Manganese-catalyzed Oxidations of 2,3-Diketogulonate*

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ABSTRACT: Autoxidations of 2,3-diketogulonate can be induced by light and a sensitizing dye or by chemical means in the dark provided manganous ions are present in the reaction mixture. 3-(*p*-Chlorophenyl)-1,1-dimethylurea specifically stimulates the flavin-sensitized photoreaction, whereas the product formed from 3-(*p*-chlorophenyl)-1,1-dimethylurea by a flavin-sensitized photooxidation usually acts as an inhibitor. The action of manganese as a catalyst is discussed on the basis of the mechanism of manganese catalysis in certain peroxidase-oxidase reactions as proposed by I. Yamazaki and L. H. Piette [*Biochim. Biophys. Acta* 77, 47 (1963)]. Trivalent manganese appears to be an essential intermediate chain carrier in all oxidations of 2,3-diketogulonate studied.

The surprising specificity of the Mn(II)–Mn(III) couple to catalyze the oxidations of 2,3-diketogulonate is very probably a result of its oxidation-reduction potential, which allows the oxidation of Mn(II) by O_2^- radicals to supplant chain-breaking removals of O_2^-. A chelation by 2,3-diketogulonate may enable Mn(III) to accomplish efficiently a splitting of the 2,3-diketogulonate molecule by attracting an electron.

In the presence of catalase, the main product of the photooxidation of ascorbate with flavin is dehydroascorbate, which in neutral solutions is hydrolyzed to 2,3-diketogulonate (DGA).[1] If manganous ions are present in the reaction mixture, the photooxidation of ascorbate is followed by a photooxidation of DGA in a subsequent reaction step (Habermann and Gaffron, 1962; Homann and Gaffron, 1963, 1964). The over-all process is interesting for three reasons: first, the specific requirement for manganous ions; second, its clear separation into two distinct parts; and, third, its sensitivity to the presence of certain herbicides such as CMU. The specific requirement for manganese, as well as a specific action of CMU-like compounds in both the photooxidation *in vitro* and the oxygen evolution *in vivo*, might be more than pure coincidence. The reciprocal regulation between two photooxidation steps resembles the necessary balancing of biochemical reactions among many possible events within the living cell.

The data reported in this paper demonstrate that the manganese-catalyzed oxidation of diketogulonate in neutral or in slightly acid solutions can be brought about not only by light and a sensitizing dye, or by illuminated chloroplasts, but under certain conditions also in the dark. Obviously the primary event is not the same in each type of reaction, yet the secondary steps appear to be common to all of them. The evidence speaks for the participation of trivalent manganese as intermediate electron acceptor in the oxidation of diketogulonate.

We also present new data on the interaction between CMU and excited flavin molecules. Under aerobic conditions this interaction establishes a photooxidation of DGA which is largely independent of the oxygen concentration (Homann and Gaffron 1963, 1964).

Materials and Methods

Experimental Conditions. Most of our methods and experimental conditions have been described in our earlier papers. In manometric experiments the temperature was usually 25°. The reaction mixture contained 6–8 μmoles of diketogulonate in 3.00 ml. The statement in our previous paper that no CO_2 is evolved during the reaction holds true only for the photooxidation of DGA under mild conditions, whereas at high light intensities or under the more vigorous oxidation processes in the dark corrections for some CO_2 evolution have to be made. To exclude other side reactions resulting from the photobleaching of the sensitizing dye itself, FMN was replaced by lumiflavin in certain experiments. Lumiflavin is resistant to photodestruction (Radda and Calvin, 1964).

Chloroplasts or chloroplast fragments were obtained from tobacco plants or from wild grown *Cassia obtrusifolia* and prepared according to Margulies and Jagendorf (1960). Mn^{3+} in its pyrophosphate complex was determined spectrophotometrically after Kenten and Mann (1955).

DGA was either prepared in solution from commercial dehydroascorbic acid (Homann and Gaffron, 1964) or crystallized as the calcium salt according to

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[1] Abbreviations used in this work: DGA, 2,3-diketogulonate; FMN, flavin mononucleotide; CMU, 3-(*p*-chlorophenyl)-1,1-dimethylurea; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; TETA, triethylenetetramine; HRP, horseradish peroxidase; NADP, nicotinamide-adenine dinucleotide phosphate.

Penney and Zilva (1945) and Curtin and King (1955). Because in aqueous solution DGA undergoes manifold structural changes, our reaction solution may have contained more than one reactive form of DGA. This may explain some irregularities in the kinetics observed. Even the structure of DGA prepared by the method of Penney and Zilva has been disputed by Euler and Hasselquist (1952), who believe it to be the enolate of dehydroascorbic acid, although no absorption appears in the ultraviolet at $\lambda > 240$ mμ, as would be expected for an enol group. It is more likely that 2,3-diketogulonate is present in aqueous solutions in its hydrated form, since the dicarbonyl moieties of the structurally related dehydroascorbate (Euler and Eistert, 1957) and diketosuccinate also add two molecules of water.

Manganese oxide catalysts were prepared by soaking unglazed porcelain chips (boiling chips) in a solution containing $MnSO_4$ and H_2O_2. Subsequently these chips were exposed to NH_3 vapors and thoroughly washed with distilled water.

Horseradish peroxidase (HRP) was a commercial preparation from the Nutritional Biochemical Corp., Cleveland, and from Boehringer and Soehne, New York, with *RZ* equal to 0.60 and 2.9, respectively. DCMU, CMU, and bromacil were kindly supplied by the du Pont de Nemours Corp., Wilmington, Del.

The incident light intensity for Figure 7 and Table II was measured with the remote probe of the ISCO Model SR spectroradiometer (Instruments Specialties Co., Lincoln, Neb.) with incandescent light passed through a blue plastic filter with 400 mμ $< \lambda <$ 600 mμ and λ_{max} = 490 mμ; 300 μw $\times$ cm^{-2} under these conditions correspond to 600 lux.

Various Types of DGA Oxidations. The oxidation of DGA in the presence of manganous ions and oxygen can be brought about not only by light and a sensitizing dye, but also nonphotochemically, i.e., by variously induced autoxidations.

TYPE I. DGA is oxidized when traces of NaOH as dispensed at the tip of a tube in a *p*H-stat titration assembly cause an autoxidation of manganous ions in solution (Homann and Gaffron, 1964).

