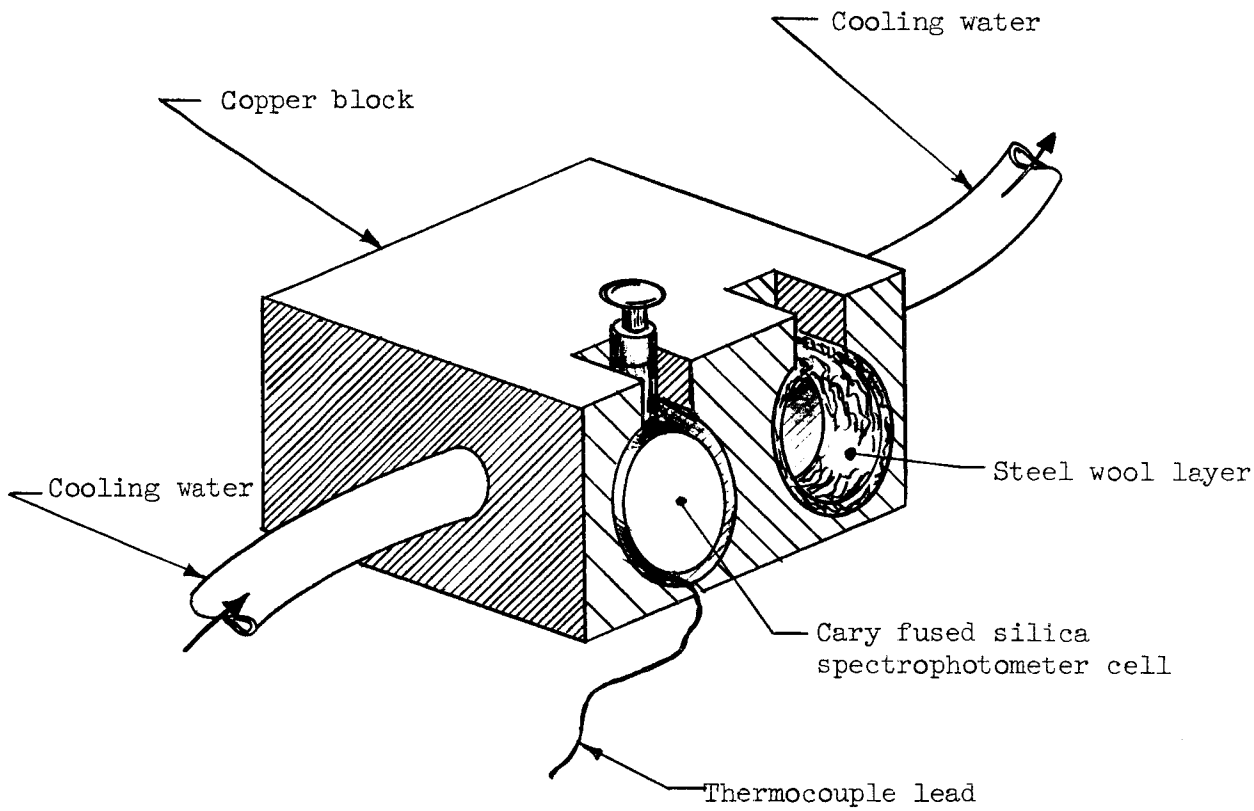

FIG. 4--Copper cooling block containing machined slots for fused silica sample cells. Samples were irradiated either singly at the exit slit of the monochromator (see Fig. 3), or in pairs with the full white light output of the xenon arc. In the former case, temperature regulation to within 1° C was achieved by circulating tap-water-cooled distilled water through the block at the rate of 1 gallon/min.

determined by horizontal integration (Leighton and Forbes, 1930) at 1 mm intervals with a bismuth-silver 12-junction surface type thermopile having a 1 mm CaF_2 window (The Eppley Laboratory, Inc., Serial No. 4963). The monochromator wavelength dial was adjusted so that maximum intensity occurred at 2575 Å. Both the entrance and exit slits of the monochromator were set at 12.4 mm so that the illuminating beam contained wavelengths from 2475 – 2675 Å at the half-power points. The xenon arc was typically operated at or near 27 amps, 30 volts (810 watts).

The integration procedure consisted in taking thermopile readings, with the light source shutter ((9) in Fig. 3) both open and closed, at 1 mm intervals spaced horizontally across the illuminating beam. The thermopile was aligned in the beam such that the receiving strip (1.5 × 16 mm) was perpendicular to the beam, centered vertically, and at a distance from the plane of the exit slit at which the width of the beam was only slightly smaller than it was at the front window of the sample cell during irradiation. A Keithly, model 149, millivolt-microvolt meter was used to measure the thermoelectric potential of the thermopile at each position. The meter was zeroed with the shutter closed prior to each reading. Vertical variations in the intensity of the beam are automatically averaged in this procedure, as shown by Leighton and Forbes (ibid.). Furthermore, at the position of the receiving strip, the height of the beam (14 mm) is comparable to the length of the strip (16 mm). However, since the sample cells are cylindrical and the cross-section of the beam is rectangular (12.4 mm wide by 20.8 mm high at the plane of the exit slit converging over a distance of 1.5 cm to 10.9 × 17.3 mm at the front window of the cell), it is necessary to take into account the area of each 1 mm-wide vertical strip of the cell window in order to calculate the average light intensity incident on the sample cell. That is, the average intensity, $\overline{I_0}$, over the circular window of the sample cell is given by

$$\overline{I_0} = (k/A) \sum_{i=1}^{i=D} r_i \, a_i \quad ,$$

where A is the area of the sample cell window ($A = 3.30\ cm^2$), D is the diameter in mm of the sample cell ($D = 20.5$ mm), r_i is the thermopile reading in microvolts at the i-th position (i.e., $r_i = 0.1 - 130$ μvolts), k is a constant which converts this reading to average light intensity over the receiving strip in ergs/sec/cm^2 ($k = 169.5$ ergs/cm^2/sec/μvolt), and a_i is the area in cm^2 of the i-th vertical strip of the sample cell window.

Contributions from stray light of $\lambda > 3000$ Å were corrected for by repeating the integration procedure with a 3000 Å-cutoff filter (see Appendix A) mounted at the source window in place of the stack of Vycor plates (⑩ in Fig. 3). This correction amounted to only 6-7% of the total intensity. A correction was also made for the reflectivity at 2575 Å (0.037)* of the front fused silica window of the sample cell. Since the horizontal beam convergence was only 1 mm/cm, it was neglected in the calculations. The beam was integrated in the above manner 3-5 times during a typical quantitative irradiation experiment. The variation between individual intensity determinations typically did not exceed 5%, and in no case did it exceed 10%. No systematic decline in intensity was observed during the runs. This observation is consistent with the rated lifetime of the type of dc xenon arc used in the present investigation (i.e., 1000 hrs; see Hanovia Form EH-274). The average value (typically 6000-7000 ergs/cm^2/sec) of the incident intensity during the run was used in the quantum yield calculations to be discussed below.

C. PHOTOPRODUCT DETECTION AND IDENTIFICATION

1. Materials and Methods

Samples of DL-phenylalanine labelled with C^{14} at the carboxyl carbon or at the side chain carbon adjacent to the benzene ring, or uniformly in the ring itself, along with several C^{14}-labeled aliphatic amino acids, were obtained from New England Nuclear Corp., Boston, Mass. DL-phenylalanine

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*The reflectivity, r , was determined by measuring the optical density of an empty sample cell on the Cary spectrometer with air only in the reference beam. Since the sample cell has two identical windows, $O.D. = \log I_0/(1-r)^2 I_0 = -2\log(1-r)$. The measured O.D. was 0.033.

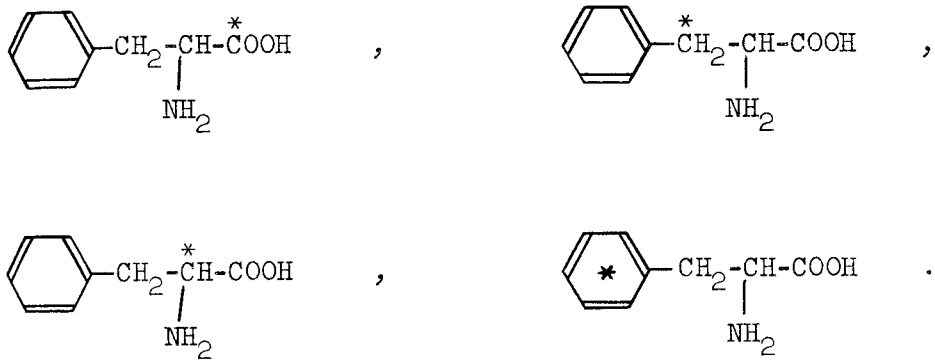
labelled with C^{14} at the alpha carbon and unlabelled DL-phenylalanine used for adjusting concentration, were obtained from Calbiochemical Corp., Los Angeles, Calif. Nuclear Chicago Corp., Chicago, Ill., supplied C^{14}-labelled benzoic acid. Unlabelled phenyllactic acid and a number of other aromatic and aliphatic compounds used as chromatographic standards were obtained from K & K Laboratories, Inc., Plainview, New York. Samples of unlabelled aminomalonic acid were kindly provided by Professor Alton Meister of Tufts University and Professor Joseph Fruton of Yale University.

Typically, a solution of DL-phenylalanine was made up in glass distilled water (Fe content < 0.2 ppm as determined by the ortho-phenanthroline method; the analysis was performed by L. Taskovich of the Stanford Biophysics Laboratory) to a concentration of $5\text{-}8 \times 10^{-4}$ M. The phenylalanine samples were labelled with C^{14}, either uniformly in the benzene ring or at one of the three carbons of the side chain, at a specific activity of 2-5 $\mu c/\mu$ mole. The pH was adjusted with KOH either to a value near 7 or to an alkaline value near 11, and the solution introduced into a Cary fused silica sample cell (light path = 1 cm).

The initial concentration of phenylalanine was estimated by means of an optical density measurement at 2575 Å ($\epsilon = 200$) . Prior to irradiation, several 20-30 microliter samples of the reaction mixture were spotted at the origins of Whatmann No. 1 paper chromatogram strips. The sample cell was then mounted in the copper cooling block (Fig. 4) and aligned at the exit slit of the monochromator. After 5-10 hrs. of monochromatic UV irradiation at 2575 ± 100 Å , 20-microliter samples were again withdrawn and spotted on separate paper strips. This process was repeated after an additional 5-10 hrs. of irradiation. The irradiated and unirradiated samples were then chromatographed using the organic phase of n-BuOH: HAc: H_2O , 4:1:5 , v/v/v (see Block, Durrum, and Zweig, 1958, Chapter V) as developer. After developing out to 10 inches, the strips were dried overnight. The distribution of radioactivity on the chromatograms was determined by means of a Vanguard, model 880, recording paper chromatogram scanner. Additional samples of the irradiated reaction mixtures were chromatographed together with standard unlabelled compounds in several other solvents systems, as discussed in detail in the Results section.

2. Results

For purposes of clarity in the discussion which follows, the sites at which phenylalanine was labelled with C^{14} in separate irradiation experiments are indicated by asterisks in the formulae shown below:

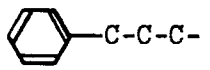
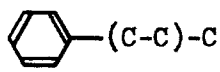
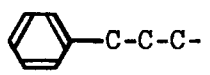
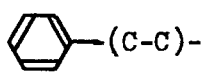
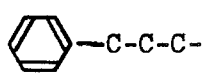
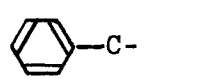


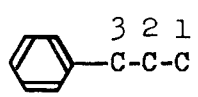
Radiochromatographic data obtained in four separate irradiation experiments using these compounds are shown in Fig. 5, together with a control scan for unirradiated, carboxyl-labelled phenylalanine.

The photoproduct peaks shown in Fig. 5 are numbered consecutively from 1 to 8 in the order of increasing R_f value. By combining the radiochromatographic data shown in scans (b) through (d), it is possible to determine which phenylalanine carbons are incorporated into each photoproduct. For example, photoproduct No. 2 occurs in scans (b) and (c), but not in (d) or (e), showing that the carboxyl carbon and the alpha carbon of phenylalanine are incorporated into this photoproduct. In the case of photoproduct No. 8, the peak occurs only in scans (d) and (e), indicating that this photoproduct contains the side chain carbon adjacent to the benzene ring of phenylalanine, and all or part of the ring itself. On the basis of this type of argument, it was possible to construct hypothetical minimum carbon skeletons for all of the photoproducts. The results are summarized in Table 1. It should be emphasized that these structures represent only the smallest carbon skeletons consistent with the labelling data and that the actual photoproducts may be larger. For example, a given photoproduct may contain carboxyl carbons derived from more than one phneylalanine molecule, or rings derived from many pnenylalanine molecules. For the sake of simplicity, it was assumed

TABLE 1

INCORPORATION OF PHENYLALANINE CARBON ATOMS INTO PHOTOPRODUCTS

Photoproduct	Phenylalanine Carbon Atoms Incorporated[+]	Hypothetical Minimum Carbon Skeleton
1.	1, 2, 3, φ*	
2.	1, 2	-C-C-
3.	1, 2, 3	-C-C-C-
4.	1, (2)≠, (3), φ	
5.	1, 2, 3, φ	
6.	(2), (3), φ	
7.	1, 2, 3, φ	
8.	3, φ	

+ The side chain carbons are numbered as follows: 

* φ = benzene ring

≠ Parentheses indicate uncertainty in the radiochromatographic data (see Fig. 5).

FIG. 5--Paper chromatographic detection of photoproducts of DL-phenylalanine. Scan (a) shows the distribution of C^{14} activity on a chromatogram of unirradiated carboxyl-carbon labelled phenylalanine. The distributions of C^{14} activity in scans (b) - (e) correspond to separate UV irradiation experiments in which phenylalanine was labelled with C^{14} at different specific sites (indicated by asterisks). The chromatograms were developed in the organic phase of n-BuOH: HAc: H_2O , 4 : 1 : 5 (v/v/v) . Samples were irradiated in dilute aqueous solution ($6 - 8 \times 10^{-4}$ M) in the presence of dissolved molecular oxygen. Other experimental conditions were as follows: Wavelength, 2575 ± 100 Å ; incident intensity, 6000 - 7000 ergs/cm^2/sec ; temperature, 20-25°C ; irradiation time, 10-20 hrs. The photoproduct peaks are numbered for purposes of reference (see text).

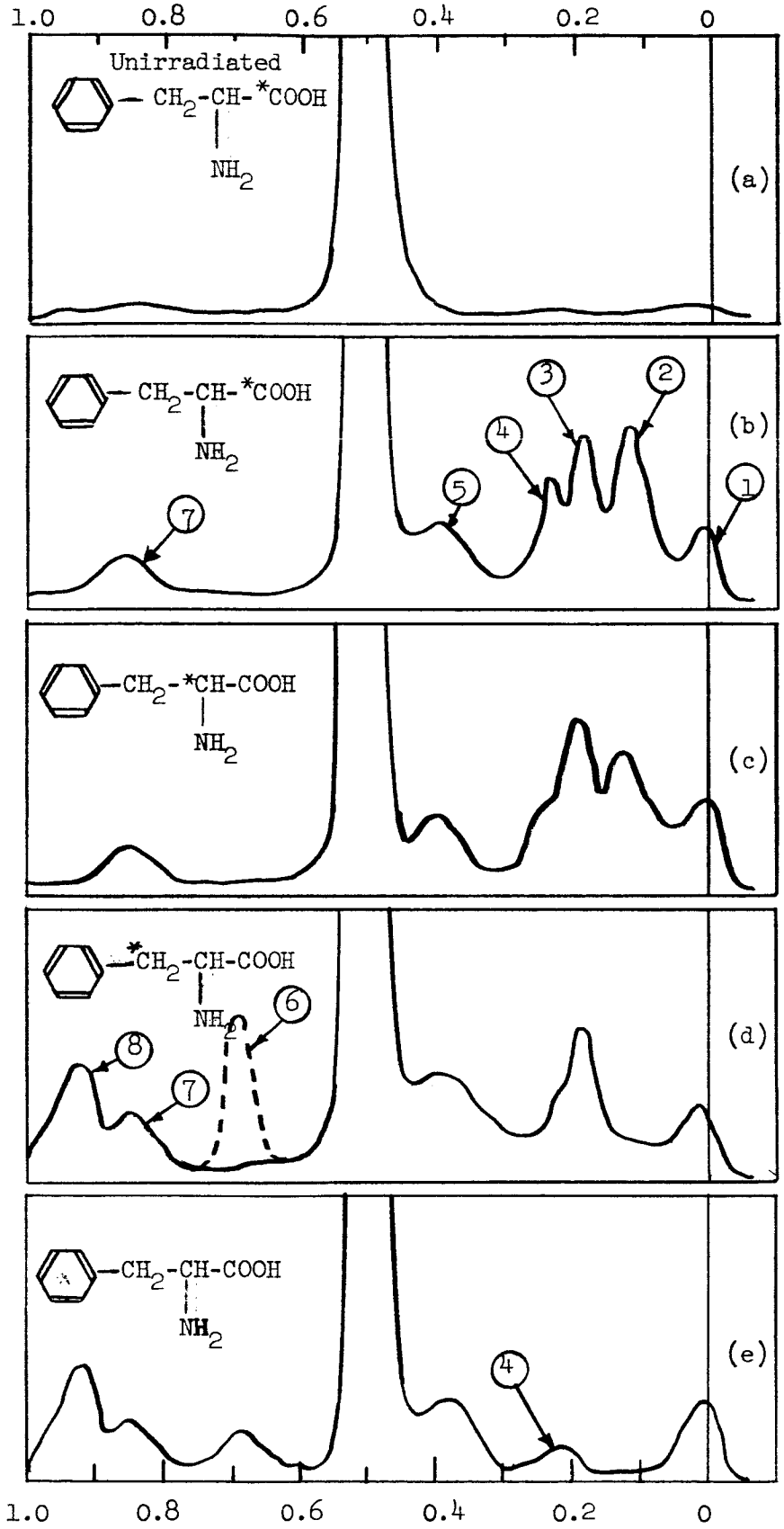

1.0
0.8
0.6
0.4
0.2
0
Unirradiated
— CH2-CH-*COOH
NH2
(a)
(b)
(c)
(d)
(e)
1
2
3
4
5
6
7
8
1.0
0.8
0.6
0.4
0.2
0
Rf Value

FIGURE 5

that in the case of the photoproduct peaks occurring in scan (e), the carbon skeleton of the benzene ring remained intact. It will be shown below that this assumption turned out to be correct.

Using standard unlabelled compounds suggested by labelling data of the type shown in Table 1, it was possible to identify the photoproducts by chromatographing samples of the irradiated solutions in several different solvent systems. Typically, several unlabelled compounds were spotted at the origins of paper chromatogram strips directly over previously applied samples of an irradiated solution in which phenylalanine had been labelled at some specific site. This spotting technique was used in order to avoid uncertainty due to small variations in R_f values from strip to strip. (These variations were particularly significant in the identification of photoproducts 2 and 3.) The chromatograms were then developed separately in different solvent systems, dried, scanned on the Vanguard strip scanner, and finally sprayed with an appropriate color reagent to reveal the chromatographic locations of the unlabelled compounds. The distribution of C^{14} activity was then compared with the positions of the color spots corresponding to the standard compounds to determine if any of the photoproduct peaks coincided with any of the color spots.

Identification of a given C^{14} peak was based upon coincidence of the peak, in several different solvent systems,* with the color spot for a given standard compound and upon consistency with the labelling data shown in Table 1. Due to the low concentrations of the photoproducts (i.e., ≤ 50 μ Molar, see Figs. 13 and 14), and since 20 λ samples of the reaction mixtures were spotted on the chromatograms, the amount of material in the photoproduct peaks were too small (0.15 μg for a molecular weight of 150) for elution and subsequent characterization by non-chromatographic analytic techniques such as absorption spectroscopy. Two-dimensional paper chromatography was impracticable due to the low photoproduct C^{14} activities (typically 1000-2000 counts/min, see Fig. 13)

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*The location on chromatograms developed in different solvent systems, of the C^{14} peaks corresponding to a given photoproduct was facilitated by the relative height of the given peak with respect to the other peaks and by its occurrence or non-occurrence, depending upon the site at which phenylalanine was labeled (see Figs. 7 and 8).

and the consequent difficulty in locating the photoproduct peaks for subsequent scanning on the Vanguard strip scanner. Photoproducts identified in the above manner, together with the solvent systems used, are listed in Table 2a. The corresponding structures are shown in Table 2b. The R_f values determined in the present investigation for the photoproducts and for a number of substances eliminated on R_f grounds are summarized in Table 3.

One possible limitation of the above technique of photoproduct identification is that a given C^{14} peak might be due to two or more different photoproducts having nearly identical R_f values. However, the following facts suggested that superposition of photoproducts was not a serious limitation in the present study. (1) The number of photoproduct peaks and the sequence of relative peak heights were identical in solvent systems A and B (see Table 2a, footnote) even though the R_f values were considerably higher in the latter solvent system (see Table 3). Chromatograms were developed to a distance of 10 inches in solvent system A and to a distance of 13-14 inches in solvent system B. (2) No resolution of any photoproduct peak was observed during prolonged development in solvent system D (Table 2a, footnote). By allowing the solvent to drip off the end of the chromatogram strip, development was carried out to an equivalent distance of 15-20 inches in some cases. Therefore, more elaborate chromatographic procedures, such as cutting out the area corresponding to a particular C^{14} peak, attaching this area to a new strip, and redeveloping in different solvent systems to test for possible resolution of the peak, were not used in the present study.

Photoproduct No. 1 was tentatively identified as a melanic polymer on solubility grounds. That is, in every solvent system used, this substance did not move from the origin of the chromatogram (Table 3), and on these grounds is considered to be identical to the pigmented, insoluble material observed in the preliminary experiments of Blois (1964). Several lines of evidence based on physical and photochemical studies of this insoluble material confirm this identification and will be discussed in detail in Chapter III.

TABLE 2a

PHOTOPRODUCT IDENTIFICATION

Photoproduct*	Chromatographic Solvent Systems+	Identification
1.	A, B, C, D, E	Melanic polymer
2.	A, B	Aminomalonic acid
3.	A, B, D	Aspartic acid
4.	A, B	DOPA
5.	A, B, C	Tyrosine
6.	A	β-Phenylethylamine
7.	A, B, F	Phenyllactic acid
8.	A, B, F	Benzoic acid

* Numbering system same as that used in Fig. 5 and Table 1.

\+ A. n-Butanol: acetic acid: water, 4:1:5, (v/v/v)

B. sec-Butanol: tert-butanol: water 4:1:3, (v/v/v) in 6N HCl atmosphere

C. 80% Aqueous Pyridine

D. 88% Aqueous Phenol

E. 77% Ethanol

F. Benzene: acetic acid: water, 2:2:1 (v/v/v).

For a general discussion of the use of these and many other solvent systems, see Block, Durrum, and Zweig (1958).

TABLE 2b

PHOTOPRODUCT STRUCTURES

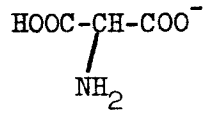
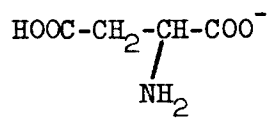
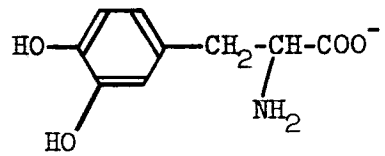
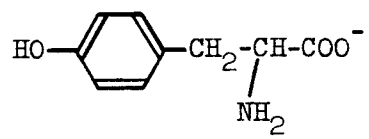
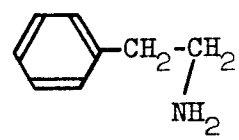
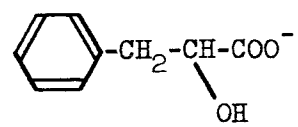
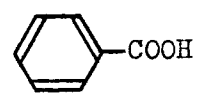
Photoproduct	Structure
Melanic polymer	Probable partial structure shown in Fig. 29.
Aminomalonic acid	
Aspartic acid	
DOPA	
Tyrosine	
β-Phenylethylamine	
Phenyllactic acid	
Benzoic acid	

TABLE 3

R_f VALUES

Photoproducts Identified	SOLVENT SYSTEMS*					
	A	B	C	D	E	F
Melanic polymer	0	0	0	0	0	0
Aminomalonic acid	0.13	0.29				0
Aspartic acid	0.19	0.37		0.07	0.18	0
DOPA	0.23	0.40				0
Tyrosine	0.39	0.66	0.87	0.81	0.48	0
PHENYLALANINE	0.51	0.75	0.74	0.95-1.0	0.56	0
β-Phenylethylamine	0.69					
Phenyllactic acid	0.85	0.90				0.93
Benzoic acid	0.92	0.96				0.81
Compounds eliminated on R_f grounds						
Glycine	0.19	0.47		0.32	0.40	
Serine	0.19	0.42		0.25	0.31	
Alanine	0.29	0.55				
Asparagine	0.15					
Oxamic acid	0.15-0.20					
Glycolic acid	0.65					
Oxalic acid	0.71-0.76					
Tyramine	0.48					
3-OH-tyramine	0.45					

*The solvent systems are listed in a footnote to Table 2a.

The identification of aminomalonic acid, which is a three-carbon compound, in UV-irradiated solutions of DL-phenylalanine required the elimination of a number of possible two-carbon compounds since the labelling data given in Table 1 suggested that the photoproduct might have a two-unit carbon skeleton. Since two-carbon compounds which are gases or liquids at room temperature, such as ethane, ethanol, acetic acid, and acetaldehyde, cannot be detected by the techniques of paper chromatography used in these studies because they would evaporate from the solution before spotting or from the paper during development and drying, these substances can be eliminated as possible photoproducts. For the purposes of this discussion, the two-carbon compounds which are stable solids at room temperature will be divided into two categories: those which contain nitrogen and those which do not.

Non-nitrogen containing two-carbon solids, such as oxalic acid and glycolic acid, are easily eliminated on R_f grounds. Substances of this type have high mobility in n-BuOH:HAc:H_2O 4:1:5, typical R_f values lying between 0.65 and 0.80 (see Table 3), whereas photoproduct No. 2 has a much lower mobility in this solvent system (R_f = 0.13, see Fig. 5).

The nitrogen containing two-carbon compounds which are stable solids at room temperature are acetamide, glycolamide, oxamic acid, and glycine. Due to the hydrophilic properties of the nitrogen, these substances have much lower mobilities in n-BuOH:HAc:H_2O than similar compounds lacking the nitrogen, and, in fact, in the case of oxamic acid and glycine, the R_f values (0.15 - 0.2 and 0.19, respectively) are close to that of photoproduct No. 2 (R_f = 0.13) . However, the resolution of the coincidence method described above is sufficiently high so that these compounds can easily be distinguished from the photoproduct. Acetamide and glycolamide were not tested as possible photoproducts due to the lack of specific color reagents for these compounds.

The clue that photoproduct No. 2 might actually be a three-carbon rather than a two-carbon compound was provided by the identification of photoproduct No. 3 as aspartic acid (see below and Fig. 7). The radiochromatographic scan data shown in Fig. 5 indicate that photoproduct No. 3 contains the three carbons of the phenylalanine side chain, but no

carbon from the benzene ring.* This raises the possibility that the fourth carbon of aspartic acid, i.e., the second carboxyl group of the molecule (Table 2b), might be derived from the carboxyl group of a second phenylalanine molecule. If this were the case, then photoproduct No. 2 might be formed by a similar process and might actually be a dicarboxylic acid. This hypothesis was confirmed by the identification of photoproduct No. 2 as aminomalonic acid. The paper chromatographic data upon which this identification is based is summarized in Fig. 6, in which the distribution of C^{14} activity on a developed and dried chromatogram of a sample of an irradiated solution in which phenylalanine was labeled with C^{14} at the carboxyl carbon is compared with the locations of color spots due to various standard compounds. Oxamic acid and glycolic acid were located by means of the pH indicator dye bromcresol green (pH 7.0) (Block, Durrum, and Zweig, 1958, p.217), which was sprayed on the chromatogram after extensive steam distillation of the latter to remove residual solvent-system acetic acid which also reacts with bromcresol green. The amino acids were detected by means of the ninhydrin reagent. The photoproduct itself was in too low a concentration to produce a visible spot with either of these color reagents. Since asparagine has been claimed as a photoproduct of phenylalanine (Tominaga, 1958), and since it could conceivably be synthesized by the coupling and subsequent rearrangement of two two-carbon fragments derived from side-chain carbons 1 and 2 of phenylalanine, the chromatographic basis of its elimination as a possible photoproduct is included in Fig. 6.

The chromatographic basis of the identification of photoproduct No. 3 as aspartic acid is shown in Fig. 7. A sample of an irradiated solution in which phenylalanine was labelled with C^{14} in the side chain at the three-carbon (the numbering scheme for side chain carbons is given in a footnote to Table 1) was chromatographed in n-butanol:acetic acid: water , 4:1:5 (v/v/v) , along with unlabelled aspartic acid, serine, and glycine. The strip was scanned for C^{14} activity and then sprayed with

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*It should be pointed out, however, that since the benzene ring is uniformly labelled with C^{14}, the specific activity of the ring carbon adjacent to the side chain, the most likely ring carbon which could conceivably be incorporated into aspartic acid, is only one-sixth the specific activity of the side chain labels in the case of scans (b) - (d).

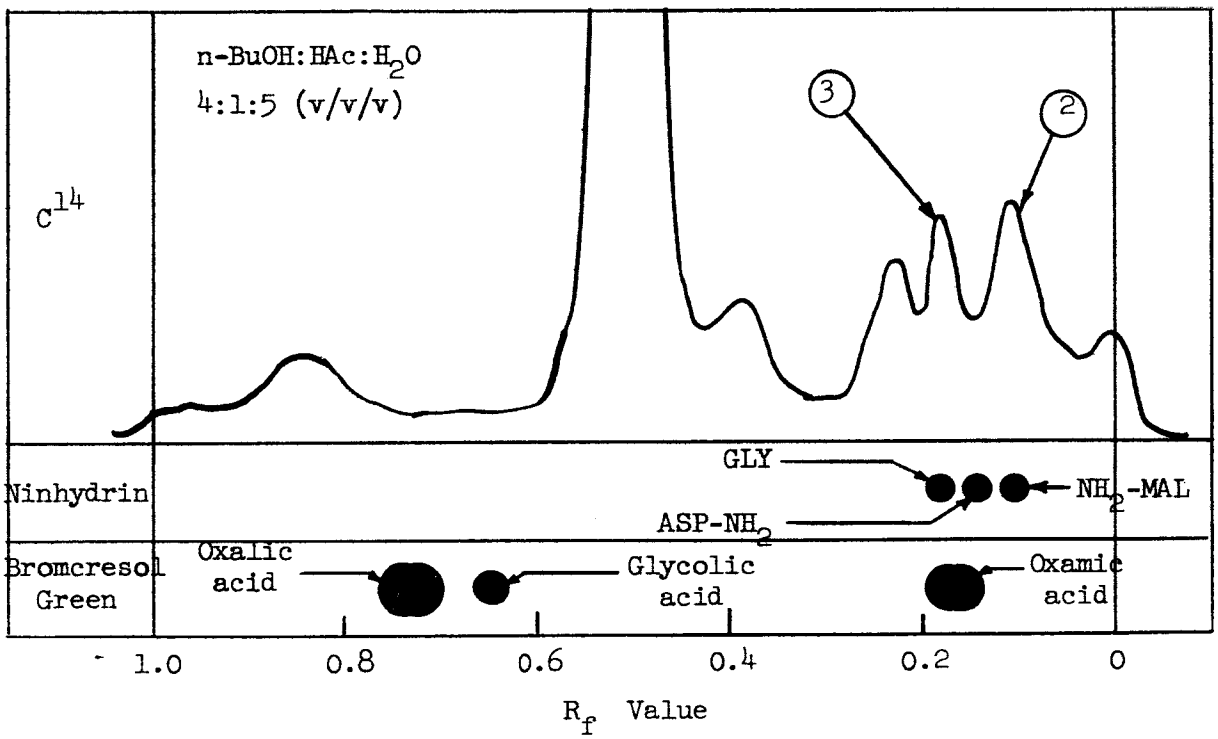

FIG. 6--Paper chromatographic identification of aminomalonic acid as a photoproduct of DL-phenylalanine irradiated in dilute aqueous solution at 2575 ± 100 Å. Other experimental conditions are the same as those given in the legend to Fig. 5. A sample of the reaction mixture was chromatographed together with several standard unlabelled amino and other aliphatic acids. The distribution of C^{14} activity on the chromatogram is compared above with the positions of the ninhydrin or bromcresol green spots of the standard compounds. Photoproduct peak No. 2 coincided with the ninhydrin spot for unlabelled aminomalonic acid. Phenylalanine was labelled with C^{14} at the carboxyl-carbon. Full details are given in the text.

