The use of galactose oxidase in lipid labeling

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Summary Galactose oxidase can be used to oxidize the terminal carbon atom of lipids containing galactose or N-acetylgalactosamine, and the resultant aldehyde group can be reduced back to the original carbinol with radioactive borohydride. The efficiency of the first reaction has been investigated systematically by using [6-^{3}H]galactosyl ceramide as substrate and measuring the amount of radioactive water formed. This enabled us to establish that the addition of catalase *and* peroxidase greatly speeded the oxidation, that phosphate and PIPES buffers were the best among those tested, that the reaction continued for 24 hr without a second addition of galactose oxidase, and that the optimum concentration of organic solvent (tetrahydrofuran) was 50%. The suggestion is made that a similar set of

Abbreviations: GO, galactose oxidase; PIPES, piperazine-N,N-bis(2-ethanesulfonic acid); THF, tetrahydrofuran.

variables be studied for each lipid or nonlipid by the same basic technique: labeling by the oxidase/borohydride method and use of the resultant compound as substrate.—**Radin, N. S., and G. P. Evangelatos.** The use of galactose oxidase in lipid labeling. *J. Lipid Res.* 1981. **22:** 536–541.

Supplementary key words peroxidase · catalase

The use of galactose oxidase (EC 1.1.3.9) to oxidize lipids containing galactose or galactosamine, followed by reduction with [^{3}H]borohydride, has been described for several glycolipids (1–8). Claims have been made for improved yields of aldehyde lipids by the addition of catalase plus peroxidase (5), taurodeoxycholate (4), or nonionic detergents (7). Catalase and peroxidase were added because of the statement (9) that hydrogen peroxide, the product of reaction between GO, oxygen, and its substrates, is an inhibitor of the enzyme. None of the publications included quantitative comparative data to indicate the magnitude of the claimed effects. In view of the frequent use of the approach to prepare labeled substrates (lipoidal and non-lipoidal) and to study the surface of membranes (10, 11), it would seem useful to examine some of the variables in a systematic way.

In most of the previous studies, the extent of oxidation was evaluated only indirectly, by determining the yield of radioactive lipid. Since the labeling step generally involved the incomplete reduction of the aldehyde with borotritide, followed by further reduction with unlabeled borohydride to destroy unreduced aldehyde, the comparisons were rather approximate. In one study (7), the amount of oxidation was measured by determining the amount of hydrogen peroxide present at the end of the incubation. This approach assumed that no catalase or peroxide-consuming material was present, although some catalase seems to contaminate GO preparations. While it is possible to determine the amount of lipid aldehyde directly by derivatization and high performance liquid chromatography (11), the procedure is complex. It is also possible to determine the amount of hydrogen peroxide formed by means of a coupled enzyme reaction, using a chromogen and peroxidase or catalase (12), but the stability of the peroxide and the effectiveness of the second enzyme could depend greatly on the medium used for the initial oxidation and thus give misleading results. Some of the above factors also limit the use of oxygen uptake measurements as an assay. We chose the use of a labeled substrate, [6-^{3}H]galactosyl ceramide, made by the GO/borohydride method, and measured the radioactivity in the water (or hydrogen peroxide) produced by the GO.

MATERIALS AND METHODS

Materials

Sigma Chemical Co. was the source of galactose oxidase (Type V) from *Dactylium dendroides* (sp act 210 o-tolidine units/mg protein); catalase from beef liver, 36,000 units/mg protein; and peroxidase (Type II) from horseradish, 150 purpurogallin units/mg. Galactocerebroside from pig brain, a mixture of the nonhydroxy- and hydroxy-types, was prepared in this laboratory (13).

The cerebroside was labeled by a modification of the method described before (2), in which 11 mg of lipid were stirred gently overnight with 70 units of GO and 50 units of peroxidase in 11 ml each of THF and of 10 mM PIPES-Na buffer, pH 7.0. The THF was freshly distilled from KOH pellets. Thin-layer chromatography with silica gel showed that the oxidation was complete. Reduction with 2.5 mCi of KBH_4 (sp act 262 Ci/mol), followed by column chromatography, yielded 8 mg of lipid, sp act 54,000 cpm/nmol; this represented a 25% yield of the activity in the borohydride.

Acidic methanolysis with HCl–methanol–water (14), together with carrier, followed by extraction of the fatty acid esters and the sphingoid bases, showed that the sugar moiety contained about 89% of the tritium, the fatty acids contained 3.6%, and the bases contained 1.8%. The tritium in the 6-position of the sugar is presumably symmetrically distributed, so 100% oxidation of the substrate should yield 44.5% of its radioactivity in the form of water or H_2O_2.