TYPE II. An oxidation of DGA is induced by adding to a solution of DGA and Mn(II) either small amounts of $KMnO_4$ or unglazed porcelain chips coated with manganese oxides.

TYPE III. In the presence of manganous ions horseradish peroxidase (HRP) catalyzes the oxidation of DGA. DGA thus can be added to the list of compounds which are attacked by HRP in an oxidase reaction.

TYPE IV. Mn(II) is complexed by triethylenetetramine (TETA). In a solution which contains this complex DGA undergoes autoxidation.

TYPE V. Illuminated chloroplasts or chloroplast fragments oxidize DGA in the presence of manganous ions. This reaction is not a chlorophyll-sensitized photooxidation because it is inhibited strongly by compounds which inhibit the oxygen evolving system in photosynthesis, such as DCMU, CMU, and bromacil (the photooxidation of DGA with chlorophyll obtained by dodecyl sulfate extraction of chloroplasts is insensitive to these poisons).

In contrast to the flavin-sensitized photooxidation of DGA which is inhibited by increasing O_2 tension, all reactions of type I through type V proceed faster with 100% O_2 than with air. As in the photosensitized reaction, approximately 1 μmole of H_2O_2 is formed per μmole of oxygen absorbed in reaction mixtures of type I, II, and III. In reaction type V any H_2O_2 formed is decomposed by the endogenous catalase of the chloroplasts. Very little H_2O_2 accumulates in reaction IV because of subsequent interactions between hydrogen peroxide and the TETA–Mn(II) complex or with the oxidation products of DGA. In the presence of catalase, the main oxidation products of DGA are always oxalate and threonate (the former was determined quantitatively and the latter was identified chromatographically). These two compounds are also formed in *alkaline* solution during the oxidation of the enolate of DGA by iodine (Herbert *et al.*, 1933) or by a copper-catalyzed autoxidation (Gosh, 1938; Kagawa, 1962).

Evidence for One Common Oxidation Mechanism. The possibility of a reversible conversion of Mn(II) to Mn(III) makes manganous ions efficient catalysts in oxidations which involve one-electron steps (Kagan and Lubarski, 1935; Waters, 1961). The specific requirement for manganous ions in all of the reactions enumerated above speaks for the participation of manganese as an intermediate electron carrier. Our observations on the effect of inhibitors support the contention that the basic mechanism remains the same.

As shown earlier, small amounts of ascorbate inhibit the sensitized photooxidation of DGA. This is also true for the HRP-catalyzed reaction (type II). In 3.00 ml of a solution of 0.2 mg of HRP (*RZ* = 0.60) and 2 μmoles of $MnSO_4$, the onset of the oxidation of DGA is delayed for 20 minutes by 0.13 μmole of ascorbate.

Copper ions inhibit not only the sensitized photooxidation of DGA (Habermann and Gaffron, 1962; Homann and Gaffron, 1964) but also the oxidation of DGA in reactions of types I (Homann and Gaffron, 1964), II (see Figure 2), and III (70% with 2×10^{-5} M Cu^{2+}).

Pyrophosphate (10^{-4} M) inhibits the flavin-sensitized photooxidation as well as the HRP-catalyzed reaction by about 25%. Pyrophosphate is known to form complexes with Mn(III); hence this observation supports the view that Mn(III) participates in the reaction. The Mn(III)–pyrophosphate complex itself is virtually ineffective as initiator of an autoxidation of DGA.

H_2O_2 is not an essential intermediate, since catalase was never found to inhibit the main course of the reaction severely. Catalase, therefore, could be used to avert side reactions which arise as a consequence of an accumulation of H_2O_2.

HRP-catalyzed Oxidation of DGA. The mechanism of various other oxidations catalyzed by HRP (for instance that of dihydroxyfumarate) has been thoroughly investigated by Yamazaki and Piette (1963). The oxidation of DGA by HRP, like its photooxidation,

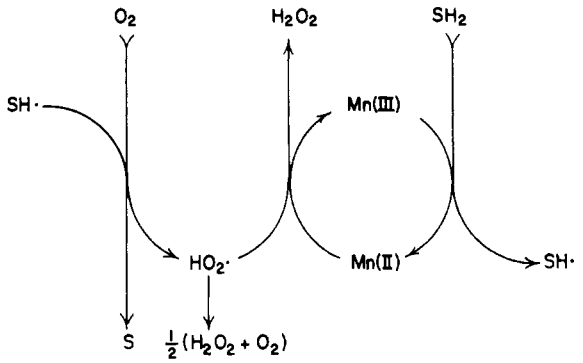

FIGURE 1: Possible mechanism of the manganese catalysis in the oxidations of 2,3-diketogulonate (SH_2) (after Yamazaki and Piette, 1963).

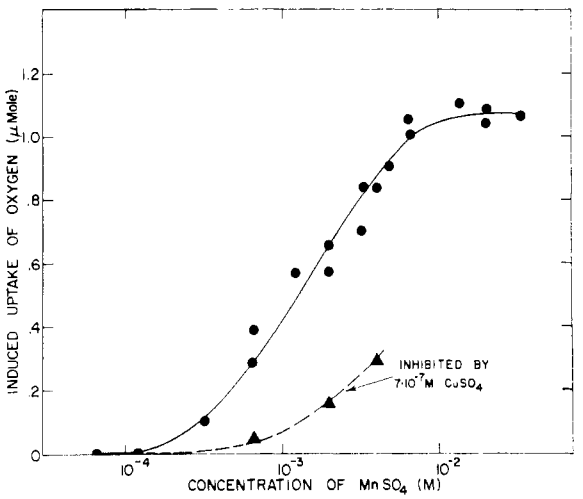

FIGURE 2: Autoxidation of 2,3-diketogulonate induced by addition of 0.01 μmole $KMnO_4$ as function of the concentration of $MnSO_4$. T = 25°; gas phase, oxygen; 3.00 ml reaction mixture contained 7 μmoles DGA, 100 μmoles succinate buffer, pH 5.0, and $MnSO_4$ as indicated. The solutions in the experiments represented by the lower curve contained in addition 0.002 μmole $CuSO_4$. $KMnO_4$ (0.01 μmole) was added from the side arm of the Warburg vessel and the subsequent oxygen uptake was measured. The background uptake of oxygen by the reaction mixture without added $KMnO_4$ was negligible. Corrections were necessary only for the two highest manganese concentrations tested.

