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Glycolate Formation in Intact Spinach Chloroplasts[1]

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ABSTRACT

Photosynthetic $^{14}CO_2$ fixation and the accumulation of photosynthetic products and the response of each process to both 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) and ascorbate were investigated in the intact spinach chloroplast.

Ascorbate increased the rate of CO_2 uptake with an increase in all photosynthetic products, but, proportionally, there was a much larger increase in glycolate formation. CO_2 fixation inhibited by DCMU was partially restored on addition of ascorbate. Under conditions not optimal for glycolate formation, such as saturating levels of CO_2 and an anaerobic atmosphere, ascorbate in the presence of DCMU restored the formation of all photosynthetic products excluding glycolate. This effect of ascorbate on glycolate synthesis in the presence of DCMU was diminished under conditions which favored glycolate formation. Externally added glycerate 3-phosphate and fructose 1,6-diphosphate depressed the appearance of radioactivity in glycolate.

The data are interpreted to indicate that glycolate is produced during photosynthesis as a result of a reaction between a 2-carbon piece derived from a sugar phosphate and an oxidant generated by the photochemical act. The oxidant may be an intermediate of photosystem 2 or a peroxide generated by a mechanism of the Mehler type involving molecular oxygen.

Glycolate has been observed by many investigators as a product of photosynthesis of algae and higher plants (2, 3) and isolated chloroplasts (5, 9, 20). This production was enhanced by high partial pressures of oxygen, high light intensities, and low concentrations of CO_2 (7, 8, 21). The immediate precursor of glycolate may be α,β-dihydroxyethylthiamine pyrophosphate (transketolase addition complex) derived from fructose-6-P (6) or xylulose-5-P (10) or possibly a 2-carbon piece split off from ribulose-1,5-diP (2) or fructose-1,6-diP yielding phosphoglycolate and subsequently the free acid (15).

It is generally accepted that a considerable fraction of carbon assimilated during photosynthesis may be metabolized via glycolate. A pathway of glycolate to glycine and serine has been demonstrated (14). A key enzyme of this pathway is glycolic acid oxidase, and therefore inhibition of the oxidase was found to be accompanied by a striking accumulation of glycolate (22). The chloroplast, however, has a limited capacity for metabolizing glycolate (18). The site of glycolate disposal in the cell has been discussed recently by Tolbert *et al.* (19) and the pivotal organelle appears to be a peroxisomal-type particle. Thus, glycolate may be regarded as an end product of the isolated chloroplast, and therefore the organelle is convenient for studying its formation during photosynthesis.

Photosynthesis may be inhibited by DCMU,[3] which is thought to block electron transport just after photosystem 2. However, CO_2 fixation in intact spinach chloroplasts inhibited by a similar herbicide is partly relieved by the addition of ascorbate (1). This finding indicates that a site of electron donation to the photosynthetic electron transport chain is between the two photosystems and prior to a phosphorylation coupling site. A similar conclusion was reached from evidence with fragmented chloroplasts preparations (12). There is evidence that glycolate synthesis is dependent on the light steps of photosynthesis (7). In this investigation the effect of ascorbate and DCMU on the incorporation of carbon-14 into products of photosynthesis and primarily into glycolate was studied in intact spinach chloroplasts. In this way, the involvement of the two photosystems in glycolate formation during photosynthesis could be evaluated.

MATERIALS AND METHODS

Spinach plants were harvested either from the field and stored up to 3 days in a refrigerator or from a growth chamber in which plants were grown at 20 C and in a photoperiod of 10 hr of light. Leaves were floated on tap water at 20 C in sunlight for 1 hr before use. This usually enhanced the rate of photosynthesis and shortened the "induction" phase (1).

