

On the Mechanism of the Photo-Oxidation of Amino Acids Sensitized by Methylene Blue

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Photo-oxidation of histidine, methionine, tryptophan, and tyrosine, and their derivatives, as the function of pH were investigated.

Photo-oxidation of histidine and methionine as a function of temperature has shown that oxidation proceeds through an initial fast reaction, which is followed by an additional light-sensitive slow reaction, the latter being more pronounced with increasing temperature. Photo-oxidation of tyrosine and tryptophan have also shown a marked temperature dependence. It was proposed that the first step in the photo-oxidation of histidine, methionine, tryptophan, and tyrosine consists of oxidation of these amino acids with a simultaneous reduction of the dye.

Photo-oxidation of the reactive amino acids obey the Michaelis-Menten reaction scheme.

A possible cyclic free radical mechanism for the photo-oxidation of amino acids mediated by methylene blue is proposed.

Photo-oxidation of amino acids sensitized by methylene blue was described by us years ago (1) and served us as the basis for further work on enzymes. These studies led to the implication of the imidazole residue as the possible catalytic site of several hydrolytic enzymes (2-5). Although extensive use of this method has been reported in the literature, relatively little is known on the mode of action of this reaction. In view of the usefulness of this procedure, continuation of the work on this problem was highly desirable.

EXPERIMENTAL

Irradiation Procedure

For the measurement of oxygen uptake during photo-oxidation, the Warburg manometric technique was used (1). A circular water bath with a transparent plastic bottom was used. Each Warburg flask was irradiated with an individual 35-W spotlight bulb placed 2 cm from the bottom, while the flask itself was 10 cm above it. The

vessels were shaken 140 strokes per minute at an amplitude of 6 cm. By synchronizing the shaking of the vessels with that of the light source, the light source always remained under the bottom of each respective vessel. To maintain a lower temperature, a copper coil was placed along the wall of the water bath through which cooled water was circulated from a thermostatically cooled refrigerated bath. Because of the extensive heat exchange which occurred when the operation was carried out at low temperature, the whole equipment was placed in the cold room (4°C). With this arrangement an even intensity of light was supplied to each Warburg vessel, as indicated by the same rates of oxygen uptake for each vessel during photo-oxidation in identical experiments in which histidine was used as the substrate.

Photo-oxidation of various amino acids and their derivatives. The substrate solutions investigated contained 5 μ moles per milliliter except in the case of 3,5-diiodo-L-tyrosine, which contained only 2.5 μ moles due to limitations of solubility. The solutions were adjusted to pH values of 2.0, 3.1, 4.0, 5.0, 6.0, 7.0, 8.4, and 10.0, respectively. The Warburg vessels were charged with 1 ml of substrate of a given pH plus 0.5 ml of 0.2 M phosphate-citrate buffer of the corresponding pH values of 2.0, 3.1, 4.0, 5.0, 6.0, 7.0, 8.4, and 10.0 (the latter

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pH was attained by adjusting the pH 8.4 buffer to pH 10.0 with 1 *N* NaOH) and with 0.5 ml of methylene blue (containing 0.1 mg) which was placed in the sidearm. The center well of the Warburg vessel was charged with 0.2 ml of 20% KOH together with a cylindrical piece of filter paper to absorb any CO₂ which might evolve during the photo-oxidation. After temperature equilibrium was reached, the vessels were closed, the methylene blue solution was tipped into the main chamber, and the irradiation was started.

RESULTS

pH Dependence of the photo-oxidation of L-histidine and glycyl-L-histidine at 40°. The results obtained with these two substrates, which are presented in Fig. 1, are in agreement with our previous findings (1) and also with those obtained recently by Sluyterman (6), who used proflavine as a sensitizer. The pH profile, which was obtained after irradiation for 10 minutes, at which time the oxygen uptake proceeded in a linear fashion, is in accord with the conclusion that only the free imidazole base is oxidized, since above neutrality the rate of photo-oxidation is essentially independent of pH. On the acid side (pH 4.0), where the imidazole base is protonated, the reaction proceeds only at a very low rate. This deduction is supported by the fact that the reported p*K* of imidazole, which is 5.8 at 37°, is in close agreement with those obtained with the oxidation curve in Fig. 1 (as indicated by the arrows). Experiments carried out at higher

substrate concentrations (30 μ M) gave essentially the same pH-profile as shown in Fig. 1.

pH Dependence of the photo-oxidation of L-tryptophan and its derivatives at 40°. Figure 2 shows the pH dependence of photo-oxidation of L-tryptophan, glycyl-L-tryptophan, and *N*-acetyl-L-tryptophan amide. The values reported were obtained after 30 minutes of irradiation. A pH optimum of about 8.5 was observed for all three of the substrates investigated, indicating that the substitution of α -amino, α -carboxyl, or both, had no effect in this respect. Since the neutral indole group is not affected by pH and the ionizable α -amino and α -carboxyl, if substituted, showed the same pH-dependence as tryptophan alone, one must assume that the observed pH-dependence is due to some other factors than those mentioned. As in the case of histidine, experiments carried out with 30 μ M tryptophan resulted in findings as presented in Fig. 2.