FIG. 7--Paper chromatographic identification of aspartic acid as a photoproduct of DL-phenylalanine in a solution irradiated for 15 hrs. at 2575 Å in the presence of oxygen at pH 7.3. Samples of the reaction mixture were chromatographed together with standard unlabelled amino acids in three different solvent systems. The distributions of C^{14} activity on the chromatograms are compared above with the positions of the ninhydrin spots of the standard amino acids. In all three cases the C^{14} peak suspected of being due to aspartic acid coincides with the ninhydrin spot of unlabelled aspartic acid. Phenylalanine was labelled with C^{14} at the side chain carbon adjacent to the benzene ring. Full details are given in the text.

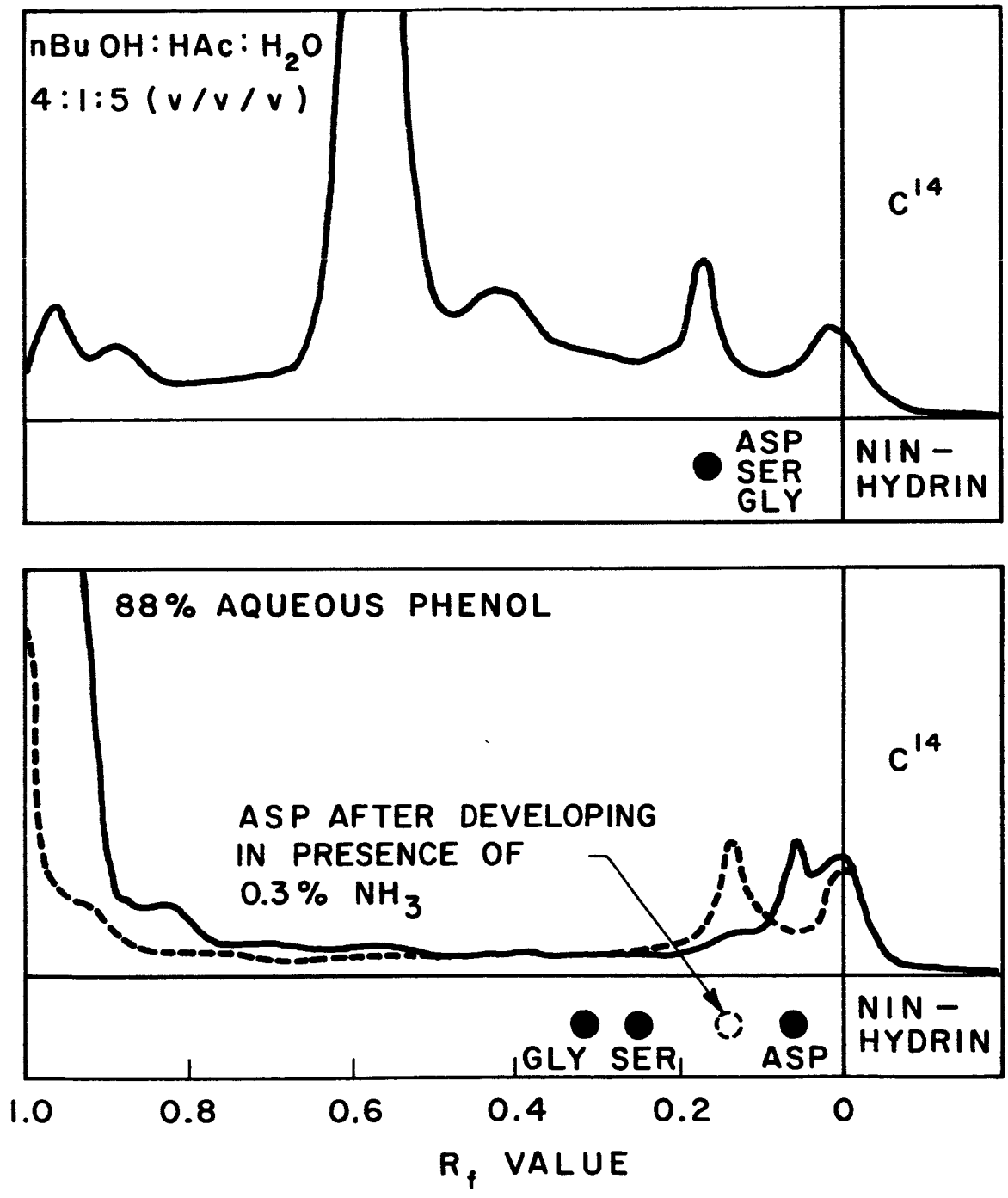
nBu OH : HAc : H_2O
4 : 1 : 5 (v / v / v)
C^{14}
ASP
SER
GLY
NIN – HYDRIN
88% AQUEOUS PHENOL
C^{14}
ASP AFTER DEVELOPING IN PRESENCE OF 0.3% NH_3
GLY SER
ASP
NIN – HYDRIN
1.0
0.8
0.6
0.4
0.2
0
R_f VALUE

FIGURE 7

ninhydrin reagent. The upper diagram in Fig. 7 shows the results. The radioactive peak suspected of being aspartic acid coincided with a single composite ninhydrin spot due to the three unlabelled amino acids. The photoproduct itself was in too low a concentration to produce a visible spot with ninhydrin. A second sample of the reaction mixture was then chromatographed in 88% aqueous phenol (Merck and Co.) together with the same three amino acids. The C^{14} trace (solid curve) is compared with the ninhydrin spots in the lower diagram of Fig. 7. In this case, the three standard amino acids are completely resolved and the radioactive peak coincides with the aspartic acid ninhydrin spot. A third sample of the reaction mixture was then chromatographed with unlabelled aspartic acid in 88% aqueous phenol in the presence of ammonia (0.3% aqueous solution placed at the bottom of the chromatogram chambers; see Block and Weiss, 1956, p. 93; Block, Durrum and Zweig, 1958, p. 153). Again the radioactive peak coincided with the aspartic acid ninhydrin spot as shown by the dotted curve in Fig. 7. Coincidence of the C^{14} peak with aspartic acid was also observed with sec-butanol:tert-butanol:water (ibid.) as solvent system. These results confirm the work of Tominaga (1958).

Serine and alanine have been reported as photoproducts of phenylalanine by Matsuda et al. (1954). The 88% aqueous phenol chromatogram trace reproduced in Fig. 7 shows that serine is not present in our reaction mixtures. This negative result was confirmed in solvent system B (see Table 3). Alanine was eliminated as a photoproduct on R_f grounds in solvents A and B (see Table 3).

Confirmation of the earlier work of Arnow (1942), Schocken (1951), and Luse (1961) is provided by the identification of photoproduct No. 4 as DOPA. This identification is based primarily on coincidence of the ninhydrin spot for DOPA with the C^{14} photoproduct peak in chromatogram scans of the type shown in Fig. 5b, e. Shoulders are present at the chromatographic location of DOPA in scans (c) and (d), although they are not well enough resolved from the aspartic acid peak to allow accurate determination of the degree of coincidence with the ninhydrin spot for DOPA.

Unequivocal identification of tyrosine (photoproduct No. 5) in UV-irradiated phenylalanine solutions posed no special problems since the C^{14} peak for this photoproduct is clearly resolved in several different solvent systems from the C^{14} peaks for both unreacted phenylalanine and the other photoproducts. Furthermore, the R_f value for tyrosine in n-BuOH:HAc:H_2O (i.e., 0.39) is less than that for phenylalanine (0.51), while in 80% aqueous pyridine the R_f value for tyrosine (0.87) is greater than that for phenylalanine (0.74) (see Table 3). Therefore, because of this reversal in R_f values and because the R_f value for tyrosine is considerably different in the two solvent systems, coincidence of the C^{14} photoproduct peak with the ninhydrin spot for tyrosine in both of these solvent systems is particularly strong evidence that the photoproduct is indeed tyrosine. Coincidence of the C^{14} peak with the color spot was also observed in sec.-BuOH:tert-BuOH:H_2O, 4:1:3 (v/v/v) in 6N HCL atmosphere. This identification confirms the previous reports of Arnow (1942), Schocken (1951), Matsuda et al. (1954), and Luse (1961).

Since β-phenylethylamine is a liquid at room temperature, it evaporates from paper chromatograms; but since its boiling point is high (212°C), it evaporates sufficiently slowly at 25°C to allow detection of the substance in UV-irradiated phenylalanine solutions, provided that the time lag between sample spotting and scanning for C^{14} activity is sufficiently short. The dotted photoproduct peak shown in Fig. 5d has an R_f value equivalent to that of authentic β-phenylethylamine . This peak was observed in an irradiation experiment in which the time lag between spotting and scanning was reduced to a minimum (i.e., 12 hrs.) consistent with development of the chromatogram to a distance of 10 inches. On subsequent chromatograms of additional samples of the same reaction mixtures (which had been exposed to air) the amplitude of the peak progressively decreased, indicating that the substance was evaporating from the solutions. The β-phenylethylamine has not been previously reported as a photoproduct of phenylalanine, although Vallentyne (1964) has shown that decarboxylation with the concommitant formation of phenylethylamine is the predominant pathway of thermal decomposition in heated phenylalanine solutions. It will be shown in Section D that decarboxylation is also the dominant photochemical process occurring in UV-irradiated phenylalanine solutions.

The chromatographic basis of the identification of photoproducts 7 and 8 as phenyllactic acid and benzoic acid, respectively, is shown in Fig. 8. In the upper trace, the chromatographic locations of the C^{14} peaks for these photoproducts are compared with the positions of the bromcresol green spots for samples of unlabelled phenyllactic acid and benzoic acid chromatographed with the radioactive sample in n-BuOH:HAc:H_2O. By chromatographing these substances individually, it was found that benzoic acid has the greater R_f value in this solvent system (see Table 3). Coincidence of the C^{14} photoproduct peaks with the bromcresol green spots in benzene:acetic acid:water , 2:2:1 (v/v/v) , is shown in the lower trace of Fig. 8. In this solvent system, the chromatographic mobility of benzoic acid (photoproduct No. 8) is smaller than that of phenyllactic acid. This result was confirmed using C^{14}-labelled benzoic acid.

The paper chromatographic identifications of the photoproducts of DL-phenylalanine are conveniently summarized by means of a composite chromatogram constructed by superimposing individual chromatogram scans of the type shown in Fig. 5, in which phenylalanine was labelled with C^{14} at different specific sites. Figure 9 shows such a composite chromatogram, in which the relative chromatographic positions of all the photoproduct C^{14} peaks in n-BuOH:HAc:H_2O are given.

3. Discussion

As the above results show, aliphatic as well as aromatic low molecular weight compounds are synthesized photochemically from phenylalanine following photoexcitation of a π-electron in the benzene ring of the molecule. Thus, it is not necessary that short wavelength UV radiation be present in the illuminating beam (as suggested by Luse and McLaren, 1963) in order to synthesize straight chain compounds from phenylalanine.

That each of the three carbon-carbon bonds in the side chain of phenylalanine can be dissociated as a result of ring photoexcitation is shown by the occurrence of β-phenylethylamine , aminomalonic acid, and aspartic acid (see labelling data in Table 1) as photoproducts. Furthermore, the occurrence of phenyllactic acid as a photoproduct shows that the carbon-nitrogen bond of the side chain is activated following ring excitation. Therefore, it is apparent that photoexcitation energy initially confined to the benzene ring can subsequently be transferred from the ring to the side chain. This result

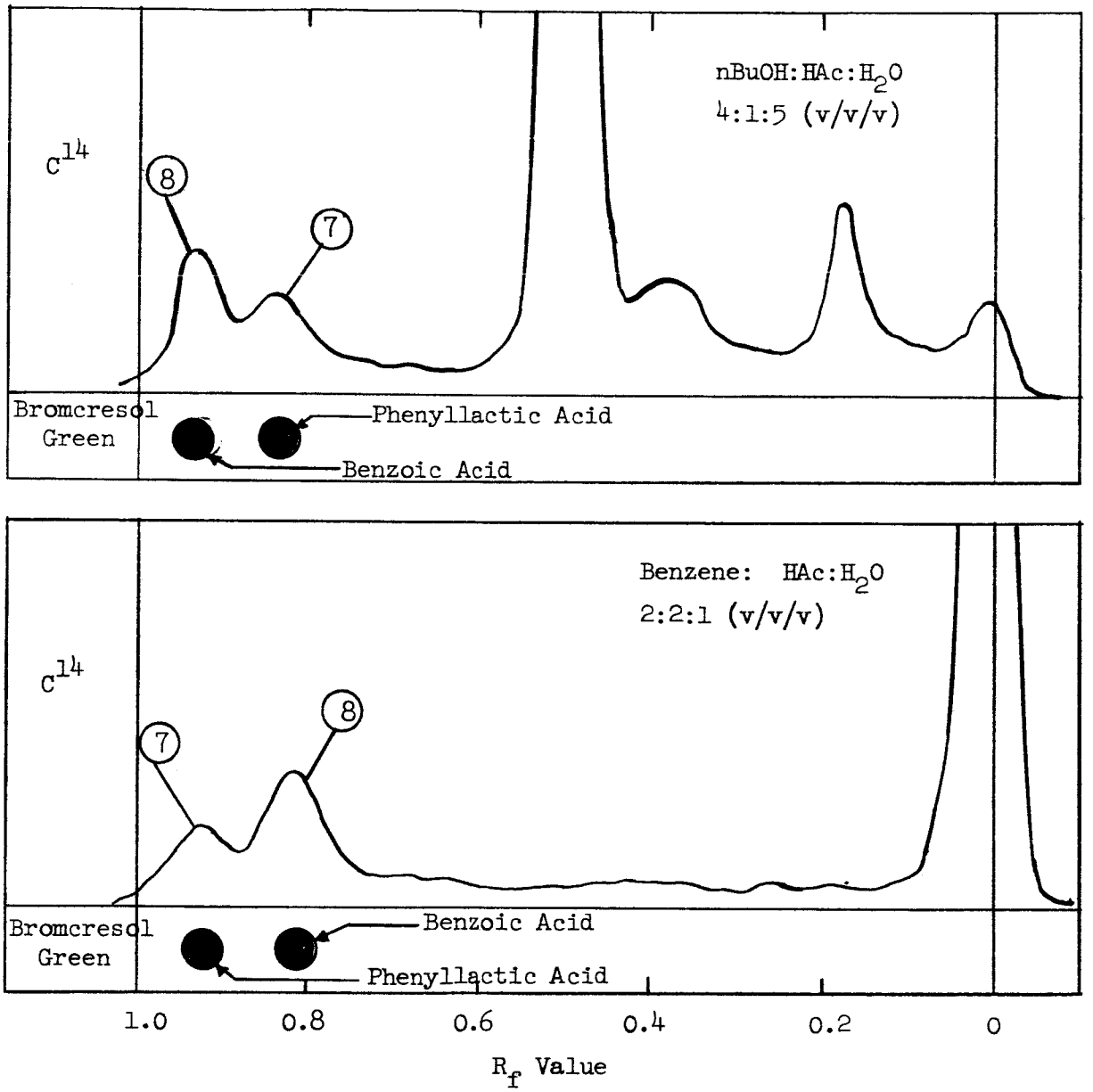

FIG. 8--Paper chromatographic identification of phenyllactic acid and benzoic acid as photoproducts of DL-phenylalanine. Experimental conditions are given in the legend to Fig. 5. Samples of the reaction mixture were chromatographed together with standard unlabelled phenyllactic acid and benzoic acid in two different solvent systems. The distributions of C^{14} activity on the chromatograms are compared above with positions of the bromcresol green spots of the standard compounds. In both cases, photoproduct peaks 7 and 8 coincide with the color spots for benzoic acid and phenyllactic acid respectively. Note the reversal in relative R_f values of the photoproducts in the two solvent systems. Phenylalanine was labelled with C^{14} at the carboxyl-carbon.

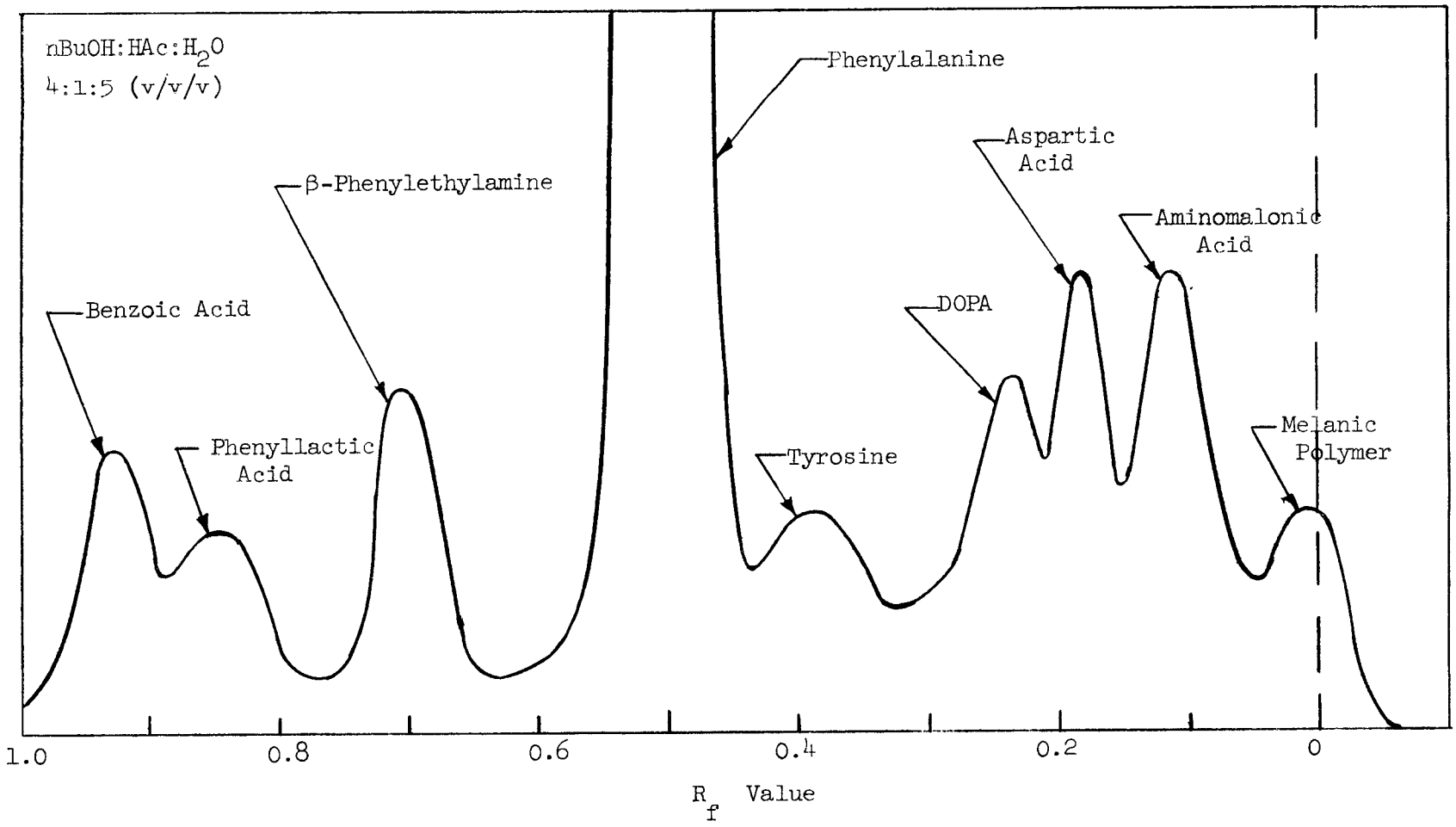

FIG. 9--Summary of paper chromatographic identification of photoproducts of DL-phenylalanine. The composite chromatogram scan shown above was constructed by superimposing individual C^{14} scans of the type shown in Fig. 5.

is consistent with the work of Porter and Strachan (1958) on side chain photolysis following ring excitation at 2537 Å in substituted benzenes having linear side chains up to four carbons in length. The exceedingly low probability that direct absorption of 2575 Å radiation by side chain electrons is responsible for cleavage of side chain carbon-carbon bonds will be discussed in Appendix C, together with possible mechanisms of energy transfer from the ring to the side chain. An example of a similar type of intramolecular energy transfer is provided by the well-known case of photolysis of acetone vapor (for example, see Noyes and Dorfman, 1948). Photoexcitation energy initially absorbed by the π-electrons of the ketone bond, (λ_{max} = 3130 Å, or photon energy = 91 Kcals/Einstein) is subsequently transferred to the adjacent carbon-carbon bond (dissociation energy, 58 Kcals/mole) where photodissociation to free radicals takes place.

That the energy of 2475 - 2675 Å photons is sufficient to dissociate any carbon-carbon bond in phenylalanine is shown in Table 4. However, the lack of any evidence that carbon-carbon bonds in the benzene ring are photochemically cleaved (see Table 1) indicates that photoexcitation energy initially delocalized over the whole ring (as is the case for the $^1B_{2u} \leftarrow {}^1A_{1g}$ transition) probably cannot subsequently be sufficiently localized in one particular bond in the ring such that that bond is dissociated, at least not with a quantum efficiency greater than 10^{-4}.*

This is consistent with the fact that ring carbon-carbon bonds are considerably stronger than side chain carbon-carbon bonds (see Table 4), partly due to their partial double bond character and partly to the high resonance energy of the benzene ring (39 Kcals/mole benzene, Pauling, 1960). Consequently, in order for a ring carbon-carbon bond to be photochemically cleaved, it is necessary that 77% of the energy of the initially absorbed 2575 Å photon be localized in such a bond long enough to guarantee dissociation. However, due to the symmetry of the benzene ring in phenylalanine, these bonds are pairwise physically equivalent, so that even if the

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*In the case of various substituted benzenes, however, Migirdicyan and Leach (1962) claim that ring carbon-carbon bonds are cleaved during illumination at 2537 Å in an organic glass at 77°K.

TABLE 4

BOND ENERGIES VS PHOTON ENERGIES

Bond*	Approximate Energy+	λ	Photon Energy
C ···— C (ring)	85 Kcals/mole	2000 Å	143 Kcals/Einstein
$\varphi - \overset{3}{C}$	60	2475	116
$\overset{3}{C} - \overset{2}{C}$	60		
$\overset{2}{C} - \overset{1}{C}$	30≠	2575	111
C - N	50		
		2675	107
C ···— O (carboxyl)	120		
O - H	110	2800	102
C - H	90		
N - H	85	3000	95

* Nomenclature as in Table 1.

\+ Primarily from Pauling, L. (1960) and Fieser and Fieser (1956) based on data obtained on simple model compounds.

≠ From J. R. Valentyne's (1964) determination of the activation energy for thermal decomposition of phenylalanine assuming that decarboxylation is the major reaction pathway.

photoexcitation energy could be localized in one of the three distinct pairs of equivalent bonds by some mechanism, it is improbable that more than half the energy could be localized in one bond of the pair.

In the side chain, the situation is entirely different. Here, the weakest carbon-carbon bond has a dissociation energy of only 30 Kcals/mole (Table 4), so that only 27% of the photon energy need be localized for cleavage of the bond. Also, the symmetry of the side chain is sufficiently low that all of the bonds are physically distinguishable from each other. Therefore, at least on symmetry grounds, there are no strong restrictions against localizing a substantial fraction of the photoexcitation energy in a given bond.

D. KINETIC STUDIES AND QUANTUM YIELD DETERMINATIONS

1. Methods

Samples were prepared as described in Section C. Prior to irradiation, the UV absorption spectrum of the reaction mixture was recorded on the Cary spectrophotometer. Small samples of known volume (usually 20 microliters) were then withdrawn from the solution with a Levy micropipette and spotted at the origin of a Whatmann No. 1 paper chromatogram strip, after which the solution was flushed vigorously for 5-10 min with either oxygen or highly purified nitrogen (Matheson Co., Inc. "prepurified" grade, O_2 content < 8 ppm). After bubbling of the gas, the quartz cell was immediately sealed with a ground glass stopper.

After the initial beam integration, the sample cell was mounted in the copper cooling block (see Fig. 4), aligned in the beam at a distance of 1.1 cm from the plane of the exit slit and irradiated at 2575 $\pm$ 100 Å for 16 - 20 hrs. Periodically during the run, the UV absorption spectrum of the reaction mixture was recorded, 20 microliter samples withdrawn, spotted on Whatmann No. 1 paper chromatogram strips and developed in n-BuOH:HAc:H_2O, 4:1:5, v/v/v , and either O_2 or "prepurified" N_2 flushed through the solution.

The distributions of C^{14}-activity on the radiochromatograms were determined by digital integration at 1 mm intervals (corresponding to 0.2 in. intervals along the chromatogram; scan speed, 12 in/hr) on the

Vanguard paper strip scanner (typical operating conditions: scale, 1000 cpm; collimation, 0.5 cm; time constant, D; Geiger cells activated, A and B). The C^{14} activity associated with each of two closely spaced peaks (i.e., aminomalonic acid and aspartic acid, photoproducts 2 and 3 in Fig. 5b) was determined geometrically by constructing a triangle for each peak such that the background activity was excluded, measuring the area of the triangles, and computing the corresponding C^{14} activities by means of a counts/unit area factor determined graphically for each trace at an integration interval coinciding with a region of one of the photoproduct peaks free from interference by neighboring peaks.

The concentrations of the photoproducts as functions of irradiation time were determined by comparing the C^{14}-activities of the photoproduct peaks at time t with the C^{14} activity of the phenylalanine peak at $t = 0$, the initial concentration of phenylalanine having been determined from optical density measurements using a molar extinction coefficient of 200. The sensitivity of the above technique is about 4×10^{-5} μMoles, which corresponds to a concentration of 2 μMoles/liter for a 20-microliter sample of the reaction mixture. That is, for a photoproduct concentration less than 2 μMoles/liter, the activity in the resulting C^{14} peak is comparable to the background activity.

Since the total amount of C^{14} activity remaining on the paper chromatograms indicates the extent to which gaseous and liquid photoproducts are formed from the labelled carton atom, the individual count values were summed over each strip and after subtracting out the background, the corresponding concentration was determined again by comparison with the C^{14} activity in the $t = 0$ phenylalanine peak.

2. Results

The optical absorbance changes which occur in solutions of DL-phenylalanine irradiated at pH 10.8, both in the presence and absence of dissolved molecular oxygen, are shown in Fig. 10. In the oxygen case, the optical density at 2575 Å rises much more rapidly than when N_2 is bubbled through the solution. In addition, the two sets of spectra differ qualitatively. In the N_2 case, the relative minimum at 2300 - 2400 Å remains pronounced and is even somewhat enhanced during

irradiation, while in the O_2 case this minimum becomes progressively less apparent. The absorbance shoulder centered near 2900 Å in oxygen-saturated solutions does not occur in nitrogen-bubbled solutions for irradiation times $\leq$ 14 hrs. If the N_2-bubbled reaction mixture is allowed to stand in the dark either at 3° C or at room temperature, the absorption band centered at 3200 Å gradually disappears, both in the presence and absence of oxygen, indicating that the substance is unstable in aqueous solution at alkaline pH.

After 20 hrs of irradiation, both solutions exhibit a faint yellow color. In a separate series of experiments (Blois and Taskovich, unpublished observations) using a low pressure mercury lamp as light source, it was shown that under O_2 the yellow color eventually disappears under prolonged irradiation while the absorbance decreases to nearly zero for all wavelengths greater than 2400 Å , but that under N_2 the color persists and the absorbance remains high in the 2400 - 3500 Å region. For this effect, however, much higher total doses of radiation are required than those used in the present experiments. In Fig. 10 the dotted curve indicates that after about 20 hrs of irradiation in the presence of O_2 , the absorbance begins to fall. In the N_2 case, the absorbance is still rising after 20 hrs of irradiation.

In Figs. 11 - 14, the concentrations of phenylalanine, of total C^{14} activity, and of several photoproducts are shown as functions of irradiation time under both O_2 and prepurified N_2 . In these experiments, phenylalanine was labelled with C^{14} at the carboxyl carbon (Figs. 11 and 13) or at the 3-carbon (Figs. 12 and 14). The data given in Fig. 11, together with the quantum yields given in Table 5 (see below for method of calculation), show that phenylalanine is somewhat more rapidly photolyzed in the presence of oxygen than in the absence of oxygen at pH 10.8. The rate at which the carboxyl carbon is lost from the solution is not markedly affected by the presence or absence of dissolved oxygen, but in either case the quantum yield for this process ($34 - 44 \times 10^{-3}$) is comparable to the total quantum efficiency for loss of phenylalanine ($37 - 55 \times 10^{-3}$) and greater by a factor of from eight to ten than the quantum yields for the most prominent non-volatile photoproducts (aspartic acid and aminomalonic acid, see Table 5).

FIG. 10--Optical absorbance changes of ultraviolet irradiated solutions of DL-phenylalanine bubbled with O_2 (left) and with prepurified N_2 (right). In both cases the illuminating beam contained wavelengths from 2475 Å to 2675 Å at the half-power points with maximum intensity at 2575 Å. Left: Initial concentration of phenylalanine (c_o), 7.1×10^{-4} M; pH 10.8; temperature (T), 21 ± 0.5°C; incident light intensity (I_o), 7,050 ergs/cm^2/sec. Right: c_o, 8.0×10^{-4}M; pH 10.8; T, 22 ± 1°C; I_o, 6570 ergs/cm^2/sec.

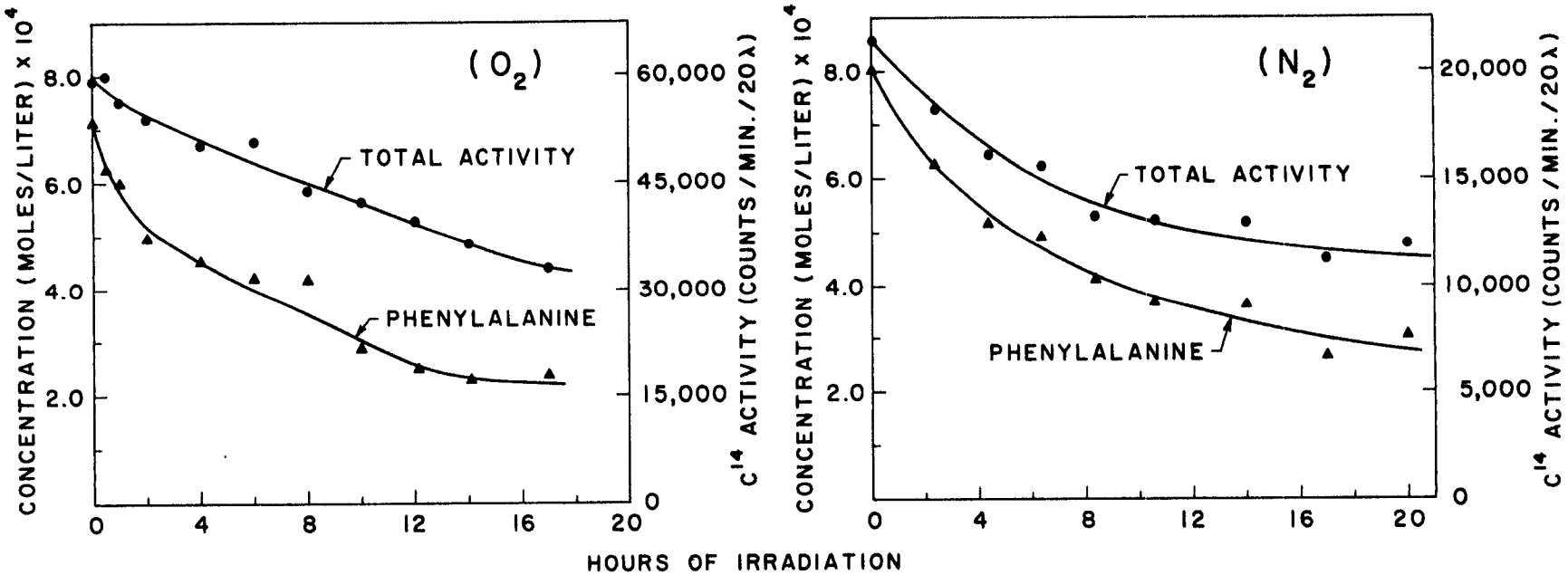

FIG. 11--Kinetics of phenylalanine loss and loss of total C^{14} activity in solutions irradiated at 2575 Å at pH 10.8 in the presence (left) and absence (right) of dissolved oxygen. "Total activity" refers to the integrated C^{14} activity on chromatograms of 20 microliter (20λ) samples of the reaction mixture. Experimental conditions are given in the legend of Fig. 10.