The isolation of chloroplasts was based on the method described by Jensen and Bassham (11). A 10-g sample of deribbed and cut leaves was blended for 5 sec in a buffer solution containing 50 mM MES adjusted to pH 6.1 with NaOH and 20 mM NaCl. The homogenate was filtered through muslin and centrifuged at 2500*g* for 45 sec. The pellet was resuspended in 1 to 2 ml of buffer solution containing 50 mM HEPES, adjusted to pH 6.7 with NaOH and 20 mM NaCl. Photosynthesis was initiated by adding aliquots of this suspension to the complete reaction mixture containing 50 mM tricine, adjusted with NaOH to pH 8.1, 2.5 mM $Na_4P_2O_7$, and the appropriate concentration of $NaH^{14}CO_3$. The final chlorophyll concentration in a reaction mixture of 2 ml was 60 to 100 μg. When ascorbate was used, a freshly prepared solution of sodium ascorbate was added to a final concentration of 10 mM. Each of the three buffer solutions (MES, HEPES, tricine) contained 0.33 M sorbitol, 2 mM $NaNO_3$, 2 mM EDTA (dipotassium), 1 mM $MnCl_2$, 1 mM $MgCl_2$, and 0.5 mM K_2HPO_4.

The appropriate gas mixtures were slowly passed through the reaction mixture before and after the addition of the chloroplasts.

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[3] Abbreviations: MES: 2-(*N*-morpholino)ethanesulfonic acid; HEPES: *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; tricine: *N*-tris(hydroxymethyl)methyl glycine; DCMU: 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

The experiments were carried out at 20 C in tubes illuminated from opposite sides with Sylvania 150-w flood lamps which gave an intensity of roughly 4000 ft-c at the point of incubation. The reaction was terminated by adding 0.02 ml of concentrated formic acid. Aliquots were pipetted onto planchets, dried, and counted with a thin window automatic gas flow counter. The CO_2 fixation rates were estimated from samples removed from the reaction mixture.

For separation of the photosynthetic products, a 0.1-ml aliquot of the reaction mixture was spotted onto Whatman No. 1 paper. The CO_2 fixation products were analyzed by one- or two-dimensional descending paper chromatography with the following solvents: (*a*) a mixture of 420 ml of phenol, 80 ml of water, and 0.5 ml of 1 M EDTA was used for one-dimensional chromatograms or for one direction in the two-dimensional chromatograms; (*b*) a mixture of 190 ml of 1-butanol, 90 ml of propionic acid, and 125 ml of water was used for the second direction. Chromatograms were sprayed with a solution of 1 M $NaHCO_3$ to minimize glycolate distillation from the paper. The radioactive spots were located by means of x-ray film and then counted directly on the paper with a thin window gas counter.

RESULTS

Effect of Ascorbate and Varying Concentrations of DCMU on Photosynthetic Products. Both a stimulation of CO_2 fixation by ascorbate and an inhibition by 0.25 μM DCMU can be seen from our experiments with intact spinach chloroplasts (Fig. 1) In this experiment the fixation rate was linear following a lag period of about 2 min. During the lag period the fixation rate was doubled when 10 mM ascorbate was added, and during the steady state phase of fixation the stimulation was even greater. The inhibition of about 50% by 0.25 μM DCMU was unchanged throughout the experimental period. When 10 mM ascorbate was added to a preparation photosynthesizing in the presence of 0.05 μM DCMU in place of 0.25 μM DCMU, CO_2 fixation was restored to the control level (Table I). In the control reaction mixtures or in those in which ascorbate alone was included, the major photosynthetic products were glycerate-3-P, triose phosphates (mainly dihydroxyacetone-P), glycolate, and a water-insoluble fraction, a polyglucan. Lesser amounts of isotope were found in the sugar monophosphates (glucose, fructose, ribose) and in the sugar diphosphates (ribulose, fructose).

Ascorbate increased the accumulation rate of most products by a factor of 2 to 3 after 10 min of photosynthesis (Fig. 2). In this experiment, carried out under N_2 and with a saturating concentration[4] of $NaHCO_3$, the effect on glycolate synthesis was striking. In spite of a lag of 2 min the rate of glycolate accumulation was stimulated with time up to a 7-fold increase after 10 min. This increase was mainly at the expense of triose and sugar phosphates. DCMU inhibited the accumulation of all products. When, however, the CO_2 fixation rate was relieved by the addition of 10 mM ascorbate, as shown in Figure 2, glycolate in contrast to the other products failed to accumulate.