pH Dependence of the photo-oxidation of L-methionine and its derivatives at 40°. Figure 3 shows the pH dependence of the photo-oxidation of L-methionine, glycyl-L-methionine, and *N*-nicotinyl-L-methionine amide. Values presented here were obtained after 20 minutes of irradiation and showed a broad pH optimum, around 8.5, as observed for tryptophan (Fig. 2). To implicate any particular group of methionine on the basis of ionization with the observed pH optimum

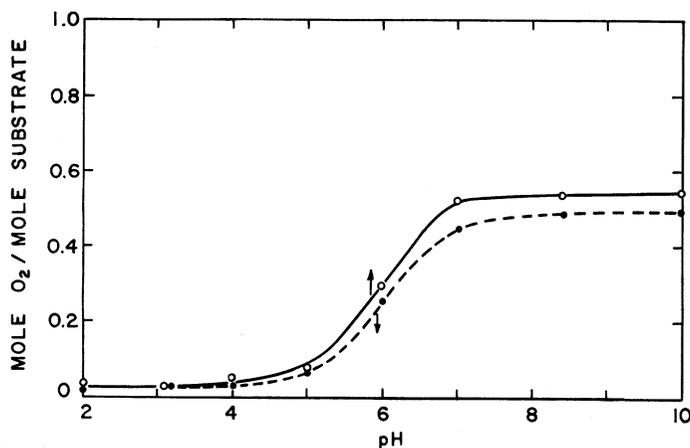


FIG. 1. pH Dependence of the photo-oxidation of histidine and its derivative at 40°. (○—○) Histidine; (●—●) glycyl-L-histidine.

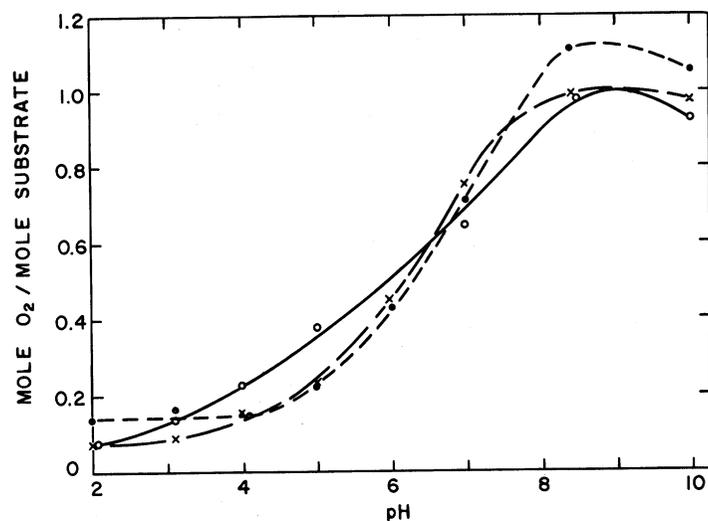


FIG. 2. pH Dependence of the photo-oxidation of L-tryptophan and its derivatives at 40°. (○—○) L-Tryptophan; (●---●) glycyl-L-tryptophan; (×—×) N-acetyl-L-tryptophanamide.

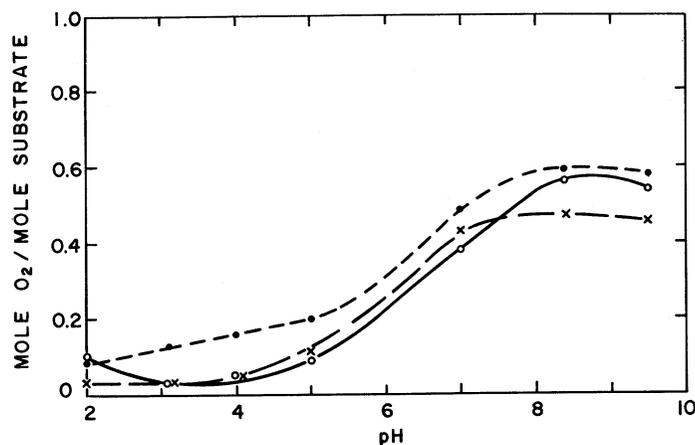


FIG. 3. pH Dependence of the photo-oxidation of L-methionine and its derivatives at 40°. (○—○) L-Methionine; (●---●) N-nicotinyl-DL-methionine amide; (×---×) glycyl-DL-methionine.

was not possible. Since the neutral thioether group in methionine is not affected by pH, and since substitution of the α -amino and α -carboxyl group of methionine gave essentially the same pH-profile as methionine itself, one must again conclude that the observed pH dependence is not due to any state of ionization but is related to some other yet unknown factor. Increase in methionine concentration ($30 \mu M$) again produced a pH profile comparable to that given in Fig. 3.

pH Dependence of the photo-oxidation of L-tyrosine and its derivatives at 40°. Figure 4 shows the photo-oxidation of L-tyrosine, glycyl-L-tyrosine, N-acetyl-L-tyrosine-ethyl-ester, N-acetyl-L-tyrosine amide, and 3,5-diiodo-L-tyrosine as the function of pH after 40 minutes of irradiation. The observed increase in photo-oxidation with increasing pH would tend to implicate the ionized phenol group of tyrosine in this reaction (since the pK of this group is near 10). The fact, however, that the ionization curve of

