Inhibition of Glycolipid Biosynthesis in Chloroplasts by Ozone and Sulfhydryl Reagents

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ABSTRACT

The metabolism of uridine 5'-pyrophosphate-galactose by spinach (Spinacia oleracea) chloroplast preparations was inhibited by ozone. The formation of digalactosyl diglyceride and trigalactosyl diglyceride was inhibited much more than the formation of monogalactosyl diglyceride, steryl glycoside, and acylated steryl glycoside. Essentially identical results were obtained when glycolipid synthesis was inhibited by N-ethyl maleimide, p-hydroxymercurobenzoate, and CdCl2. Iodoacetate and iodoacetamide affected neither the total incorporation of sugar from uridine 5'-pyrophosphate-galactose nor distribution of the incorporated sugar in the various glycolipids.

When these preparations were included during chloroplast preparations, it was also found that malonaldehyde was produced and glutathione was oxidized.

It was concluded that ozone inhibits glycolipid biosynthesis in chloroplast preparations by way of oxidation of enzyme sulfhydryl groups but that this reaction may be a secondary effect of oxidation of unsaturated fatty acids.

The synthesis of galactosyl diglycerides by cell-free preparations from leaves of higher plants was first reported by Neufeld and Hall (18). Their results have been substantiated and extended by subsequent work. This work has shown that in addition to the predominant naturally occurring monogalactosyl diglyceride and digalactosyl diglyceride, there is at least one additional compound, trigalactosyl diglyceride, which becomes labeled during the incubation of chloroplast preparations with 3H-UDP galactose (11, 17, 20).

The ratio of monogalactosyl diglyceride, digalactosyl diglyceride, and trigalactosyl diglyceride, synthesized by cell-free preparations, can be changed by variations in pH and temperature (17). Preparation of an acetone powder of chloroplasts tends to leave intact only the capability to synthesize MGDG

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MATERIALS AND METHODS

Chloroplasts. Chloroplasts were prepared from spinach (Spinacia oleracea) purchased at local markets. The leaves were washed in distilled water and the petioles and midribs were removed. The leaves (75 g) were ground in a Waring Blender (70 v, 5 sec) with an equal weight of ice-cold homogenizing medium (0.5 M sucrose, 10 mM phosphate, pH 7.5). The homogenate was filtered through cheesecloth and the filtrate was centrifuged at 200g for 2 min. The pellet was discarded and the supernatant was centrifuged at 1000g for 7 min. The supernatant was discarded and the pellet was resuspended in 0.1 M phosphate, pH 7.5. Chlorophyll concentration was determined by the method of Arnon (2), and the chloroplast suspension was diluted so that addition to the reaction mixtures would give 1 mg of chlorophyll per vessel.

Reaction Mixtures. Reaction mixtures for the study of glycolipid biosynthesis consisted of 100 µmoles of tris-Cl, pH 7.5, or phosphate, pH 7.5; 0.4 ml of the broken chloroplast suspension containing 1 mg of chlorophyll; and 1 µmole of UDP-3H-galactose (44,000 dpm) in a final volume of 1.60 ml. When inhibitors were used, they were preincubated with the chloroplast preparation for 5 min at 30°C. In all cases the reaction was started by the addition of UDP-3H-galactose (in 0.10 ml). Reaction mixtures were incubated for 1 hr at 30°C. The reaction was stopped and the lipid was extracted by the method of Bligh and Dyer (5). Aliquots of the chloroform solution were dried on planchets and counted in a thin window gas flow counter. The remainder of the lipid samples was chromatographed in one dimension with the acidic solvent of Nichols (19). The thin layer plates were exposed to x-ray film in order to determine an accurate outline of the radioactive spots. The radioactive areas were scraped into scintillation counter vials and counting solution was added.

Quenching was corrected for by the channels ratio method.

In the assay of radioactivity by planchet counting, the devia-
tion of the duplicate samples from the mean was ±2%. The deviations of the duplicate samples from the mean after thin layer chromatography in a typical experiment were: MGDG, ±3%; DGDG, ±6%; and TGDG, ±10%.