When conditions were more favorable for glycolate formation, such as a rate-limiting (0.1 mM) concentration of $NaHCO_3$, the effect of ascorbate became more substantial (Table II). Glycolate formation was stimulated appreciably while all other products were increased only slightly and glycerate-3-P even decreased. The increase in glycolate on a relative basis is compensated for by a decrease in most products but again mostly at the expense of triose phosphates. In contrast to the experiment

[4] In our preparations, the fixation rate became maximal with a bicarbonate concentration of about 2 mM. A value of 3 mM bicarbonate was recorded by Jensen and Bassham (11). The bicarbonate level for half-maximal rate is roughly 0.8 mM.

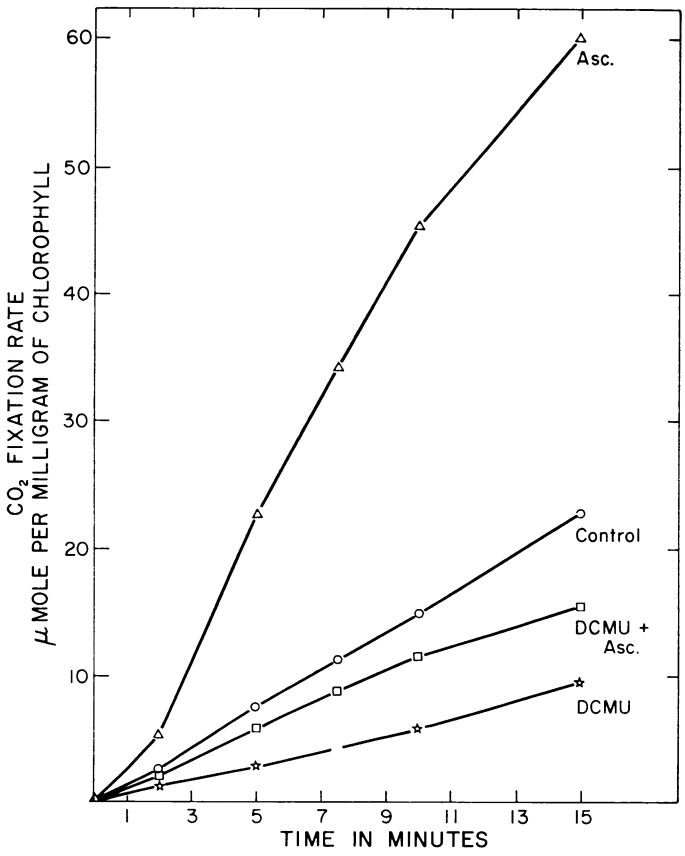

FIG. 1. Effect of ascorbate (Asc.) and DCMU at a saturating level of $NaHCO_3$ and under N_2 on CO_2 fixation of spinach chloroplasts. CO_2 fixation was carried out in 2-ml reaction mixtures at 20 C and 4000 ft-c. The reaction mixture contained 50 mM tricine adjusted with NaOH to pH 8.1, 0.33 M sorbitol, 2 mM $NaNO_3$, 2 mM EDTA (dipotassium salt), 1 mM $MnCl_2$, 1 mM $MgCl_2$, 0.5 mM K_2HPO_4, 2.5 mM $Na_4P_2O_7$, 2 mM $NaH^{14}CO_3$ (specific radioactivity 5 μc/μmole), and 100 μg of chlorophyll. Where indicated, 10 mM ascorbate or 0.25 μM DCMU or both were added.

Table I. *Effect of Ascorbate at a Saturating Level of $NaHCO_3$ and under N_2 on Fixation Rates and Photosynthetic Products*

Chloroplast isolation and experimental conditions as outlined in Figure 1 with the exception that the DCMU concentration was only 0.05 μM. Products were sampled after 6 min of photosynthesis. The data represent the radioactivity detected on the paper chromatogram.

Experimental Conditions	CO_2 Fixation Rate	Radioactivity in Photosynthetic Products					
		Glycerate-3-P	Triose-P	Sugar monophosphates	Sugar diphosphates	Glycolate	Insolubles
	μmoles/mg chlorophyll·hr	*cpm/0.1 ml reaction mixture*					
Control	38	950	1540	90	170	40	790
Ascorbate, 10 mM	62	3580	2370	600	330	330	2720
DCMU, 0.05 μM	29	440	720	140	170	0	390
Ascorbate, 10 mM, + DCMU, 0.05 μM	64	2490	2980	770	410	510	2500

in which the reaction mixture was saturated with respect to bicarbonate (see Fig. 2), the addition of ascorbate to this DCMU-inhibited preparation increased glycolate synthesis 10-fold.