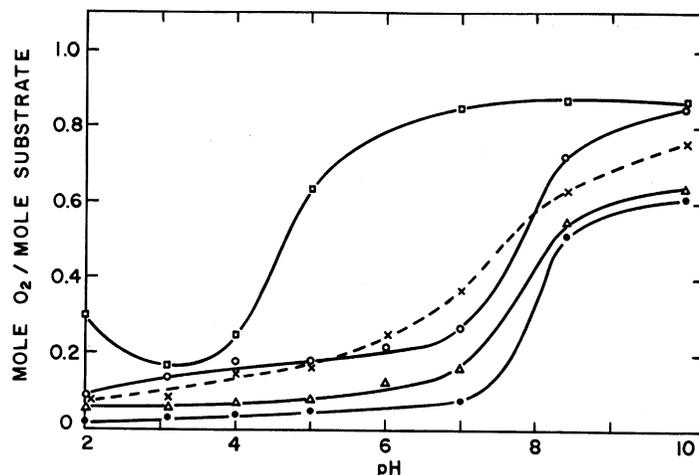


Fig. 4. pH Dependence of the photo-oxidation of L-tyrosine and its derivatives at 40°. (○—○) L-Tyrosine; (●—●) N-acetyl-L-tyrosine ethyl ester; (×---×) glycyl-L-tyrosine; (△—△) N-acetyl-L-tyrosine amide; (□—□) 3,5-diiodo-L-tyrosine.

tyrosine is distinct from the curve obtained by photo-oxidation casts some doubt on this assumption. At pH 7.0 the phenolic group of tyrosine is essentially nonionized, while at this pH a reduced but distinct photo-oxidation takes place. The sharp increase in the photo-oxidation rate up to 8.6 and only a moderate increase up to pH 10.0 (at the pK of the phenolic group) is not in quantitative accord with the ionization of this substance. On the other hand, photo-oxidation of 3,5-diiodo-L-tyrosine, which has a pK of about 6.5 due to the increased acidity of the hydroxyl group, shows a broad pH optimum around neutrality (where it is largely ionized). This would tend to support the conclusion that the ionization of the phenolic group of tyrosine is required for the photo-oxidation of this substrate. One must assume, therefore, that, in addition to the ionization of this group, some other yet undefined factors might be required for the photo-oxidation of tyrosine. The participation of the other polar groups (α -amino and α -carboxyl) must be excluded since substitution of these groups had no effect on the pH profile. Due to the limited solubility of tyrosine, experiments with higher concentrations could not be carried out.

pH Dependence of the photo-oxidation of L-cystine and —S—S—glutathione at 40°. Although we previously reported (1) that

cystine was susceptible to photo-oxidation sensitized by methylene blue, repeated experiments with this amino acid, and also with S—S—glutathione (with 60 minutes irradiation time) showed them to be very sluggish to photo-oxidation (Fig. 5). Due to the limited solubility of cystine, the photo-oxidation of this substrate was carried out only at pH 8.4 and 10.0, with LiOH as an alkalinizing agent.

Whether or not the previously observed reactivity of cystine was due to impurity of the particular preparation used cannot be answered now.

Photo-oxidation of histidine, methionine, tryptophan, and tyrosine at pH 7.0 as the Function of temperature. Results reported here were obtained at pH 7.0 with the same experimental and irradiation conditions as those described before. The findings obtained with histidine (Fig. 6) show that, at 2°, the oxygen uptake levels off exactly at 1 mole per mole of amino acid. Above this temperature (10°, 20°, and 40°) an additional slow reaction takes place, the rate of which appears to be a function of temperature.

Similar experiments, which were carried out with methionine (Fig. 7), show that, at 2°, the photo-oxidation levels off at about 0.6 mole of O₂ per mole of amino acid; at 10° the oxygen uptake stops when 1 mole of oxygen is consumed by 1 mole of methionine;

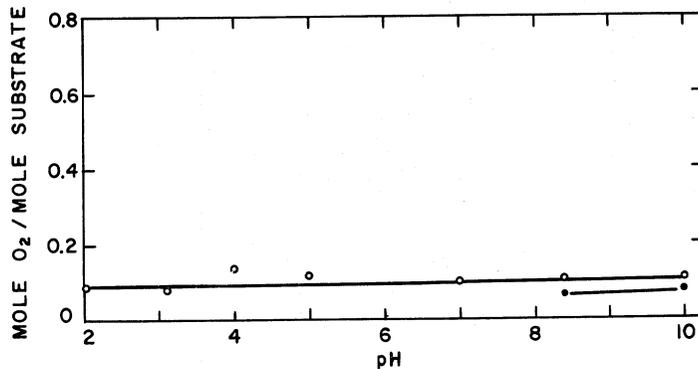


FIG. 5. pH Dependence of the photo-oxidation of —S—S—glutathione and L-cystine at 40°. (○—○) —S—S—glutathione; (●—●) L-cystine.