**Ozonization Experiments.** Ozone was generated by a silent electric discharge apparatus. Oxygen was passed through the apparatus at a flow rate of 20 ml/min and the high voltage was adjusted so that the ozone content was equivalent to 1 to 2 μmoles/min. Ozone concentration in the effluent was assayed by liberation of iodine from potassium iodide solution (15). Ozonization of chloroplast suspensions or of the model systems was done simply by bubbling the ozone from a capillary tip through the test solution. At the end of the experimental period 0.1 ml of 2 N HCl was added to the reaction mixtures, which were then extracted with two 2.5-ml portions of hexane. The aqueous phase was assayed for (a) malonaldehyde by the method of Wilbur et al. (30), with a molar extinction coefficient of 1.58 × 10⁴ (24); (b) sulfhydryl by the method of Ellman (10); and (c) peroxide by mixing a suitable aliquot with 0.1 ml of 2 N HCl and making the sample up to volume with 0.1 M KI. Liberation of iodine was calculated from the change in absorbance at 350 nm. In the cases of experiments with chloroplasts, an aliquot from the hexane phase was assayed for chlorophyll by the method of Arnon (2).

**Preparation of Egg Lecithin.** Egg lecithin was isolated from egg yolk as the cadmium chloride adduct. It was purified by column chromatography on silica gel, eluting with various percentages of methanol in chloroform, and the purity of the product was checked by thin layer chromatography.

**RESULTS**

**Inhibition of Glycolipid Synthesis by Ozone.** Treatment of chloroplast preparations with ozone caused inhibition of the ability to incorporate radioactivity from UDP-14C-galactose into glycolipids. In the experiment summarized in Table I, only galactolipids became radioactive, and it was clear that the inhibition of the synthesis of DGDG and TGDG was much greater, on a percentage basis, than the synthesis of MGDG. In other experiments preparations were obtained which labeled steryl glucoside and acylated glucoside as well as the galactolipids. In these cases also, the inhibition of DGDG and TGDG was greatest, while the synthesis of steryl glucoside and acylated steryl glucoside was relatively resistant to inhibition by ozone. This differential susceptibility to ozone suggested a comparison with enzyme inhibitors of known chemical reactivity in order to understand the chemistry of the ozone inhibition.

**Inhibition of Glycolipid Biosynthesis by Sulphydryl Reagents.** A number of sulphydryl reagents have been tested with respect to their effect on metabolism of UDP-galactose. Typical results for NEM are shown in Table II. The most obvious characteristic is the particular susceptibility of the syntheses of DGDG and TGDG. The syntheses of MGDG, SG, ASG, and X are affected by NEM but at higher concentrations than those required for effects on DGDG and TGDG.

Essentially similar data are shown in Table III for inhibition by CdCl₂. In this experiment the compound X was not detected by exposure to x-ray film and so the area was not scraped and counted. Inhibition by p-hydroxymercuribenzoate is also similar to that observed with NEM and CdCl₂. All three of these inhibitors simulate the effect of ozone.

Iodoacetate and iodoacetamide were also tested for inhibitory activity but showed no significant effect over the concentration range used (1 μM to 1 mM). Aliquots from these samples were chromatographed on thin layers of silica gel to check whether the radioactivity was redistributed among the

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**Table I. Inhibition of Galactolipid Synthesis by Ozone**

Spinach chloroplasts were prepared as described in "Materials and Methods." Two 7.0-ml volumes of chloroplast suspension were exposed to ozone and at 2-min intervals 0.6-ml aliquots (1 mg of chlorophyll) were taken for assay of UDP-galactose metabolism. Duplicates consisted of aliquots taken from the two 7-ml volumes of chloroplast suspension. Reaction mixtures for assay of galactolipid synthesis and procedures for analysis are described in "Materials and Methods." Data presented are averages of duplicate samples.