Assay procedure

Portions of a solution of labeled cerebroside were evaporated to dryness in 13 × 100 mm borosilicate culture tubes, then dissolved in 250 μl of THF (freshly distilled from KOH). To this was added 125 μl of 0.1 M aqueous buffer, pH 7.0, and 125 μl of aqueous GO, 5 units. Substances to be tested were included in the GO solution. Care was taken to mix the buffer with the THF solution before adding the enzymes, which were mixed promptly after pipetting to minimize the exposure of the enzymes to the relatively concentrated organic solvent.

The tubes, prepared in duplicate, were closed with three layers of Parafilm and incubated at room temperature with gentle shaking. At the end of the incubation period, 1.5 ml of water–THF 1:1 and 4 ml of chloroform were added to partition the enzymatically generated radioactive water into the upper layer. After vortexing and centrifuging, a portion

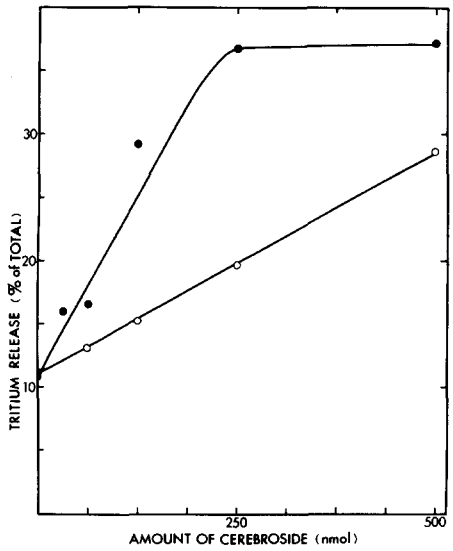

Fig. 1. Relationship between cerebroside concentration and fraction of substrate oxidized. The lipid was oxidized in phosphate buffer (upper curve) or PIPES buffer (lower curve), using 5 units of galactose oxidase and 20 units of peroxidase in water–tetrahydrofuran 1:1. Incubation was for 3 hr at room temperature.

(450 μl) was removed from the upper layer with a 0.5 ml gas-tight syringe and mixed with 10 ml of a scintillation fluid containing xylene and Triton X-100 (15). The observed activity was corrected by dividing it by the volume of the aliquot counted since the volume of the upper layer is very close to 1 ml. Because the chloroform layer is quite radioactive, it is important to centrifuge long enough (at least 10 min at 600 g_{av}) to clear the upper layer completely.

Each incubation tube contained 54,000 cpm of substrate and the blanks (no enzyme) were approximately 0.7% of this, after correcting for aliquot size.

RESULTS

Effect of adding peroxidase

Using a very small amount of substrate in phosphate or PIPES buffer and a 3-hr incubation, we found that 20 units of peroxidase produced a tripling in activity in PIPES buffer and a 170% increase in activity with phosphate buffer. A saturating level for peroxidase was not achieved in this experiment.

Effect of substrate concentration

In a similar experiment, using 20 units of peroxidase in all tubes and increasing amounts of unlabeled cerebroside, a saturating level was found in the presence of phosphate but not in PIPES (**Fig. 1**). The maximal concentration of cerebroside shown (0.4 mg per tube or about 1 mM) could not be increased appreciably because of solubility limitations. In this study, phosphate was superior to PIPES.

Effect of incubation duration and buffer composition

Two time points were chosen, 4 and 24 hr. Each tube contained 0.4 mg of cerebroside, 5 units of GO, and 20 units of peroxidase. With Na phosphate as buffer, 54% of the cerebroside was oxidized by 4 hr and 98% by 24 hr. With N-tris(hydroxymethyl)-methyl-2-aminoethanesulfonic acid (TES), the corresponding values were 72% and 97%, respectively. With HEPES, the values were 64% and 73%, respectively. Imidazole-Cl was poor, yielding only 34% and 40% oxidation, respectively.

Effect of tetrahydrofuran concentration

In a 4-hr incubation, using phosphate buffer under the above conditions, lowering the organic solvent concentration to 40% produced a turbid suspension and decreased the amount of oxidation by 33%. Raising the THF concentration did not precipitate any detectable material but it drastically reduced the amount of substrate oxidized: by 90% with 60% solvent, and by 89% with 80% solvent. Similar differences were seen with 24-hr incubations. In the latter experiment, the tubes containing 50% THF released 48.9% of the cerebroside tritium, rather than the 44.5% expected.