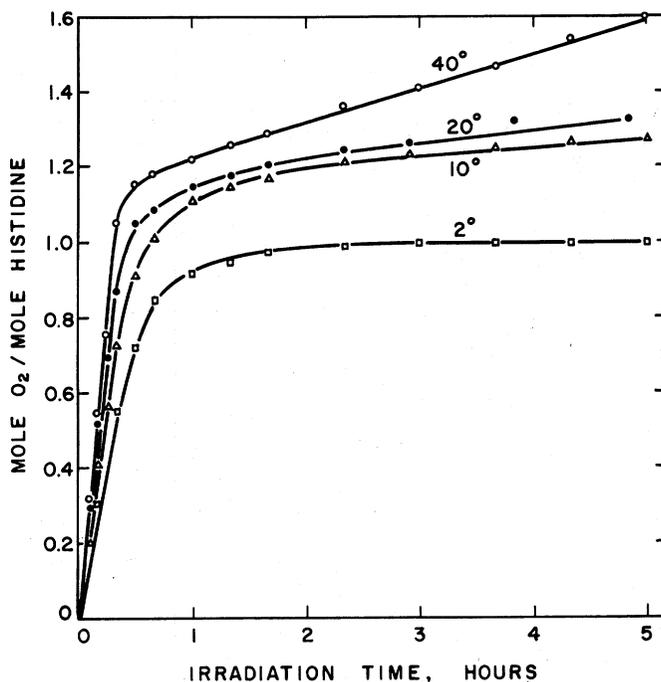


FIG. 6. Photo-oxidation of L-histidine at various temperatures.

and at 20° a slow additional reaction could be observed above the 1 mole level; this reaction became more marked if the temperature was raised to 40°.

Photo-oxidation of tyrosine (Fig. 8) showed a very low reactivity of this amino acid at 2° and progressively increased rates as the temperature was raised up to 40°. The slow rate observed at 2° was not due to partial precipitation of this amino acid at this temperature.

The rates of photo-oxidation of tryptophan as the function of temperature (Fig. 9) also indicate that both the rates and extent of oxygen uptake are governed by the temperature employed.

In our previous report (1) photo-oxidation of histidine resulted in a progressive decrease in the Pauly reaction (7), with complete disappearance at 1 mole of oxygen uptake. Similarly, photo-oxidation of methionine showed a progressive decrease if

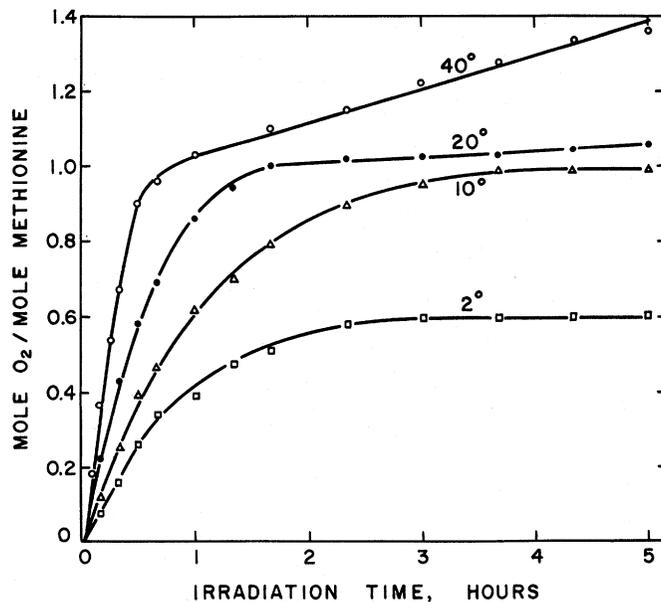


FIG. 7. Photo-oxidation of L-methionine at various temperatures.

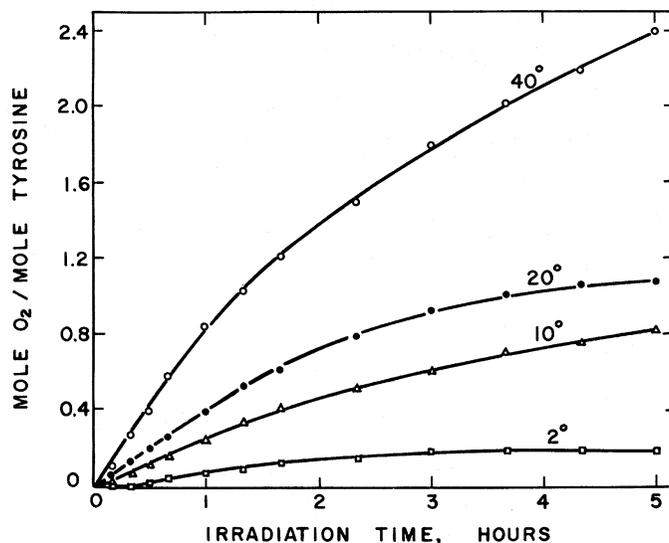


FIG. 8. Photo-oxidation of L-tyrosine at various temperatures.

followed by the McCarthy and Sullivan method (8), and reached zero value if 1 mole O₂ per mole of amino acid was reached.

The photo-oxidation of 1 mole of tryptophan required 2 moles of oxygen uptake before complete conversion was reached, as indicated by the Shaw and McFarlane reaction (9) (although the reaction did not stop at this level), but in the case of tyrosine

2 moles of oxygen uptake per mole of amino acid was needed before the color reaction of Bernhart (10) was negative.