<table>
<thead>
<tr>
<th>O₃ Applied</th>
<th>Incorporation into:</th>
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<tr>
<td>μmoles</td>
<td>Total</td>
<td>MGDG</td>
<td>DGDG</td>
<td>TGDG</td>
</tr>
<tr>
<td></td>
<td>dpm</td>
<td>%</td>
<td>dpm</td>
<td>%</td>
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<td>10</td>
<td>6,920</td>
<td>41</td>
<td>6,040</td>
<td>60</td>
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**Table II. Inhibition of UDP-Galactose Synthesis by NEM**

Reaction mixtures consisted of 94 μmoles of tris-Cl, pH 7.5; 1 μmole of 14C-UDP-galactose (44,000 dpm); 0.4 ml of chloroplast preparation (1 mg of chlorophyll); and inhibitor in a final volume of 1.60 ml. The inhibitor was preincubated for 5 min with the chloroplast preparation at 30°C before starting the reaction. Samples were incubated for 1 hr at 30°C and then extracted by the method of Bligh and Dyer (4). An aliquot of the chloroform extract was dried on a planchet and counted in a thin window gas flow counter. The remainder of the sample was subjected to chromatography on thin layers of Silica Gel G (Merck Damstadt) with chloroform-methanol-acetic acid-water (170:25:25:60) as a solvent (19). The chromatogram was exposed to x-ray film and the film was developed to locate the radioactive compounds. Corresponding areas from the thin layer plate were scraped into vials, scintillation fluid was added, and the samples were counted in a scintillation counter. Quenching was corrected by the channels ratio method. Data presented are the averages of duplicate samples.

| Final Conc of NEM | Incorporation into: | | | |
| --- | --- | --- | --- | --- | --- | --- | --- |
| | Total | MGDG | DGDG | TGDG | ASG | SG | X |
| mM | dpm | % | dpm | % | dpm | % | dpm | % | dpm | % |
| 0 | 24,800 | 100 | 4,500 | 100 | 2,370 | 100 | 2,080 | 100 | 1,600 | 100 | 1,090 | 100 | 900 | 100 |
| 0.01 | 22,500 | 91 | 4,870 | 108 | 2,150 | 91 | 1,910 | 92 | 1,800 | 113 | 1,100 | 101 | 900 | 100 |
| 0.30 | 12,900 | 52 | 3,210 | 71 | 400 | 17 | 190 | 9 | 950 | 59 | 420 | 39 | 440 | 49 |
| 0.60 | 6,400 | 26 | 2,380 | 53 | 340 | 14 | 150 | 7 | 690 | 43 | 370 | 34 | 330 | 37 |
| 1.00 | 220 | 9 | 780 | 17 | 150 | 6 | 110 | 5 | 240 | 15 | 270 | 25 | 140 | 16 |

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labeled compounds, but it was found that at all concentrations of these two reagents the distribution of label was identical with the control.

**Reaction of Ozone with Lipids and Lipoproteins.** Although the action of sulfhydryl reagents in some cases simulates the action of ozone, causing inhibition of glycolipid synthesis, it is not clear that ozone reacts directly with sulfhydryl groups of the enzymes. It is possible that ozone reacts primarily with unsaturated compounds such as unsaturated fatty acids, and the products of this reaction may react further with the sulfhydryl compounds. Earlier experiments have shown that simultaneous exposure of linoleate and GSH to ozone causes the formation of malonaldehyde as well as the oxidation of the sulfhydryl groups, and the amount of malonaldehyde produced is practically the same either in the absence or the presence of GSH. These results show that linoleate reacts equally well with ozone whether GSH is present or not and that the oxidation of sulfhydryl in the presence of linoleate depends at least to some extent on the hydrogen peroxide produced when linoleate reacts with ozone (16). Formation of hydrogen peroxide when biological materials are treated with ozone has been previously noted by Baker and Wilson (3).