Analysis of the reaction product from the 24-hr incubation in 50% THF by thin-layer chromatography showed that all of the galactosyl ceramide had been converted to the aldehyde.

Effect of catalase concentration

Each incubation tube contained 5 units of GO, 20 units of peroxidase, and 0.4 mg of galactosylceramide in phosphate/THF, as well as various amounts of catalase: zero, 50, 100, 200, and 400 units. The fraction of cerebroside tritium released by 4 hr was, respectively: 23, 31, 38, 33, and 37%. The optimum amount of catalase was 100 units, which produced a 65% improvement in yield and brought the total yield (corrected for the assumed 44.5% available tritium) to 85%.

Effect of peroxidase concentration

Various amounts of peroxidase were added to the standard incubation tubes, containing 5 units of GO and 0.4 mg of cerebroside. Maximal activities were observed in the region of 45 units, with either phosphate or PIPES buffer (**Fig. 2**).

Effect of sodium taurodeoxycholate

This bile salt was tested in the presence and absence of THF, using phosphate buffer and GO as the only enzyme in the system. By 4 hr, the control incubation containing THF yielded 39.4% oxidation and the tubes containing bile salt plus THF produced 41.7% oxidation. However, the latter showed no increase during the next 20 hr while the controls yielded 48.9% oxidation. Evidently the enzyme had become denatured by the bile salt during the first 4 hr.

The bile salt could not make the use of THF unnecessary, as shown by the low reaction yields when water was the only solvent with the taurodeoxycholate: 20.5% at 4 hr and 19.4% at 24 hr. Under these conditions, too, the GO had become inactivated within 4 hr.

DISCUSSION

The major finding of this study is that the addition of catalase and peroxidase produced considerable acceleration of the action of GO, in confirmation of the claim of Novak et al. (5) with respect to the labeling of ganglioside GM_2 and its asialo derivative. These authors, however, used this combination of enzymes in the apparent belief that peroxidase and catalase hydrolyze hydrogen peroxide, the product of GO action. It is the general belief that catalase decomposes the peroxide to water and oxygen and that peroxidase, on the other hand, uses peroxide to oxidize various substrates. It is conceivable that the action of peroxidase in this system is to use the H_2O_2 to oxidize the organic solvent, THF, or the sphingolipid itself. The latter possibility might need additional study in some applications, since reduction of the ketone group would yield a mixture of optical isomers.

In some circumstances catalase can act as a peroxidase, catalyzing the oxidation of ethanol, for example. Thus this enzyme, too, might oxidize the galactolipid at some susceptible site. It is unlikely that catalase helps GO reactions by blocking the inhibitory action of hydrogen peroxide on GO, since this reported effect was noted only at concentrations above 3 mM (9) and few labeling methods have utilized such high substrate concentrations.

The stimulatory effect of peroxidase on GO has been noted before in studies utilizing galactose as the substrate in an aqueous medium (16–18). The mechanism of the effect is still unclear. Perhaps it involves the oxidation by peroxidase of the copper in GO to the Cu^{3+} state, which is probably the enzymatically active form (19). Catalase, acting as a peroxidase, might exert its stimulatory effect the same way. A recent description of an improved isolation method for GO (20) attributed the stimulatory effect to neutralization of an inhibitor that contaminates GO preparations.