In view of the above findings, it was of interest to find out whether the oxygen uptake above the required levels (as observed in Figs. 6-9) might be due to a dark reaction accelerated by elevated temperature. To decide this possibility, experiments pre-

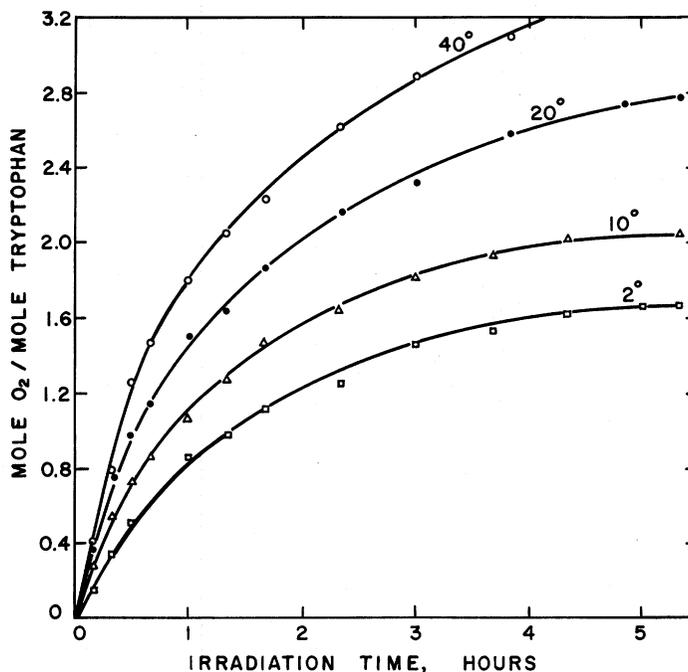


Fig. 9. Photo-oxidation of L-tryptophan at various temperatures.

sented in Figs. 6–9 were repeated at 40°. After the desired oxygen uptake was reached (1 mole of oxygen per mole of histidine or methionine and 2 moles of oxygen per mole of tryptophan or tyrosine), the reaction was allowed to proceed in the dark for a certain period, and subsequently the light was turned on again. The results (Fig. 10) clearly show that the oxygen uptake above the given level is not due to any dark reaction (since the reaction does not proceed without light) but to an additional light-sensitized process.

Irradiation of the various amino acids in the presence of methylene blue under anaerobic conditions at pH 8.0. In a 12 × 1.6-cm Thunberg type test tube were placed 8 ml of 0.01 M amino acid solution (containing 0.02 M phosphate buffer of pH 8.0; final pH checked with glass electrode) and 2 ml of methylene blue solution (0.1 mg). In the case of tyrosine the concentration was 0.005 M due to the limited solubility. The tube was then evacuated until boiling and filled with nitrogen. This operation was repeated three times. Under these conditions the system was strictly anaerobic. The tube was

then placed in a 37° glass-walled water bath and irradiated with a 375-W spotlight bulb from a 10-cm distance from the water bath. At various time intervals the tube was quickly removed and the optical density was measured at 640 m μ . This operation required only about 10 seconds. After a given irradiation period the tube was wrapped in tinfoil, and optical densities were measured during the dark period. The entire operation was carried out in a darkened room. After completion of the dark period, the tube was opened and aerated by bubbling air through it to check whether the original color density was restored. The results (Fig. 11) show that only those amino acids which were susceptible to photo-oxidation (histidine, methionine, tryptophan, and tyrosine) showed a marked reduction of the dye. The fact that during the dark period the optical densities remained unchanged proves that the reaction is not reversible. The return of optical density to the original values upon aeration indicate that the dye was reduced to its leuco form and was not irreversibly bleached.

In addition to the four photoreactive

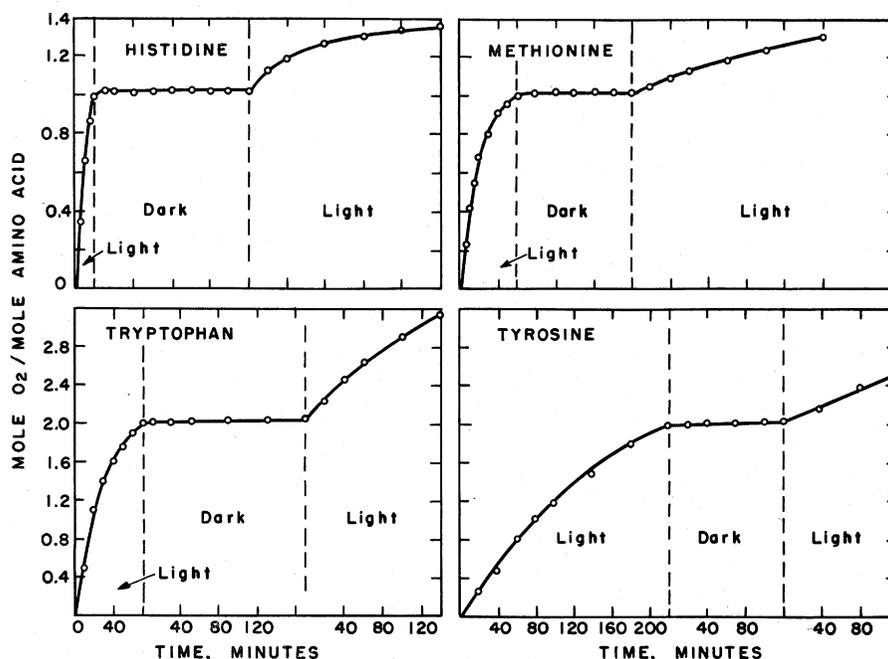


FIG. 10. The influence of light and darkness on the oxygen uptake of various amino acids in the presence of methylene blue (at 40°).