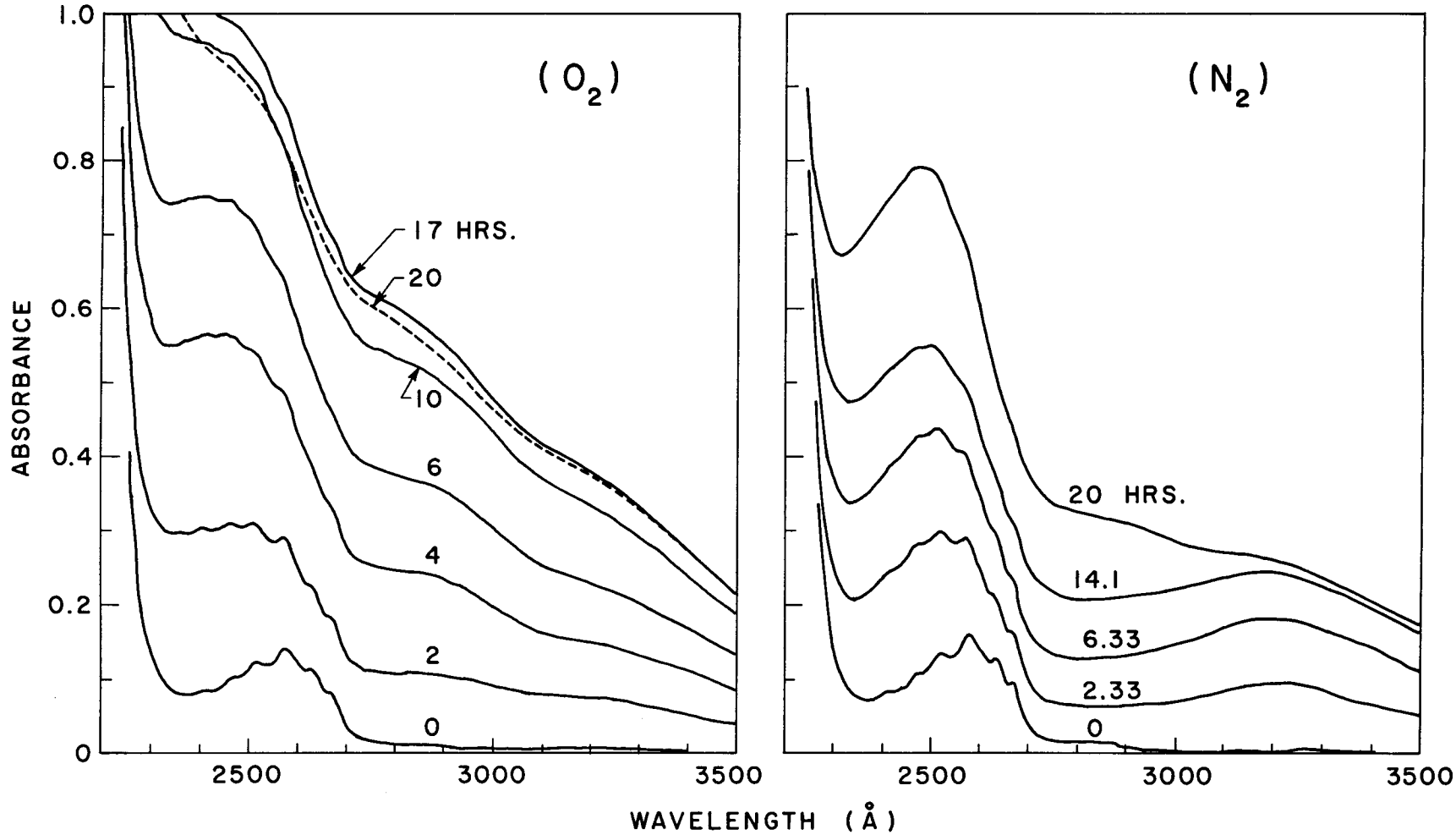
1.0
0.8
0.6
0.4
0.2
0
ABSORBANCE
(O_2)
17 HRS.
20
10
6
4
2
0
2500
3000
3500
(N_2)
20 HRS.
14.1
6.33
2.33
0
2500
3000
3500
WAVELENGTH (Å)

FIGURE 10

Figure 12 shows the rate of phenylalanine photolysis at pH 7.3 in the presence of dissolved oxygen. The net quantum efficiency for all the processes contributing to phenylalanine loss is considerably lower at pH 7.3 ($\Phi = 27 \times 10^{-3}$) than at pH 10.8 ($\Phi = 55 \times 10^{-3}$). Luse and McLaren (1963) report a quantum efficiency for phenylalanine loss of 13×10^{-3} at pH 1.0, which is consistent with this trend of increasing photochemical stability with decreasing pH. The rate at which carbon No. 3 is lost from the solution at pH 7.3 is considerably less than the rate at which carbon No. 1 is lost from the solution at pH 10.8. This result is due in part to the synthesis of non-volatile photoproducts incorporating carbon No. 3 but not carbon No. 1 (such as benzoic acid), but also reflects the increased photochemical stability of phenylalanine at neutral pH.

The rates at which the stable, non-volatile photoproducts accumulate at alkaline pH both in the presence and absence of oxygen are shown in Fig. 13. In the presence of oxygen, the concentration of tyrosine reaches a steady state value after 6 hrs or irradiation. DOPA first appears in the reaction mixture in detectable concentrations (i.e., ≥ 2 μ moles/liter) after 3-4 hrs of irradiation and thereafter continues to accumulate at a relatively rapid rate while the melanic polymer slowly accumulates after 6 hrs of irradiation. In solutions bubbled with prepurified nitrogen, the yields of tyrosine, DOPA and polymer were too low to be measured accurately so that no kinetic data could be obtained.

The data given in Fig. 13 show that aspartic acid is synthesized at comparable rates in the presence and absence of oxygen, although the quantum yield is somewhat greater in the oxygen case (Table 5). In the nitrogen case, the rate of accumulation of aspartic acid decreases with increasing irradiation time, while in the presence of oxygen at both pH 10.8 (Fig. 12) and pH 7.3 (Fig. 14), aspartic acid accumulates at a nearly constant rate.

Phenyllactic acid is synthesized in higher net concentrations under N_2 than under O_2, but this may be a result of further oxidation of the compound in the presence of O_2. So far, kinetic data for benzoic acid synthesis have only been obtained for the oxygen case at pH 7.3 (Fig. 14).

FIG. 12--Kinetics of phenylalanine photolysis and loss of total C^{14} activity in solutions irradiated at pH 7.3 in the presence of dissolved oxygen. Initial concentration of phenylalanine, 7.85×10^{-4}M; temperature, 21 ± 1°C; incident light intensity, 6,410 ergs/cm^2/sec.

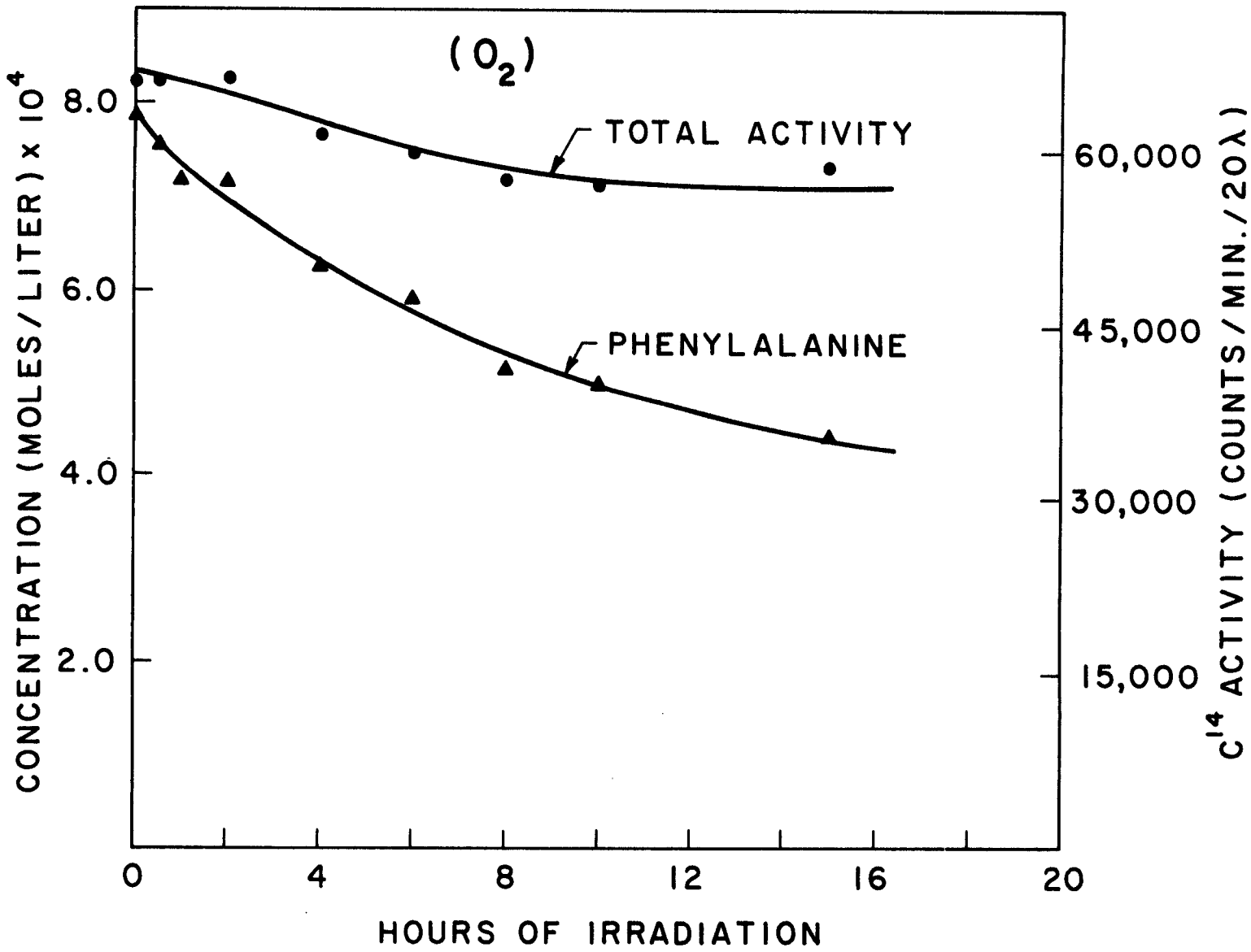
(O2)
TOTAL ACTIVITY
PHENYLALANINE
CONCENTRATION (MOLES/LITER) x 10^4
8.0
6.0
4.0
2.0
C^14 ACTIVITY (COUNTS/MIN./20λ)
60,000
45,000
30,000
15,000
0
4
8
12
16
20
HOURS OF IRRADIATION

FIGURE 12

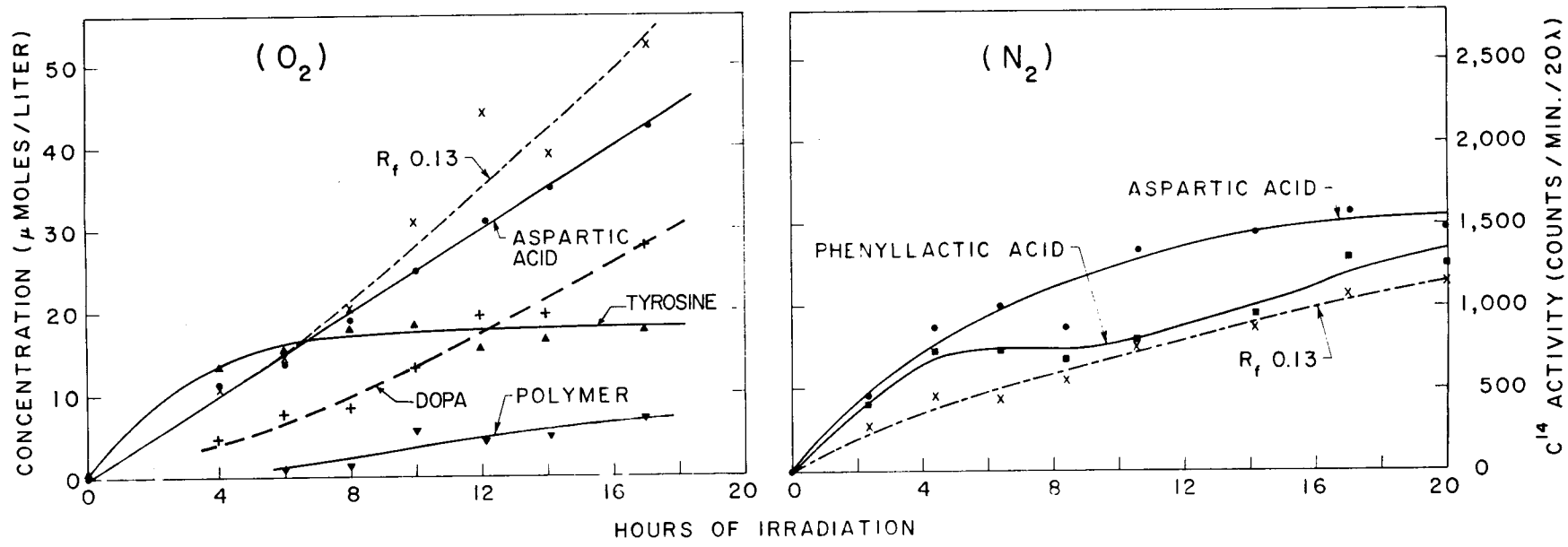

FIG. 13--Kinetics of photoproduct accumulation in dilute solutions of DL-phenylalanine irradiated at 2575 Å in the presence (left) and absence (right) of dissolved oxygen. Experimental conditions are given in the legend to Fig. 10, (after Kenyon and Blois, 1964).
Note: R_f 0.13 has been identified as aminomalonic acid (see text).

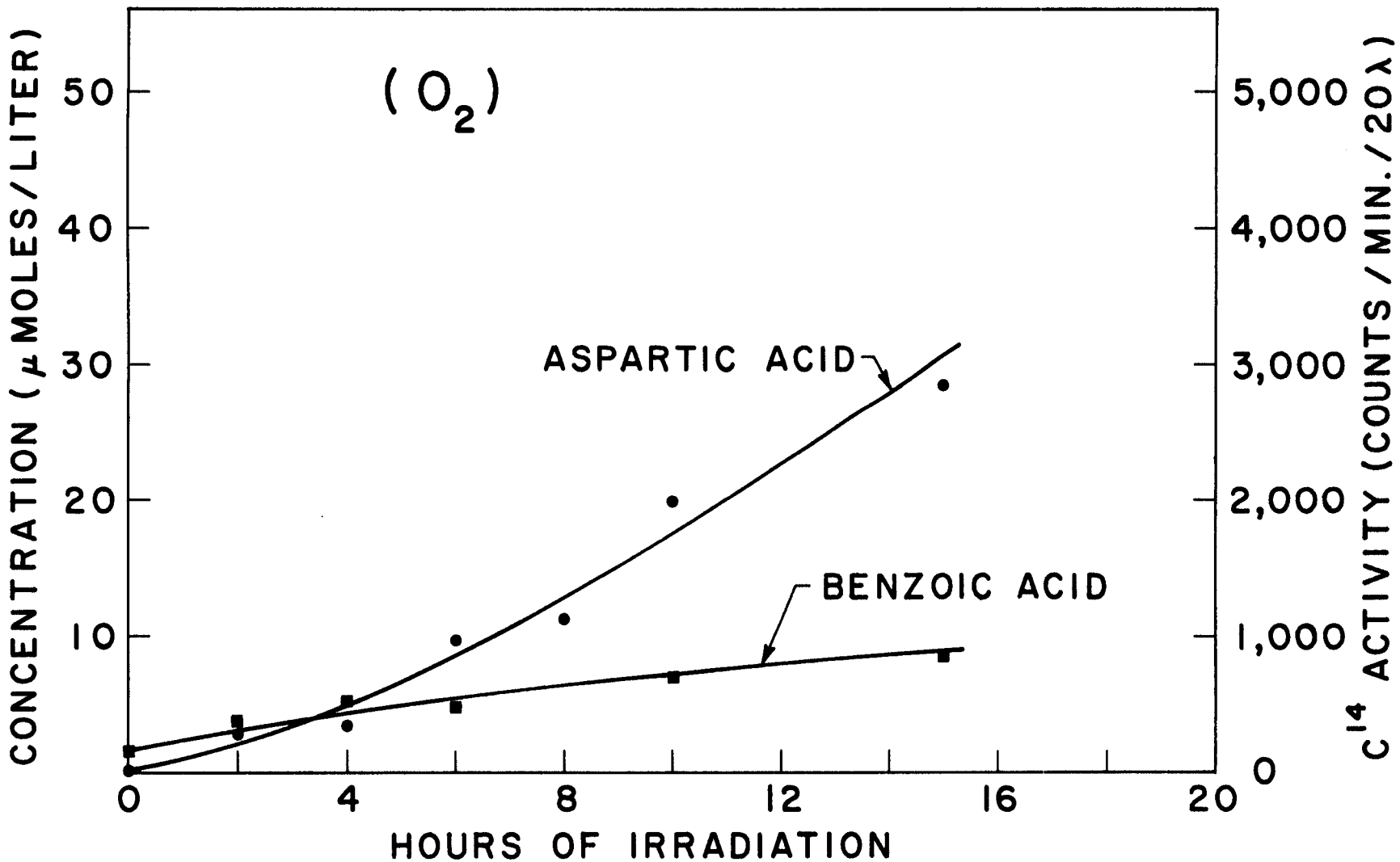

FIG. 14.—Kinetics of aspartic acid and benzoic acid synthesis in phenylalanine solutions irradiated at 2575 Å in the presence of dissolved oxygen at pH 7.3. Experimental conditions are given in the legend of Fig. 11.

Calculation of Quantum Yields

In order to calculate the quantum yields for photochemical reactions of the type discussed in this study, it is necessary to know the concentration of the reacting species as a function of irradiation time under specified conditions of pH, temperature, incident monochromatic light intensity, and concentration of dissolved molecular oxygen. Approximate methods based on the assumption that the concentration of the reacting species does not change appreciably during the irradiation period (S. Claesson, 1964) are not applicable here, since the doses required to synthesize detectable quantities of the photoproducts result in the depletion of an appreciable fraction of the starting material (see Figs. 11 and 12). In addition, since the absorbance at 2575 Å increases appreciably during the irradiation period (Fig. 10), corrections must be made for absorption of light by the photoproducts (S. Claesson, 1964; Luse and McLaren, 1963). In order to calculate quantum yields, it is necessary to know the following experimental quantities as function of irradiation time: the concentration of the reacting species, the concentrations of the photoproducts, and the total absorbance of the reaction mixture at 2575 Å .

Since the concentration of phenylalanine is not a simple function of irradiation time, it is necessary to integrate graphically in order to calculate the total number of photons absorbed by phenylalanine molecules during a time, t . In order to do this, the time axis in plots of the type shown in Figs. 11 and 12 were divided into n equal intervals, Δt_i , (e.g., Δt_i = 30 min.) and the concentration of phenylalanine, c_i , was assumed to be constant during each Δt_i and equal to the value at the center of the i-th interval. The corresponding values of the absorbance of the reaction mixture, A_i , were obtained from plots of absorbance versus irradiation time using spectral data of the type shown in Fig. 10.

By applying the Beer-Bouguer absorption law to solutions containing several molecular species, each of which contributes to the observed absorbance, it can easily be shown that the number of photons, N_i , absorbed per unit volume by phenylalanine during each Δt_i is given to a

close approximation by the following equation:

$$N_i = (I_0 \, \Delta t_i / h\nu)(1 - 10^{-A_i})(\epsilon c_i \, d/A_i) \quad ,$$

where I_0 is the incident light intensity at 2575 Å in ergs/cm^2/sec, $h\nu$ is the energy of a photon of wavelength 2575 Å ($h\nu = 7.72 \times 10^{-12}$ ergs), ϵ is the molar extinction coefficient for DL-phenylalanine ($\epsilon = 200$), and d is the thickness of the sample cell ($d = 1.0$ cm). The N_i's are then calculated using the experimental values of c_i and A_i. The total number of photons absorbed by phenylalanine molecules during time t is computed by summing the N_i's:

$$N(t) = \sum_{i=1}^{t/\Delta t} N_i = (I_0/h\nu) \, \Delta t \sum_{i=1}^{t/\Delta t} (1 - 10^{-A_i}) \, \epsilon c_i \, d/A_i \quad .$$

The net quantum yield for any given photoproduct at time t is defined as the ratio of the concentration of the photoproduct at time t, expressed in molecules /cm^3, and $N(t)$:

$$\Phi(t) \equiv c(t) \, / \, N(t) \quad .$$

It is important to note that the quantum yields will, in general, depend on the value of t for which they are computed, since the photoproducts may undergo secondary reactions. This is particularly true in the case of aromatic photoproducts such as tyrosine, phenyllactic acid, and benzoic acid. In the case of these substances, only lower limits for the quantum yields can be calculated. Quantum yields are given in Table 5, together with the values of t for which they were computed. The time interval of 5-10 hrs. was used in the quantum yield calculations for loss of phenylalanine and loss of the 1-carbon and 3-carbon from the reaction mixture, since it was found that the quantum yield for

TABLE 5

QUANTUM YIELDS AT pH 7.3 AND 10.8

Reaction or Photoproduct	Time Interval Used In Calculation	Quantum Yield $\times 10^3$		
		(O_2)pH 7.3*	(O_2)pH 10.8+	(N_2)pH 10.8+
1. Loss of phenylalanine	5-10 hrs.	27	55	37
2. Loss of carboxyl carbon from solution	5-10	--	44	34
3. Loss of 3-carbon from solution	5-10	12	--	--
4. Incorporation of carboxyl carbon into polymer	6-10	--	1.4	Negligible
5. Incorporation of 3-carbon into polymer	0-1	2.7	--	--
6. Aminomalonic acid	0-10	--	4.0	1.5
7. Aspartic acid	0-10	1.8	3.6	2.9
8. DOPA	0-10	--	1.9	Negligible
9. Tyrosine	0-8	--	> 2.8	Negligible
10. Phenyllactic acid	0-2	> 1.1	--	> 2.7
11. Benzoic acid	0-10	> 0.7	--	--

*Experimental conditions given in legend to Fig. 12.

+See legend to Fig. 10.

phenylalanine loss for both the O_2 and N_2 cases at pH 10.8 (calculated for each Δt) decreased during the interval 0-5 hrs,* but then remained nearly constant from 5 to 10 hrs.

3. Discussion

The prominent absorbance shoulder centered near 2900 Å in the absorption spectra for the O_2 case (Fig. 10) suggests that aldehydes are present in the reaction mixture. Since the characteristic aldehyde peak occurs at 2900 Å for phenylacetaldehyde (Kumler et al., 1950) and since this substance has been reported as a photoproduct of phenylalanine (Arnow, 1942), it is probable that the absorbance shoulder shown in Fig. 10 reflects the photochemical synthesis of phenylacetaldehyde.

The substance responsible for the unstable absorbance peak centered at 3200 Å in nitrogen-flushed solutions has not been identified. Claeson (1956) has observed a similar absorption band centered at 3100 Å in solutions of phenylalanine irradiated in the presence of O_2 at slightly acidic pH. This absorption peak also slowly decreases if the solution is allowed to stand in the dark, so that the same compound is probably being observed in the two cases.

The contributions which the aromatic photoproducts listed in Table 5 make to the observed absorbance changes during ultraviolet irradiation of phenylalanine can be estimated from known extinction coefficients and concentrations. This was done in the oxygen case at pH 10.8 for tyrosine, DOPA, benzoic acid, and phenyllactic acid for an irradiation time of 10 hrs. Extinction coefficients for these aromatic compounds over the wavelength range 2200 - 3500 Å were determined in a separate series of experiments. The net absorbance due to these compounds was then added to the absorbance due to the remaining phenylalanine. The results are compared in Fig. 15 with the observed absorbance. It is clear that these four photoproducts, together with the remaining phenylalanine, account for only a small fraction of the observed absorbance.

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*I.e., at $t = 0$, $\Phi = 80 \times 10^{-3}$ for the O_2 case and $\Phi = 62 \times 10^{-3}$ for the N_2 case .

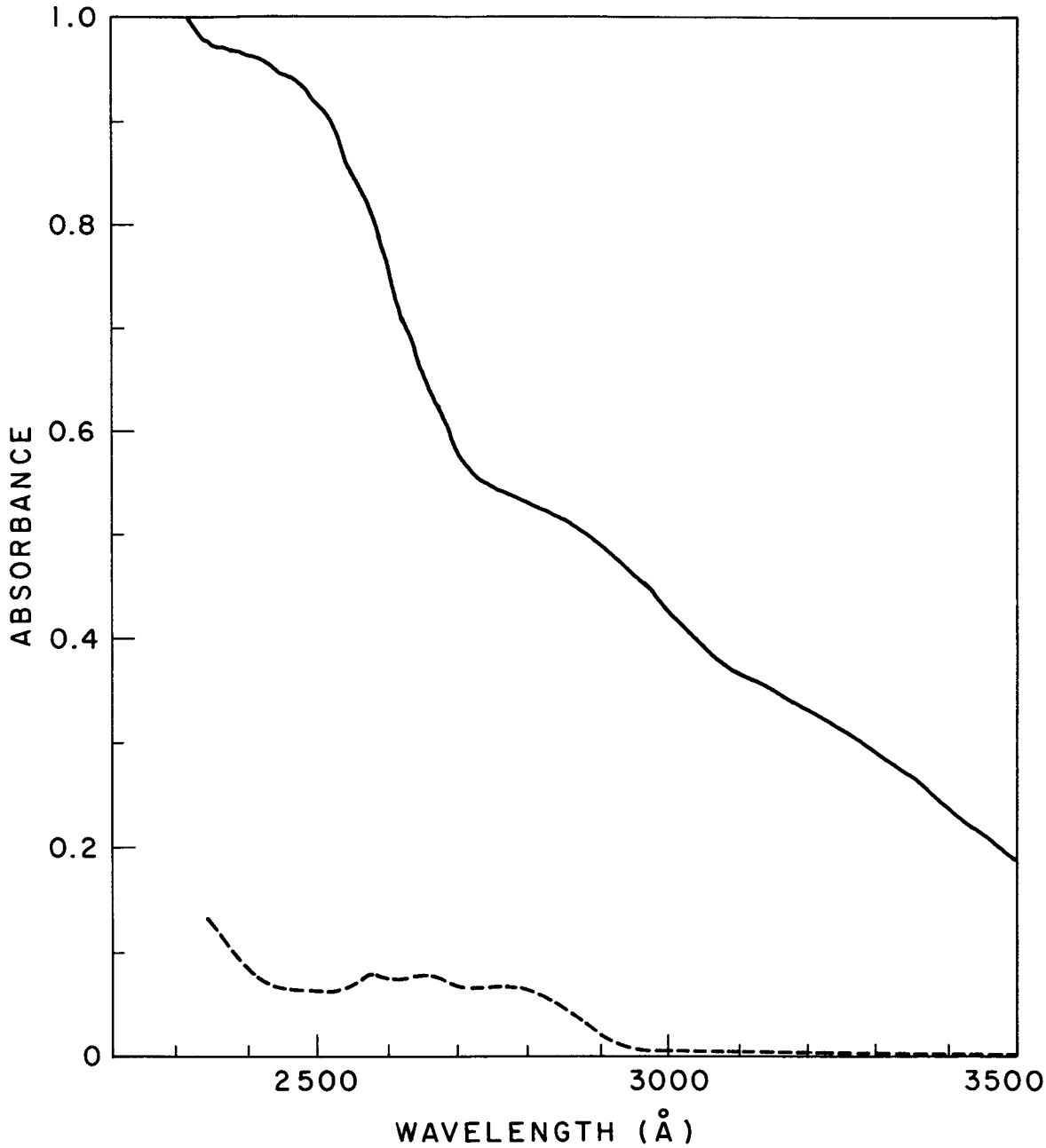


FIG. 15--Observed optical absorbance of phenylalanine solution after 10 hrs. irradiation (solid curve) compared with absorbance calculated from contributions of tyrosine, DOPA, benzoic acid, phenyllactic acid and the remaining phenylalanine (dotted curve). Experimental conditions are the same as those for the oxygen case given in the legend to Fig. 10.

Even if it is assumed that concentrations of β-phenylethylamine and phenylacetaldehyde as high as 10^{-4} M (i.e., one-third the concentration of the remaining phenylalanine) contribute to the observed absorbance, the calculated optical density at 2575 Å is only increased to 0.12 (Phenylethylamine, $\epsilon_{2575\text{Å}} = 240$, Salomon and Bina, 1946; Phenylacetaldehyde, $\epsilon_{2575\text{Å}} = 190$, Kumler et. al., 1950). Therefore, it is apparent that the large optical absorbance increases occurring in UV-irradiated phenylalanine solutions are not due to the low molecular weight aromatic photoproducts. That the increased light absorption is associated with only a small fraction of the material in the reaction mixture is shown by the fact that the sum of the concentrations of all the low molecular weight photoproducts detected chromatographically accounts nearly quantitatively for the amount of phenylalanine converted to non-volatile substances, as determined by the difference in quantum yields for loss of phenylalanine and loss of carboxyl carbon from the solution (see Table 5). The only other non-volatile substances in the reaction mixture are the melanic polymer and the low molecular weight quinones and semiquinones likely to be present as intermediates in the system of photopolymerization reactions leading to the formation of the melanic polymer (see Chapter III). These compounds, however, are not detectable by the methods of chromatography used in the present investigation, since they are present individually in very low concentrations and are unstable in the presence of oxygen even in the dark (Walaas, 1963; Horner, 1961, pp 205-208). Since the observed optical absorbance shown in Fig. 15 is unaffected by bubbling oxygen through the solution in the dark, it is probable that the apparent high absorbance is due primarily to the combined absorption and scattering of the melanic polymer itself, rather than to the oxygen-labile intermediates.

The kinetic data for photoproduct accumulation shown in Fig. 13 and the quantum yields given in Table 5 show that molecular oxygen is required for the synthesis of tyrosine, DOPA, and the melanic polymer. The kinetics of accumulation of these substances (Fig. 13, O_2 case) suggest that tyrosine and DOPA are intermediates in melanic polymer synthesis. Schocken's (1951) finding that tyrosine is photo-oxidized to DOPA which in turn spontaneously oxidizes to form melanins lends support to this conclusion.

Aspartic acid accumulates at comparable rates both in the presence and in the absence of oxygen (see Figs. 13 and 14), showing that O_2 is not required for the synthesis of this substance, although the rate is somewhat greater in the O_2 case. This difference in kinetic behavior may be due in part to the formation of aspartic acid from both tyrosine and DOPA in the oxygen case. Aspartic acid has been detected in UV-irradiated solutions of tyrosine by Suzutani (1958) and by Luse (1961). In N_2 - flushed solutions, the concentrations of tyrosine and DOPA are negligible (i.e., less than 2 μ Molar after 20 hrs irradiation), so that these substances would not contribute appreciably to the aspartic acid concentration, with the result that as the concentration of phenylalanine decreases, the rate of aspartic acid synthesis also decreases. The increased rate of synthesis of aminomalonic acid in the presence of oxygen might be explained by a similar argument if this compound is also a photoproduct of tyrosine and DOPA.