Reaction of ozone with free fatty acids clearly is not a suitable comparison with the physiological situation. In order to test a more suitable model, egg lecithin was prepared and exposed to ozone either in the absence or the presence of GSH. At the end of the reaction period, samples were taken for the analysis of malonaldehyde, peroxide, and sulfhydryl. The results are presented in Figure 1 and are comparable in many respects to the results obtained with free fatty acid. In the absence of GSH, malonaldehyde and peroxide are produced. In the presence of GSH, no peroxide is detectable and the amount of malonaldehyde produced is about 50% of the control. The latter observation contrasts with the results obtained with free fatty acid, where the presence of GSH did not markedly affect the production of malonaldehyde. We conclude from these results with egg lecithin that the hydrogen peroxide produced when unsaturated fatty acids react with ozone is partially responsible for the oxidation of GSH. The peroxide measured in the control vessels would not be sufficient to account for the sulfhydryl oxidation which is observed, since independent tests show that 1 mole of hydrogen peroxide reacts with 2 moles of GSH. The GSH which reacts may also be accounted for in this case by direct reaction with ozone or perhaps by reaction with some of the oxidation products described by Schauenstein (25).

This experience with egg lecithin was followed by reexamination of the reaction of ozone with a lipoprotein system, the chloroplast. The results of analysis of such an experiment are shown in Figure 2. The most notable difference in this experiment as compared with those where fatty acid or lecithin was exposed to ozone is that peroxide is not detectable under any conditions. This could be due to a number of circumstances: (a) peroxide is not formed, (b) the peroxide formed is decomposed enzymically, or (c) the peroxide formed reacts chemically with other components of the chloroplast system.

**Table III. Inhibition of UDP-Galactose Metabolism by CdCl₂**

Experimental procedure was as described for Table II.

<table>
<thead>
<tr>
<th>Final Concentration of CdCl₂</th>
<th>Incorporation into:</th>
</tr>
</thead>
<tbody>
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<td>Total</td>
</tr>
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<td>6,770</td>
</tr>
<tr>
<td>0.50</td>
<td>2,300</td>
</tr>
</tbody>
</table>

**Fig. 1. Reaction of ozone with egg lecithin.** Five micromoles of egg lecithin were dried from chloroform solution on the walls of a test tube. Five milliliters of 0.1 M phosphate buffer, pH 7.2, were added and the lecithin was suspended by agitation with a wrist action shaker. In one set of tubes no further additions were made; in a second set 20 μmoles of GSH were added. The reaction mixtures were exposed to varying amounts of ozone and were subsequently analyzed for malonaldehyde, GSH, and peroxide as described in "Materials and Methods."

**Fig. 2. Reaction of ozone with chloroplast lipids.** Chloroplasts were isolated from spinach leaves as described in "Materials and Methods." Chloroplast suspensions equivalent to 1 mg of chlorophyll were added to reaction mixtures (0.1 M with respect to phosphate buffer, pH 7.2) either in the presence or absence of 20 μmoles of GSH. Reaction mixtures were exposed to varying amounts of ozone and then assayed for malonaldehyde, GSH, and peroxide as described in "Materials and Methods."

**Removal of Ozone from the Gas Stream.** The products of reaction of ozone with components of chloroplasts can be measured directly and compared with the amounts of ozone introduced into the reaction vessel. But such experiments do not provide information concerning the amount of ozone which dissolved or reacted in the reaction vessel. Experiments...
tube was two vessels 5.0 ml and gas at 5.0 m. The Notable points shown in Figure 3 are that the chloroplast suspension efficiently traps ozone, whereas the lecithin suspension traps relatively little.

The low reactivity of the lecithin may be a consequence of the formation of micelles or laminar sheets in the aqueous suspension. In this form, the fatty acyl groups would be associated in the center of the lipid structure and the surface of the structure would be composed of the hydrophilic phosphoryl choline moiety which may prevent to some extent the access of the ozone to the fatty acyl groups. If this were the case, ozone should react better with lecithin if the formation of micelles or laminar sheets is prevented. Such a situation exists when lecithin is dissolved in a nonpolar solvent. Experiments such as that presented in Figure 4 illustrate the fact that the reaction of ozone with lecithin is much greater in hexane than in aqueous media, and this may be a reflection of the amount of micelles in the two media. It is also noticeable that, when lecithin is suspended in aqueous medium, the amount of ozone absorbed from the gas stream is independent of the amount of lecithin used to make the suspension. But when the lecithin is dissolved in hexane, the amount of ozone absorbed is proportional to the amount of lecithin dissolved.