occurs equally well between pH 6.0 and 4.0 and resembles the oxidation of dihydroxyfumarate in many respects: manganous ions serve as strong activators in both reactions, and both reactions are inhibited by copper ions, by ascorbate, and by hydroquinone. The reaction mechanism proposed for the HRP-catalyzed oxidation of dihydroxyfumarate can also serve to explain the HRP-catalyzed oxidation of DGA. This means that manganous ions catalyze the oxidation of DGA because they supplant the dismutation of intermediate $HO_2\cdot$ radicals by a much faster reaction in which Mn(II) is oxidized to Mn(III). The possible reaction sequences are summarized in Figure 1. If this reaction mechanism is valid for the oxidation of DGA we may postulate that the various types of DGA oxidations are chain reactions which involve the Mn(II)–Mn(III) couple as chain carrier and differ only with respect to the initiating reaction. The length of the chain may be controlled by chain-breaking substances, for example, by certain organic molecules or copper ions. One way to test the validity of the reaction scheme (Figure 1) would be to initiate the oxidation by introducing small amounts of Mn(III) into a solution containing a relatively large amount of Mn(II). Once the reaction has started, an excess of Mn(II) should assure the continued oxidation of Mn(II) by $HO_2\cdot$ in place of the chain-breaking dismutation reaction.

Mn(III)-*induced Oxidation of DGA.* Porcelain chips carrying manganese oxides can be used to induce an oxidation of DGA, but more quantitative results are obtained by generating Mn(III) from Mn(II) with permanganate in the "Guyard reaction." Neglecting any formation of Mn(IV) (which of course can dismutate to Mn(II) and Mn(III)), 5 μmoles of Mn(III) is formed per μmole of permanganate according to the equation:

$$4\,Mn^{2+} + MnO_4^- + 4\,H^+ \longrightarrow 5\,Mn^{3+} + 4\,OH^- \quad (a)$$

Table I gives some data on the permanganate-induced oxidation. The data for the oxygen uptake are corrected for a very slow background autoxidation of DGA. It can be seen that in the presence of relatively high Mn(II) concentrations more oxygen is taken up than would be expected from a stoichiometric oxidation of DGA (written as SH_2) by Mn(III) and oxygen to oxalate

TABLE I: Induction of a Chain Oxidation of 2,3-Diketogulonate by the Guyard Reaction.[a]

$KMnO_4$ Added (μmole)	$MnSO_4$ Present (μmoles)	Induced Uptake of O_2 (μmole)	H_2O_2 Formed (μmoles)	μmoles O_2 Consumed/ μmole $KMnO_4$ Added
0.400		0.7	0.7	2
0.040		Nil	Nil	
0.400	20	2.25	2.30	6
0.200	20	1.37	1.40	7
0.020	20	0.75		38
0.004	20	0.35		90

[a] Reaction mixture (3.00 ml) in Warburg vessels at 25°, under pure oxygen, with 8 μmoles 2,3-diketogulonate, 100 μmoles succinate buffer, pH 6.0, and $MnSO_4$ as indicated. $KMnO_4$ was tipped from the side arm.

and threonate (written as S) according to the following equations:

$$2\,SH_2 + 2\,Mn^{3+} \rightarrow 2\,SH\cdot + 2\,Mn^{2+} + 2\,H^+ \quad (b)$$

$$2\,SH\cdot + 2\,O_2 \rightarrow 2\,S + 2\,HO_2\cdot \quad (c)$$

$$2\,HO_2\cdot \rightarrow H_2O_2 + O_2 \quad (d)$$

i.e.,

$$2\,DGA + 2\,Mn(III) + O_2 \longrightarrow 2\,oxalate + 2\,threonate + 2\,Mn(II) + H_2O_2 \quad (e)$$

or

$$\frac{\mu moles\ O_2\ consumed}{\mu moles\ KMnO_4\ added} = \frac{\mu moles\ H_2O_2\ formed}{\mu moles\ KMnO_4\ added} = 2.5$$

Thus the addition of permanganate to a solution containing DGA and manganous ions induces an oxidation of DGA via a reaction chain. In the presence of sufficient Mn(II) the oxidation does not appear to be initiated predominantly by a direct attack of permanganate on DGA. This can be deduced from the observation that, in 5 ml of solution, 0.4 μmole of $KMnO_4$ is decolorized by 15 μmoles of DGA more slowly in the absence than in the presence of 2 μmoles of $MnSO_4$. Manganese(III), therefore, can be assumed to play the role of initiator as well as of chain carrier as postulated by the mechanism shown in Figure 1. In agreement with this mechanism, the efficiency of a given amount of $KMnO_4$ to induce an oxidation of DGA increases with the amount of Mn(II) present until a constant turnover is reached (Figure 2).

Less than the calculated amount of oxygen is taken up when the oxidation of DGA is induced by permanganate in absence of manganous ions. Lack of Mn(II) cuts reactions (a–d) short. An inefficient Guyard reaction (a) favors more direct, i.e., oxygen-independent, oxidations of DGA or DGA radicals by permanganate.

$KMnO_4$–Mn(II) efficiently induces the oxidation of DGA between *p*H 6.0 and 4.5, but not at *p*H 2. Thus the *p*H dependence resembles that found for the photooxidation of DGA.