Since the minimum quantum yield for phenyllactic acid is higher in N_2- flushed solutions than in oxygen saturated solutions (see Table 5), it is clear that oxygen is not required for the photochemical synthesis of this substance.

By combining the quantum yield data given in Table 5 with the C^{14} incorporation data given in Table 1, it is possible to estimate the relative photochemical stabilities of the side chain carbon-carbon bonds in phenylalanine. That the most photolabile bond is that between carbons 1 and 2 is suggested by the high quantum yield ($34\text{-}44 \times 10^{-3}$, see Table 5) for loss of the carboxyl carbon from the solution. However, it should be pointed out that this value represents only an upper limit on the quantum yield for decarboxylation of phenylalanine, since it is likely that CO_2 is also photochemically liberated by some of the aromatic photoproducts (e.g., tyrosine, DOPA, and phenyllactic acid). Also, it has been shown that decarboxylation occurs in the system of reactions involved in the synthesis of melanic polymers (see e.g., Clemo, Duxbury, and Swan, 1952). Nevertheless, the high quantum yield for carboxyl carbon loss is consistent with the anomalously low activation energy for decarboxylation of phenylalanine (i.e., 30 Kcals/mole, see Table 4).

That the remaining two carbon-carbon bonds, as well as the carbon-nitrogen bond in the side chain, are considerably more photochemically stable than the bond between carbons 1 and 2 is suggested by the relatively low quantum yields for aminomalonic acid, aspartic acid, and phenyllactic acid (4×10^{-3}, 3.6×10^{-3}, and 2.7×10^{-3}, respectively; see Tables 1 and 5). These results are summarized in Fig. 16.

The probability that a given side chain carbon-carbon bond dissociates following ring π-electron excitation varies inversely with the distance of the bond (along the carbon backbone) from the ring. That is, the relative magnitudes of the photostabilities of the side chain carbon-carbon bonds are as follows: $(\text{ring} - \overset{3}{C}) > (\overset{3}{C} - \overset{2}{C}) \gg (\overset{2}{C} - \overset{1}{C})$. The possible relevance of energy migration from the ring to the end of the side chain in phenylalanine for the general problem of intermolecular energy transfer in proteins will be discussed in Chapter IV.

E. LOW TEMPERATURE ELECTRON SPIN RESONANCE EXPERIMENTS

The occurrence of side chain photolytic reactions in phenylalanine following ring π-electron excitation (see Sections C and D) suggested that free radicals might be formed as primary photodissociation products. In order to test this hypothesis, low temperature electron spin resonance (e.s.r.) experiments were carried out.

1. Methods

First, DL-phenylalanine (Calbiochemical Corp., Grade A) was dissolved in a mixture consisting of 40% (by volume) propylene glycol and 60% water, at a concentration of 5.0×10^{-3} M. A small sample of the solution (2-3 ml) was introduced into a quartz capillary tube (O.D., 4 mm; thickness, 0.5 mm) which was first flushed with nitrogen to remove molecular oxygen and then sealed. A control sample consisting of the solvent alone was prepared in a similar manner. The samples were irradiated in liquid nitrogen in a quartz Dewar which was mounted in the cavity of a Varian, model V4500-10A, X-band e.s.r. spectrometer. A medium pressure, 140 watt mercury lamp (Hanovia No. 30620), together with a short focal length quartz lens provided an intense beam of UV radiation focussed on the light-admitting slit

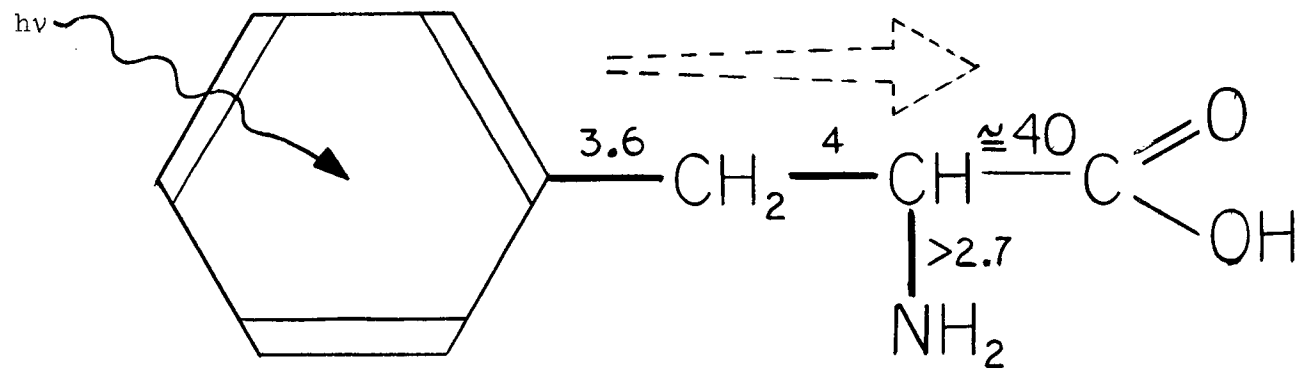


FIG. 16--Relative photochemical instabilities of the side chain carbon-carbon and carbon-nitrogen bonds of phenylalanine. The number opposite each bond is proportional to the quantum yield for the photochemical reaction in which the given bond is dissociated (see Tables 1 and 5) following ring π-electron excitation at 2575 Å (represented by the wavy arrow). The dotted arrow indicates the direction of energy transfer following photoexcitation.

system of the cavity. Wavelengths less than 2400 Å were filtered out of the beam by means of a Corning Cs 7-54 "UV window" filter mounted on the cavity (see Appendix A). The electron spin resonance spectra of both the control and phenylalanine samples were measured in the $g = 2$ region before and continuously during irradiation.

2. Results

The resulting e.s.r. spectra are shown in Figs. 17 and 18. Both samples exhibit a weak signal at $g = 2.0$ before irradiation (Fig. 17). This signal, however, was shown in a separate run to be associated with the quartz Dewar. After 30 min of irradiation, the amplitude of the control signal increased only slightly. However, after only 9.5 min of irradiation, there is a marked increase in the amplitude of the signal associated with the phenylalanine sample. This amplitude increases with further irradiation, and after 22 min the signal exhibits several fine structural features. The rates at which the amplitudes of the two signals increase are shown in Fig. 19.

In Fig. 18, the e.s.r. signal of the phenylalanine sample after 44 min of irradiation is shown with an expanded horizontal scale in order to exhibit clearly the hyperfine structure. The signal appears to consist of a broad triplet (or doublet) with a splitting of 155 gauss and a linewidth of 10 gauss, superimposed on one or more strong, narrow absorptions centered at or near $g = 2.0$. Also, six or more hyperfine lines spaced at intervals of 11.4 gauss are discernible in the spectrum.

3. Discussion

These results clearly demonstrate that free radicals are formed from phenylalanine during UV irradiation at wavelengths longer than 2400 Å. That short wavelength UV radiation is, in fact, effectively filtered out of the illuminating beam is shown by the almost negligible rate of increase of the control signal amplitude. That is, the solvent begins to absorb strongly below about 2300 - 2400 Å, so that if wavelengths less than 2400 Å are present in the illuminating beam, then large increases in the amplitude of the control signal would be expected due to solvent photolysis.

FIG. 17--Low temperature free radical electron spin resonance spectra of UV-irradiated DL-phenylalanine. Samples were irradiated at 77°K in an organic glass (40% propylene glycol: 60% water) in the cavity of a Varian, model V4500-A, X-band spectrometer having 100 Kc field modulation. The light source was a medium pressure, 140 watt mercury lamp (Hanovia No. 30620). Wavelengths less than 2400 Å were filtered out of the illuminating beam by a Corning Cs 7-54 filter mounted on the cavity.

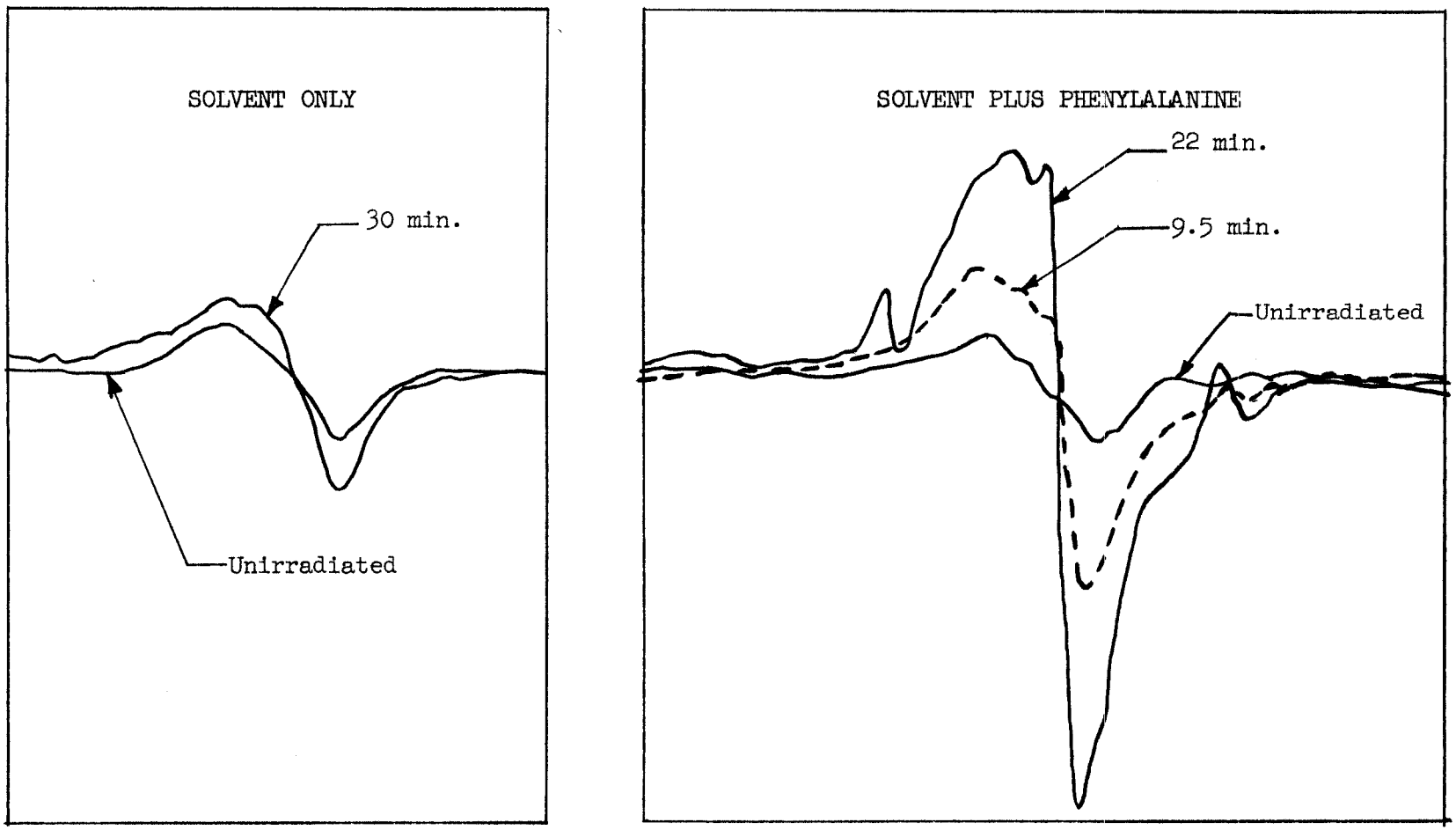

SOLVENT ONLY
30 min.
Unirradiated
SOLVENT PLUS PHENYLALANINE
22 min.
9.5 min.
Unirradiated

FIGURE 17

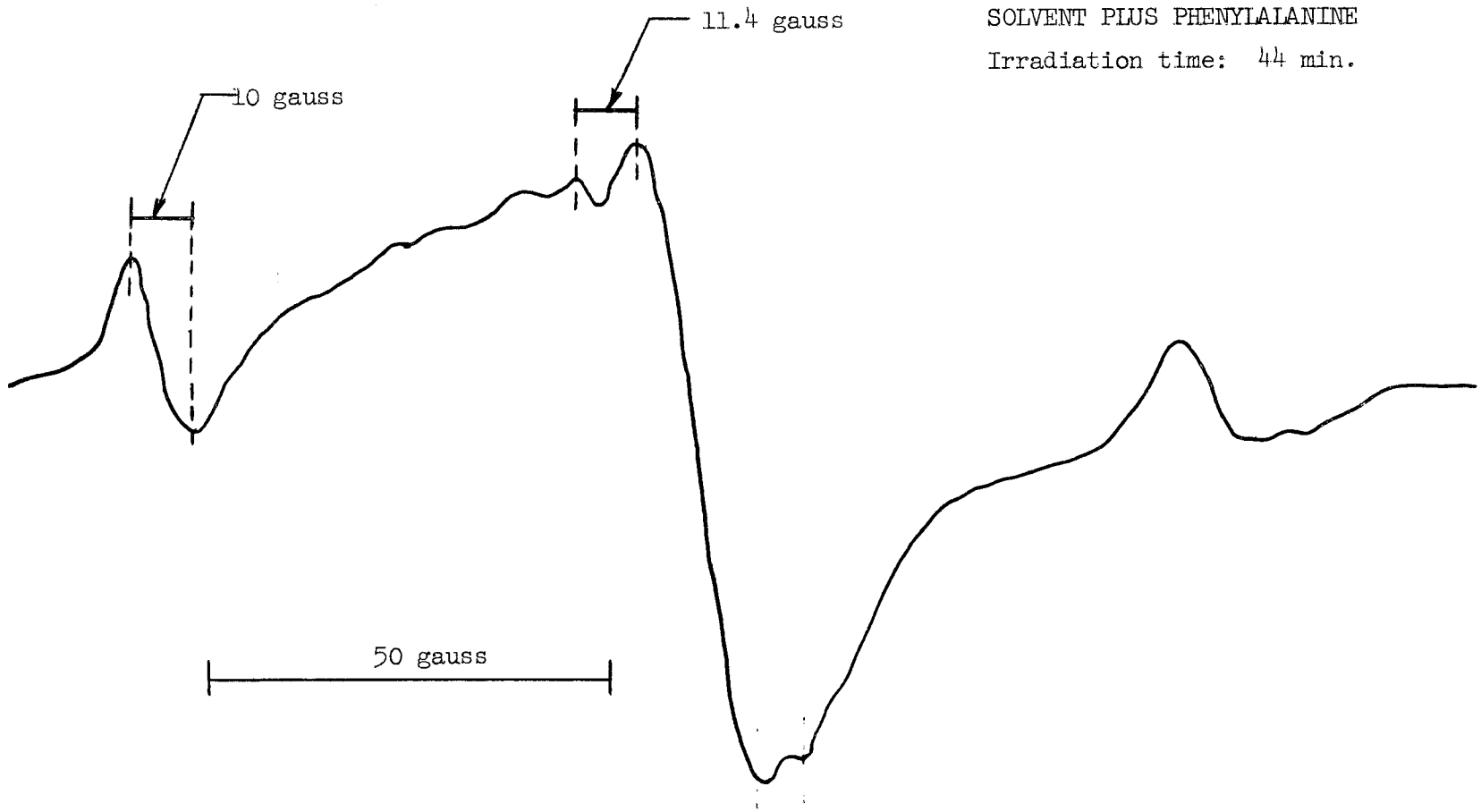


FIG. 18--Hyperfine structure of the low temperature composite e.s.r. signal of UV-irradiated DL-phenylalanine. The vertical (amplitude) scale is reduced twofold and the horizontal (D.C. magnetic field strength) scale expanded about threefold with respect to Fig. 17. Experimental conditions are given in the legend to Fig. 17.

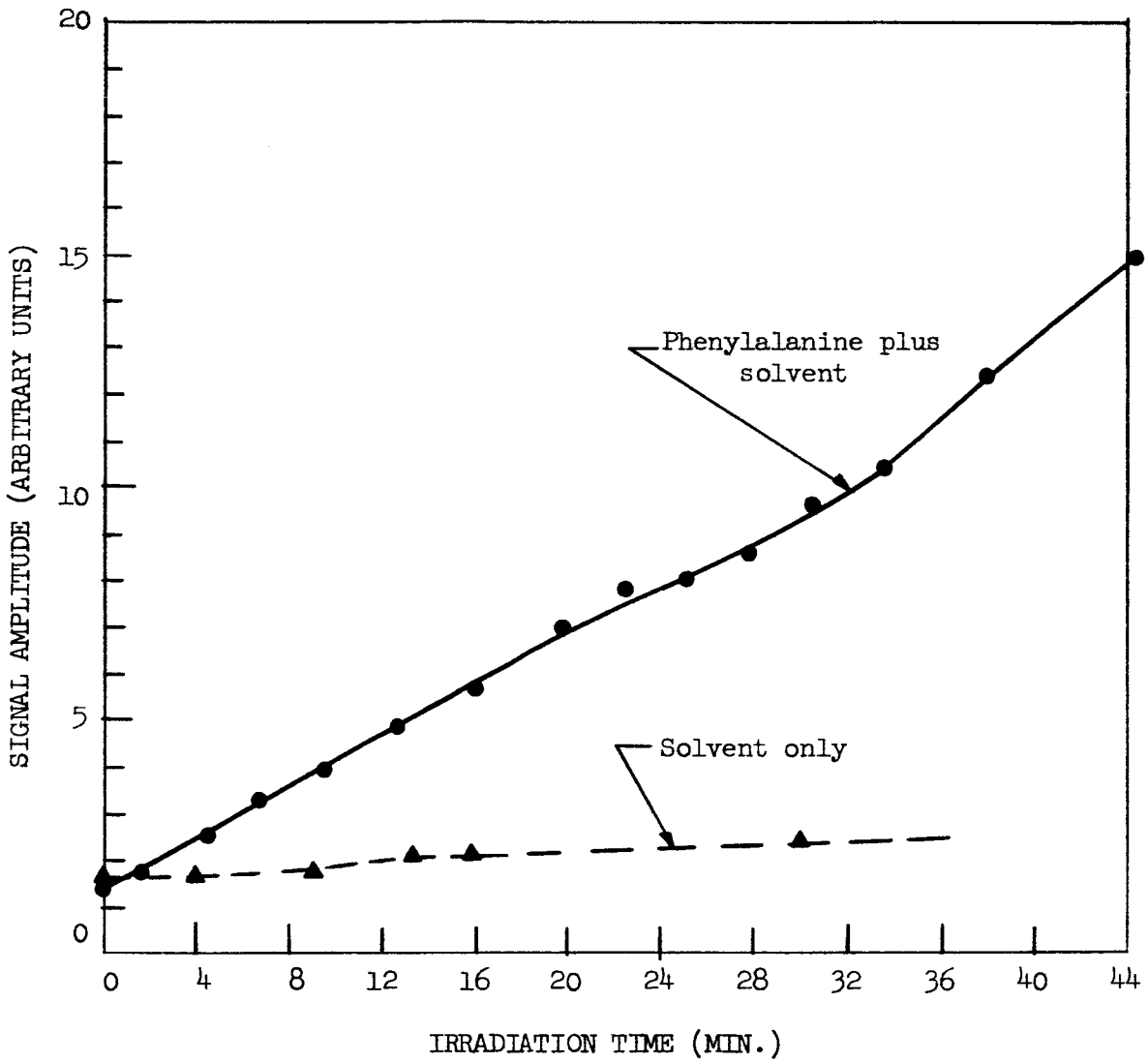


FIG. 19--Rate of increase in amplitude of the phenylalanine low temperature e.s.r. signal during UV-irradiation. Experimental conditions are given in the legend to Fig. 17.

Due to the high viscosity (at 77° K) of the organic glass (40% propylene glycol: 60% water) in which phenylalanine was irradiated, it is highly probable that all of the trapped free radicals which contribute to the observed signal are primary photodissociation products of phenylalanine. This conclusion is supported by the fact that all components of the signal are stable at 77° K in the dark, indicating that secondary processes are not occurring to any appreciable extent. Semiquinones and OH. radicals postulated as intermediates in the room temperature photochemistry of UV-irradiated phenylalanine solutions do not contribute to the signal since they arise from reactions in which phenylalanine does not directly participate. However, the possibility remains that once a given free radical is formed from phenylalanine, it may subsequently abstract a hydrogen atom from the solvent, and the resulting solvent radical would then contribute to the observed signal (see Ingram, 1958, pp 174-175). The asymmetry of the signal, together with the fact that the hyperfine lines are not sharp peaks, but merely shallow ripples (Fig. 18), indicates that a mixture of several different free radical species is present. Therefore, it is apparent that following ring π-electron excitation, several different alternative photodissociation processes are possible. Furthermore, the fact that the shape of the signal changes during irradiation (compare the 9.5 min and 22 min signals shown in Fig. 17) indicates that the different free radical species are accumulating at different rates. Therefore, the various photodissociation processes following π-electron excitation are not equiprobable, i.e., do not occur with the same quantum efficiency.

F. PROBABLE REACTION PATHWAYS

In the present investigation of the photochemistry of DL-phenylalanine, paper chromatography was the principal technique used for the detection of photoproducts. Normally, this technique is limited to the detection of those substances which are stable solids at room temperature, although under favorable conditions (i.e., high concentration, rapid development and analysis of the chromatogram; see discussion in Section C, "Results" on detection of β-phenylethylamine), certain

liquids may also be detectable. The sensitivity of the radiochromatographic technique used in the present investigation is 4×10^{-5} μ Moles, corresponding to a concentration of 2μ Moles/liter (see Section D, "Methods"). Therefore, it is apparent that photoproducts present in the reaction mixture will not be detected if (1) they are liquids or gases at room temperature, or (2) they are stable solids at room temperature but occur at concentrations less than 2 μM, or (3) they are solids at room temperature but are unstable in the presence of O_2 . These non-detectable substances may be either primary photoproducts in the sense that they are formed directly from phenylalanine, or they may be derived from primary or higher order photoproducts. One such class of substances are the quinones and semiquinones thought to occur as intermediates in the synthesis of melanic polymer (see Chapter III, Section D).

It is also possible that non-detectable intermediates occur in photochemical reaction pathways involved in the synthesis of the low molecular weight photoproducts listed in Table 2. That is, in a reaction sequence of the type shown below

$$A \xrightarrow[(1)]{h\nu} B \xrightarrow[(2)]{(h\nu)} C \xrightarrow[(3)]{(h\nu)} D \quad ,$$

where A represents phenylalanine, only D may accumulate at detectable concentrations in the reaction mixture, and reactions (2) or (3) may not require light, so that D cannot be regarded as a photoproduct. If reaction (3) does not require light, then the quantum yield for D based upon the number of photons absorbed by A is not relevant to the mechanism of the photochemical synthesis of D .

Since reaction sequences involving low concentrations of intermediates are not discernible by the methods of chromatography used in this investigation, the reaction pathways proposed below were constructed on the basis of the following types of evidence:

1. The degree of structural similarity between the photoproduct and phenylalanine.

2. Kinetic data for photoproduct accumulation of the type shown in Figs. 13 and 14.

3. Carbon-14 incorporation data of the type given in Table 1.

4. Requirements for oxygen (see Fig. 13 and Table 5).

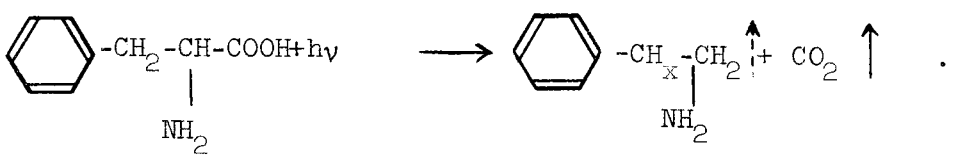
5. Low temperature (77° K) electron spin resonance spectra of UV-irradiated phenylalanine.

6. Known or probable pathways and mechanisms for synthesis of substances similar to the photoproducts.

In the case of the low molecular weight photoproducts of phenylalanine, the criterion that the reaction pathways be as simple as possible consistent with all of the available evidence was applied where possible.

1. β-Phenylethylamine

Although no kinetic data are available for the accumulation of β-phenylethylamine in UV-irradiated phenylalanine solutions, the high quantum yield for carboxyl carbon loss from the solution both in the presence and absence of O_2 (see Table 5) suggests that this substance is formed in the following reaction:

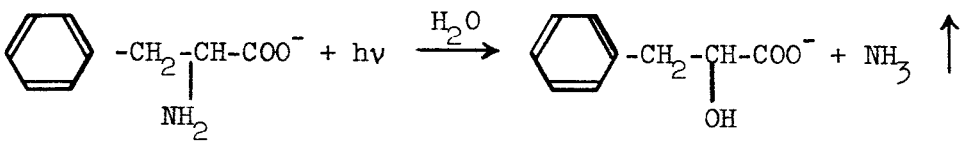


The dotted arrow indicates that β-phenylethylamine evaporates from paper chromatograms. Subject to the qualifications discussed above (Section D), this reaction probably represents the dominant photochemical process occurring in the system. That this reaction is also the dominant process occurring in heated aqueous solutions of phenylalanine has been shown by Vallentyne (1964).

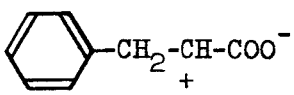
2. Phenyllactic acid

Although no attempt was made in the present investigation to detect free ammonia in UV-irradiated phenylalanine solutions, it has been

detected by a number of investigators in UV-irradiated solutions of aliphatic amino acids. For aspartic acid, alanine, and glycine, it has been shown that photochemical ammonia liberation is accompanied by synthesis of the α-hydroxy derivative of the amino acid (Henri et al.. 1934; Weizmann et al., 1936; Mandl and McLaren, 1951; McLaren, 1949). Since phenyllactic acid accumulates both in oxygen-saturated and oxygen-free UV-irradiated phenylalanine solutions, it is likely that this substance is synthesized via photochemical hydrolysis of the C-N bond, as has been proposed in the case of the aliphatic amino acids (Weizmann et al.. 1938):



It is possible that the ionic species



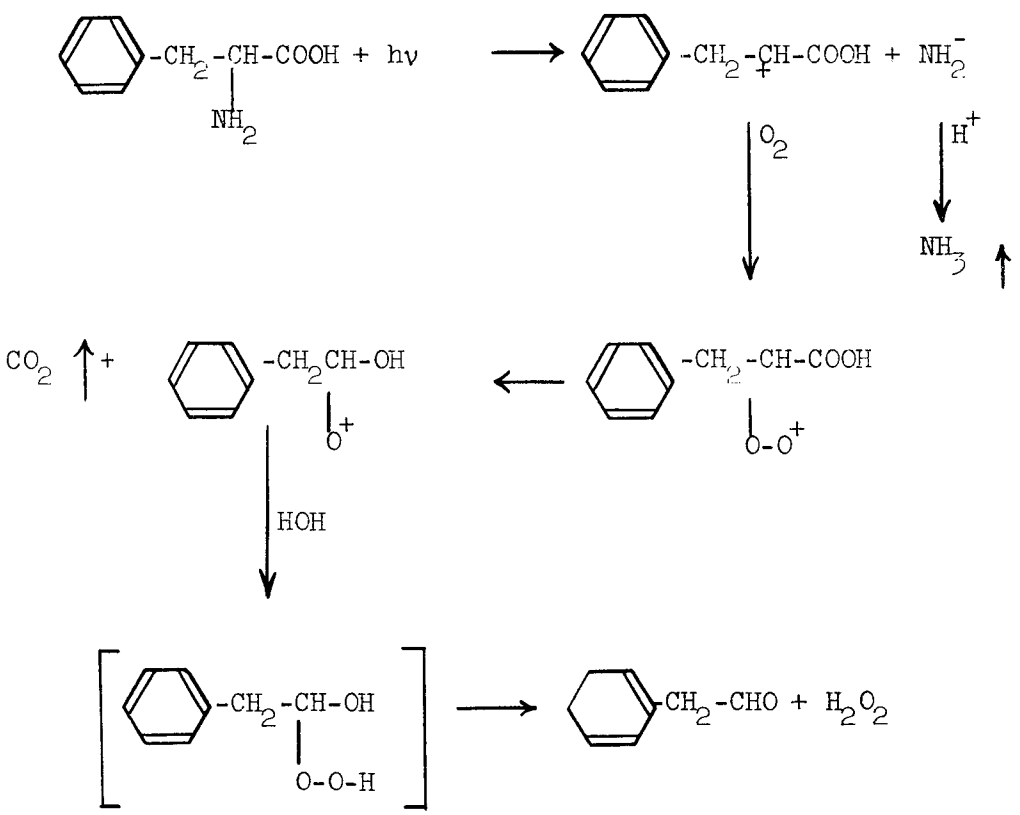
and NH_2^- occur as intermediates in the above reaction as suggested in the case of aliphatic amino acids by Weizmann et al.. (1939). At any rate, if the overall reaction shown above takes place, the quantum yield for ammonia should be comparable to that of phenyllactic acid. And in fact, Luses' (1961) value of 2×10^{-3} for ammonia agrees fairly well with the value of 2.7×10^{-3} for phenyllactic acid (Table 5) obtained in the present investigation, although the phenylalanine irradiations were carried out at different pH's and somewhat different methods of computing quantum yields were used.

3. Phenylacetaldehyde

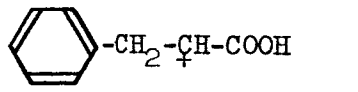
Although phenylacetaldehyde was not detected chromatographically, it is likely that this substance was present in the reaction mixtures, since an absorption band characteristic of aromatic aldehydes (Kumler et al., 1950) occurs at 2900 Å in the absorption spectra shown in Fig. 10 for the oxygen case. Also, the characteristic odor of

phenylacetaldehyde (Arnow, 1942; Tominaga, 1958) was detected in most of the oxygen containing reaction mixtures. Arnow (1942) positively identified this substance in UV-irradiated phenylalanine solutions by means of the Schiff and phenylhydrazine tests, and Tominaga, on the basis of its characteristic odor, claimed that it was present in his reaction mixtures.

That aliphatic α-amino acids yield aldehydes upon exposure to ultraviolet irradiation in the presence of oxygen has been shown by a number of investigators (see, for example, Allen et al., 1937; Weizmann et al., 1939). A system of reactions by which this synthesis might occur has been proposed by Weizmann et al. (ibid.). In analogy with this scheme, it is plausible that phenylacetaldehyde is photochemically synthesized from phenylalanine as follows:



It is apparent that if phenyllactic acid is synthesized via the ionic mechanism discussed above, then the oxygen consuming reaction in the above scheme will compete with the hydrolytic reaction which results in the formation of phenyllactic acid. That is, O_2 and H_2O (more correctly, OH^- ions) will compete for the ionic species



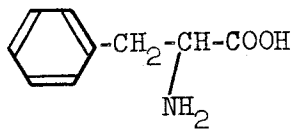
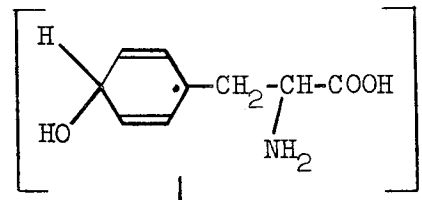
Therefore, other factors being equal, the quantum yield for phenyllactic acid should be greater in oxygen-free than in oxygen-saturated solutions. The quantum yields for phenyllactic acid given in Table 5 are consistent with this view (i.e., $\geq 1.1 \times 10^{-3}$ in the presence of O_2 ; $\geq 2.7 \times 10^{-3}$ in the absence of O_2), although it is probable that the low quantum yield in the O_2- case is partially accounted for by the low concentration of OH^- ions since the experiment was conducted at neutral pH.