amino acids, the following amino acids were tested: alanine, arginine, aspartic acid, glutamic acid, glycine, leucine, isoleucine, lysine, phenylalanine, proline, serine, threonine, valine, and —S—S—glutathione. The results obtained with these amino acids were essentially the same, and for the sake of simplicity only our findings with alanine as a representative example are given in Fig. 11. Although a moderate bleaching of the dye is quite obvious, the fact that aeration did not restore the original optical density would indicate that the observed reduction in color density in these cases was not due to reduction but to irreversible bleaching of the dye. This is confirmed by the experiment presented in Fig. 11, where essentially the same result was obtained if the buffer solution alone was irradiated in the presence of methylene blue. It would appear that, in the presence of photoreactive amino acids (histidine, methionine, tryptophan, and tyrosine), methylene blue is protected against photodestruction since the original color densities were restored upon aeration (Fig. 11).

Effect of substrate concentration on the rate of photo-oxidation of histidine, methionine, tryptophan, and tyrosine at pH 7 and 10°. Since the photo-oxidation of amino acids sensitized by methylene blue resembles in some respect an enzymic reaction, we investigated the effect of substrate concentration on the reaction velocity at constant methylene blue concentration, as postulated by Michaelis and Menten (11) for enzymes. One-ml aliquots of amino acid solution, at pH 7.0, containing 1.5–30 μ moles, were placed in the Warburg flask, to which was added 0.5 ml of 0.2 *M* phosphate-citrate buffer, pH 7.0. The sidearm of the vessel was charged with 0.5 ml of methylene blue (containing 0.1 mg), and the irradiation was started after temperature equilibrium at 10°. In the case of tyrosine, due to the limited solubility of this amino acid in water, the photo-oxidation was carried out in 8 *M* urea which contained the same concentration of buffer as described above. With this solvent, experiments in the concentration range from 1.5 to 12.0 μ *M* were possible. The same amount of methylene

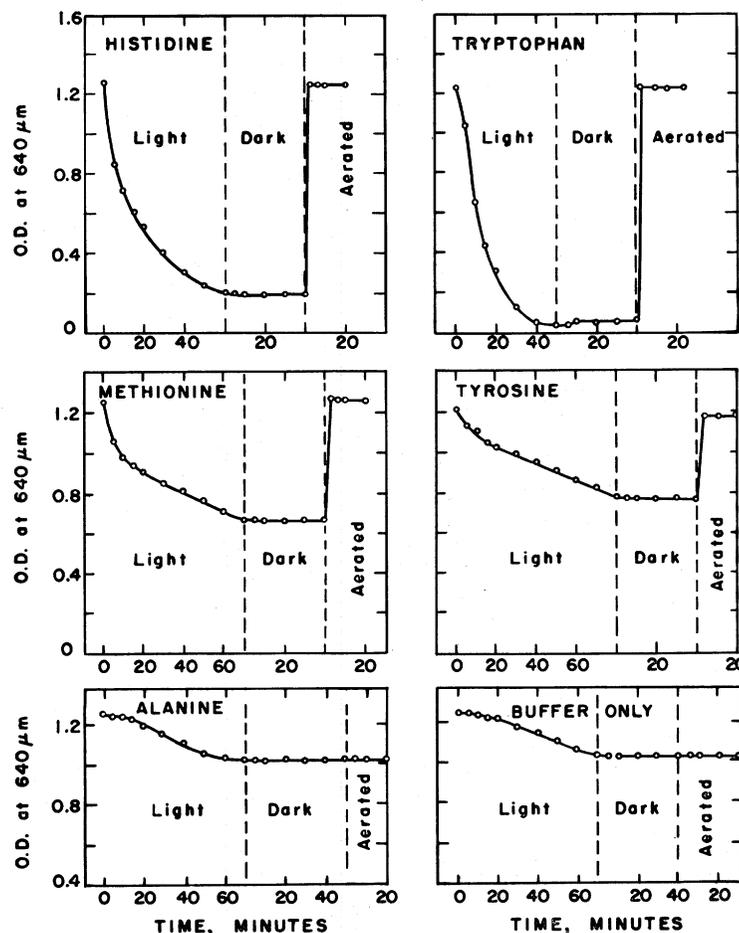


FIG. 11. Photodehydrogenation of various amino acids in the presence of methylene blue at pH 8.0 under anaerobic conditions. Alanine serves as a representative example for other amino acids listed in the text.

blue was used as above but was dissolved in 8 *M* urea. Our findings, to be reported subsequently, have shown that the rate of photo-oxidation of the reactive amino acids was identical whether 8 *M* urea or water was used as a solvent.