*Oxidation of DGA by the TETA–*Mn(II) *Complex.* In the presence of TETA, Mn(II), and oxygen, a fast autoxidation of DGA will eventually start in the dark after a lag period of variable lengths. The time of onset of oxidation can, however, be controlled by preilluminating a buffered solution of TETA, Mn(II), and a dye, usually lumiflavin. An autoxidation starts immediately upon addition of DGA in the dark (Figures 3 and 4). The initiating species formed in the light is not a radical because an autoxidation of DGA can still be observed when the latter is added 3 minutes after illumination. A prolonged illumination of the solution without DGA leads to a precipitation of brown manganese oxides. At least part of these oxides contain

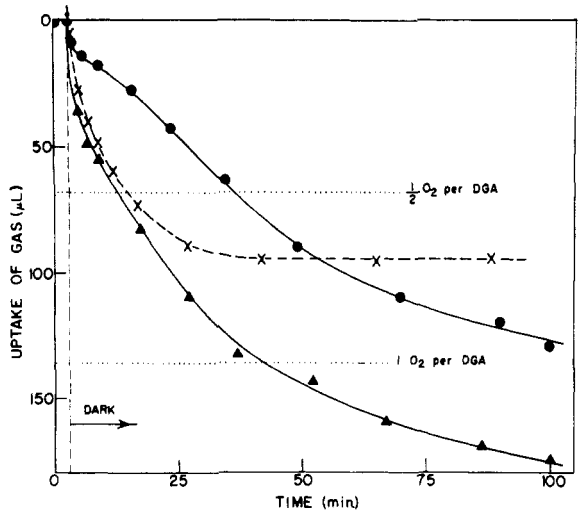

FIGURE 3: Autoxidation of 2,3-diketogulonate in the presence of TETA and Mn(II); dependence on concentration of Mn(II) and effect of catalase. $T = 30°$; gas phase, oxygen; 3.00 ml reaction mixture contained 0.07 μmole umiflavin, 18 μmoles TETA, 100 μmoles succinate buffer, *p*H 6.2, and 5.0 μmoles $MnSO_4$ (▲–▲–▲), or 1.0 μmole $MnSO_4$ (●–●–●), or 5.0 μmoles $MnSO_4$ plus 2 mg catalase (×–×–×). After illumination for 2 min with 20 klux incandescent light, 5.5 μmoles of DGA was added in the dark at point indicated by arrow. The gas uptake is not corrected for a small CO_2 evolution. No gas exchange was observed with 0.08 μmole and 0.3 μmole $MnSO_4$.

trivalent manganese, since the sulfuric acid solution of the oxides gives the spectrum of the sulfato complex of Mn^{3+}.

The autoxidation of DGA in the presence of TETA and manganese is a very complex process. Some observations merit discussion, although we do not yet have enough data for a complete description of the reaction mechanism.

The most serious complications arise from side reactions. There is often an uptake of oxygen surpassing that required for an oxidative splitting of DGA into threonate and oxalate. Some CO_2 evolution during the reaction is due in part to an oxidation of oxalate.

The susceptibility to autoxidation of a solution of DGA, manganous ions, and TETA appears to result from the dual role of manganese as chain carrier (see Figure 1) and, in a complex with TETA, as participant in the initiating reactions. The following observations suggest that catalytic amounts of hydrogen peroxide can act as chain initiators. If prior to the addition of DGA a little H_2O_2 is added to a solution of Mn(II) and TETA, an autoxidation of DGA occurs as promptly as in the case of preillumination. If H_2O_2 is added when the solution already contains DGA, an autoxidation of DGA may or may not follow. This means that H_2O_2 can initiate a TETA–Mn(II)-catalyzed oxidation of

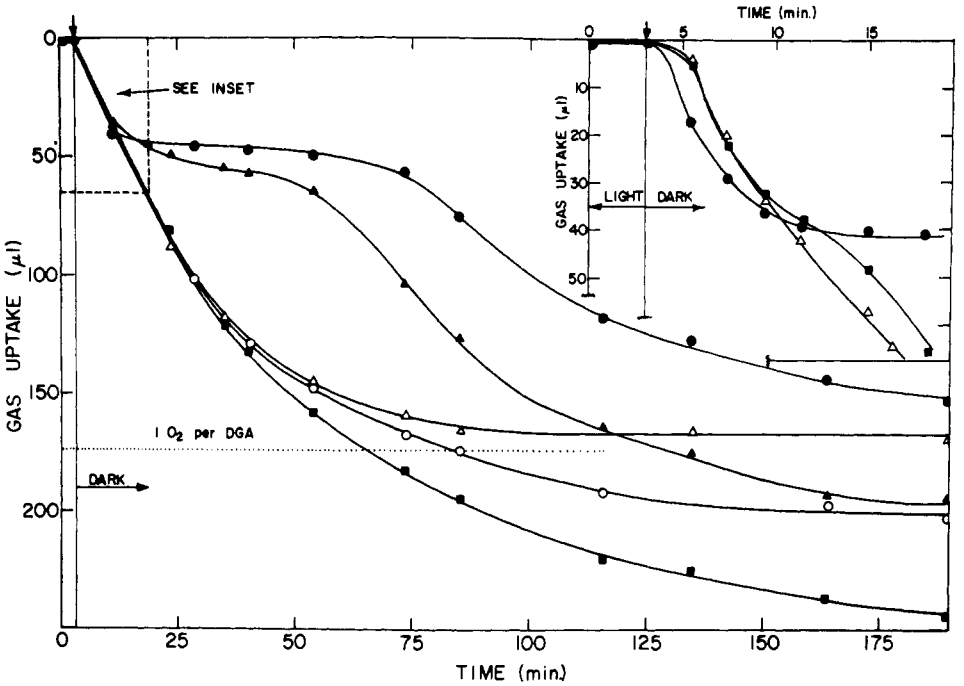

FIGURE 4: Autoxidation of 2,3-diketogulonate in the presence of TETA and Mn(II); dependence on TETA concentration. T = 30°; gas phase, oxygen; 3.00 ml reaction mixture contained 0.37 μmole FMN, 4.0 μmoles $MnSO_4$, 100 μmoles succinate buffer, pH 6.2, and 2.6 μmoles TETA (Δ–Δ–Δ), 6.5 μmoles TETA (○–○–○), 33 μmoles TETA (■–■–■), 65 μmoles TETA (▲–▲–▲), 130 μmoles TETA (●–●–●). After illumination for 3 min with 20 klux incandescent light, 7 μmoles DGA was added in the dark at point indicated by arrow. The gas uptake is not corrected for a small CO_2 evolution. No gas exchange was observed with 0.75 μmole TETA.