When more extreme conditions were established favoring glycolate synthesis, namely a rate-limiting concentration of bicarbonate and air in place of N_2, the effect of ascorbate and DCMU on glycolate formation was diminished (Table III). The photosynthetic fixation rates were very low under these conditions (see also Reference 9). In the control, about two-thirds of the isotope fixed in photosynthesis was already found in glycolate. Nonetheless, ascorbate increased the total formation of glycolate, but its amount relative to the total isotope assimilated was unchanged. In DCMU-treated chloroplasts, both in the absence and in the presence of ascorbate, a large part of the glycolate-forming capacity was still maintained. The enhancement of glycolate synthesis under low bicarbonate and air, even without ascorbate, was also at the expense of triose and sugar phosphates. Of the total isotope assimilated, the amounts of radioactivity located in the triose and sugar phosphates were decreased from about 50 and 20% at high bicarbonate and N_2 to 23 and 4%, respectively, under rate-limiting bicarbonate and air.

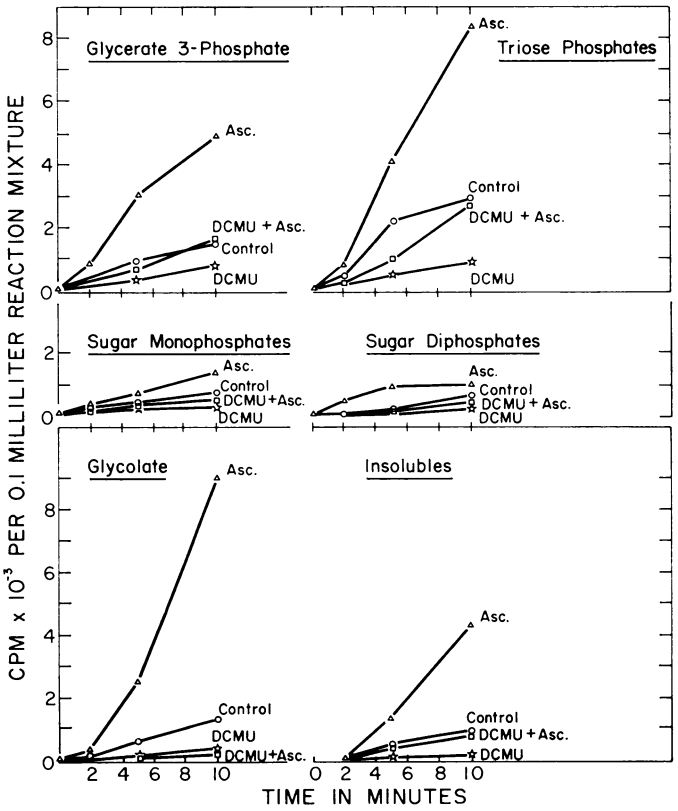

FIG. 2. Effect of ascorbate (Asc.) and DCMU at a saturating level of $NaHCO_3$ and under N_2 on photosynthetic products. Chloroplast isolation and experimental conditions as outlined in Figure 1 with the exceptions that the 2-ml reaction mixture contained 90 μg of chlorophyll and 2 mM $NaH^{14}CO_3$ with a specific radioactivity 60 μc/μmole. Where indicated, 10 mM ascorbate or 0.25 μM DCMU or both were added. An aliquot (0.1 ml) of the reaction mixture was analyzed by paper chromatography. The ordinate represents the radioactivity detected in the compounds on the paper. Insolubles (H_2O-insoluble fraction) represents a polyglucan since after acid hydrolysis (1 N HCl) over 90% of the radioactivity was located in glucose. Triose phosphates are essentially dihydroxyacetone-P; sugar monophosphates are the monophosphates of fructose, glucose, and ribose; sugar diphosphates are the diphosphates of fructose and ribulose.