Figure 12 shows the reciprocals of reaction velocities (mm^3 of oxygen per minute, using the initial rates only) plotted against the reciprocal of substrate concentrations according to Lineweaver and Burk (12), where the slope of the line is K_M/V and its intercept on the ordinate is $1/V$.

The numerical values obtained for K_s and V_{\max} , as calculated from the plots presented in Fig. 12, are shown in Table I.

V_{\max} values were calculated on the basis that 1 mole of oxygen is required to photo-oxidize 1 mole of histidine, or methionine, while in the case of tryptophan and tyrosine, 2 moles of oxygen are needed to photo-oxidize these amino acids.

It should be noted that the above values were obtained at pH 7.0 and at 10°, and not under optimal conditions. It will be shown in a subsequent paper on the photo-oxidation of various proteins that, under these conditions, the reaction proceeds in a more specific fashion, and the kinetic parameters obtained under these conditions represent a better approximation of the processes which have taken place.

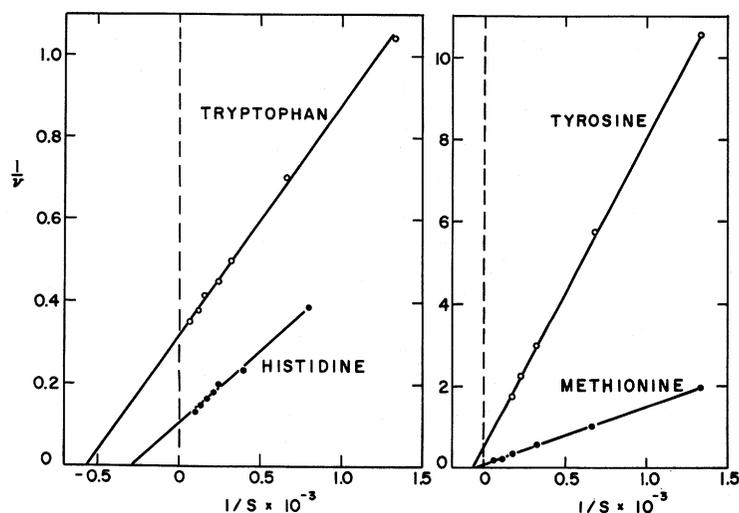


Fig. 12. Effective of substrate concentration on the velocity of photo-oxidation ($\text{mm}^3 \text{O}_2/\text{minute}$) of various amino acids at pH 7.0 and at 10° (Lineweaver-Burk plot).

TABLE I
KINETIC DATA OF PHOTO-OXIDATION

	Histidine	Methionine	Tryptophan	Tyrosine
$K_s(M)$	0.00344	0.0200	0.00178	0.0166
V_{\max} (moles/min)	0.446×10^{-6}	0.651×10^{-6}	0.07×10^{-6}	0.044×10^{-6}

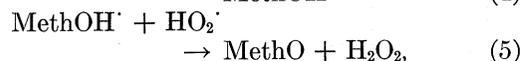
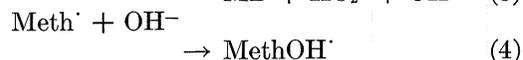
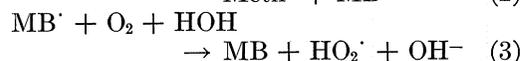
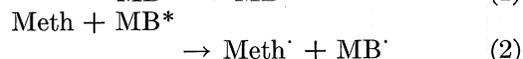
DISCUSSION

The susceptibility of certain amino acids to photo-oxidation sensitized by dyes was observed by Lieben (13), Harris (14), Gaffron (15), and Weil *et al.* (1). In addition, it was demonstrated (1) that, in the case of histidine, tryptophan, and tyrosine, the photo-oxidation mediated by methylene blue was confined to the imidazole, indole, and phenol groups, respectively.

Consistent with the oxidation reduction characteristics of methylene blue, the photo-oxidation mediated by this dye might function through a cyclic free-radical mechanism, whereby the light-excited dye is reduced by the substrate (see Fig. 11) through a semiquinone formation while the latter is being oxidized. Such a reactive semiquinone radical formation as an intermediate product formed during organic oxidation-reduction reactions has been demonstrated by Michaelis *et al.* (16) and Rabinowitch (17). The semiquinone formed possesses an odd number of electrons and in

solution exists in equilibrium with a molecular species containing one more electron and another with one electron less. Thus, a solution containing the semiquinone radical of methylene blue is in equilibrium with the dye and its leuco-base (16). Applying this concept to the photo-oxidation of amino acids, one might assume that the light-excited dye can act as an electron acceptor (being reduced), and is thus converted into its semiquinone radical, while the reactive amino acid acts as an electron donor (being oxidized). Under aerobic conditions the formed semiquinone and amino acid radicals are further oxidized by molecular oxygen, and the regenerated dye can re-enter the cycle again. Applying this reaction mechanism to methionine, where we know from our previous work (1) that the end product is methionine sulfoxide, and that the photo-oxidation is accompanied by 1 mole of oxygen uptake and in the formation of 1 mole of H_2O_2 per mole of substrate, the following tentative reaction scheme is

obtained:



where MB = methylene blue; MB* = light-excited methylene blue; MB[·] = semiquinone of methylene blue; Meth = methionine; Meth[·] = methionine radical; MethOH[·] = hydroxyl methionine radical; and HO₂[·] = peroxide radical.