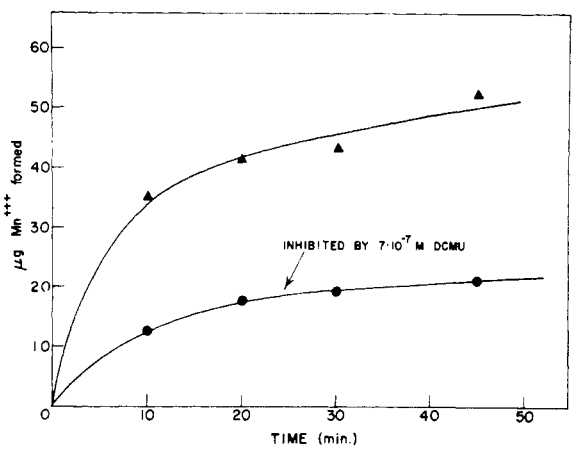

FIGURE 5: Oxidation of Mn(II) by illuminated chloroplasts. T = 25°; gas phase, air; 4.00 ml reaction mixture contained 20 μmoles $MnSO_4$, 200 μmoles pyrophosphate buffer, pH 7.0, and chloroplasts from *Cassia obtrusifolia* (200 μg chlorophyll); illumination with 4.5 klux incandescent light from which $\lambda < 600$ mμ was removed by a red plastic filter.

DGA, but not directly, i.e., it has to build up the actual initiator by reacting with the TETA–Mn(II) complex. The complex of Mn(II) and TETA, like other metal chelates of TETA (Jonassen and Ramanujam, 1959), decomposes H_2O_2 with oxygen evolution. This process is accompanied by the formation of colored higher valency states of manganese. If these are allowed to accumulate during a preillumination of a flavin–TETA–Mn(II) solution as well as during a preincubation in the presence of H_2O_2, any added DGA will be oxidized immediately (see Figures 3 and 4). The complexity of the process becomes evident in reaction mixtures which are unbalanced with respect to the concentrations of TETA and Mn(II), e.g., when high concentrations of TETA do not leave enough chain-carrying free manganese in the solution. Because the chains are very short, the concentration of TETA–Mn(III) present at the time of the addition of DGA will determine the extent of its oxidation. After this fast initial oxidation the reaction comes more or less to a stop and is resumed at a slower rate after a variable length of time (Figure 4). A new steady-state condition had to be built up while side reactions could occur with the oxidation products of DGA and with the complexing TETA molecules. Oxidative self-destructions of chelates are known (e.g., Fallab, 1958), and can be observed also in the autoxidation of Co^{2+} complexed by TETA. One experiment presented in Figure 3 was made in the presence of catalase. As in experiments of Yamazaki and Piette (1963), catalase apparently did not decompose the intermediate H_2O_2 fast enough to abolish its catalytic action completely, but did so in the slower side reactions.

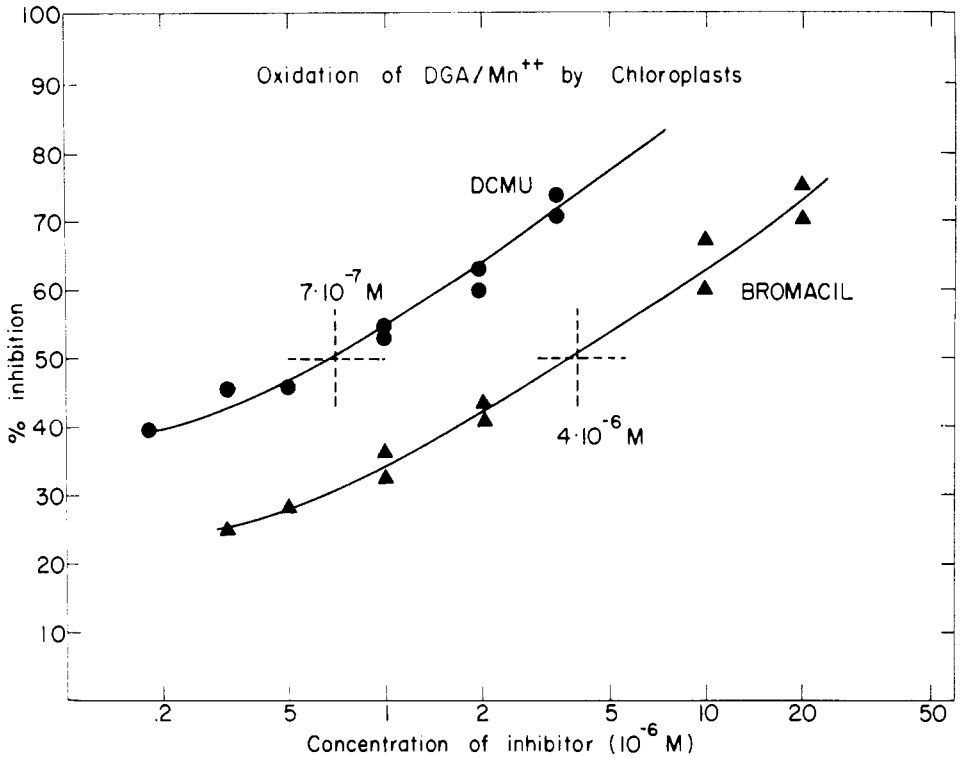

FIGURE 6: Inhibition by DCMU and bromacil of the oxidation of 2,3-diketogulonate by illuminated chloroplasts. T = 25°; gas phase, air; 3.00 ml reaction mixture contained 7 μmoles 2,3-diketogulonate, 6 μmoles $MnSO_4$, 100 μmoles succinate buffer, *p*H 6.2, chloroplasts from *Nicotiana tabacum* (175 μg chlorophyll), and DCMU or bromacil as indicated. Illumination with 4.5 klux incandescent light from which $\lambda < 600$ mμ was removed by a red plastic filter.

Under our usual experimental conditions (see legend of Figure 3), the autoxidation of DGA by the TETA–Mn(II) system is very *p*H sensitive. A fast reaction occurred at *p*H 5.9, but no reaction was observed at *p*H 5.5. This *p*H dependence may reflect the stability of the manganese–TETA chelate.

Oxidation of DGA by Illuminated Chloroplasts. Habermann and Gaffron (1962) reported a manganese-dependent two-step oxidation of ascorbate by *Helianthus* chloroplasts in red light for solutions containing FMN. In 1964 Muneeruddin, and recently in independent studies Habermann and Hayward (unpublished), found that certain chloroplast preparations catalyze this reaction also in absence of FMN. In the course of our studies on the manganese-dependent second step, the oxidation of DGA, we reinvestigated the action of illuminated chloroplasts.

