Permeability changes induced by peroxidation in liposomes prepared from human erythrocyte lipids

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Abstract The diffusion of [2-^{14}C]glucose out of liposomes prepared from extracted human erythrocyte lipids was examined. Increased glucose efflux was observed when the lipids were treated with hydrogen peroxide and $CuCl_2$ before liposome formation, and this phenomenon required both peroxide and metal. Peroxidation of these lipids also resulted in the destruction of polyunsaturated fatty acids and the generation of conjugated dienes, but neither of these processes appeared to be the sole cause of increased glucose efflux. Thin-layer chromatography and the effects of aqueous washes suggested that surface-active lysophosphatides or other lipid degradation products were responsible for the increased permeability of the treated liposomes. It is suggested that the behavior of this liposome model system may be relevant to the high permeability and fragility of vitamin E-deficient erythrocytes.

Supplementary key words glucose efflux

Aqueous lipid dispersions ("liposomes") have been developed for use as model membranes (1, 2). Reproducibility and simplicity have favored these structures for studies on lipid bilayer permeability, lipoprotein complexes, immunological reactions, steroid action, antibiotics, toxins, and anesthetics (3–16). However, it has been noted recently that liposome permeability is extremely sensitive to the autoxidation of lipids, both before and after dispersion, and that stringently anaerobic procedures are required to ensure reproducible results (12, 17). While autoxidation is not a severe problem with synthetic lipids or purified lecithins containing mostly saturated or monounsaturated fatty acids, the increasing use of complex lipid mixtures of biological origin (8–10, 18) is accompanied by greater hazards in this respect.

Lipid peroxidation and its effects upon membrane permeability may be particularly relevant to vitamin E-deficient erythrocytes, which hemolyze readily in the presence of free radicals or ionizing radiation (19). With this in mind, we have studied the effects of peroxidation on the glucose permeability of liposomes formed from normal human red blood cell lipids. Liposomes were formed from H_2O_2-treated and control lipids by mechanical dispersion in ^{14}C-labeled glucose (0.15 M), and the rate of glucose efflux was determined. Variations in permeability were then related to both the phospholipid and fatty acid compositions of the component lipids.

In general, we have found that lipid peroxidation results in greater liposome permeability and that this change in permeability appears to be associated with the production of surface-active lipid degradation products.

MATERIALS AND METHODS

Materials

EGSS-X (an ethylene glycol succinate polyester combined with a silicone), Gas-Chrom P, and 10% boron trifluoride in methanol were supplied by Applied Science Laboratories, State College, Pa. Butylated hydroxytoluene, or BHT (2,6-*tert*-butyl-*p*-cresol), was a gift from the Shell Chemical Corp. Sephadex G-25 (coarse) was supplied by Pharmacia Fine Chemicals, Piscataway, N.J., and [2-^{14}C]glucose by New England Nuclear, Boston, Mass. All other solvents and reagents used were reagent grade. Thin-layer plates coated with silica gel F-254 for phospholipid separations were obtained from Brinkmann Instruments, Westbury, N.Y.

Cell collection and preparation

Blood from random human donors was anticoagulated and stored in acid–citrate–dextrose. It was then washed

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Abbreviations: LPC, lysophosphatidylcholine (lysolecithin); TLC, thin-layer chromatography.

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three times in Krebs-Henseleit buffer (20) at 4°C, and lipid was extracted immediately.

Lipid extraction

The washed erythrocytes were extracted with isopropanol and chloroform according to the method of Rose and Oklander (21). All solvents were flushed with nitrogen prior to use. The extract was dried on a rotary evaporator and resuspended in chloroform–methanol 2:1 at a concentration of 50 μg of lipid phosphorus/ml. This solution was flushed with nitrogen and stored at −28°C until used.

Lipid analysis

Lipid phosphorus assays, TLC, preparation of fatty acid methyl esters, and GLC were performed by minor modifications of standard methods (22–24), which will be published elsewhere.[2]

Lipid peroxidation

Erythrocyte lipid extracts containing 45 μmoles of phospholipid were dried on a rotary evaporator and redissolved in 5 ml of chloroform–methanol 2:1. Copper chloride was added to this solution in a final concentration of 5 mM. Hydrogen peroxide (30% stock solution) was then added to the lipid extract to final concentrations ranging from 0.15% to 0.60%. The mixture, which consisted of a single organic phase, was then incubated at 20°C for 5 or 24 hr, after which it was either washed with 1 ml of 0.05 M KCl or used directly to prepare liposomes.

Formation of liposomes

Lipid dispersions with a particle size of 5–50 μm (2) were prepared by a modification of the method of Bangham, Standish, and Watkins (1). 45 μmoles of peroxidized or untreated lipids were thoroughly dried on a rotary evaporator. 1 ml of 0.15 M glucose, containing 1 μCi/ml of [2-^{14}C]glucose, was added to the tube. The solution was deaerated, and excess lipid was dislodged from the sides of the tube by a stream of nitrogen. Two glass beads were added, and the tube was sealed and vigorously agitated on a Vortex mixer for 5 min. The turbid solution was then allowed to equilibrate for 2 hr.