Reactions 1-5 would be in full accord with the experimentally observed data. Application of the above reaction scheme to the other reactive amino acids, however, has to await the chemical identification of their photo-oxidative end products. Under anaerobic condition, on the other hand, the reoxidation of the reduced dye does not take place, and the formed semiquinone radical undergoes a disproportionation reaction and forms the leuco-base and the dye, the latter of which can act again as an electron acceptor until all the dye is converted into the leuco-base. Thus, the observed rates of photobleaching of methylene blue by the reactive amino acids, as shown in Fig. 11, might be only apparent due to the disproportionation reaction, and the actual reduction rates of the dye might be much faster than the observed ones.

In view of the above concept, the pH dependence of the photo-oxidation of histidine (Fig. 1) would indicate that only the free imidazole base can act as a reducing agent (electron donor), while its protonated form is essentially inactive. In the cases of tryptophan (Fig. 2) and methionine (Fig. 3), the observed optimal pH for photo-oxidation was about 8.5. Since the neutral indole and thioether groups are not affected by pH, and since the first step in these reactions, as shown in Fig. 11, consists of a reduction of the dye by the substrate, it might be assumed that the observed pH effect is due to the proper oxidation-reduction potential between the amino acid and the light-excited

dye. In the case of tyrosine (Fig. 4), the required redox potential might be facilitated by the ionization of the phenolic group. The ionization of the α-amino and α-carboxyl groups of these amino acids are of no consequence since the substitution of these groups had no effect on the pH profile of the photo-oxidation.

The photo-oxidation of histidine and methionine (Figs. 6 and 7) as a function of temperature at pH 7.0 would indicate that, in addition to the main fast reaction, an additional slow reaction takes place, the latter of which becomes more pronounced as the temperature increases. Thus, the photo-oxidation of histidine (Fig. 6) at 2° is strictly confined to 1 mole of oxygen per mole of amino acid, while above this temperature a progressive increase in an additional slow reaction takes place. In the case of the photo-oxidation of methionine (Fig. 7), at 10° the reaction stops at 1 mole of oxygen per mole of amino acid, while above that temperature, as in the case of histidine, a progressive increase in the slow reaction is observed. At 2°, a definite plateau is reached at about 0.6 mole of oxygen uptake; this finding requires further investigation. Similarly, the rate and extent of the photo-oxidation of tyrosine and tryptophan (Figs. 8 and 9) appear to be a function of temperature. Results presented in Figs. 6-9 would indicate that photo-oxidation around 10° confines the photo-oxidation of histidine, methionine, and tryptophan only to the extent where specific color reactions for these amino acids become negative (1 mole of oxygen per mole of histidine or methionine and 2 moles of oxygen per mole of tryptophan or tyrosine). In the case of tyrosine, a drastic reduction in the photoreactivity of this amino acid at 2° was noted. In addition, it was demonstrated that at 40° the oxygen uptake observed above the value that is required to eliminate the specific color reaction of these amino acids is not due to a dark reaction but to additional light-sensitized oxidation (see Fig. 10). The fact that this latter reaction becomes more apparent at a higher temperature would indicate a higher activation energy requirement.

Our studies on the dependence of photo-

oxidation on substrate concentration (Fig. 12) have shown that they follow the scheme proposed by Michaelis and Menten (11) for enzyme reactions, and that the obtained K_m values compare favorably with those obtained for numerous enzymic reactions (18).

The reaction scheme described in this paper, as far as methylene blue is concerned, differs from that proposed by Oster *et al.* (19), Schenck (20), and Livingston and Owens (21). These investigators assume that the irradiated dye is converted into a metastable long-lived excited state which reacts with oxygen or forms a labile peroxide which in turn oxidizes the substrate. Tentative evidence for such a labile oxygen-dye complex formation was presented recently by Simpson *et al.* (22) and was based on electron spin resonance measurements in solid methylene blue crystals. Our own findings (1), however, do not support the existence of such an intermediary peroxide dye complex. We have shown previously (1) that, if methylene blue solution is irradiated in the presence of sodium pyruvate (a highly effective trapping agent for peroxides) under aerobic conditions, neither oxygen uptake nor CO_2 evolution is observed. The first would be indicative for the peroxide-dye complex formation, and the second for the oxidative decarboxylation of pyruvic acid by the assumed dye-peroxide complex in a manner as described by Wieland and Winkler (23) and Sevag (24). However, if the above experiment was carried out in the presence of susceptible amino acids, marked oxygen uptake and CO_2 evolution were observed, indicating that the oxygen uptake was not due to binding by the light-excited dye and that the observed CO_2 evolution was the result of the interaction of the intermediately formed peroxide radical (see proposed scheme of reactions in this paper) with the pyruvic acid present in the system. Thus the evidence presented appears to support the concept that at the first step the light-excited dye might act as an electron rather than an oxygen acceptor, and the subsequent steps in the photo-oxidation process proceed in the manner described in our proposed reaction scheme. A similar conclusion has been

obtained by Bolland and Cooper (25) on the photosensitized oxidation of ethanol; they used anthraquinonoid derivatives as photosensitizers.

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