Effect of Fructose-1,6-diP and Glycerate-3-P on Glycolate Formation. Finally, experiments were conducted in order to gain further information about the relation between glycolate formation and intermediates of the photosynthetic carbon reduction cycle. In the first experiment, unlabeled glycerate-3-P was added to the reaction mixture, and fructose-1,6-diP in the second, before the light was turned on (Table IV). Glycerate-3-P strongly depressed the appearance of isotope in glycolate both in the control and in the ascorbate-treated chloroplasts. In spite of a lower rate of glycolate synthesis in the fructose-1,6-diP experiment, a similar effect was observed when the hexose diphosphate was added.

DISCUSSION

The present study indicates that the oxidation of a 2-carbon moiety derived from the photosynthetic carbon reduction cycle to glycolate is associated with the photochemical act of photosynthesis. Our data suggest two sites for oxidant formation in the electron transport chain at the regions indicated in Figure 3.

α,β-Dihydroxyethylthiamine pyrophosphate, the intermediate in the transketolase-catalyzed reaction, can be converted to glycolate with ferricyanide (6) or 2,6-dichlorophenolindophenol (10) as oxidant. To account for an inhibition of photosynthesis at low concentrations of CO_2 by high partial pressures of oxygen due to the drainage of carbon in the form of glycolate from the photosynthetic carbon reduction cycle, Coombs and Whittingham (7) have proposed a mechanism envisaging an oxidation of the 2-carbon compound by an oxidant, probably hydrogen peroxide. Under these conditions, the ratio of oxidized pyridine nucleotide to reduced pyridine nucleotide would be small, and reduced ferredoxin would accumulate and then react with molecular oxygen to yield hydrogen peroxide. That reduced ferredoxin (4, 17) or ferredoxin-TPN reductase (Shain and Gibbs, unpub-

Table II. *Effect of Ascorbate and DCMU at a Limiting Level of $NaHCO_3$ and under N_2 on Fixation Rates and Photosynthetic Products*

Chloroplast isolation and experimental conditions as outlined in Figure 1 except that the concentration of $NaH^{14}CO_3$ was reduced to 0.1 mM and the chlorophyll concentration was 68 μg. Products were sampled after 20 min of photosynthesis.

Experimental Conditions	CO_2 Fixation Rate	Radioactivity in Products					
		Glycerate-3-P	Triose-P	Sugar monophosphates	Sugar diphosphates	Glycolate	Insolubles
	μmoles/mg chlorophyll·hr	*cpm/0.1 ml reaction mixture*					
Control	20	1810	5940	290	440	670	430
Ascorbate, 10 mM	34	1120	6180	310	880	7930	400
DCMU, 0.25 μM	8	280	1680	90	270	110	180
Ascorbate, 10 mM + DCMU, 0.25 μM	30	1630	7380	500	1090	1010	880

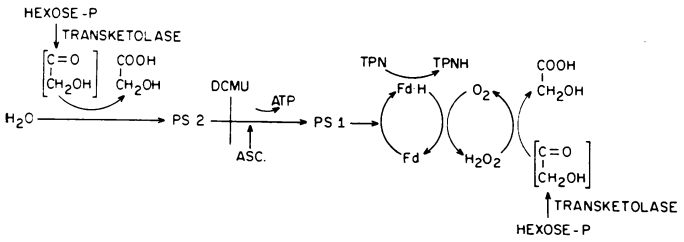

FIG. 3. Proposed mechanism for glycolate biosynthesis. The oxidation of $\begin{bmatrix} C=O \\ | \\ CH_2OH \end{bmatrix}$ the transketolase addition complex of α,β-dihydroxyethylthiamine pyrophosphate or possibly a phosphorylated 2-carbon fragment from ribulose-1,5-diP, to glycolate or phosphoglycolate is envisaged to occur either by donation of electrons between the photosystems or by a reaction involving hydrogen peroxide. The hydrogen peroxide may be formed either as the result of the oxidation by O_2 of reduced ferredoxin, ferredoxin-TPN reductase, or by photoxidation of ascorbate. Fd and FdH represent the oxidized and reduced forms of ferredoxin, respectively. A site of ATP formation is designated after the site of DCMU inhibition since ascorbate restores CO_2 fixation.

lished data) can react with molecular oxygen in the presence of reduced pyridine nucleotide has been demonstrated. The experimental findings recorded in Tables II and III are consistent with their proposal (7). However, to account for the experimental findings recorded in Figure 2, we suggest that under conditions of photosynthesis such as a saturating concentration of bicarbonate and low partial pressures of oxygen, glycolate is produced as a result of a reaction between the 2-carbon moiety and an oxidant formed in photosystem 2. Clearly, both mechanisms could be effective simultaneously.