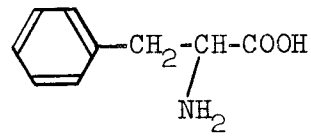
4. Tyrosine and DOPA

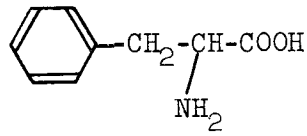
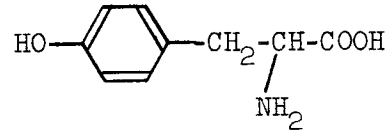
Of particular interest in the above reaction scheme is the proposed liberation of hydrogen peroxide in the final step. This substance undergoes photochemical dissociation into OH· radicals via a free radical chain mechanism (Kornfeld, 1935; Lea, 1949; Uri, 1952). Stein and Weiss (1949) have shown that OH· radicals attack the benzene ring, resulting, in the case of substituted benzenes, in the hydroxylation of certain sites on the ring. Therefore, if OH· radicals are produced photochemically from H_2O_2 in oxygen-containing UV-irradiated solutions of DL-phenylalanine, it is likely that these radicals will react with the benzene ring of phenylalanine, resulting in the formation of phenolic substances. This type of free radical attack would provide an oxygen-dependent mechanism for the synthesis of tyrosine and DOPA. If such a mechanism is operating in UV-irradiated phenylalanine solutions, it would not be required that phenylalanine molecules absorb 2575 Å photons

in order to react to form tyrosine. Adapting the scheme proposed by Stein and Weiss (ibid.) for OH· radical attack on benzoic acid to the case of phenylalanine, the formation of tyrosine might occur as follows:

(a) $H_2O_2 + h\nu \longrightarrow 2\ OH\cdot$

(b) $OH\cdot +$  

$H_2O +$ 

(c) $OH\cdot +$  

A similar set of reactions can be constructed for the formation of DOPA from tyrosine.

An alternative mechanism by which tyrosine might conceivably be photochemically synthesized from phenylalanine involves simply the direct reaction of molecular oxygen with phenylalanine in its lowest lying excited singlet state. Such a process can be represented as follows:

$$S_0 + h\nu_{2575\ \text{Å}} \rightleftharpoons S_1$$

$$S_1 \xrightarrow{\kappa} O_2$$

$$O_2 \longrightarrow \text{Tyr} + O$$

where S_0 is phenylalanine in its ground state, S_1 is phenylalanine in which the π-electron system of the benzene ring is excited to its

$^1B_{2u}$ singlet state (Fig. 2), and κ is the rate constant for the bimolecular reaction (the dotted arrow represents return to the ground state by radiationless processes or fluorescence emission; see Appendix C).

That the maximum rate at which tyrosine could be synthesized by the above mechanism is considerably higher than the observed initial rate of tyrosine accumulation is shown in Appendix B. The calculated maximum rate of tyrosine accumulation is compared below with the observed initial rate estimated from the tyrosine kinetic data given in Fig. 13.

Calculated	Observed
$\left(\frac{d[Tyr]}{dt}\right)_{max} = 41 \times 10^{-9}$ moles/liter/sec	$\frac{d[Tyr]}{dt} = 1.2 \times 10^{-9}$ moles/liter/sec

It is apparent that the proposed singlet state mechanism would be consistent with the kinetic data if the probability of reaction per encounter of S_1 with O_2 were less than unity (i.e., $\cong 0.03$).* That singlet-excited benzene reacts directly with molecular oxygen is shown by Bowen and Williams' (1939) finding that the quantum yield of fluorescence of benzene at 2537 Å in oxygen-free hexane ($\varphi_f = 0.11$) is approximately equsl to the sum of the quantum yields for both fluorescence ($\varphi_f = 0.04$) and oxygen uptake ($\varphi = 0.08$) in air-saturated hexane. This result, together with McLean and Giese's (1950) finding that UV-irradiated aqueous benzine solutions exhibit optical density increases indicative of hydroxylation, lend support to the proposed mechanism.

Since the lowest lying triplet state of phenylalanine is probably much longer-lived than the lowest-lying singlet state (the lifetimes of aromatic triplet states are 10^{-5} - 10^{-3} sec in low viscosity solvents at room temperature; Porter, 1961), it is possible that tyrosine is synthesized upon encounter with triplet-excited phenylalanine with O_2 . Such a mechanism has been shown to be consistent with the kinetic data in the case of anthracene photo-oxidation (Livingston, 1961). However, the stable end

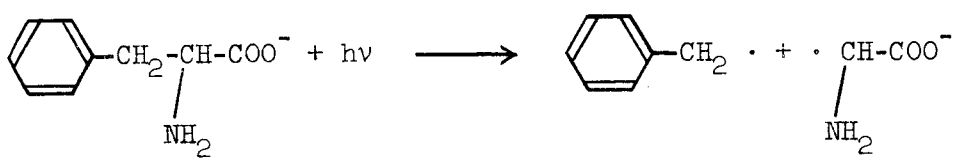
*It was assumed in the kinetic calculation that the probability of reaction per encounter is equal to unity (see Appendix B).

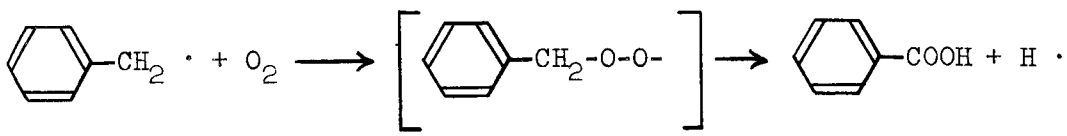
product of the reaction is a transannular peroxide (ibid.), and not hydroxy-substituted anthracene.

In order to evaluate the possibility that triplet-state phenylalanine might be involved in the synthesis of tyrosine, it is necessary to know the steady-state concentration of triplet states under the given experimental conditions. However, since the probability of intersystem crossing (Kasha, 1960) is not known, this concentration cannot be calculated. Direct measurements of phenylalanine triplet state concentrations in aqueous solution have not been reported.

5. Benzoic Acid

That the benzyl radical is a likely intermediate in the synthesis of benzoic acid is suggested by the results of flash photolytic studies carried out by Porter and his associates (Porter and Strachan, 1958). These investigators have shown that upon exposure to UV-radiation benzene derivatives having linear side chains up to four carbons in length undergo side-chain photolysis to yield the benzyl radical. It has been shown that such radicals will react readily with molecular oxygen to yield a hydroperoxyl radical, which in turn rearranges to the corresponding carboxylic acid (Livingston, 1961). In view of these observations, the following mechanism for the photochemical synthesis of benzoic acid from phenylalanine is proposed:

(a) 

(b) 

Since aminomalonic acid is known to contain only carbons 1 and 2 of the phenylalanine side chain, it is likely that this substance results from a secondary reaction of the glycyl radical produced in the benzyl yielding reaction in the above scheme (see discussion below).

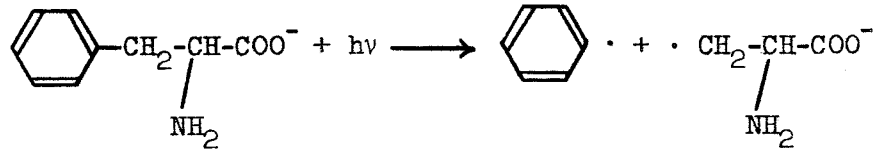
6. Aspartic Acid and Aminomalonic Acid

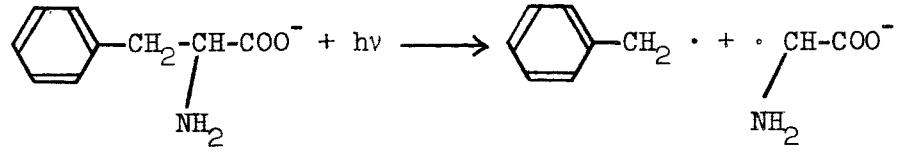
The problem of the mechanism of aspartic acid synthesis is similar to that of aminomalonic acid synthesis, since the C^{14} incorporation data given in Table 1 show that both compounds (photoproducts 2 and 3) incorporate one less carbon atom from a given phenylalanine side chain than the actual number of carbons present in each of these compounds. That is, aspartic acid, which is a four carbon compound, incorporates all three phenylalanine side chain carbons while the three carbon compound, aminomalonic acid (structures are given in Table 2b) incorporates only carbons 1 and 2. That the benzene ring does not contribute the fourth carbon of aspartic acid nor the third carbon of aminomalonic acid is suggested by the absence of the C^{14} peaks for these photoproducts in scan (e) in Fig. 5. Therefore, two phenylalanine side chains may be involved in the synthesis of each of these photoproducts.

Due to the high net quantum efficiency for decarboxylation in UV-irradiated phenylalanine solutions (Table 5), it is possible that both aspartic acid and aminomalonic acid result from reactions of aliphatic free radicals (derived from the side chain of phenylalanine) with dissolved CO_2 . The fact that the quantum yields for these substances are higher in oxygen-saturated than in oxygen-free solutions is consistent with this hypothesis since the net quantum yield for decarboxylation is higher when oxygen is present in the solution (Table 5).

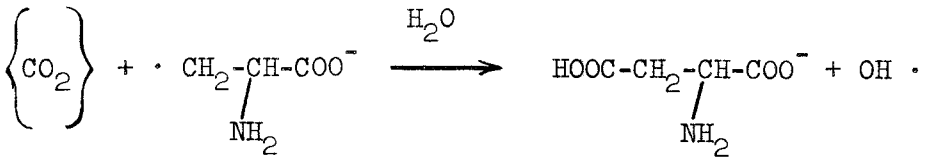
The proposed mechanisms for the synthesis of aspartic acid and aminomalonic acid are shown below.

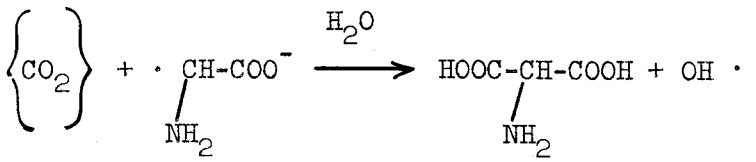
1. Side chain photodissociation reactions following ring π-electron excitation:





2. Secondary reactions of the aliphatic radicals:





The brackets around CO_2 indicate uncertainty about the actual form of carbon dioxide which participates in the reactions. The reactive species may be vibrationally excited CO_2, the COOH· radical, HCO_3^- or $CO_3^=$ ions; or some unknown form of CO_2 possibly associated with some carrier species to which the CO_2 must be bound in order to react with the aliphatic radicals.

If the proposed mechanism is correct, then the amount of radioactivity incorporated into aspartic acid when phenylalanine is labelled with C^{14} at the 3-carbon should be one-half the radioactivity incorporated when phenylalanine carries C^{14} at the carboxyl carbon. That is, both carboxyl carbons would be labelled in the latter case, whereas in the former case only the 3-carbon would be labelled. Thus, the finding that the apparent quantum yield of aspartic acid is 1.8×10^{-3} when the 3-carbon is labelled, while the yield is $2.9 - 3.6 \times 10^{-3}$ in the case of carboxyl carbon labelling (Table 5) is consistent with the proposed mechanism. However, since the experiments in which phenylalanine was labelled at the 3-carbon were conducted at pH 7.3, while those in which phenylalanine was labelled at the carboxyl carbon were carried out at pH 10.8, these results must be interpreted with caution.

7. Low Temperature e.s.r. Data

In the reaction mechanisms discussed above for the synthesis of aminomalonic acid, aspartic acid, and benzoic acid, free radicals are proposed as primary photodissociation products of phenylalanine.

Evaluation of the extent to which the low temperature e.s.r. results discussed in Section E confirm the details of the proposed mechanisms for synthesis of these photoproducts in aqueous solution at room temperature must await positive identification of the individual contributing free radical species. However, the general features of the e.s.r. data are consistent with the proposed mechanisms. That is, several different free radical species are formed following ring π-electron excitation, each of which is associated with a different characteristic quantum efficiency. Furthermore, it is likely that the primary free radicals formed in a viscous organic glass at 77° K are identical with those formed in aqueous solution at room temperature, since the process of internal conversion from the excited singlet electronic state to a high vibrational state of the electronic ground state, which probably must occur before photodissociation can take place (see Appendix C), presumably occurs at both temperatures (Kasha, 1960). However, the quantum efficiencies are probably lower at room temperature since, once internal conversion has taken place, temperature dependent processes, such as vibrational cascading due to collisions with solvent molecules, may de-activate the high vibrational state before photodissociation can occur.

III. CHARACTERIZATION OF THE PHOTOPOLYMER

The problem of chemically and physically characterizing the insoluble pigmented photoproduct (i.e., photoproduct No. 1, $R_f = 0$; see Tables 2a and 3) is made especially difficult by the exceedingly low solubility of the material. Attempts to dissolve small samples of the substance, which were prepared by centrifugation followed by lyophilization, were uniformly unsuccessful for a wide variety of organic solvents (Taskovich, unpublished observations), thus precluding the possibility of characterizing the material by finite chromatographic R_f values. Furthermore, the material does not melt or decompose below 300°C (the highest temperature tested), suggesting the possibility of high molecular weight or molecular heterogeneity, since the vast majority of low molecular weight organic substances have decomposition or melting points below 300°C (Frankel et.al., 1960). X-ray powder photographs show no diffraction lines (Bezrukov, unpublished observations), indicating that the substance is non-crystalline.

Direct evidence that the pigmented photoproduct is actually a high molecular weight, possibly polymeric, substance was obtained by Blois (1964) in an elegant series of dialysis experiments. An unirradiated control solution of C^{14}-labelled DL-phenylalanine (5×10^{-4} M) was dialyzed against running water. The rate at which phenylalanine diffused through the dialysis membrane was determined by measuring the radioactivity remaining inside the bag as a function of dialysis time. The expected exponentially decreasing rate of dialysis is shown in Fig. 20. If, however, the solution is first irradiated, either in the presence of high or low concentrations of dissolved oxygen, at 2537 Å with a dose sufficient to produce the maximum increase in optical absorbance under the given conditions (Chapter I, Section D) and then dialyzed in the above manner, the radioactivity is lost much less rapidly from the

dialysis bag.* Minimum rates of dialysis are observed for solutions through which standard grade N_2 , which contains only small amounts of O_2 , is bubbled during irradiation. This result is correlated with the earlier finding that maximum yields of pigment are obtained in N_2-flushed solutions (Blois, 1964). If the irradiated solutions are dialyzed in the presence of NaCl , no change is observed in the rates of dialysis, showing that the radioactivity is not retained inside the bag by charged, low molecular weight photoproducts. These results show that the pigment synthesized in UV-irradiated solutions of DL-phenylalanine is a high molecular weight, most probably polymeric material, although they reveal nothing about the chemical structure of the material.

A. PHYSICAL STUDIES

Having established that the photopigment is a high molecular weight substance, the next step in characterizing it consisted in comparing it with samples of natural and synthetic melanin by means of several types of physical measurements.

1. Sample Preparation

Purified samples of unlabelled photopigment were prepared by irradiating large volumes (200-500 ml) of phenylalanine solutions (10^{-3} - 10^{-2}M) with 2537 Å radiation from a low pressure mercury arc lamp, or with all UV wavelengths above 2400 Å using a modified version of the xenon arc irradiation apparatus (Fig. 3) in which the monochromator was not used. The pigment was separated from the reaction mixtures by centrifugation followed by extensive dialysis against glass-distilled water, and finally by freeze drying. Samples of natural and synthetic melanins were purified in a similar manner.

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*After these doses, the solutions were yellow-brown, but not significantly turbid due to the low initial concentration of phenylalanine (5×10^{-4}M). The pigmented material remained inside the bag during dialysis.

FIG. 20--Rate of dialysis of phenylalanine plus photoproducts. Dilute (5×10^{-4}M) aqueous solutions of DL-phenylalanine labelled with C^{14} at the carboxyl-carbon were dialyzed against running water after irradiation at 2537 Å had resulted in the maximum increase in optical absorbance attainable under the given conditions, the required dose depending on whether the solution was flushed with standard grade N_2, or with O_2, periodically during irradiation (see text). During dialysis, samples were withdrawn from the bag and assayed for C^{14} activity. The rate of dialysis of unirradiated (control) phenylalanine is included for comparison (after Blois, 1964).

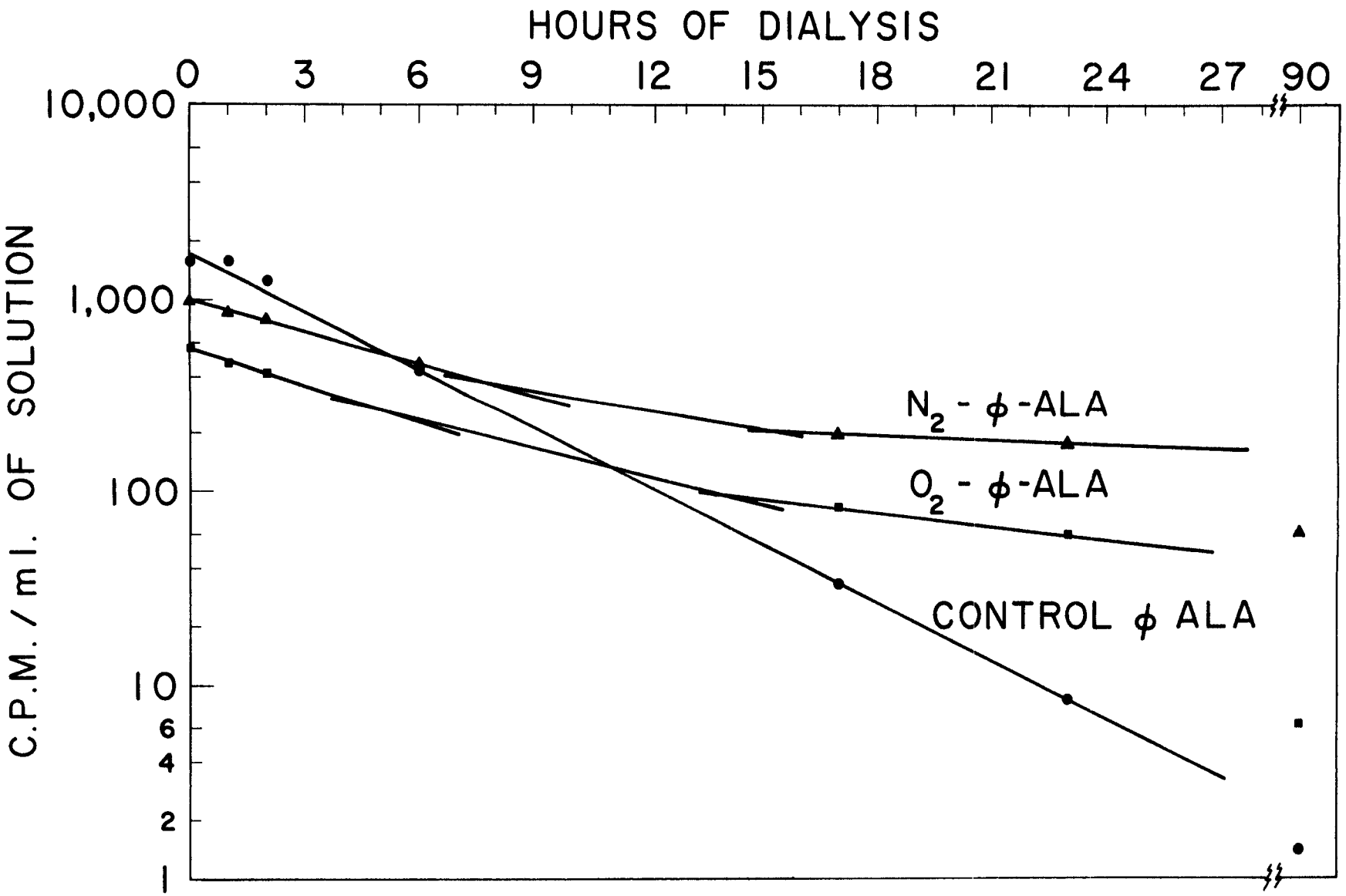
HOURS OF DIALYSIS
0
3
6
9
12
15
18
21
24
27
90
10,000
1,000
100
10
6
4
2
1
C.P.M. / ml. OF SOLUTION
N2 - ϕ -ALA
O2 - ϕ -ALA
CONTROL ϕ ALA

FIGURE 20

2. Infrared Spectra

In Fig. 21 the infrared (IR) spectrum of the photopigment is compared with the IR spectra of a sample of autoxidized DOPA-melanin and a sample of naturally occurring melanin obtained from the ink sack of the squid, Loligo opalescens.* These spectra were obtained on a Perkin-Elmer, model 221, recording infrared spectrophotometer, using 0.5 mg samples imbedded in 300 mg KBr pellets.

It is apparent that the photopigment spectrum closely resembles the spectra of the two types of melanin, not only in number and position of the absorption bands, but also in relative amplitudes of the bands. However, the wealth of fine-structural detail normally contained in the IR spectrum of a molecularly homogeneous organic substance, e.g., phenylalanine, and upon which structural identification is usually based, is absent from these spectra in which only a few broad absorption bands occur. Consequently, the great similarity of these spectra does not necessarily imply that the three substances are structurally identical. In this connection, the work of Nicolaus (1962) on the structure of natural melanins is of considerable interest. Using various degradation procedures, he has shown that natural melanins are most probably high molecular weight, randomly cross-linked polymers of as many as 10 different monomer units linked together by four different types of bonds. Therefore, what the IR spectra of Fig. 21 reflect is (1) the molecular heterogeneity of the three types of substance, and (2) the general "statistical" similarity of the photopigment and the two types of melanins with respect to types and relative abundances of certain characteristic bonds and chemical structures.

3. Visible and UV Absorption Spectra

The visible and UV absorption-scatter spectrum of an aqueous suspension of the photopolymer is compared in Fig. 22 with that of DL-DOPA-melanin.

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*Included for comparison are the IR spectra of DL-phenylalanine and two insoluble high molecular weight carbonaceous substances.

These spectra were measured on a Cary, model 14M, recording spectrophotometer. Although the curves are similar in shape, neither shows any characteristic absorption maxima. The difference in apparent absorbance below 3500 Å probably reflects a different average particle size in the two samples. However, the high apparent light absorption above 2500 Å suggests the presence of aromatic structures in both substances.

4. Electron Spin Resonance Spectra

That natural melanins exhibit a stable electron spin resonance (e.s.r.) signal at $g \cong 2.0$ was first shown by Commoner, Townsend, and Pake (1954). These investigators attributed the e.s.r. signal to free radicals trapped within the pigment particles. Recent detailed e.s.r. studies of natural and synthetic melanins (Vivo-Acrivos and Blois, 1958; Blois, Zahlan, and Maling, 1964) show that these trapped free radicals most probably result from the non-enzymatic, free radical mode of synthesis of these polymers. Therefore, in view of the probable structural similarity of the photopigment to natural melanin as revealed by the IR data discussed above, and in view of the fact that DOPA is photochemically synthesized from phenylalanine (Chapter II) and is known to undergo spontaneous auto-oxidative free radical polymerization to melanins (Wertz, Reitz and Dravnieks, 1961), it appeared likely that the photopolymer should also exhibit a stable e.s.r. signal, due to trapped, unpaired electrons.

This hypothesis was fully confirmed by examining a 3-mg lyophilized sample of the photopolymer in a Varian X-band, model V 4500-10 e.s.r. spectrometer with 100 Kc modulation. The resulting spectrum is shown in Fig. 23, together with a similar spectrum for squid melanin. The over-all similarity of the two spectra, both in shape and linewidth, is evident, although neither exhibits hyperfine structure. This lack of hyperfine structure is further evidence that both types of substance are molecularly heterogeneous and consist of several different types of monomer. However, the relatively small line widths (8 - 10 gauss) of the absorptions indicates the over-all similarity of the monomer types (Blois, Zahlan, and Maling, 1964).

FIG. 21--Infrared (IR) absorption spectra of the photopolymer synthesized in UV-irradiated phenylalanine solutions and of samples of natural and synthetic melanins. The spectra were measured on a Perkin-Elmer, model 221, recording IR spectrophotometer, using 0.5 mg lyophilized samples imbedded in 300 mg KBr pellets. The IR spectra of DL-phenylalanine, graphite, and charcoal are included for comparison (see text; adapted from Blois, 1964; and Blois, Zahlan, and Maling, 1964).

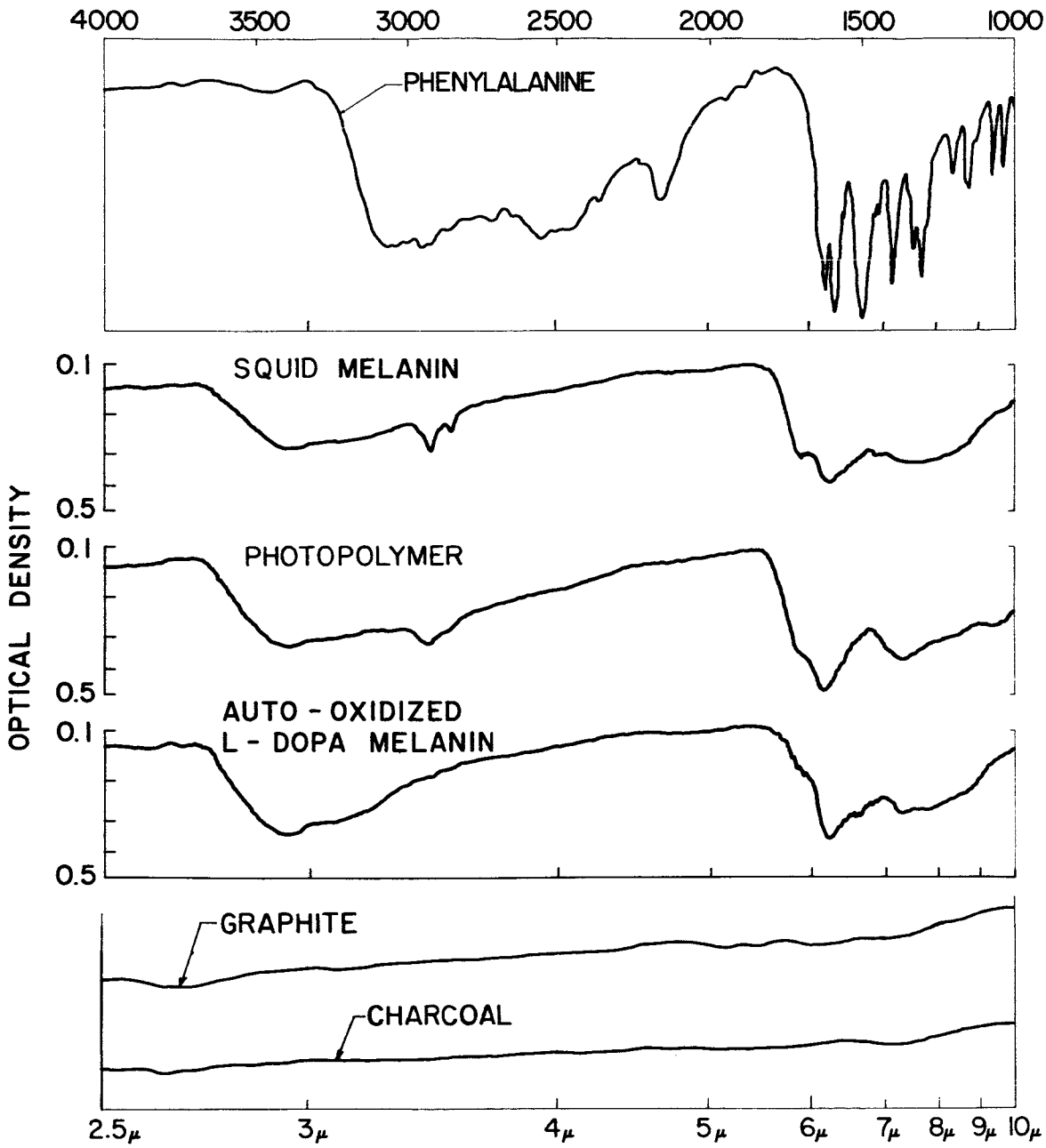

4000
3500
3000
2500
2000
1500
1000
PHENYLALANINE
SQUID MELANIN
PHOTOPOLYMER
AUTO - OXIDIZED
L - DOPA MELANIN
GRAPHITE
CHARCOAL
OPTICAL DENSITY
0.1
0.5
2.5μ
3μ
4μ
5μ
6μ
7μ
8μ
9μ
10μ

FIGURE 21

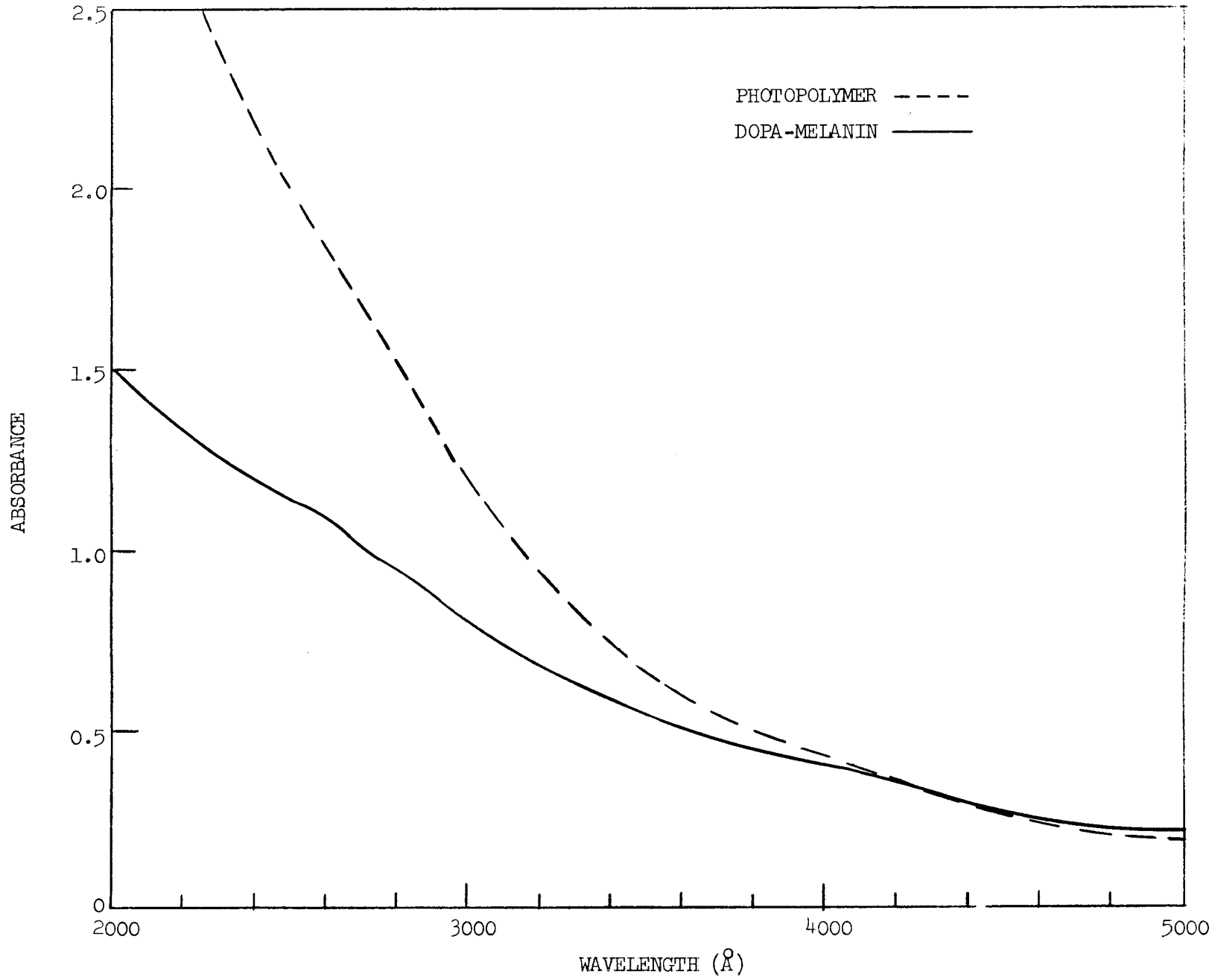


FIG. 22--Visible and UV absorption-scatter spectra of aqueous suspensions of the photopolymer and autoxidized DOPA-melanin measured on a Cary, model 14M, recording spectrophotometer.