In the light and in the presence of manganese, DGA is rapidly oxidized by whole or fragmented chloroplasts from *Nicotiana tabacum* or *Cassia obtrusifolia*. No FMN is needed in the reaction mixture. The rate of the reaction does not depend on the ability of the chloroplast preparations to perform the Hill reaction with quinone or ferricyanide, but our preparations were still capable of reducing oxygen to hydrogen peroxide (Mehler, 1951) or, in the presence of pyrophosphate, to oxidize Mn(II) to Mn(III) in the light (Kenten and Mann, 1955). With fresh chloroplast preparations these three reactions (reduction of oxygen, oxidation of Mn(II) or of DGA) are sensitive to low concentrations of DCMU or bromacil (Figures 5 and 6), poisons which act specifically in the oxygen-evolving pathway (Bishop, 1958; Hoffmann *et al.*, 1964; unpublished data from our laboratory). The oxidation of DGA by illuminated chloroplasts does not depend on an intermediate formation of H_2O_2. When hydrogen peroxide is generated in the dark by glucose oxidase and glucose in the reaction mixtures just mentioned, no DGA oxidation and only a slight oxidation of Mn(II) could be observed. It is more likely, therefore, that the oxidation of Mn(II) and of DGA are initiated by the first product of the reduction of oxygen, the $HO_2\cdot$ radical.

According to Yamazaki and Piette (1963) the oxidation-reduction potential of O_2–$HO_2\cdot$ is between zero and −0.3 v. If this value is correct (see, however, George and Griffith, 1959), oxygen may be reduced to $HO_2\cdot$ by a reduced one-electron carrier in the electron transport chain of the chloroplast on the way to NADP, e.g., by ferredoxin, which is known to be readily autoxidized (Tagawa and Arnon, 1962).

Details of the Sensitized Photooxidation of DGA.

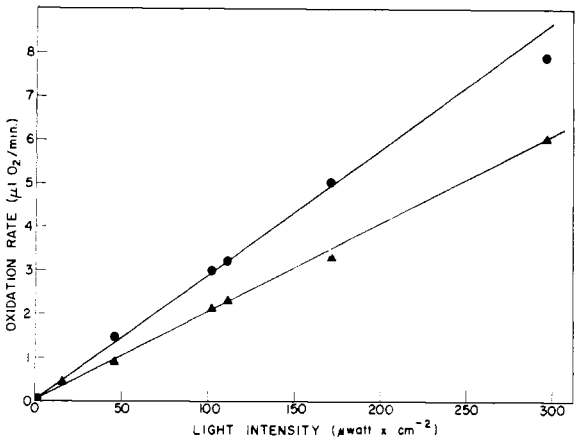

FIGURE 7: Sensitized photoxidation of DGA; dependence of the rate on the light intensity. $T = 25°$; gas phase, air. The center well of the vessels held 0.1 ml 20% KOH; 4.00 ml reaction mixture contained 0.1 μmole lumiflavin, 4.0 μmoles $MnSO_4$, 100 μmoles succinate buffer, *p*H 5.4, and 14.0 μmoles 2,3-diketogulonate (●–●–●), or 9.5 μmoles 2,3-diketogulonate (▲–▲–▲). 2,3-Diketogulonate was added as its calcium salt.

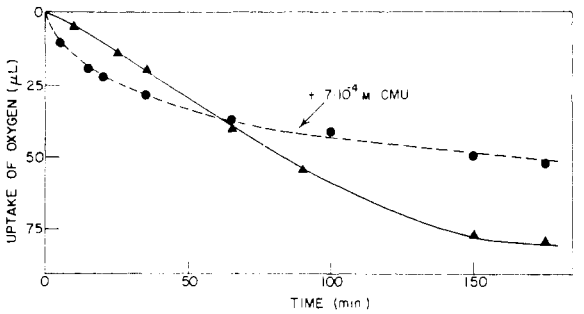

FIGURE 8: Effect of CMU on the flavin-sensitized photooxidation of 2,3-diketogulonate in the presence of small amounts of manganese. $T = 26°$; gas phase, air; 3.00 ml reaction mixture contained 0.37 μmole FMN, 6.5 μmoles DGA, 0.2 μmole $MnSO_4$, 1 mg catalase, 100 μmoles phosphate buffer, *p*H 6.0. Illumination with 4.2 klux white incandescent light.

TABLE II: Dependence of the Oxidation Rate on 2,3-Diketogulonate concentration.[a]

Concn of 2,3-Diketogulonate (10^{-3} M)	Rate of Oxidation (μl gas/min) + KOH in Center well	Rate of Oxidation (μl gas/min) No KOH in Center well	Induction Period (min)
9.0	−5.8	−4.9	20
4.5	−4.2	−3.8	15
2.5	−2.7		13
1.5	−1.4	−1.4	10

[a] $T = 25°$; gas phase, air; 4.00 ml reaction mixture contained 0.1 μmole lumiflavin, 4 μmoles $MnSO_4$, 100 μmoles succinate buffer, *p*H 4.6, and the calcium salt of 2,3-diketogulonate as indicated. Illumination with 120 μw × cm^{-2} blue light. The rate was measured during the period of constant maximal rate, and the induction period includes the initial lag and the period of acceleration.

We are now in a position to discuss the initiating reaction in the photosensitized oxidation of DGA where the rate of oxygen uptake decreases with an increase of the oxygen concentration (Homann and Gaffron, 1963, 1964). The rate of photooxidation is proportional to the light intensity when the latter is increased tenfold (Figure 7). Catalase was omitted from the reaction mixtures to avoid any reaction between catalase and the illuminated lumiflavin Anaerobically catalase reduces flavins (Radda and Calvin, 1964), and aerobically it catalyzes a photooxidation of manganous ions by flavin.

Some data on the oxidation rate as function of DGA concentration are given in Table II. The table also shows that the approximate length of an induction period preceding the condition of steady maximum rate increases with increasing DGA concentration. This points to the presence of inhibitory contaminations. Moreover, high concentrations of DGA favor side reactions, as can be seen from the increasing evolution of CO_2, determined from the difference of gas exchange in presence or absence of KOH. Therefore the data are not reliable enough for a detailed discussion.