**DISCUSSION**

It has been reported previously that sulfhydryl reagents inhibit galactolipid synthesis by spinach chloroplasts (6). Our results are in agreement with these previous results, but we have extended the analysis of the experiment and discovered differential inhibition of the synthesis of the various products. It is clear that the synthases of DGDG and TGDG are more susceptible to inhibition both by sulfhydryl reagents and ozone. Exposure of plants to concentrations of ozone in the range observed in polluted atmospheres causes an increase in the amount of steryl glycoside (H. Tomlinson and S. Rich, personal communication). Our results are consistent with this observation to the extent that sulfhydryl reagents slightly increase the labeling of SG and ASG. In this case the effect of ozone may be the inhibition of one pathway of utilization of nucleotide sugar, making available substrate for alternative reactions, that is, synthesis of steryl glycosides.

We have already reported that ozone reacts with a number of amino acids and proteins (15). The sulfhydryl group was particularly susceptible to oxidation and was therefore suggested as a possible primary point of attack of ozone on biological systems. It is apparent from the studies in this paper that even in the presence of sulfhydryl compounds, ozone oxidized unsaturated fatty acids of the chloroplast. It is probable that the hydrogen peroxide produced in the oxidation of the fatty acids is in turn responsible for the oxidation of sulfhydryl groups.

The results in this paper may have some bearing on the mechanism of toxicity of ozone. The questions are (a) whether the ozone reacts with the plasmalemma or penetrates into the cytosol and then to the subcellular components and (b), regardless of the site of action, whether the primary effect of ozone is on protein or on lipid components. Thomson et al. (27) have shown that the earliest damage by ozone perceivable with the electron microscope is in the chloroplast, but it is easily conceivable that this effect has been preceded by a number of chemical events which are not detectable by the electron microscope. For example, the antibiotic filipin can cause great changes in the permeability of red beet discs without causing changes detectable by the electron microscope (14). It may be
argued that if ozone passes through the plasmalemma it should come in contact with unsaturated fatty acids and react with them. However, oxygen passes through these membranes and yet gives rise to little detectable autoxidation. The construction of the biological membrane may prevent the oxidation of unsaturated fatty acids. Although serious questions have been raised concerning the Danielli and Davson concept of the biological membrane (4, 9), recent studies reiterate the importance of the lipid bilayer in the construction of the membrane (26, 29). Even so, the latter studies with Mycoplasma laidlawii need not be conclusive in consideration of the structure of the chloroplast membranes. The studies in this paper show that the bilayer configuration of lecithin does not readily react with ozone (Fig. 4); in fact, the reaction which is observed may be of monomers of lecithin which are exchangeable with the bilayer or even of fatty acids which have been released by hydrolysis. If ozone were to react with the unsaturated lipids of the plasmalemma, the products would be glycerolipids with relatively short chain aldehydic substituents. It has been shown that lecithins with short chain acyl substituents are very lytic (22). This potential lytic activity would be compounded by toxic effects of other products such as hydrogen peroxide (21) and malonaldehyde (7, 13). Resistant varieties of plants may have the capability to detoxify these products.

There have been previous reports of formation of malonaldehyde as a result of exposing biological materials to ozone (1, 12). Goldstein et al. (12) have referred to this process as lipid peroxidation. It should be pointed out that the formation of malonaldehyde need not be by the same process in ozonation and in peroxidation (autoxidation). Autoxidation of linoleate does not produce malonaldehyde (8), whereas ozonation of the same acid does (16). This is an indication that there is direct attack of ozone on the double bond system. In addition, autoxidation reactions may be initiated. Although reactions of ozone both with proteins and with unsaturated lipids have now been observed, it is still too early to decide whether the symptoms of toxicity can be attributed to either or both of these reactions.

LITERATURE CITED