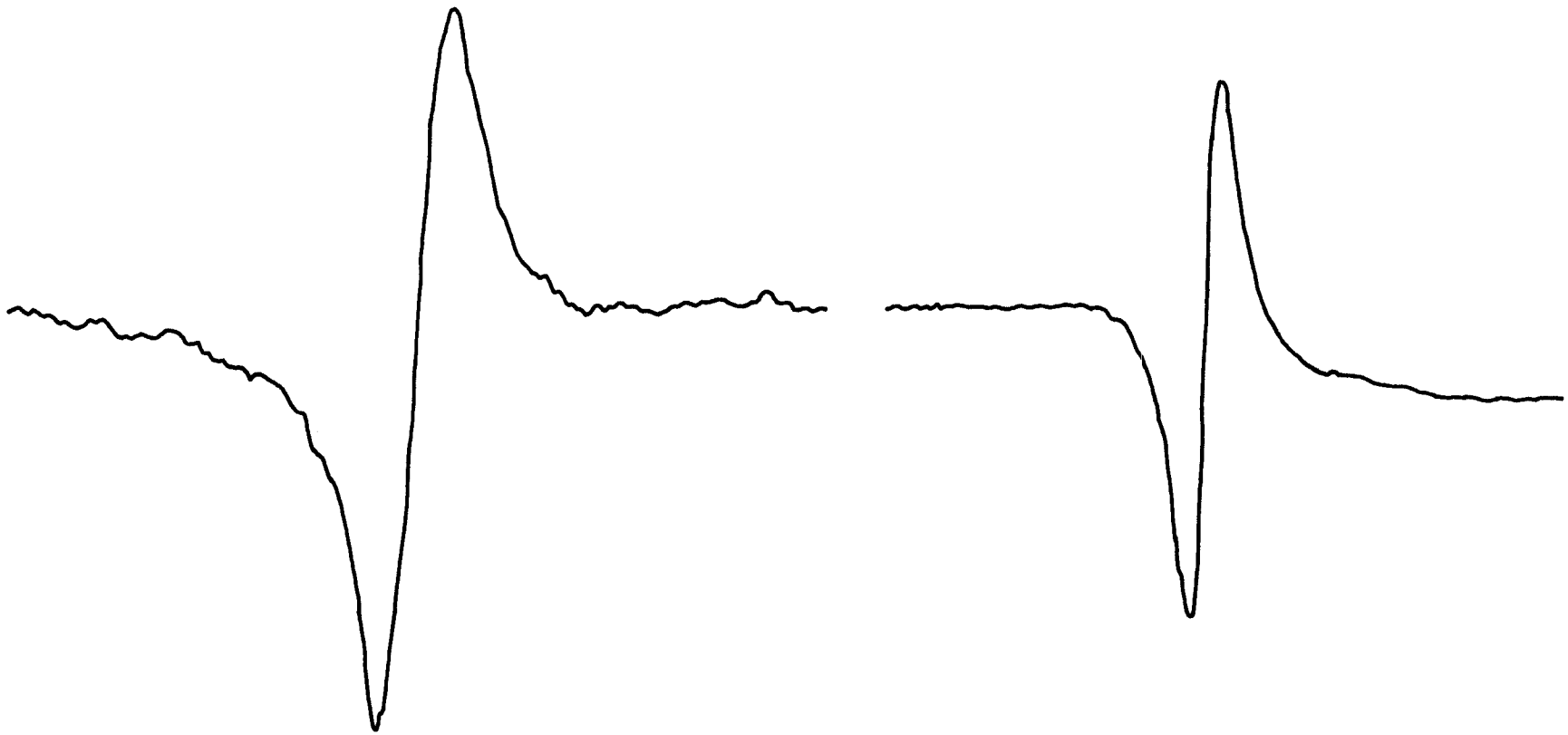


FIG. 23--Electron spin resonance spectra of lyophilized samples (3-5 mg) of the photopolymer, and natural melanin isolated from the ink sack of the squid, Loligo opalesencs, recorded at room temperature on a Varian, model V-4500-10, X-band spectrometer.

B. PHOTOCHEMICAL DEGRADATION STUDIES

Further evidence that the photopolymer closely resembles natural and synthetic melanins was obtained in a comparative study of the photo-oxidation of the two types of substance. This study was undertaken primarily in order to test the hypothesis that the photopolymer synthesized in oxygen-saturated, UV-irradiated phenylalanine solutions is subsequently photo-oxidizable with continued irradiation. That is, one plausible explanation of the observation that the optical absorbance first increases to a maximum and then falls to nearly zero for all wavelengths greater than 2400 Å in UV-irradiated phenylalanine solutions saturated with oxygen (Blois, 1964) is that photopolymerization reactions leading to the formation of the strongly absorbing pigment, and photo-oxidative breakdown of the pigment particles, are occurring simultaneously. During the initial stages of irradiation, while the concentration of phenylalanine is still relatively high, the photopolymerization reactions presumably predominate, while later in the process when the concentration of phenylalanine has been considerably reduced, the photo-oxidative degradation reactions would predominate. Demonstration in a separate series of experiments that the photopigment is indeed bleached by UV irradiation of $\lambda > 2400$ Å in the presence of dissolved molecular oxygen would lend support to this hypothesis. At the same time, it is possible to compare quantitatively the photo-oxidative behavior of purified samples of the photopigment and DOPA-melanin in dilute aqueous suspension using C^{14}-labelled material. In this way, further evidence could be obtained on the extent of similarity of the molecular structures of the two substances.

1. Sample Preparation

A purified sample of C^{14}-labelled photopigment was prepared as follows: 160 ml of a 5×10^{-3}M solution of DL-phenylalanine (Calbiochemical Corp., Grade A) in glass-distilled water containing 30 - 40 μc of DL-phenylalanine-3-C^{14} (New England Nuclear Corp.) was irradiated in a quartz reaction vessel with 2537 Å radiation from a low pressure

mercury resonance lamp (Braun-Knecht-Heimann Co., No. 36554) equipped with two Vycor filters having cut-off points at 2350 Å. The distance from the lamp to the front window of the reaction cell was about 2 cm. During irradiation, an effort was made to keep the concentration of dissolved oxygen low by continuously flushing the solution with standard grade N_2, although occasionally the solution was exposed to air. The solution became intensely pigmented after 24 hours of exposure and the turbidity gradually increased throughout the run.

After about one week of continuous exposure, part of the photopigment fraction of the reaction mixture was concentrated by centrifugation (8000 rpm; 30 min). The supernatant remained strongly pigmented, indicating the presence of smaller photopolymer molecules which were either in stable colloidal suspension or were actually small enough to be water soluble. The residual supernatant fluid at the bottom of the centrifuge tube was agitated to suspend the photopolymer particles, and a sample was chromatographed in n-BuOH: HAc: H_2O; the resulting chromatogram was scanned for C^{14} activity. The results indicated the presence of a substantial yield of phenylethylamine (see dotted peak in Fig. 5d) in addition to photopolymer and unreacted phenylalanine. No other major components were present. The photopolymer suspension was dialyzed for four days against approximately 500 times its volume in glass-distilled water, the water being changed three times daily. Periodically during dialysis, small samples were withdrawn from the dialysis bag and chromatographed to follow the progress of the purification. When more than 90% of the total C^{14} activity on the chromatogram was concentrated in the photopolymer peak at the origin and no other minor peaks were discernible, the sample was considered sufficiently pure for use in the photochemical experiments described below.

Carbon-14 labelled DL-DOPA-melanin was prepared by auto-oxidation of 50 μc of DL-DOPA-2-C^{14} in aqueous solution (20 ml) at alkaline pH. After sufficient pigmentation developed, the reaction mixture was dialyzed until radiochromatographic purity was achieved as described above.

2. Irradiation Procedure

The concentration of pigment was adjusted to be roughly the same in both suspensions by diluting with glass-distilled water. After adjustment of the pH to 7.0 with KOH, the suspensions were transferred to Cary fused silica spectrophotometer cells and flushed vigorously with oxygen for 5-10 min. Prior to irradiation, samples of known volume (0.10 - 0.12 ml for the photopolymer and 0.048 ml for DOPA-melanin) were withdrawn from each suspension and chromatographed in solvent system A (Table 2a). After determination of the initial absorption-scatter spectra on the Cary spectrophotometer, the two suspensions were irradiated simultaneously in contact with air in the xenon arc apparatus with the full white output of the arc. Wavelengths less that 2400 Å were filtered out of the beam as discussed above (Chapter II, Section B). Periodically during the run, samples were removed for paper chromatography and the absorption-scatter spectra of the reaction mixture determined. Due to the high infrared content of xenon arc radiation, the temperature of the sample cells rose considerably above room temperature, but was maintained constant at 46 $\pm$ 2°C by the copper block cooling system (See Fig. 4).

The amount of radioactivity remaining at the origin of the chromatograms was determined by means of the digital integration accessory to the Vanguard paper strip scanner as described above (Chapter II, Section D). This activity was taken as a measure of the amount of insoluble, high molecular weight polymer remaining in each suspension. Also, in order to assess the extent to which gaseous or liquid photoproducts were produced from the C^{14}-labelled carbon atoms, the total activity on each chromatogram was determined by summation over the entire strip of the individual one-minute count values.

3. Results and Discussion

That both the photopigment and DL-DOPA-melanin are photochemically bleached by UV radiation of wavelengths greater than 2400 Å is shown in Fig. 24, in which the apparent optical densities of the suspensions at 4000 Å and at 5000 Å are plotted as functions of irradiation time. It is apparent that after about 3 hours of irradiation

(total intensity, 2.5×10^6 ergs/sec/cm^2) the rates of photobleaching of the two substances are nearly identical, although the photopigment curve is somewhat steeper than the curve for DOPA-melanin. The reason for the initial difference in rates of photobleaching is not known. It is possible, however, that this difference reflects a different initial average particle size in the two suspensions such that the contribution of scatter to the apparent optical density is greater in the case of the photopolymer.

Quantitative radiochromatographic data corresponding to the photobleaching kinetics are shown in Fig. 25. These data provide additional evidence that high molecular weight photopolymer is degraded at substantially the same rate as high molecular weight DOPA-melanin under identical conditions of pH, temperature, incident light intensity, and concentration of dissolved oxygen, although again the photopolymer appears to be slightly more rapidly photo-oxidized than DOPA-melanin. In the case of DOPA-melanin, however, the total C^{14} activity per unit volume of the suspension declines considerably more rapidly than the total C^{14} activity per unit volume of the photopolymer suspension. Since DOPA-melanin was synthesized from DL-DOPA labelled with C^{14} at the 2-carbon of the side chain, whereas the photopolymer was prepared from phenylalanine labelled at the 3-carbon of the side chain, this result suggests (provided the detailed molecular structures of the two polymers are similar; see discussion below, Section C) that polymer carbon atoms derived from the 2-carbon of the starting material are much more photochemically labile than those derived from the 3-carbon.

Although the radiochromatogram traces showed no well-defined photoproduct peaks, the amount of radioactivity leaving the origin did progressively increase with increasing irradiation time in the case of the photopolymer. This C^{14}-activity corresponds to the difference between total activity and activity associated with high molecular weight photopolymer (see Fig. 25). As shown in Fig. 26, this chromatographically mobile C^{14} activity tails out from the photopolymer peak

FIG. 24--Rates of photobleaching of the photopolymer and DL-DOPA-melanin in aqueous suspension. The two samples were irradiated simultaneously in open fused silica cuvettes mounted in the copper cooling block shown in Fig. 4. Other experimental conditions were as follows: Wavelengths, full white light output of the xenon arc above 2400 Å ; incident intensity, 2.5×10^6 ergs/cm^2/sec.; temperature, 46 ± 2°C; pH, 7.0. The apparent optical densities (i.e., including contributions from scatter, see Fig. 22) of the suspensions at 4000 and 5000 Å were followed on a Cary, model 14M, recording spectrophotometer.

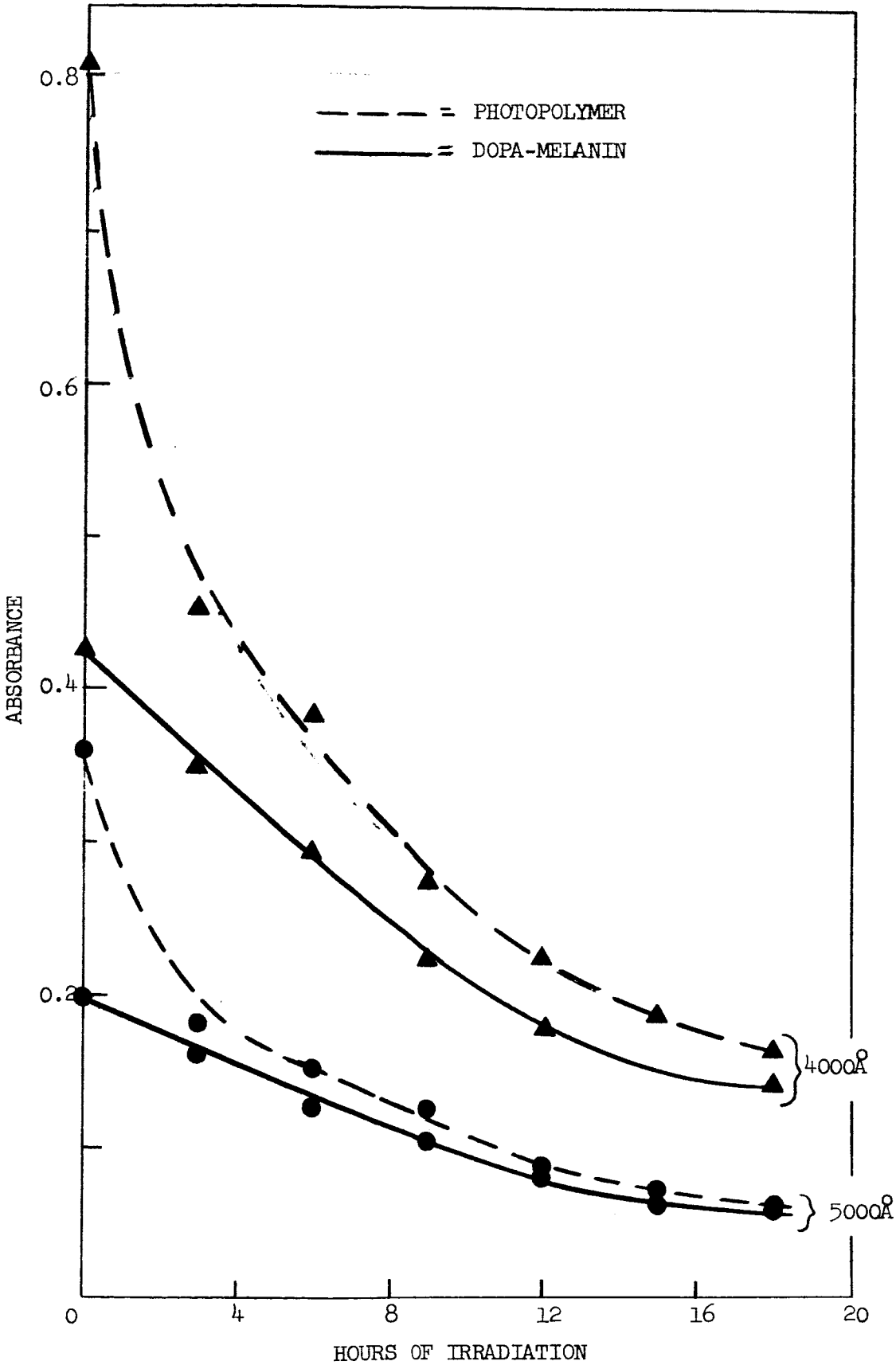

= PHOTOPOLYMER
= DOPA-MELANIN
ABSORBANCE
0.8
0.6
0.4
0.2
4000Å
5000Å
0
4
8
12
16
20
HOURS OF IRRADIATION

FIGURE 24

FIG. 25--Radiochromatographic data corresponding to the photobleaching kinetics shown in Fig. 24. The photopolymer sample was synthesized from DL-phenylalanine labelled with C^{14} at the 3-carbon. The DOPA-melanin sample was prepared by dark autoxidation of DL-DOPA-2-C^{14} (see text). After each optical density determination, samples were withdrawn from the suspensions and chromatographed in the organic phase of n-BuOH: HAc: H_2O, 4:1:5 (v/v/v). The C^{14} activity remaining at the origin and the total activity on the resulting chromatograms were determined by digital integration on a Vanguard, model 880, recording paper strip scanner.

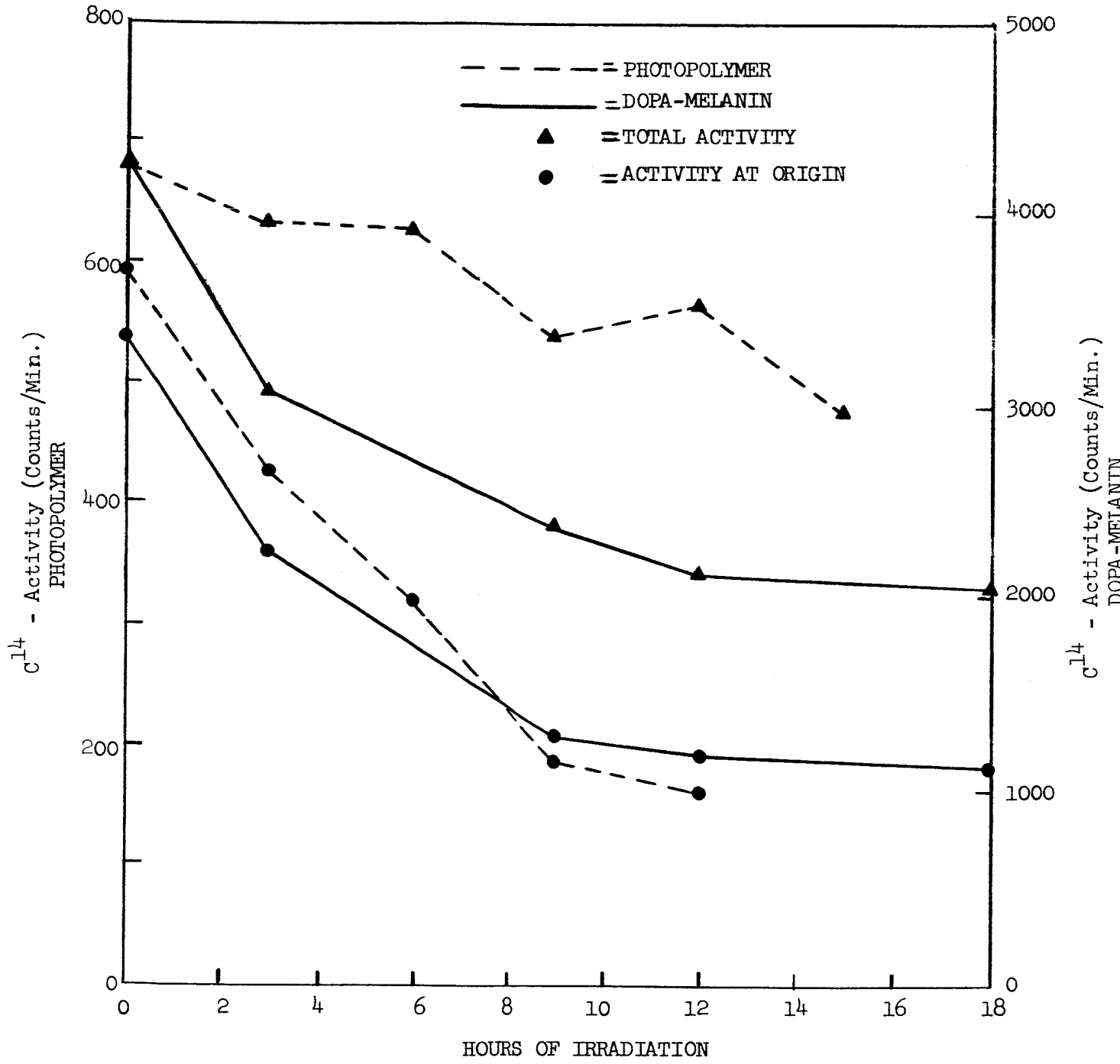

800
600
400
200
0
5000
4000
3000
2000
1000
0
= PHOTOPOLYMER
= DOPA-MELANIN
= TOTAL ACTIVITY
= ACTIVITY AT ORIGIN
C^{14} - Activity (Counts/Min.)
PHOTOPOLYMER
C^{14} - Activity (Counts/Min.)
DOPA-MELANIN
0
2
4
6
8
10
12
14
16
18
HOURS OF IRRADIATION

FIGURE 25

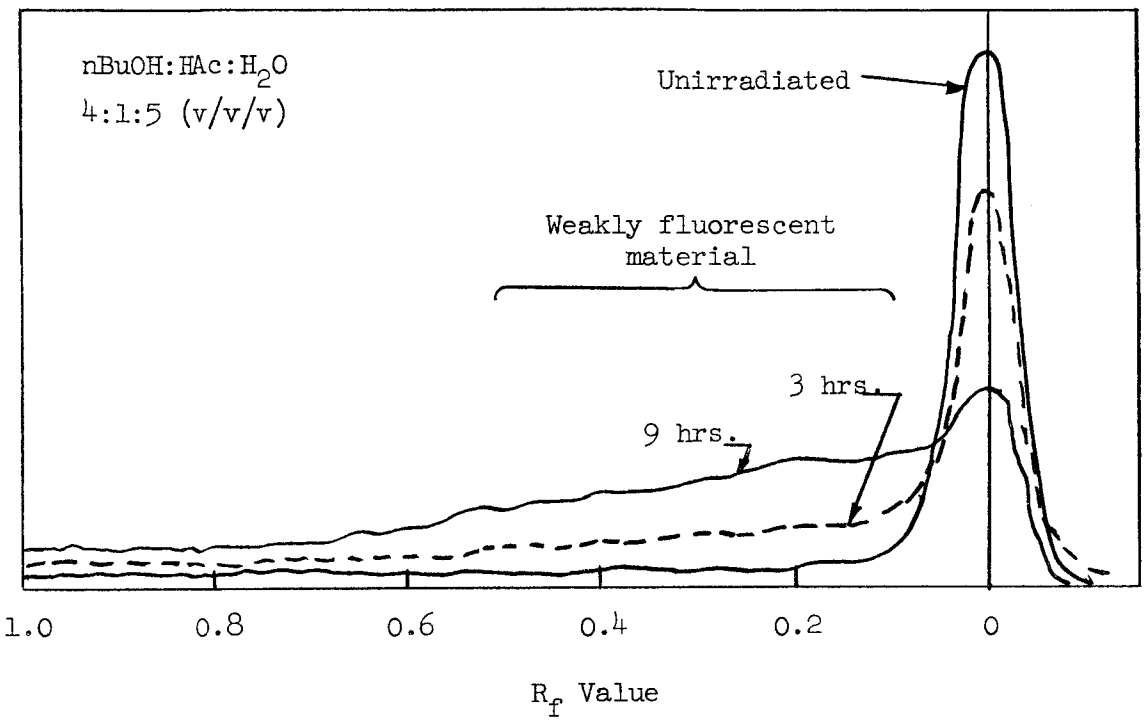


FIG. 26--Distributions of C^{14} activity and fluorescent material on paper chromatograms of samples of an irradiated photopolymer suspension. Experimental conditions are the same as those given in the legend to Fig. 24.

at the origin. In the case of DOPA-melanin the difference between total activity and polymer activity is much less marked (see Fig. 25), and consequently the tailing effect is much less pronounced.

Examination in UV light (Mineralight, Ultraviolet Prod., Inc. No. R51) of the paper chromatogram strips corresponding to the traces for t = 6 and 9 hrs. shown in Fig. 26, as well as similar chromatograms of the irradiated DOPA-melanin suspension reveals the presence of traces of weakly fluorescent material distributed more or less uniformly over the region indicated. The t = 0 strip showed no fluorescent material. For both the irradiated photopolymer and DOPA-melanin suspensions, chromatographically mobile fluorescent material was detected near the origin on strips developed in 88% aqueous phenol. However, with benzene:HAc:H_2O, 2:2:1 as solvent system, the fluorescent material remained at the origin distributed around the periphery of the sample spot. These results show that upon UV irradiation, the photopolymer and DOPA-melanin yield weakly fluorescent substances of similar chromatographic behavior which are somewhat soluble in the moderately polar solvents n-butanol and phenol, and insoluble in the non-polar solvent, benzene. That the fluorescent photoproducts are water soluble is suggested by the appearance, as a function of irradiation time, of the sample spots at the origins of the chromatogram strips before development, as shown schematically in Fig. 27.

The samples were withdrawn directly from the aqueous reaction mixtures and spotted with micropipettes. The distribution of material in these spots is actually the result of a microscale chromatographic separation occurring during application of the sample and in which water acts as the developer. In the t = 0 spot, no fluorescent material is present and the brown polymer is concentrated near the center, as expected, since this material is non-dialyzable, of high molecular weight, and water insoluble. However, in the 6-hr spot, the pigment is distributed over the whole area with a small excess of the material at the periphery suggesting that much of the pigment now has some degree of water solubility and that a small fraction is highly water soluble. Also, the total amount of pigment in the spot is less than in the t = 0

t = 0 6 hrs. 12 hrs.

FIG. 27--Appearance of sample spots at the origins of Whatmann No. 1 paper chromatogram strips before development as a function of irradiation time for both the photopolymer and DOPA-melanin suspensions. The distribution of brown pigment within the spot is represented by differential shading, the amount of pigment being proportional to the degree of shading. In the t = 0 spot, no fluorescent material was visible. In both the 6-hr. and 12-hr. spots, a peripheral ring of fluorescent material was present which was somewhat thicker and more intense in the 12-hr. spot.

spot as expected from the bleaching kinetics shown in Fig. 24. A thin ring of presumably water soluble fluorescent material is present around the perimeter of the spot. In the 12-hr. spot, the small amount of pigment remaining is concentrated entirely at the periphery together with a ring of fluorescent material somewhat more concentrated than in the 6-hr. spot.

One possible explanation of these results is that UV radiation in the presence of dissolved molecular oxygen breaks down the large pigment particles into progressively smaller and more water soluble fragments, the smallest of which are fluorescent. Alternatively, it is possible that one or more water soluble, low molecular weight fluorescent photoproducts are liberated continously during irradiation, and that the polymer is degraded into increasingly water soluble, but non-fluorescent, smaller fragments. That the fluorescent material is highly water soluble was confirmed by chromatography of samples of the irradiated suspensions using distilled water as developer. The fluorescent material was concentrated in a compact spot near the solvent front which was easily distinguished from fluorescent artifacts occurring at the solvent front by running control chromatograms on which no sample had been spotted. For both the photopolymer and DOPA-melanin, C^{14} activity was associated with the fluorescent spot.

Preliminary attempts to characterize the fluorescent material in irradiated suspensions of photopolymer and DOPA-melanin by means of fluorescence emission and excitation spectroscopy suggested the presence of a mixture of different fluorophores. However, such results must be treated with great caution due to the high probability that artifacts are introduced by the presence of uncharacterized light scattering material in the suspensions.

Attempts were made to isolate and purify the fluorescent substances by means of thin layer chromatography on silica gel G (E. Merck, Darmstadt). Some 0.2 ml samples of both irradiated and unirradiated reaction mixtures were lyophilized and resuspended in 0.01 ml of 30% H_2O - 70% acetone. The concentrated suspensions were taken up in a micropipette and spotted at the origin of thin layer plates which had previously been

activated at 110°C for 30 min. Before development, the spots of the irradiated samples showed wide peripheral annuli of fluorescent material similar to those which had been observed on paper chromatograms (see Fig. 27). Residual brown pigment was concentrated at the center of the spots. No fluorescent material was visible in the control (t = 0) spots. After development in n-BuOH:HAc:H_2O , 6:2:2 (Randerath, 1963), part of the material in the irradiated samples was distributed over long fluorescent tails which diminished continuously in concentration and width with increasing R_f value. This material was only visible under ultraviolet light. The unirradiated sample remained at the origin. These results are summarized in Fig. 28. Anthranilic acid was used as a fluorescent marker in order to check the performance of the system.

The thin layer chromatographic results, together with the paper chromatographic data discussed above, support the view that UV radiation degrades high molecular weight, non-dialyzable photopolymer and DOPA-melanin into smaller, weakly fluorescent fragments of continuous molecular weight distribution and continuously variable solubility in polar solvents. Within the limits of these studies, the fluorescent material derived from the photopolymer is indistinguishable from that derived from DOPA-melanin.

C. MOLECULAR STRUCTURE OF PHOTOPOLYMER

In this chapter, physical and photochemical studies in which the photopigment (photoproduct No. 1) was systematically compared, both qualitatively and quantitatively, with natural and synthetic melanins have been discussed in detail. The results of these comparative studies show that the detailed molecular structure of the photopigment derived from DL-phenylalnine is very similar to that of the melanic pigments. However, the lack of crystallinity as determined by X-ray diffraction methods, the lack of a melting point (below 300°C), as well as the relatively structureless UV, visible, and infrared absorption spectra and the lack of hyperfine structure in the electron spin resonances, all suggest that both substances are molecularly heterogeneous.

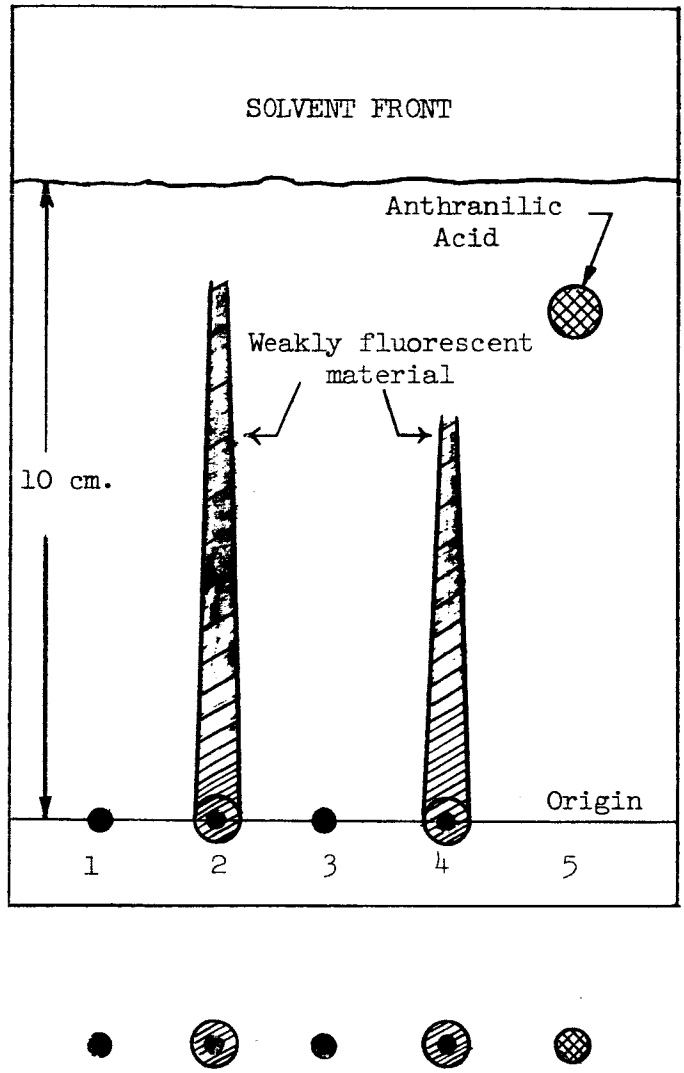


FIG. 28--Thin layer chromatogram of samples of irradiated photopolymer and DOPA-melanin suspensions. One-tenth ml samples of the reaction mixtures were concentrated by freeze drying, resuspended in 0.005 ml 30% acetone: 70% water, spotted on an activated silica gel G thin layer plate, and developed in n-BuOH:HAc:H_2O, 6:2:2 (w/w/w). 1. Photopolymer, unirradiated. 2. Photopolymer irradiated for 12 hrs. 3. DOPA-melanin, unirradiated. 4. DOPA-melanin irradiated for 12 hrs. 5. Anthranilic acid marker (see text). Conditions of irradiation are given in the legend to Fig. 24.

Nevertheless, the appearance of several characteristic (although broad) absorption bands in the IR spectra, and the relative narrowness (8-10 gauss) of the e.s.r. signals indicate that there is some degree of molecular order in both polymers.