Flavin and other dyes are able to sensitize the oxidation of Mn(II) to Mn(III), yet it is not likely that this is the initiating step we are looking for because the reaction requires the presence of electron donors like phenol (Andreae, 1955), or amines such as TETA (see above), or proteins. No Mn(III) could be detected after illumination of a plain buffered solution of Mn(II) and lumiflavin, and flavins do not sensitize a manganese-catalyzed oxidation of indoleacetic acid, although Mn(III) acts as catalyst during the oxidation of this substrate by peroxidase. We prefer to think, therefore, that the photooxidation of DGA starts not with an oxidation of Mn(II) but with an abstraction of an electron from the substrate molecule. Oxygen inhibits such electron-transfer reactions simply by quenching the primary excited state on a competing basis. Photoreactions involving electron transfer processes between dye and substrate are usually inhibited also by compounds which form charge-transfer complexes with the excited dye molecule (Homann and Gaffron, 1963, 1964; Radda and Calvin, 1963). However, our DGA photooxidation does not follow this simple rule. With DGA as substrate these same compounds accelerate the photooxidation. The most interesting

example for us is the stimulating effect of CMU which we have described and discussed earlier. The following section tells how the charge-transfer complex reacts with oxygen and thereby initiates the DGA oxidation.

Photodestruction of CMU by Flavin. An aerobic photodestruction of CMU by flavin was discovered by Sweetser (1963). Under our experimental conditions, using either manometry or spectrophotometry, this photooxidation remained unobservable. Yet we have been able to show in a roundabout way that Sweetser's reaction does happen to some extent. A solution of 4×10^{-4} M CMU and 5×10^{-5} M FMN or lumiflavin is illuminated for 15 minutes at 1.3 klx in the absence or presence of DGA; 0.5 ml of this solution is then added to a mixture in which the oxidation of DGA is catalyzed by peroxidase according to reaction type III. Addition of preilluminated samples causes an 80% inhibition in the reaction rates as compared with the rates found with unilluminated controls. The same effect can be achieved with a methanolic solution of the photoproduct of CMU and FMN isolated according to Sweetser. This preparation also inhibits slightly the flavin-sensitized photooxidation of DGA, which is stimulated by the unoxidized CMU. That CMU is changed into an inhibitor can also be seen by adding a second batch of DGA to a reaction mixture in which the photooxidation of some DGA has just gone to completion. We can expect, therefore, the rate of the flavin-sensitized photooxidation of DGA to decrease measurably with time if CMU is present, due to the accumulation of the inhibiting photoproduct. This could not be seen in our earlier experiments because a high Mn(II) concentration tends to mask the self-inhibition of the reaction. With low Mn(II) concentrations it is, however, easy to demonstrate the phenomenon (see Figure 8).

It seems that the interaction between oxygen and the CMU–flavin complex leads unavoidably to some oxidation of CMU also under the conditions of our experiments. Very likely radicals are formed initially. This would explain the appearance of high molecular products in strongly illuminated solutions of FMN and CMU (Sweetser, 1963). By reacting with either DGA or Mn(II), the radicals may induce the oxidation of DGA. Thus the photooxidative destruction of the CMU–flavin complex could be viewed as an integral part of the CMU effect. Our earlier concept implied that the active species is a reactive oxygen molecule which originates in contact with the excited flavin–CMU complex. Such a reactive oxygen molecule, however, can hardly be expected to be set free from an intermediate triple complex of flavin, CMU, and oxygen, which would more likely react within itself. Recently Foote and Wexler (1964) have revived an older theory of Kautsky's (1937; Kautsky and de Bruÿn, 1931), who suggested that in photooxidations singlet oxygen is formed by energy transfer from the triplet excited dye molecule. Thus if "reactive" (singlet) oxygen molecules are generated they may not arise from an intermediate oxygen sensitizer adduct ("moloxide").

According to Sweetser (1963), the inhibition of photosynthesis in green microorganisms is relieved when FMN is added to the illuminated suspension medium. The herbicide is destroyed by photooxidation and the photoproduct formed does not inhibit photosynthesis. On the other hand, CMU-poisoned cells can be exposed to light for hours while the inhibition persists. No significant photooxidation of CMU appears to occur inside the cells, hence no relation can be seen between the inhibitory action of CMU *in vivo* and of its photooxidation product *in vitro*.

We also tested the action of Sweetser's photoproduct on the DGA photooxidation by chloroplasts. No inhibition occurred with an amount which, on the other hand, inhibited the rate of the HRP-catalyzed reaction by 70%. The chloroplast reaction (type V), therefore, is affected by CMU and its photoproduct in the same way as photosynthesis, but not as the peroxidase-catalyzed reaction (type III). This may also indicate that the chloroplast-induced oxidation of manganese and consequently that of DGA is not helped much or at all by chloroplast peroxidase, as was once suggested by Kenten and Mann (1955), but aged or otherwise inactivated chloroplasts which no longer catalyze the DGA oxidations as described may, during a prolonged illumination, acquire this property again, even in the presence of large amounts of DCMU. This chloroplast reaction cannot yet be explained, although several hypotheses are possible on the basis of the data presented in this paper.

Discussion

The experiments reported in this paper are concerned with the specific role of manganous ions during the oxidation of DGA by molecular oxygen in neutral or slightly acid solutions. Oxalate, threonate, and hydrogen peroxide are formed. Manganous ions are required regardless whether the oxidation is initiated by light and a sensitizing dye, or by chemical means, or by peroxidase. Manganous ions are known to catalyze one-electron transfer reactions via a reversible oxidation of bivalent manganese to trivalent manganese (see Waters, 1961). Since the manganese-catalyzed dark autoxidation of DGA is a chain reaction (see Figure 2), manganous ions may act as intermediate one-electron carriers. This could be shown directly by inducing the oxidation of DGA with trivalent manganese. Yamazaki and Piette (1963) assigned a similar role to the Mn(II)–Mn(III) pair in the aerobic oxidation of dihydroxyfumarate and other substrates by peroxidase. We found that DGA is another of those substrates which are attacked by a combination of Mn and peroxidase. Moreover, there seems to be a close relationship between the mechanisms of the various manganese-catalyzed oxidations here described.