Regardless of the experimental conditions, ascorbate stimulated the rate of photosynthesis and of glycolate formation. Ascorbate could increase the rate of photosynthesis either by electron donation to the photosynthetic electron transport chain or by undergoing a photoxidation of the Mehler type (13), resulting in the formation of hydrogen peroxide. By either mechanism dehydroascorbate would result. The normal redox potential (pH 7) of the ascorbate-dehydroascorbate couple is +60 mv, and the corresponding value of the system O_2/H_2O_2 is +270 mv. Therefore, the latter system should be the more effective in a direct oxidation of a 2-carbon compound. This could account for the higher accumulation of isotope in glycolate relative to the rate of photosynthesis when the partial pressure of oxygen was high (Table III). In low partial pressures of oxygen, peroxide formation would be limited, and the preferred oxidant would be

Table IV. *Effect of Externally Added Glycerate-3-P and Fructose-1,6-diP on Glycolate Formation in Absence or Presence of Ascorbate*

	2 min		5 min	
	Total	Glycolate	Total	Glycolate
	cpm/0.1 ml reaction mixture			
Experiment 1				
Control	1,250	100	4,390	560
Control + glycerate-3-P	1,730	0	3,940	140
Ascorbate	2,680	280	12,690	2,500
Ascorbate + glycerate-3-P	2,660	20	10,720	620
Experiment 2				
Control			8,040	240
Control + fructose-1,6-diP			5,670	60
Ascorbate			10,940	550
Ascorbate + fructose-1,6-diP			10,780	230

either dehydroascorbate or a substance proposed to be a component of photosystem 2. Dehydroascorbate was apparently most effective only under certain abnormal conditions such as CO_2 starvation and low O_2 (Table II) when there would be an increase in the size of the pools of the intermediates of the photosynthetic carbon reduction cycle and subsequently of the immediate precursor of glycolate (21).

A stimulation of the rate of glycolate formation could, in turn, affect photosynthesis. The isolated chloroplast in contrast to the intact cell has few metabolic sinks, *i.e.*, reactions which drain carbon from the photosynthetic reduction carbon cycle. That glycolate may be thought of as an end product of chloroplast metabolism similar to the insoluble polyglucan is indicated by our experiments. When the formation of glycolate was stimulated by ascorbate, its relative amount increased with time similarly to the insoluble fraction, while other products decreased (Fig. 2). A stimulation of glycolate formation by ascorbate may enhance the assimilation of carbon, perhaps by decreasing the level of a photosynthetic cycle intermediate such as sedoheptulose 7-P, an inhibitor of glyceraldehyde-3-P dehydrogenase (16).

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Table III. *Effect of Ascorbate and DCMU at a Limiting Level of $NaHCO_3$ and under Air on Fixation Rates and Photosynthetic Products*

Chloroplast isolation and experimental conditions as outlined in Figure 1 with the exception that the $NaH^{14}CO_3$ concentration was 0.1 mM, the chlorophyll concentration was 60 μg, and the atmosphere was air. Products were sampled after 20 min of photosynthesis.

Experimental Conditions	CO_2 Fixation Rate	Radioactivity in Photosynthetic Products					
		Glycerate-3-P	Triose-P	Sugar monophosphates	Sugar diphosphates	Glycolate	Insolubles
	μmoles/mg chlorophyll·hr	*cpm/0.1 ml reaction mixture*					
Control	3.5	1,200	2,810	110	370	8,000	210
Ascorbate, 10 mM	8.2	2,040	3,000	180	700	11,100	580
DCMU, 0.25 μM	1.3	770	1,510	40	210	1,980	180
Ascorbate, 10 mM + DCMU, 0.25 μM	1.9	1,720	2,650	140	480	4,870	480

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