That the type of molecular order present in melanins lies between complete homogeneity and complete heterogeneity has recently been shown by Nicolaus (1960, 1962; see also, Piattelli and Nicolaus, 1961; Nicolaus and Piatelli, 1962) in a remarkable series of chemical investigations. By using various degradation procedures (i.e., treatment with $KMnO_4$, Br_2-NaOH, Cl_2-K_2CO_3, H_2O_2, followed by paper chromatography to isolate and identify the low molecular weight products), this investigator was able to reconstruct an average partial structure, statistically representative of the type of structure occurring within natural melanin particles. This structure is shown in Fig. 29. On the basis of these studies, Nicolaus (1962) has concluded that melanins are irregular, three-dimensional, cross-linked copolymers of several different monomer types including 5,6 indole quinone, 5,6 indole quinol, the 2'-carboxyl derivatives of these, and various pyrrole carboxylic acids, linked together by various types of covalent bonds such as -C-C- , -C-O-C- , -C-O-O-C- and hydrogen bonds of the type >NH----O-C- . The general features of this conclusion are confirmed by the recent e.s.r. studies of Blois, Zahlan, and Maling (1964) on a variety of natural and synthetic melanins, although no data bearing on the identity of the various monomers and bond types was obtained.

One probable consequence of the molecular heterogeneity of melanic pigments is that a given molecule of the photopolymer synthesized in UV-irradiated solutions of DL-phenylalanine probably resembles a given molecule of natural or synthetic melanin (of the DL-DOPA type) as much as any two molecules within a given preparation of melanin resemble each other.

In the structure shown in Fig. 29, it is apparent that the indole nucleus is the predominant monomer type. On the basis of the work of Mason (1948) and of Raper (1928) on the intermediates involved in

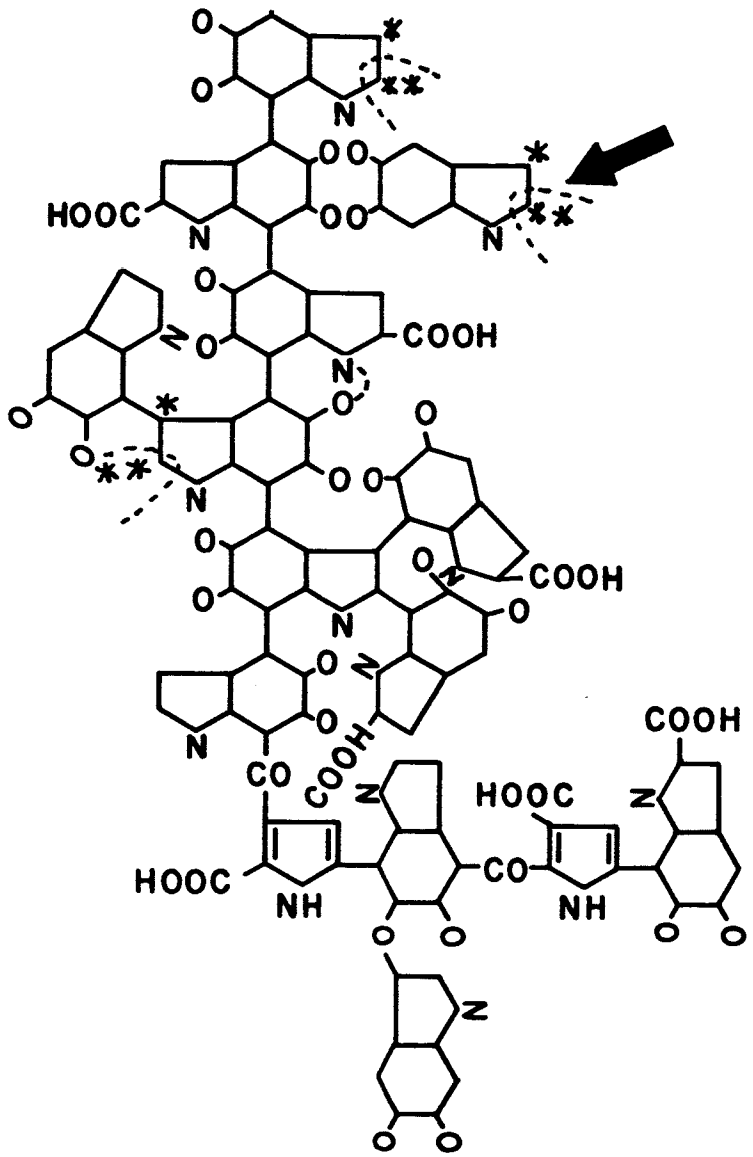


FIG. 29--Average partial molecular structure present in sepiomelanin (adapted from Nicolaus, 1962). The asterisks (see arrow) indicate the sites within the indole units at which the photopolymer (*) and DOPA-melanin (**) were presumably labelled with C^{14} in the photochemical experiments discussed in the text. The dotted lines indicate the labelled carbon atom which is preferentially lost from the polymer suspensions during UV-irradiation (see Fig. 25).

in vitro melanin synthesis, it is probable that the indole nuclei are formed by an intramolecular reaction of the side chain of the starting material in which the amino nitrogen is bound to the benzene ring. Assuming that this is correct, it is possible to indicate which carbon atoms in the structure of the photopolymer and of DOPA-melanin were presumably labelled with C^{14} in the photo-oxidation experiments discussed above. This has been done by means of asterisks in Fig. 29. The photo-oxidation kinetics given in Fig. 25 show that carbon No. 2 is more rapidly lost from the reaction mixture than is carbon No. 3, suggesting that carbon No. 2 is more photochemically labile as indicated schematically by the dotted line in Fig. 29.

D. PROBABLE PATHWAYS OF PHOTOPOLYMER SYNTHESIS

That the photopolymer not only closely resembles natural and synthetic melanins with respect to molecular structure, but also with respect to mode of synthesis, is suggested by the following lines of evidence.

1. The large optical density increases which occur in UV-irradiated solutions of phenylalanine (Fig. 10) are associated primarily with photopolymer synthesis and contain only minor contributions from the low molecular weight aromatic photoproducts (Chapter II, Section D). Claesson (1956) has shown, and we have confirmed and extended her findings, that the rate of absorbance increase in oxygen-saturated, UV-irradiated phenylalanine solutions is maximal at alkaline pH (8.2, Claesson, ibid.; 8-11, present investigation). The rate at neutral and slightly acidic pH (6.8) is considerably smaller and is independent of the concentration of dissolved O_2. In addition, we have shown that irradiation of solutions of DL-phenylalanine at acidic pH's (3-4) results in only slight increases in optical density at 2575 Å for irradiation times up to 5 hrs at 6000-7000 ergs/cm^2/sec. Therefore, OH^- ions as well as molecular oxygen participate in photopolymer synthesis. These results parallel the known roles of both OH^- ions and molecular oxygen in the in vitro autoxidation of DOPA to melanin (Monder et al., 1957).

2. The kinetic data given in Chapter II, Section D show that both tyrosine and DOPA are intermediates in the synthesis of photopolymer. Tyrosine is the starting material for in vivo melanin synthesis (Raper, 1928; Mason, 1948) and DOPA undergoes spontaneous autoxidation to melanin (Monder et al., 1957).

3. UV radiation does not significantly alter the pathways of melanin synthesis from DOPA (or from adrenalin and noradrenalin, which are closely related structurally to DOPA), but rather enhances the rates of the reactions.

4. In vivo, melanin synthesis involves enzymatic conversion of tyrosine to DOPA (or possibly further to DOPA-semiquinone; Nakamura, 1960), followed by autoxidative transformation of DOPA to a variety of quinones and semiquinones which undergo free radical polymerization. The stable electron spin resonance signal exhibited by both natural and synthetic melanins indicates the presence within the polymer particles of trapped, unpaired electrons which reflect the free radical mode of synthesis of these substances (Blois, Zahlan, and Maling, 1964). The presence of trapped, unpaired electrons within the photopolymer particles (see Fig. 23) suggests that free radicals are also involved in the synthesis of this substance.

The reaction scheme for in vivo melanogenesis proposed by Nicolaus (1962) is shown in Fig. 30. On the basis of the evidence listed above, it is likely that many of the reactions in this scheme also occur in UV-irradiated phenylalanine solutions. Of particular interest is the decarboxylation reaction which occurs during the transformation of 5,6-indole quinone-2-carboxylic acid to 5,6-dihydroxyindole. Since the carboxyl group which is liberated is derived from the carboxyl group of tyrosine (see Fig. 30), and, therefore, ultimately from the carboxyl group of phenylalanine in the photochemical experiments discussed in Chapter II, it is possible that this reaction contributes to the total loss of carboxyl carbon C^{14} label from the reaction mixture (see Fig. 11). Furthermore, this reaction could conceivably contribute some reactive form of CO_2 which might participate in the synthesis of aminomalonic

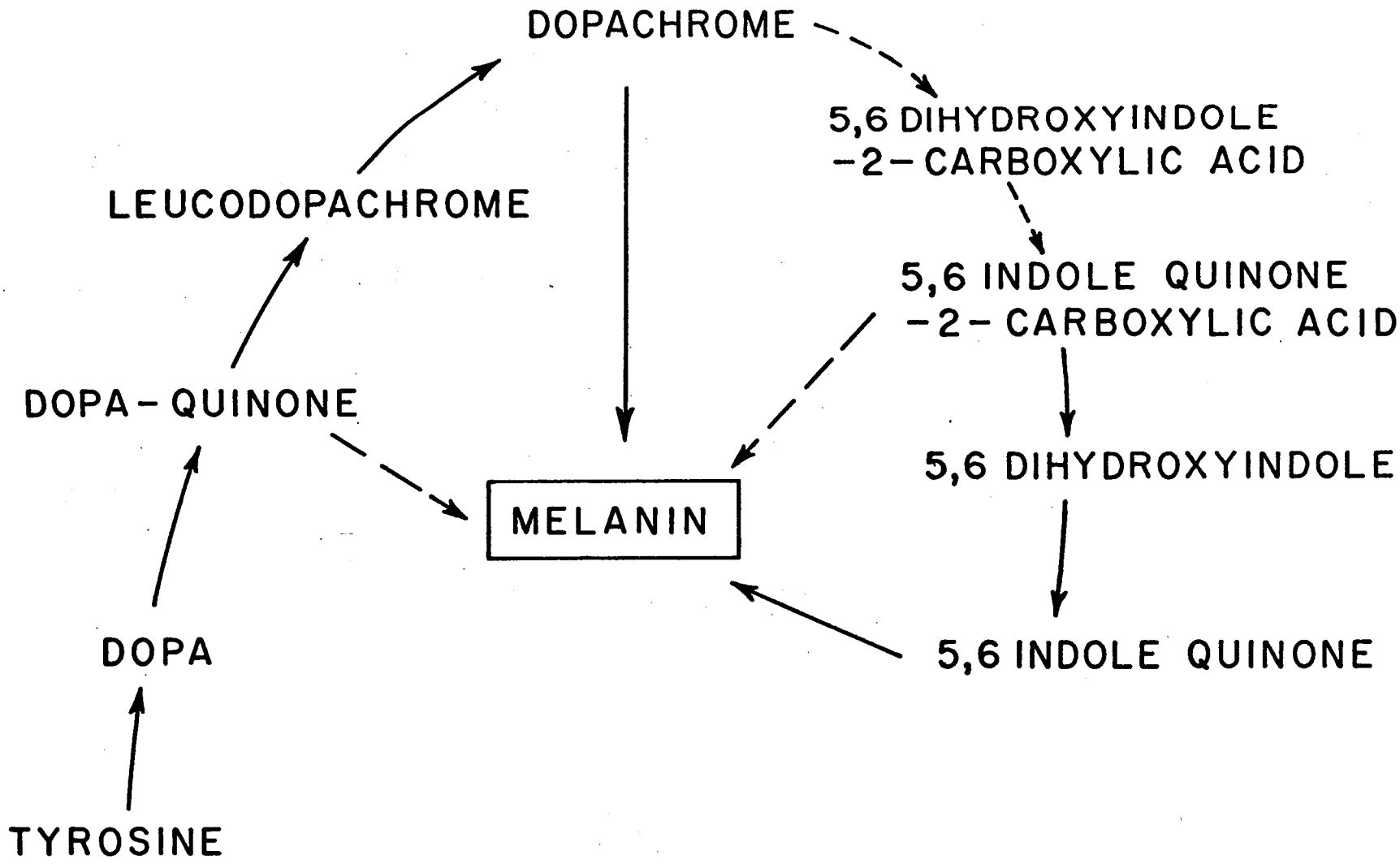
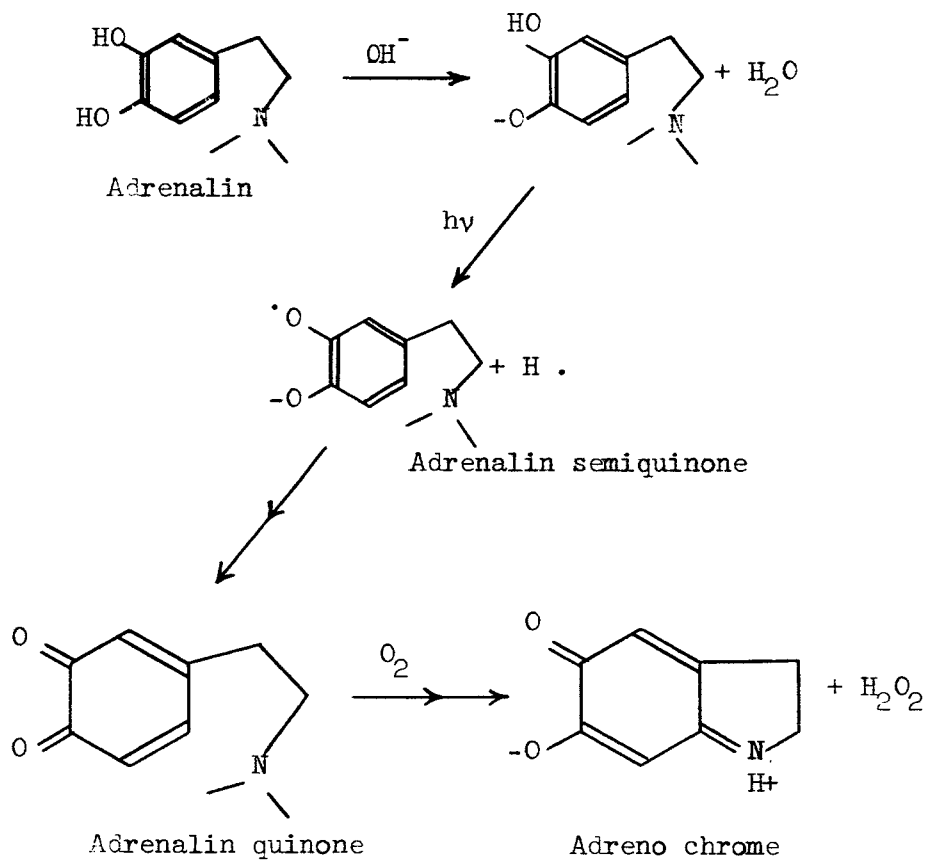


FIG. 30--Reaction scheme for in vivo melanin synthesis. This scheme is a modified version of the pathways proposed by Raper (1928) and Mason (1948) and is based partly upon chromatographic identifications of degradation products of sepiomelanin. The dotted arrows represent hypothetical reactions (from Nicolaus, 1962).

acid and aspartic acid according to the mechanisms proposed above (Chapter II, Section F).

Walaas (1963) has proposed that the requirement for molecular oxygen in the UV-promoted synthesis of melanin from adrenalin is confined to the indolization step which occurs during the transformation of adrenalin quinone into adrenochrome, and that H_2O_2 is liberated during the reaction as shown below.



(The earlier reactions have been included in order to indicate the probable roles of OH^- ions and UV radiation in the photopolymerization reactions; Walaas, ibid.). If the analogous reaction (i.e., indolization of DOPA-quinone to dopachrome via leucodopachrome, see Fig. 30) occurs in the system of reactions leading to the formation of photopolymer in UV-irradiated phenylalanine solutions, then the resulting H_2O_2 would provide a source of OH· radicals for the synthesis of tyrosine and DOPA according to the mechanism proposed above (Chapter II, Section F). If this is the case, then the photopolymerization reactions exhibit a kind of rudimentary autocatalysis. That is, for a given concentration of molecular oxygen and a given intensity of UV radiation, the rate of indolization depends on the concentration of DOPA-quinone, which in turn depends indirectly on the rate at which H_2O_2 is produced in the indolization reaction, since presumably phenylalanine and tyrosine are converted to the precursor of DOPA-quinone (i.e., DOPA, see Fig. 30) by means of OH· radicals derived photochemically from the H_2O_2 .

IV. SPECULATIONS ON POSSIBLE IMPLICATIONS OF THE RESULTS

A. UV INACTIVATION OF ENZYMES

Due to the occurrence of oxygen-dependent, non-photochemical hydroxylation reactions of the type discussed above, the total quantum efficiency for photolysis of free phenylalanine is considerably higher in oxygen saturated than in oxygen free solutions ($\Phi = 55 \times 10^{-3}$ and 37×10^{-3} respectively, see Table 5). On the basis of this result, one might expect that the quantum yield for UV inactivation of phenylalanine containing enzymes should be higher in the presence than in the absence of dissolved molecular oxygen. However, no such oxygen effect has been observed (McLaren, 1964). This apparent contradiction is easily resolved if the following four factors are taken into account:

1. The UV inactivation of those enzymes which have been investigated in detail is due primarily to the photochemical cleavage of disulfide bridges. This process occurs with a quantum efficiency (0.13) two to three times greater than the total quantum efficiency for phenylalanine photolysis obtained in the present investigation (Table 5) and ten times greater than the yield for phenylalanine loss measured under the same conditions as those used in the enzyme irradiation (Luse and McLaren, 1963; McLaren, 1964), and is nearly independent of the presence or absence of molecular oxygen (Dose and Rajewsky, 1962).

2. Phenylalanine may not be involved significantly in the maintenance of those aspects of structure required for enzyme activity.

3. Even though the number of phenylalanine residues in a given enzyme may be comparable to the number of cystine, tryptophane, and tyrosine residues, it absorbs only a relatively small fraction of the incident UV radiation due to its low molar extinction coefficient (McLaren, 1964).

4. The photochemical reactions which phenylalanine undergoes when it is part of the structure of an enzyme may be both qualitatively and quantitatively different from the photochemical reactions which free phenylalanine molecules undergo in aqueous solution. In particular, it is likely that the phenylalanine residues of a given enzyme will not undergo the type of oxygen-dependent hydroxylation discussed above, at least not to any appreciable extent for doses of UV radiation sufficient to produce high percentages of inactivation* (see, e.g., McLaren, 1949), since these reactions probably depend to some extent upon the prior production of H_2O_2 in reactions involving the complete side chain of phenylalanine. However, hydroxylation of phenylalanine residues might occur after prolonged irradiation, provided that sufficient amounts of phenylalanine (or tyrosine) have been photochemically liberated from the enzyme.

The extent to which the photochemistry of free phenylalanine is likely to resemble the photochemistry of phenylalanine residues in a given enzyme will be discussed in more detail below.

B. PHOTOCHEMISTRY OF PROTEIN PHENYLALANINE RESIDUES

That transfer of excitation energy from the benzene ring to the side chain does take place in the case of free phenylalanine is shown by the occurrence of β-phenylethylamine, phenyllactic acid, aminomalonic acid, and aspartic acid as photoproducts (Chapter II, Section C). It is probable that this process involves internal conversion of the excited singlet electronic state of the benzene ring π-electron system to a high vibrational level of the ground electronic state in which a substantial fraction of the vibrational energy is localized either in a given side chain carbon-carbon bond or in the carbon-nitrogen bond (see discussion in Appendix C). When phenylalanine is incorporated into the structure of a protein, the side chain is coupled through both the 1-carbon and the nitrogen to the polypeptide backbone, as shown in Fig. 31.

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*It is possible, however, that if other sources of H_2O_2 are present in the enzyme system, hydroxylation of phenylalanine residues might occur early during irradiation.

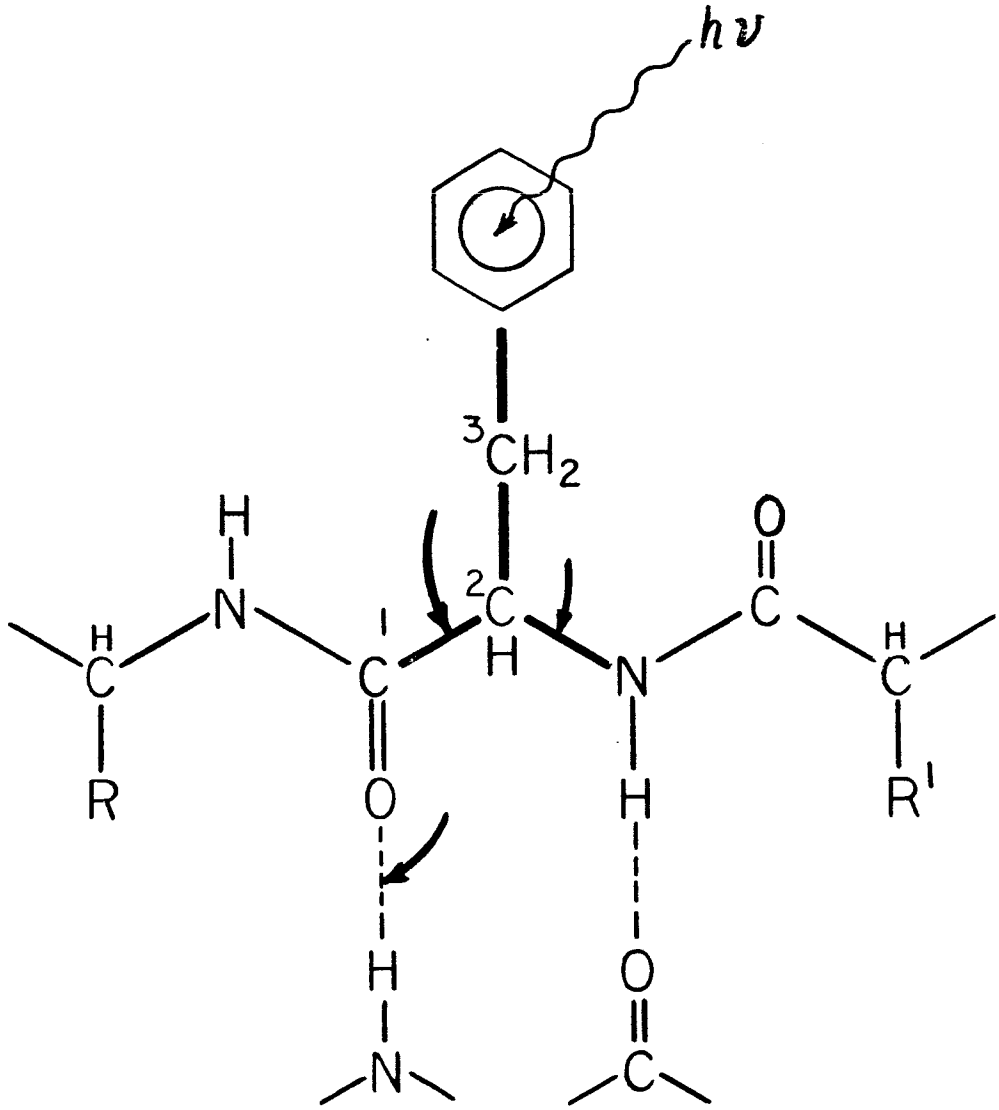
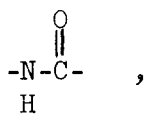


FIG. 31--Energy transfer at polypeptide phenylalanine residues during UV irradiation at 2575 Å. The above structure shows the coupling of the aliphatic portion of phenylalanine to the polypeptide backbone, and the two hydrogen bonds in which the 1-carbon and the nitrogen of the residue participate. R and R' denote other amino acid residues. The wavy arrow denotes initial photoexcitation of the ring π-electron system. The other arrows indicate the bonds to which vibrational energy might be transferred following internal conversion of the excited singlet state (see text).

If the above mentioned mechanism of energy transfer is applicable to the case of proteins, then the extent to which such transfer occurs at phenylalanine residues in a given protein exposed to UV radiation at 2500 - 2600 Å depends upon the existence of a certain class of vibrational modes. That is, the coupling of the side chain to the rest of the polypeptide backbone must be such that there exist energetic (i.e., 30 - 100 Kcals/mole) vibrational states of the protein in which the vibrational energy is localized in the phenylalanine residues. Futhermore, these vibrational states must be at least roughly similar to those of free phenylalanine so that upon internal conversion of the excited singlet state, a substantial fraction of the vibrational energy is localized in a given bond in the aliphatic portion of the residue.

That proteins have low lying vibrational states in which the vibrational energy is localized in particular bonds and groups of atoms (such as



N-H , C-H , and C-C) is shown by the occurrence of characteristic absorption bands in the IR spectra of proteins in the 2-20 μ region (Beer, et. al., 1959). Although the wavelengths of the characteristic IR absorptions of a given type of bond differ somewhat depending on whether the bond occurs in a synthetic polypeptide or in a protein, in which it is coupled through the polypeptide backbone to a large number of other bonds, or in a simple model compound in which it is coupled to only a few bonds, the variation is generally less than 0.25 μ . The C-H stretching vibration for N-methylacetamide occurs at 3.40 μ (Asai et al., 1955), while the corresponding values for poly-l-leucine-dl-phenylalanine and bovine serum albumin are 3.42 μ (Darmon and Sutherland, 1947) and 3.41 μ (Beer et al., ibid.), respectively.

Therefore, it is likely that the higher vibrational states of the phenylalanine residues of proteins resemble the higher vibrational modes of free phenylalanine, so that a substantial fraction of the photo-excitation energy initially confined to the benzene ring of a given phenylalanine residue can subsequently be transferred to a given bond

in the aliphatic portion of the residue. Since CO_2 liberation is the dominant photochemical process following ring excitation in the case of free phenylalanine, it is possible that in the case of proteins, the polypeptide backbone can be cleaved at the bond between carbons 1 and 2 (see Fig. 31) following absorption of a 2575 Å photon by a phenylalanine residue. Photodissociation could also conceivably take place at the following bonds: $\overset{2}{C}$-N , $\overset{2}{C}$-$\overset{3}{C}$, and $\overset{3}{C}$-ring; however, on the basis of the quantum yield date given in Table 5, it is likely that these processes would occur with much lower probabilities than that for dissociation of the $\overset{1}{C}$-$\overset{2}{C}$ bond.

Although the phenylalanine residues of a given protein might be expected to undergo primary photodissociations during UV-irradiation at 2500 - 2600 Å which are similar to those which occur in free phenylalanine, the resulting stable photochemical products are probably quite different in the two cases due to the coupling of the phenylalanine side chain to the polypeptide backbone. In particular, even though photodissociation may take place at the ring-$\overset{3}{C}$ and $\overset{3}{C}$-$\overset{2}{C}$ bonds, the aliphatic photoproducts, aspartic acid and aminomalonic acid would not be synthesized, since (1) the side chain aliphatic radicals would remain coupled to the polypeptide backbone, and (2) the decarboxylation reaction which presumably provides the extra carbon atoms for the synthesis of these two products (see Chapter II, Section F) cannot occur, since the carboxyl group has been modified to a substituted amide group (Fig. 31).

Also, it is apparent on structural grounds that neither phenylethylamine nor phenyllactic acid could be photochemically synthesized directly from the phenylalanine residues of proteins. However, if a source of OH· radicals is available, and if such radicals are not prevented sterically from interacting with the benzene rings of phenylalanine residues, it is likely that these rings will be hydroxylated to diphenols and further oxidized to quinones and semiquinones during UV irradiation, since these reactions depend only upon the electronic properties of the benzene ring. Synthesis of melanic photopolymer cannot

occur, however, except possibly at very high doses,* since indolization is prevented by the constraints on the nitrogen atom, and since, in general, the phenylalanine residues do not come into direct contact with each other.

The extent to which peptide bonds adjacent to phenylalanine residues are broken during UV irradiation of proteins at 2500 - 2600 Å is difficult to evaluate on the basis of currently available data, but probably depends upon the details of the vibrational coupling of this bond to the side chain bonds of phenylalanine. Since the bond energy of the peptide linkage is 75 - 80 Kcals/mole (Pauling, 1960), photodissociation following ring π-electron excitation requires localization of 70% of the photon energy. This value is considerably higher than the energy required for the dissociation of the bonds between carbons 1 and 2 (30 Kcals/mole) in free phenylalanine and is comparable to the value required for dissociation of the other C-C bonds of the side chain. Therefore, if peptide bond cleavage does occur, the quantum efficiency for the process is probably less than 5×10^{-3} (see Table 5).** McLaren (1964) claims that dissociation of peptide bonds is not quantitatively significant at 2537 Å. However, the mechanism of dissociation which he discusses involves direct absorption of 2537 Å photons

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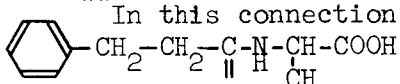
*The brown color exhibited by many proteins after prolonged UV irradiation (McLaren, 1949) is probably due to the synthesis of melanic photopolymer from liberated phenylalanine, tyrosine, and tryptophan residues and aromatic fragments of the residues. Both liberation of aromatic residues and direct production of OH· radicals from water probably occurred to an appreciable extent in many of the early photochemical studies of proteins, since, in general, precautions were not taken to filter out 1849 Å mercury resonance radiation present in the illuminating beam.

**In this connection, it is of interest that phenylpropionyl-alanine,  , undergoes peptide bond cleavage with a quantum yield of 4×10^{-3} following ring excitation at 2537 Å (Estermann, Luse, and McLaren, 1956).

by the π-electron system of the peptide linkage, so that the lack of quantitative significance of the process for enzyme inactivation is easily explained by the vanishingly small extinction coefficient for the peptide linkage at 2537 Å (McLaren, ibid.).

That hydrogen bonds are involved in the maintenance of the secondary structure required for enzyme activity has long been recognized (Mirsky and Pauling, 1936). On the basis of UV irradiation studies of chymotrypsin at different temperatures, McLaren (1964) has concluded that H-bond rupture is involved in inactivation, although he proposed no mechanism for the process. Since the dissociation energy of hydrogen bonds in α-helical segments of protein, polypeptide backbones is only 1.5-5 Kcals/mole (Scheraga, 1963; see also, Davies, 1957), it is plausible that H-bonds involving the 1-carbon (or the nitrogen) of phenylalanine (see Fig. 31) are broken following ring excitation by means of the type of vibrational energy transfer mechanism discussed above. That is, if at least 30 Kcals/mole of vibrational energy can be localized in the bond between carbons 1 and 2 following internal conversion of the excited singlet state of the ring, it seems likely that the same type of mechanism could result in the localization of 1.5-5 Kcals of vibrational energy in the adjacent H-bond, and that such a process could contribute significantly to UV inactivation of enzymes.

C. INTRAMOLECULAR ENERGY MIGRATION IN PROTEINS

In connection with the above discussion it is of interest to evaluate briefly the possibility that transfer of energy from the ring to hydrogen bonds via vibrational coupling might play a role in the proposed semiconductor models of long range energy migration in proteins (Evans and Gergely, 1949; Suard, Berthier, and Pullman, 1961; Yomosa, 1964). In these models, it is proposed that there exist π-electron orbitals which are delocalized over the hydrogen bonds of the polypeptide backbone. The energies of these delocalized orbitals fall into three distinct bands,

two of which are conduction bands.* On the basis of this result, it is argued that highly efficient long range energy transfer of the type observed by Broser and Lautsch (1956) in the UV-induced dissociation of CO from CO-mesohemin-IX-poly-DL- (phenylalanine-glutamic acid) is due to promotion of an electron from the non-conducting to a conducting band. Once in the conduction band, the electron can presumably migrate over the whole polypeptide backbone of the system until it is trapped (i.e., at the heme moiety in the experiment of Broser and Lautsch).

In order for the above type of electron promotion to occur, it is necessary that photoexcitation energy initially confined to the aromatic rings of phenylalanine residues be efficiently transferred to the system of hydrogen bonds in the polypeptide backbone. However, the minimum calculated value for the energy gap which separates the non-conducting band from the lowest lying conduction band is 3 - 3.5 eV, or 70 - 80 Kcals/mole (Evans and Gergely, ibid.). Therefore, it seems highly improbable that the vibrational mechanism of energy transfer discussed above in connection with the rupture of hydrogen bonds adjacent to phenylalanine residues can account for the required electron promotion, since:

1. The maximum quantum efficiency for transfer of 27% of the photoexcitation energy to the bond between carbons 1 and 2 , which is adjacent to one of the hydrogen bonds in question, is only 0.044 in the case of free phenylalanine (see Table 5), while the semiconductor model requires that 70% (i.e., ~ 75 Kcals/mole) of the photoexcitation energy be transferred to the vicinity of the hydrogen bond with a quantum efficiency of 0.89 (Broser and Lautsch, ibid.).