For the manganese catalysis of oxidations by peroxidase, Yamazaki and Piette (1963) assume a chain reaction propagated by the rather unstable hydrogen peroxy radical $HO_2\cdot$. This would parallel the well-proven role of substrate peroxy radicals $RO_2\cdot$ as chain carriers in many other autoxidation processes. The $HO_2\cdot$ radicals are rapidly removed by dismutation

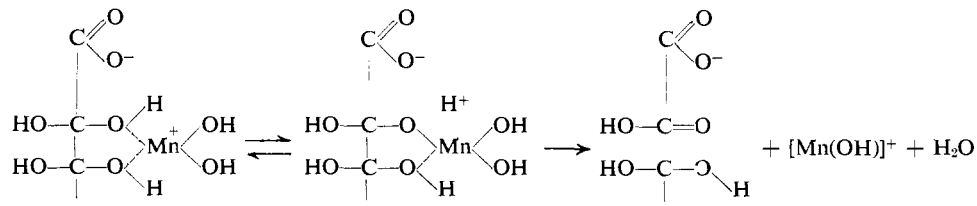

FIGURE 9: Possible mechanism of the oxidation of 2,3-diketogulonate by trivalent manganese.

(Yamazaki and Piette, 1963) or upon collision with the walls of the reaction vessel (Cooper and Melville, 1951). In the presence of sufficient manganous ions, however, the chain-propagating capacity of the $HO_2\cdot$ radicals may be preserved by the formation of trivalent manganese (Yamazaki and Piette, 1963):

$$Mn^{2+} + HO_2\cdot + H^+ \rightarrow Mn^{3+} + H_2O_2 \qquad (f)$$

There is the difficulty, however, that in solutions with a *p*H higher than 3 the $HO_2\cdot$ radical is dissociated and therefore present as the peroxy anion $O_2^{\cdot-}$ (Baxendale *et al.*, 1951). A direct electron transfer to $O_2^{\cdot-}$ is not possible, because the free divalent anion O_2^{2-} does not exist (George and Griffith, 1959). The peroxy anion, therefore, is an electron donor rather than an electron acceptor (Uri, 1952). Considering that manganous salts are at least partially hydrolyzed in neutral aqueous solutions, a hydrogen transfer reaction may occur between $O_2^{\cdot-}$ and hydroxylated manganous ions $[Mn(OH)]^+$. It is very likely that the electron-transfer processes between $O_2^{\cdot-}$ and $[Mn(OH)]^+$ and subsequently between Mn(III) and DGA happen within one complex. In this complex the OH groups of the hydrated α,β-dicarbonyl moiety of DGA would have to provide the site for a coordination binding of the manganese. Free Mn^{3+} ions are not likely to play any role in our reaction because they are stable only in highly acid solutions. The reaction complex may be similar to that believed to be formed between pinacol and Mn(III) in the oxidation of pinacol by the pyrophosphate complex of Mn(III) (Drummond and Waters, 1953). Figure 9 shows a possible description of the reaction sequence.

With these modifications of Yamazaki and Piette's reaction mechanism we are able to explain how the trivalent manganese, once it has been formed, escapes reduction by $O_2^{\cdot-}$, although this reaction is highly exergonic (Uri, 1961). The potential of the couple Mn(II)–Mn(III) lies between the potentials of $O_2^{\cdot-}$–O_2 and of $HO_2^{\cdot}$–$[O_2^{\cdot-} + H^+]$. Thus both the oxidation of Mn(II) as well as the reduction of Mn(III) by $O_2^{\cdot-}$ are theoretically possible. In our reaction the oxidation of Mn(II) by $O_2^{\cdot-}$ and the reduction of Mn(III) by DGA must be dominant over the reduction of Mn(III) by $O_2^{\cdot-}$. This may be a result of the low steady-state concentration of Mn(III) and of $O_2^{\cdot-}$ and of the enhanced reactivity of the trivalent manganese in the reaction complex with DGA. The surprising specificity of manganese therefore appears to be the result of the efficiency of the Mn(II)–Mn(III) couple as a one-electron carrier in reactions involving peroxy radicals and of the favorable structure of DGA for complexing with manganese, which facilitates the transfer of an electron from DGA to Mn(III).

Attempts to confirm this hypothesis more directly by kinetic measurements were not successful because of the instability of DGA. For example, in neutral solutions of DGA there occurs a slow formation of a compound with an absorption maximum at λ 295 mμ (Euler and Eistert, 1957) which, in the *absence* of manganous ions, is photooxidized by lumiflavin and destroyed by peroxidation with peroxidase. That compound will certainly interfere with the reaction chain in the oxidation of DGA. In addition, as described above, our solutions of DGA are not free from oxidation-inhibiting contaminations.

The strongly retarding action of Cu^{2+} in oxidations of DGA probably involves an intermediate reduction to cuprous ions, which are very strong reductants and are likely to cut the reaction chain short. Hydroquinone and ascorbate probably interfere with the radical chain in the DGA oxidation because they so easily form electron-deficient intermediates such as quinhydrone or monodehydroascorbate (the latter in the form of a monodehydroascorbic-ascorbate dimer (Levandoski *et al.*, 1964)).

The reaction rate of the linear phase of the photooxidation is proportional to the incident light intensity (Figure 7). This can be discussed on the basis of the general set of equations for the description of autoxidation processes as reviewed by Waters (1961). If Mn(III) is included as intermediate electron donor, the concentration of Mn(III), like that of the radicals SH· and $O_2^{\cdot-}$ [see equations (a) through (d)], would be constant under steady-state conditions. If by the action of a light quantum only one reaction-promoting radical were formed and if the chain were predominantly terminated by the dismutation of $O_2^{\cdot-}$ or the reduction of Mn(III) by $O_2^{\cdot-}$, we would expect the rate to be proportional to the square root of the light intensity. This is not the case. Therefore either two chain-promoting radicals are formed per quantum, or the prevalent chain termination involves only one of the chain-promoting intermediates. These questions cannot be answered because of the complication arising from unknown side reactions in our system.

In order to give a convincing description of every single step in our system, we will have to look for a compound less labile than DGA, yet with the same manganese specific reactivity toward oxygen in spon-

taneous or induced oxidations. A complete understanding of the surprisingly specific action of manganese in a reaction which can be brought about with light and a sensitizing dye, or with an enzyme like peroxidase, or by purely chemical means, may also help us in attempts to elucidate specific actions of manganese in the living cell.

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