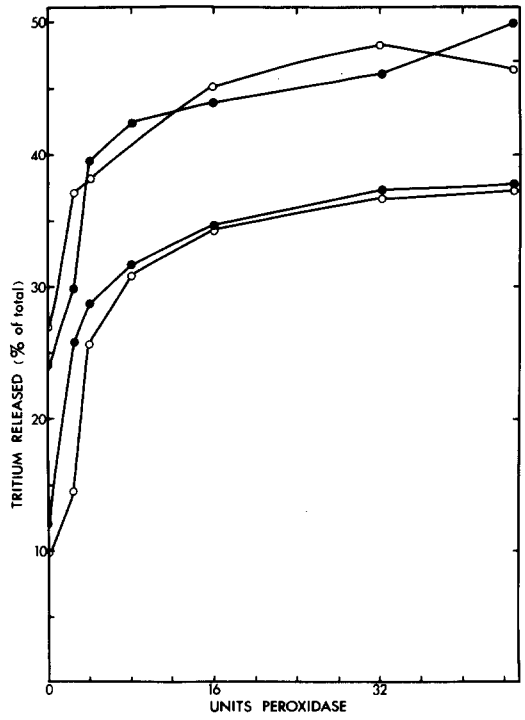

Fig. 2. The effect of increasing amounts of peroxidase in the oxidation of cerebroside. The lower pair of curves was obtained with a 4-hr incubation; the upper pair, with a 24-hr incubation. Galactose oxidase (5 units) was used with 0.4 mg galactosyl ceramide under the conditions described in Methods. Data from phosphate buffer tubes: ○ – – – ○; PIPES buffer: ● – – – ●.

Ferricyanide also stimulates GO (17, 19) and reactivates "auto-inactivated" GO that was used in an immobilized form (21). Its mode of action might be similar to that of peroxidase and catalase. It might be worth study as a substitute for peroxidase and catalase in GO use.

Other agents reported to promote GO action are bovine serum albumin (16) and the chelator, ethylenediaminetetraacetate (19). These might act by removing substances that block the oxidation of the copper in GO. While such interference is unlikely to be a problem in the labeling of pure substances by the GO/borohydride method, it might be important in the labeling of membranes. In the case of gangliosides, which may very well be isolated with heavy metals as contaminants, EDTA might improve the yield of aldehyde.

Chloride ion has been found to inhibit GO in a catalase-containing system (18) so its use in buffers should be avoided. Possibly it is oxidized to chlorine, which would be expected to inactivate enzymes.

An incidental point of interest is that many assays for GO or galactose utilize peroxidase and an easily oxidized chromogen. It is likely that these methods inadvertently stimulate the GO. However, one chromogen has been claimed (without supporting evidence) to yield an inhibitory product (22).

Our experiment with various concentrations of THF suggests that the solvent is inhibitory to one or more of the enzymes present although it had initially been found essential for the practical oxidation of cerebroside by GO (23). Therefore it would seem helpful to reduce its concentration to the minimum acceptable, depending on the concentration of the lipid to be oxidized and its number of sugar residues. Substituting a bile salt for THF, as suggested by Uda et al. (4), was inefficient in our system but it may have merit for the more polar lipids, which are easier than cerebroside to emulsify. Nonionic detergents have been used similarly (7), but this involves an extra step since one must remove the detergents before carrying out the reduction. (Most nonionic detergents are polyethers and are therefore usually contaminated with peroxides.)

One feature of our findings is that GO is quite stable, even in water–THF 1:1, and there is no need to add a second portion of the enzyme during the incubation, as so many workers have done. In addition, the claim that phosphate buffer is inhibitory (12) does not seem to be relevant to the oxidation of lipids. However, imidazole is to be avoided, possibly because it forms a coordinate complex with the copper in the oxidase. The copper normally present appears to be bound to four nitrogenous groups (17). Therefore one should avoid amines capable of binding copper strongly.

The assay system used in this study could be extended to other lipids and nonlipids for which optimum labeling conditions would be useful to know. All that is needed is a simple method for separating the substrate from the labeled water formed in the system. Solvent partitioning is effective for the nonacidic glycolipids, and gangliosides can be directed into the chloroform-rich layer by the addition of $CaCl_2$ (24, 25). A more general method for separation is the evaporation to dryness of the reaction mixture, followed by counting the remaining, unoxidized substrate. While this approach is undesirable at low levels of oxidation, for statistical reasons, it is quite satisfactory for determining how to obtain maximal oxidation.

It should be noted that commercially available GO is impure and that "stabilizing agents" are added to the Sigma product. It is therefore likely that the precise optimal oxidation conditions will vary from batch to batch. An additional complication is that the GO from different suppliers may actually be different enzymes (17). It is therefore important to determine the optimal oxidative conditions not only for each compound but for each GO source. While our own study did not examine every possible variation, it did show that virtually complete oxidation of cerebroside, at least, can be produced with a low, economical amount of enzyme. This makes it feasible to determine galactosyl ceramide, and possibly related lipids, by reducing the aldehyde completely with borotritide and counting the radioactive product.

Ecological note on the use of labeled borohydride

Judging from the literature, users of borotritide make no effort to prevent the escape of tritium gas into the atmosphere. While the gas no doubt rises into the upper atmosphere, it is very likely converted to radioactive water in the presence of sunlight, and thus returned to our environment. Effort ought to be exerted to minimize this release.

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