2. The energy required for the electron promotion greatly exceeds the dissociation energy of the hydrogen bond, so that bond rupture would effectively compete with electron promotion even if the vibrational coupling were such that the required energy transfer could take place.

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*In the ground state of the system, the non-conducting band is filled with electrons associated with the ^2p_z atomic orbitals of the carbon, oxygen, and nitrogen atoms which participate in the hydrogen bonds (Evans and Gergely, ibid.).

It is of course possible that other types of coupling exist in CO-meshohemin-IX-poly-DL- (phenylalanine-glutamic acid), which would allow the energy transfer required for electron promotion to occur (i.e., spatial proximity of the benzene rings of phenylalanine residues to hydrogen bonds). However, it seems improbable that such "conformational coupling" could result in highly efficient energy transfer for all of the phenylalanine residues of the model protein. On the basis of the above arguments, it is apparent that the semiconductor model of intramolecular energy migration in proteins is subject to serious doubt.

V. SUMMARY

The photochemistry of DL-phenylalanine in dilute aqueous solution has been investigated in detail by means of quantitative paper chromatography using C^{14}-labelled material. Solutions were irradiated at the exit slit of a grating monochromator using a high pressure xenon arc as light source. In separate experiments, phenylalanine was labelled with C^{14} at different specific sites in the side chain, or uniformly in the benzene ring, allowing the determination of which phenylalanine carbon atoms were incorporated into each photoproduct.

By using six different chromatographic solvent systems, the following photoproducts were identified: aminomalonic acid, aspartic acid, DOPA, tyrosine, β-phenylethylamine, phenyllactic acid, benzoic acid, and a high molecular weight photopolymer.

Kinetics of photoproduct accumulation and phenylalanine loss were determined for both oxygen-saturated and oxygen-free solutions under well-defined conditions of incident light intensity, wavelength, temperature, and pH. The data were used to estimate quantum yields for the photoproducts and for phenylalanine loss. Low temperature ($77^{\circ}K$) electron spin resonance experiments on UV-irradiated phenylalanine were carried out. The major conclusions derived from the results are as follows:

1. Photoexcitation energy initially confined to the benzene ring can be subsequently transferred to the side chain, where it either promotes cleavage of a carbon-carbon bond or activates the carbon-nitrogen bond for subsequent hydrolysis.

2. The most labile side chain carbon-carbon bond is that between the carboxyl and alpha carbons. Decarboxylation is the predominant photochemical process.

3. Molecular oxygen is required for the synthesis of tyrosine, DOPA, and the photopolymer. Tyrosine and DOPA are intermediates in photopolymer synthesis.

4. The optical density increases occurring in UV-irradiated solutions of phenylalanine are associated with the synthesis of photopolymer and contain only minor contributions from the low molecular weight aromatic photoproducts.

5. Several different free radical species are formed as primary photodissociation products of phenylalanine.

Probable reaction pathways for synthesis of the low molecular weight photoproducts are discussed.

The photopolymer was characterized by infrared, visible and UV absorption spectra, electron spin resonance spectra, and photo-oxidation kinetics. From these studies, it is concluded that the molecular structure of the photopolymer closely resembles the molecular structure of natural and synthetic melanins. Evidence is presented which suggests that the photopolymer also resembles melanins with respect to mode of synthesis.

Possible implications of the results obtained in the present investigations for the following problems have been discussed: (a) UV inactivation of enzymes, (b) photochemistry of protein phenylalanine residues, and (c) intramolecular energy transfer in proteins.

APPENDIX A

UV ABSORPTION SPECTRA OF FILTERS

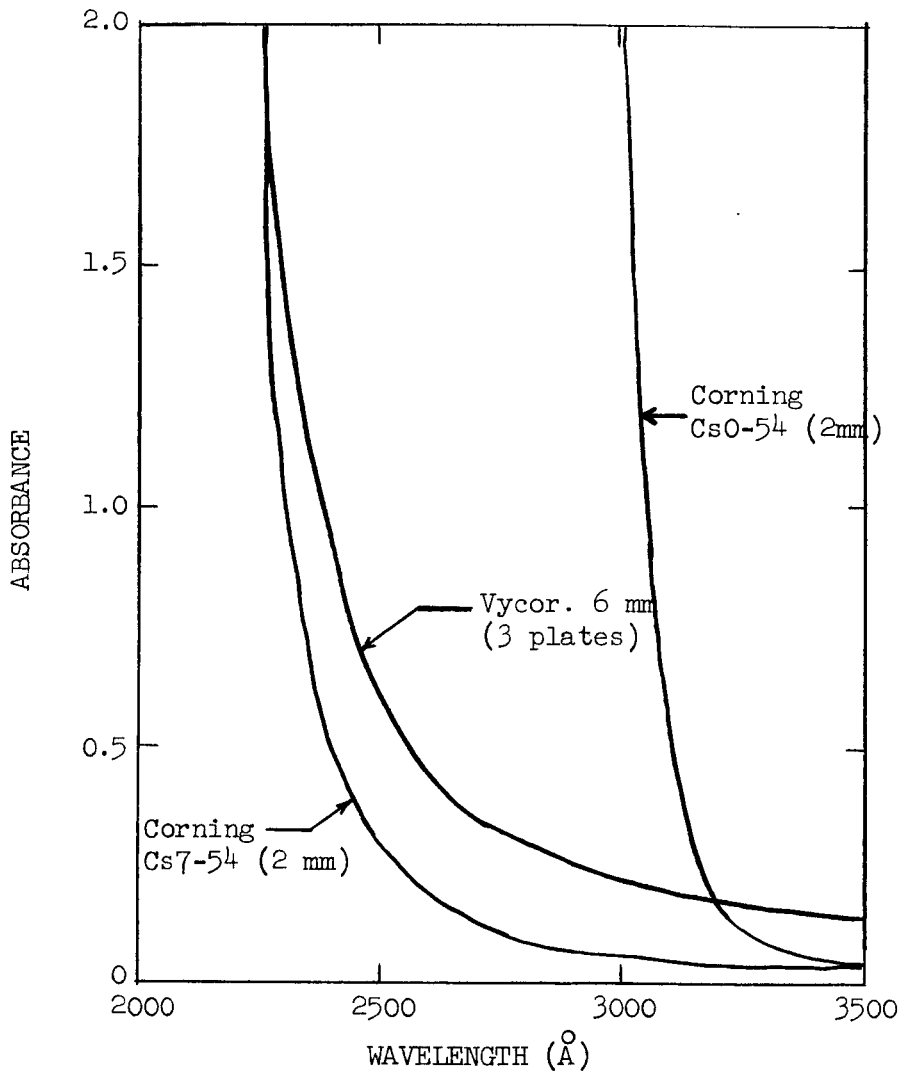

FIG. A.1--Spectra were recorded on a Cary 14M spectrophotometer against air in the reference beam. Contributions to the absorbance due to surface reflectivity have not been subtracted out.

APPENDIX B

KINETIC CALCULATION FOR A HYPOTHETICAL SINGLET STATE MECHANISM OF TYROSINE SYNTHESIS

The mechanism proposed in Chapter II is the following:

$$S_0 + h\nu \rightleftharpoons S_1$$
$$\downarrow O_2$$
$$\text{Tyr} + O \quad ,$$

where S_0 and S_1 are the ground ($^1A_{1g}$) and the first excited singlet ($^1B_{2u}$) states, respectively. The dotted arrow represents return to the ground state via fluorescence emission of radiationless processes (see Appendix C).

The above reaction is bimolecular, so that the initial rate of tyrosine accumulation would depend upon both the concentrations of S_1 and of dissolved molecular oxygen. Furthermore, the observed rate near $t = 0$ should depend only upon these two concentrations, since the secondary reactions of tyrosine, e.g., conversion to DOPA, probably do not appreciably affect the rate of tyrosine accumulation for irradiation times shorter than 2-4 hrs (see Fig. 3). This conclusion is consistent with the low total quantum yield for tyrosine photolysis ($\Phi = 0.002$; Luse and McLaren, 1963). At any rate, even if it is assumed that the initial observed rate of tyrosine formation represents the minimum rate which the proposed reaction scheme must account for (i.e., that the secondary reactions of tyrosine do appreciably affect the slope), the conclusion derived from the calculations given below remains unchanged. According to the proposed scheme, the initial rate of tyrosine accumulation is given by

$$\frac{d[\text{Tyr}]}{dt} = \kappa [S_1] [O_2] \quad . \qquad (1)$$

The steady state concentration of S_1 near $t = 0$ can be estimated from the observed lifetime of the excited state. That is, the rate of change of the concentration of S_1 may be represented by the following equation:

$$\frac{d[S_1]}{dt} = I_{abs} - \kappa'[S_1] \cong 0 \quad , \qquad (2)$$

where I_{abs} is the intensity of light absorbed by phenylalanine molecules per unit volume and κ' is the total first order rate constant for loss of the excited state due to fluorescence emission, non-radiative decay to the ground state, and photochemical reactions. It can easily be shown that $\kappa' = 1/\tau$, where τ is the observed mean lifetime of the excited state as determined from measurements of the quantum yield of fluorescence (see Udenfriend, 1962). Setting Eq. (2) approximately equal to zero (i.e., assuming that the concentration of S_1 very rapidly rises to a maximum value after illumination begins and thereafter remains nearly constant, at least for $t \leq 4$ hrs; see Fig. 13) and solving for $[S_1]$ yields

$$[S_1] \cong \tau \cdot I_{abs} \quad . \qquad (3)$$

The value of I_{abs} at $t = 0$ can be calculated from the initial concentration of phenylalanine using the Beer-Bouguer absorption law (see discussion of calculation of quantum yields, Chapter IV):

$$I_{abs} = I_o (1 - 10^{-A}) \quad , \qquad (4)$$

where A is the initial optical absorbance of the solution. For $I_o = 7{,}030$ ergs/cm^2/sec and $A = 0.142$ (see Fig. 10, O_2-case), $I_{abs} = 1960$ ergs/cm^2/sec $= 2.54 \times 10^{14}$ photons/cm^2/sec. Using Karreman, Steele, and Szent-Györgyi's (1958) value of τ (1.1×10^{-8} sec), we obtain $[S_1] \cong 2.8 \times 10^6$ molecules/cm^3 $= 4.65 \times 10^{-15}$ moles/liter.

The concentration of molecular oxygen in the reaction mixture at $t = 0$ can be calculated by means of Henry's law, assuming that the pressure of oxygen above the solution inside the reaction vessel is 1 atmosphere. This assumption is reasonable in view of the method used to saturate the solution with oxygen (see Chapter II, Section D). The mole fraction of O_2 in the solution X_{O_2} is given by

$$X_{O_2} = P_{O_2} / c = 2.57 \times 10^{-5}$$

or (5)

$$[O_2] = 1.41 \times 10^{-3} \text{ moles/liter},$$

where P_{O_2} = partial pressure of O_2 = 760 mm Hg , c = Henry's law coefficient for O_2 in water at $20^{\circ}C$ ($c = 2.95 \times 10^{7}$; Handbook of Chemistry and Physics, 42^{nd} edition, 1960 - 1961 p. 1708).

In order to calculate the maximum rate at which tyrosine could be formed by the proposed mechanism, it will be assumed that every encounter between an S_1 molecule and an O_2 molecule results in the formation of a tyrosine molecule. Although the frequency of collisions between S_1 and O_2 is nearly independent of the viscosity of the solution, the number of encounters per unit time does depend on the viscosity, η , due to the cage effect, and is, in general, much lower than the collision frequency. The maximum value of the bimolecular rate constant, κ , in Eq. (1) is given by the following equation (Umberger and La Mer, 1945; Livingston, 1961):

$$\kappa_{max} = 8 RT/30\eta \quad . \qquad (6)$$

At $T = 20^{\circ}C$, we have $\kappa_{max} = 6.26 \times 10^{9}$ liters/mole/sec.

By using the values of $[S_1]$, $[O_2]$, and κ_{max} computed above, the maximum rate at which tyrosine could be expected to accumulate according to the proposed singlet state mechanism was computed by means of

Eq. (1) and is compared below with the value determined from the initial slope of the tyrosine curve in Fig. 13.*

Calculated	Observed
$\left(\frac{d[Tyr]}{dt}\right)_{max} = 41 \times 10^{-9}$ moles/liter/sec	$\frac{d[Tyr]}{dt} = 1.2 \times 10^{-9}$ moles/liter/sec.

*The initial slope was taken to be equal to the slope of the straight line drawn from the origin to the t = 4 hrs point on the tyrosine curve.

APPENDIX C

QUANTUM THEORETICAL DISCUSSION OF THE PRIMARY PHOTOPHYSICS AND PHOTOCHEMISTRY OF DL-PHENYLALANINE

The possible photophysical and photochemical processes which can occur in phenylalanine molecules following photoexcitation of the ring π-electron system at 2575 Å are conveniently summarized in the state diagram shown in Fig. C.1. This diagram is a modification of the more general scheme given by Hammond and Turro (1963). The electronic energy levels labelled $^1A_{1g}$ and $^1B_{2u}$ are the ground and first excited singlet states, respectively (see discussion of spectroscopy of phenylalanine, Chapter II, Section A). The level designated by T_1 is the lowest lying triplet state. Vibrational states are represented by a series of levels above each electronic state. For simplicity, the corresponding rotational levels have been omitted.

During illumination at 2575 Å, phenylalanine undergoes an electronic transition from the ground state ($^1A_{1g}$) to the first excited singlet state ($^1B_{2u}$) of the benzene ring π-electron system (see Fig. 2). In order for this transition to occur, an asymmetric vibrational mode of the ring must be excited simultaneously, since the purely electronic transition is symmetry forbidden (Sklar, 1937). This excited vibrational level is rapidly deactivated through collisions with solvent molecules in times which are usually very short (i.e., 10^{-13} - 10^{-12} sec; Förster 1959) compared to the actual mean lifetime of the excited singlet state ($\tau = 1.1 \times 10^{-8}$ sec; Karreman, Steele, and Szent-Györgyi, 1958). Following vibrational cascading (represented by wavy lines in Fig. C.1, the excited singlet state can undergo a number of alternative transformations. One possibility is that the electronic excitation energy is re-emitted as a fluorescent photon, such that the molecule undergoes a transition to a low lying asymmetric vibrational mode of the ground

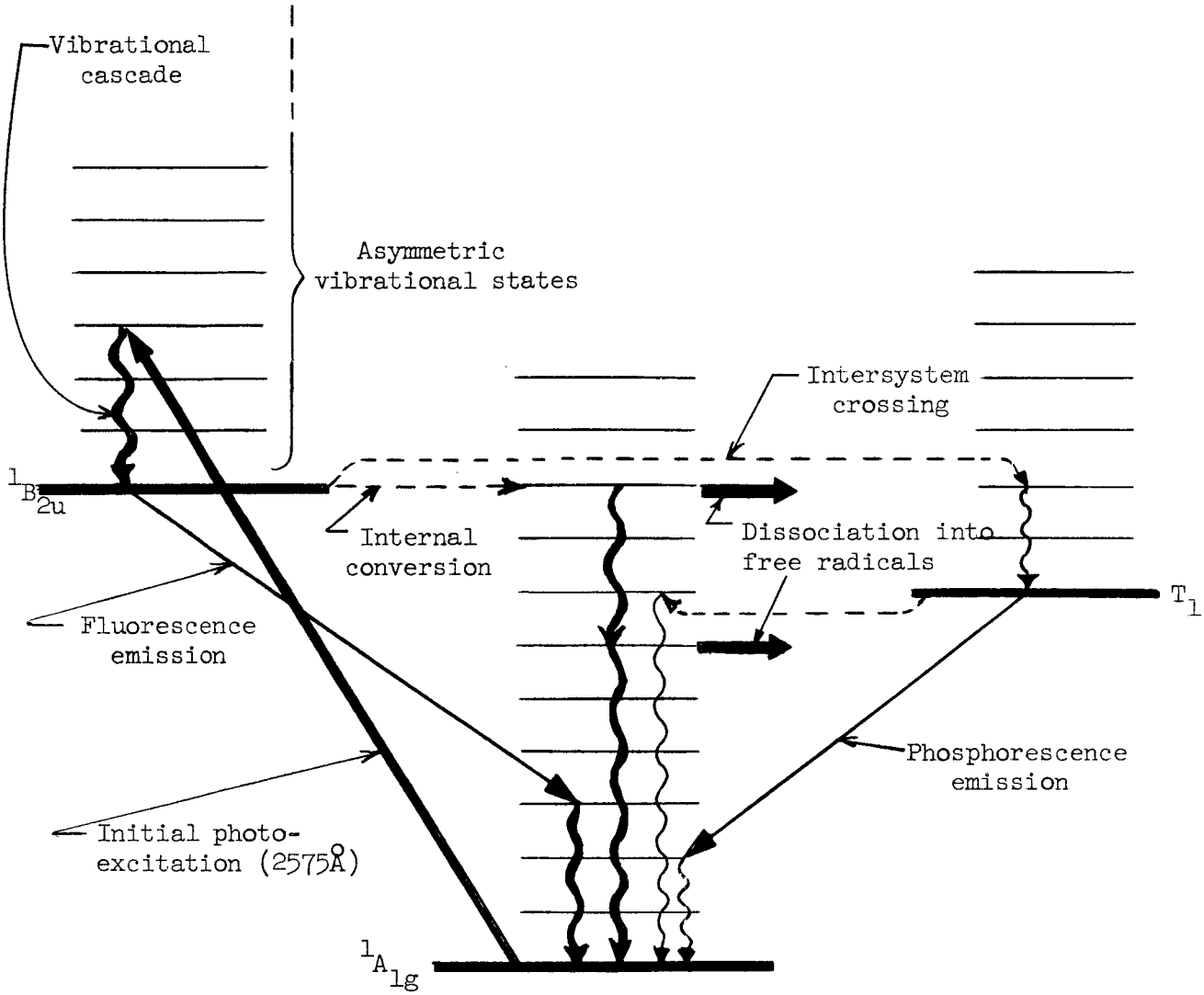

FIG. C.1--Pathways of de-excitation of the lowest lying ($^1B_{2u}$) singlet excited state of DL-phenylalanine. The above energy level diagram is a modified version of the general scheme of Hammond and Turro (1963). The levels designated $^1A_{1g}$ and T_1 are the ground and lowest lying triplet states, respectively. The series of levels above each electronic state represent vibrational states. Rotational levels have been omitted for the sake of clarity.

electronic state, followed by a vibrational cascade during which the system again comes to thermal equilibrium with the surrounding solvent. However, due to the symmetry forbidden character of the $^1B_{2u} \leftarrow\!\!\!- \; ^1A_{1g}$ electronic transition, the intrinsic mean lifetime of the excited singlet state is sufficiently long that other processes can effectively compete with fluorescence emission for deactivation of the excited state. The result is that the fluorescence efficiency for phenylalanine in aqueous solutions at room temperature is only 0.04 (Teale and Weber, 1956). Therefore, 96% of the initial photoexcitation events occurring in UV-irradiated phenylalanine solutions are followed by radiationless transitions resulting either in eventual deactivation to the ground state or in photochemical reactions. Phosphorescence (see Fig. C.1) has not been detected at room temperature in aqueous phenylalanine solutions.

In this connection, it is of considerable interest that the total quantum yield for tyrosine photolysis is considerably smaller than the corresponding yield for phenylalanine, suggesting that the photochemical stabilities of these substances depend upon the extent to which radiationless processes contribute to disappearance of the excited singlet states. That is, the fluorescence efficiency of tyrosine is higher by a factor of five than that of phenylalanine, so that radiationless processes take place correspondingly less frequently. Therefore, it appears likely that the relative total quantum yields of photolysis for similar aromatic compounds, such as DOPA, will be found to depend inversely on the fluorescence efficiencies and directly on the natural lifetimes of the excited singlet states.

Through the radiationless process of internal conversion (Kasha, 1960), the excited singlet state may be converted to a high vibrational level of the ground electronic state. Whether or not side chain carbon-carbon bonds will be cleaved while the molecule is in this vibrational state will depend on the way in which vibrations of side chain carbon atoms are coupled to the ring vibrations. If the vibrational energy is initially delocalized over the whole molecule, dissociation will not take place. However, other lower lying vibrational levels of the system may have vibrational energy distributions favorable to dissociation.

That is, suppose that within the manifold of vibrational states through which the system passes during thermal equilibration with the solvent, there exist certain vibrational levels in which a substantial fraction of the excitation energy is localized in a given side chain carbon-carbon bond. Cleavage of such a bond would then occur if (1) the localized energy were equal to or greater than the activation energy for dissociation, and (2) the energy remained in the bond long enough for at least one complete vibration to occur.

Side chain dissociation by such a mechanism would account for the production of free radicals both at room temperature in aqueous solution and at 77°K in an organic glass (see Fig. 17), since the necessary prior process of internal conversion is essentially temperature independent and vibrational cascading would be expected to occur at both temperatures, although at a much lower rate at 77°K. Since collisional transfer of vibrational (and rotational) energy to the solvent effectively competes with photodissociation at room temperature due to the relatively high collision frequency, the quantum efficiencies for dissociation will be much lower at room temperature than at 77°K. The resulting free radical pairs will then either recombine due to the cage effect (Franck and Rabinowitch, 1934; Livingston, 1955) to reform vibrationally excited phenylalanine, or diffuse away from each other to undergo secondary reactions with other molecular species.

The other radiationless process which contributes to deactivation of the excited singlet state is intersystem crossing, by which the excited electron may be considered to undergo an inversion in the direction of its intrinsic spin vector with the result that the π-electron system acquires one unit of spin angular momentum (Kasha, 1960). Since the resulting triplet state is less energetic than the singlet state (Fig. C.2) (see Robinson, 1961), the molecule will be in an excited vibrational state immediately following intersystem crossing. This excess vibrational energy can be estimated by comparing the energies of the two electronic states. Although the absorption maximum for the $^1B_{2u} \longleftarrow {}^1A_{1g}$ transition of phenylalanine occurs at 2575 Å, the actual electronic energy of the excited state corresponds to a wavelength of about 2720 Å, as can

easily be shown by means of an analysis of the vibrational structure of the absorption spectrum. That is, since the 0 - 0 transition is absent from the spectrum (Sandorfy, 1964), the wavelength corresponding to the purely electronic transition is about 50 Å greater than the wavelength maximum for the least energetic fine structural peak (see Fig. 1).

The energy of the triplet state, T_1, can be estimated from the wavelength at which the multiplicity forbidden $T_1 \longleftarrow {}^1A_g$ transition occurs in the case of benzene. Although this transition is exceedingly weak ($\epsilon \cong 10^{-3}$; Pitzer, 1953), it has been shown to occur near 3400 Å by Sklar (1937) using very long optical paths. Therefore, the energy difference between the excited singlet and triplet states is given approximately by $\Delta E \cong hc\,(1/\lambda_s - 1/\lambda_t) = hc\,(1/2720 - 1/3400) \cong 0.9$ eV, or 20 Kcals/mole. This value represents the maximum vibrational energy which the triplet state can have immediately following intersystem crossing, assuming that the latter process is isoenergetic (Kasha, 1960). It is apparent that this energy is insufficient to cause cleavage of a side chain carbon-carbon bond even if it could all be localized in one bond, since the lowest side chain dissociation energy is 30 Kcals/mole (i.e., for the bond between carbons 1 and 2, see Table 4).* Therefore, the vibrational energy will be transferred to the solvent. The triplet state can then decay to a low lying vibrational level of the ground electronic state by emission of a phosphorescent photon, or, alternatively, it can undergo intersystem crossing to an isoenergetic vibrational level of the ground state (see Fig. C.1). In the latter case, dissociation into free radicals is again possible if the system reaches a sufficiently energetic vibrational state in which energy is localized in a given bond as discussed above. At room temperature, the triplet state is rapidly depopulated by intersystem crossing and phosphorescence emission is not observed (Porter, 1961).

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*A similar calculation for the maximum possible vibrational energy of the excited singlet state immediately following photon absorption results in a value of only 10.6 Kcals/mole (i.e., assuming that the vibrational mode corresponding to the absorption peak at 2470 Å is excited; see absorption spectrum shown in Fig. 1).

Using the quantum yield data given in Table 5 (Chapter II) for the synthesis of aspartic acid, aminomalonic acid, and benzoic acid, and for the decarboxylation reaction resulting in the formation of β-phenylethylamine, together with the physical arguments given above, it is possible to construct a general, quantitative scheme for the primary photophysical and photochemical processes taking place in aqueous phenylalanine solutions illuminated at 2575 Å. Such a scheme is shown in Fig. C.2. It is apparent that the predominant process which takes place following initial photoexcitation to the excited singlet state is radiationless return to the ground state. Therefore, most (i.e., ≧ 90%) of the photoexcitation energy is eventually converted to heat through transfer of vibrational and rotational quanta to water molecules (Robinson, 1961). Collisional transfer of electronic excitation energy (Porter, 1961), as well as resonance energy transfer (Förster, 1948, 1959, 1960), are not quantitatively significant in the system due to the low concentration of phenylalanine (i.e., $6 - 8 \times 10^{-4}$ M).

Although the evidence obtained in the present investigation suggests that CO_2 liberation involves a localization of vibrational energy in the bond between carbons 1 and 2 following internal conversion, as shown in Fig. 31, the detailed physical basis of such a process is not understood. Since the photoexcitation energy is initially localized in the benzene ring, it is essential that some type of coupling exist between the ring and the end of the side chain which would allow part (i.e., ≧ 30%) of the energy to be transferred to the side chain. It seems reasonable that this energy transfer could take place along the carbon backbone of the side chain via vibrational coupling of the side chain to the ring. Evaluation of this possibility must await detailed analysis of the higher vibrational levels of phenylalanine.

Alternatively, the energy transfer may depend upon a "conformational coupling" of the carboxyl group to the ring. That is, in aqueous solution at room temperature, the side chain may bend back onto the ring such that the carboxyl group is weakly bound, at least part of the time. If this is the case, transfer of energy from the ring to the bond between carbons 1 and 2 might be expected to occur with relatively

high probability due to the spatial proximity of the carboxyl group to the π-electron system. However, since the absorption spectrum of phenylalanine is very similar to that of benzene (see Fig. 1), it is apparent that such conformational coupling, if it exists, has very little effect on the electronic properties of the ground and excited singlet states. Direct absorption of 2575 Å photons by the π-electron system of the carboxyl group can easily be ruled out as a possible mechanism of CO_2 liberation, since the extinction coefficient for the carboxyl group is negligible above 2300 Å (i.e., for alanine, $\epsilon_{2300\,\text{Å}} \leq 5$; Saidel, Goldfarb and Waldman, 1951; also see Fig. 1; for formic acid and acetic acid, $\epsilon_{2500} \leq 1$, Ramart-Lucas, 1942).

FIG. C.2--Quantitative scheme for primary photophysical and photochemical processes occurring in phenylalanine molecules following initial photoexcitation of the benzene ring π-electron system at 2575 Å. The symbols are defined as follows: $\underline{S_0}$, ground state ($^1A_{1g}$ level in Fig.C.1); $\underline{S_1}$, first excited singlet state ($^1B_{2u}$ level in Fig.C.1); $\underline{T_1}$, lowest lying triplet state; a single asterisk denotes a low lying vibrational level of the given electronic state (i.e., energy $\leq$ 25 Kcals/mole) and a double asterisk denotes a high vibrational level (energy $\geq$ 25 Kcals/mole, see text); $\underline{\Delta}$, rotational and vibrational energy transferred to the solvent; $\underline{h\nu}$, initially absorbed photon (2575 Å); $\underline{h\nu'}$ and $\underline{h\nu''}$, fluorescent and phosphorescent photons, respectively. Solid arrows indicate radiative processes or photodissociation events. Wavy arrows denote vibrational cascading. Dotted arrows indicate radiationless processes or free radical recombination. The numbers opposite various processes indicate the probable number of initial photoexcitation events which are followed by the given process, per 100 photons absorbed.

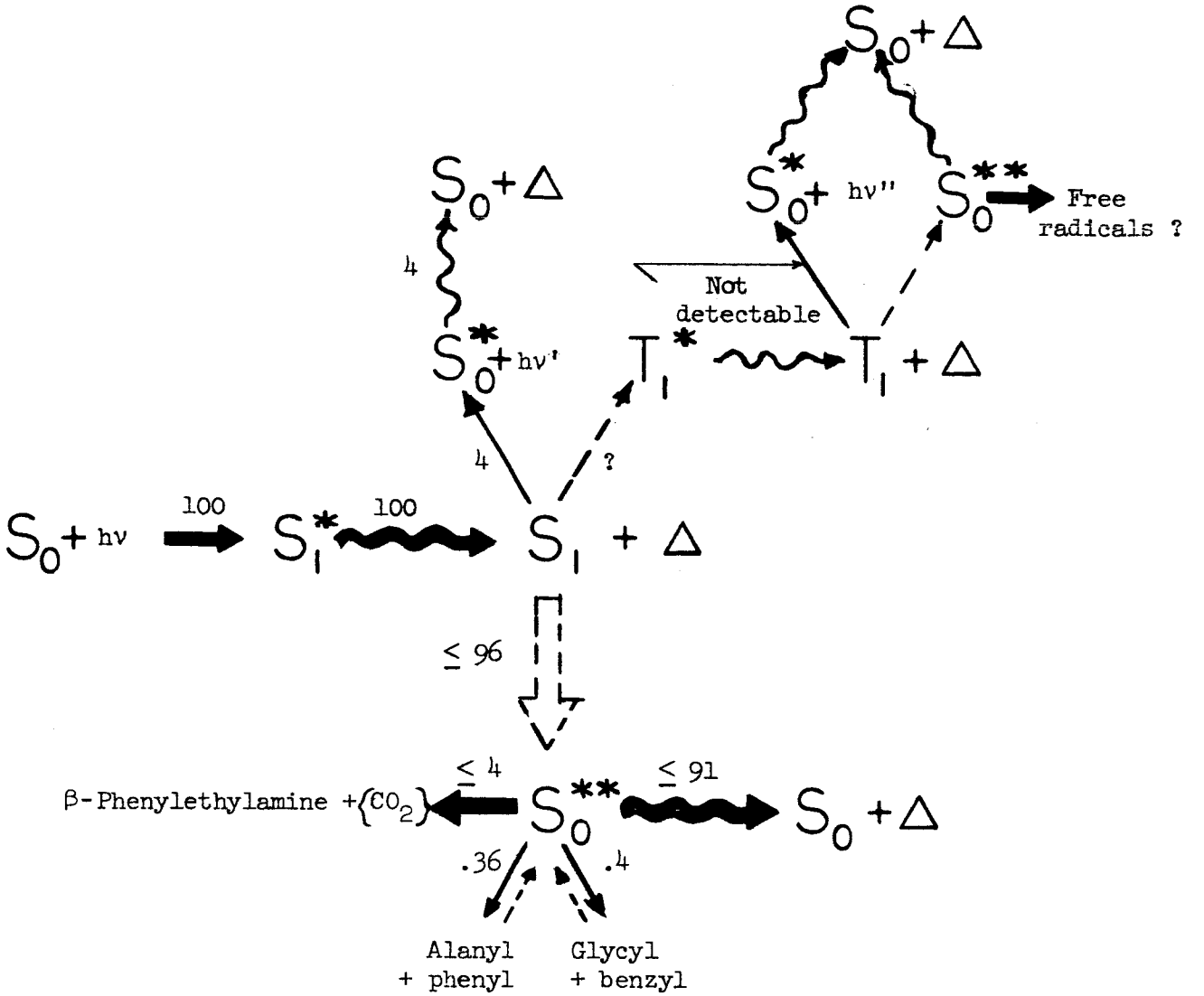
$S_0 + \Delta$
$S_0^* + h\nu''$
S_0^{**}
Free radicals ?
4
Not detectable
$S_0 + \Delta$
$S_0^* + h\nu'$
T_1^*
$T_1 + \Delta$
4
?
$S_0 + h\nu$
100
S_1^*
100
$S_1 + \Delta$
≤ 96
≤ 4
≤ 91
β-Phenylethylamine + (CO_2)
S_0^{**}
$S_0 + \Delta$
.36
.4
Alanyl + phenyl
Glycyl + benzyl

FIGURE C.2

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