Removal of untrapped glucose

A 1 × 20 cm column was formed with Sephadex G-25 (coarse) that had swollen overnight in 37.5 mM NaCl–37.5 mM KCl (isotonic salt solution). The lipid dispersion was layered on the column and eluted with isotonic salt solution at a flow rate of 0.5 ml/min; liposomes emerged in the void volume, and the first 3 ml of turbid suspension was collected. Four samples of 0.1 ml each were placed in scintillation vials and counted in Buhler's solution (25).

Determination of glucose efflux

Two 1.0-ml samples of the liposome suspension were sealed in presoaked dialysis tubing. Dialysis with rocker platform agitation was performed in screw-capped tubes containing 10 ml of isotonic salt solution. The tubes were inverted several times and thoroughly mixed every 10 min. Every hour, 0.2-ml aliquots of the dialysate were taken in duplicate for assay of radioactivity, and 0.4 ml of fresh salt solution was returned to the tubes. At the end of 5 hr, the dialysis bags were washed briefly in isotonic salt solution, and duplicate samples of the liposome suspension were removed for counting.

Peroxidation of liposomes prepared from fresh lipids

In an effort to simulate the peroxidation of intact cell membranes, peroxidation and subsequent glucose efflux measurements of preformed liposomes were undertaken in some experiments. For these studies, liposomes containing [^{14}C]glucose were prepared from fresh unperoxidized erythrocyte lipids as described above. The unpurified liposome suspension was then added to 2 ml of an incubation medium containing a final concentration of 0.45% H_2O_2, 0.5 mM $CuCl_2$, and sufficient NaCl to give a final osmolarity (by freezing point depression) of 295–305. The liposomes were then preincubated at 20°C for 1 and 5 hr. They were then freed from both untrapped glucose and released glucose by passing them through the G-25 Sephadex column as described previously. Glucose efflux from these purified liposomes was then measured in the same way as previously described for liposomes originally made from peroxidized lipids.

Calculations

The amount of glucose trapped within the liposome preparation was determined from the predialysis counts contained within the dispersion relative to the specific activity of the 0.15 M glucose solution. Glucose remaining in the liposomes at the end of the experiment (5 hr) was similarly calculated. The average rate of glucose leakage (nmoles/hr) was then determined from these two measurements. Counts from the dialysate were corrected for loss resulting from the sampling procedure and plotted. The initial rate of glucose loss (nmoles/hr) was determined from the dialysate counts at hr 1 and 2. Beyond hr 2 or 3, the leakage rates decreased as glucose accumulated in the dialysates, leading to nonlinear plots. We did not calculate the initial rate from the background count of the isotonic salt solution and the first point because of the possible presence of untrapped glucose. Others have shown that this is mostly released during the first hour (1, 13). Since the initial amount of untrapped marker varied

[2] Smolen, J. E., and S. B. Shohet. 1974. Remodeling of granulocyte membrane fatty acids during phagocytosis. *J. Clin. Invest.* In press.

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TABLE 1. Glucose efflux rates[a] and unsaturation indices for liposomes prepared from unperoxidized erythrocyte lipids

Sample[b]	Treatment	Average Rate	Initial Rate	Unsaturation Index
a	None	2.0 ± 0.79[c]	1.3 ± 0.51	115.2 ± 6.5
b	Cu^{2+}, 24 hr	2.2 ± 0.05	1.7 ± 0.17	115.6 ± 6.9
c	0.6% H_2O_2, 24 hr	1.3 ± 0.57	1.4 ± 0.30	113.2 ± 6.5

[a] Efflux rates are reported as percentages of the original trapped glucose released to the medium per hour.
[b] Results were obtained from 16 experiments for sample a and 6 experiments for samples b and c. Lipids were treated and liposomes prepared as in Materials and Methods. "Treatment" gives the concentration of hydrogen peroxide, the presence of 5 mM $CuCl_2$, and the duration of incubation. The two rate measurements in %/hr representing the glucose efflux were calculated as noted in Materials and Methods. Unsaturation indices were calculated as the cumulative number of double bonds in each fatty acid species multiplied by the mole percentage of each. All lipids were washed with 0.05 M KCl before liposome formation.
[c] The data are presented in this and subsequent tables as the mean values ± SD of separate experiments.

greatly with the preparation, all rates were standardized according to the glucose concentration gradient and converted to terms of percentage of trapped glucose released per hour (%/hr).

The unsaturation index (UI) was calculated as the cumulative number of double bonds in each fatty acid species multiplied by the mole percentage of each fatty acid in the lipid extract as determined by GLC.

Estimation of the extent of lipid peroxidation

The amount of induced lipid peroxidation that took place in the organic phase was estimated by the method of Klein (26). Samples containing 2 μmoles of lipid were suspended in 1 ml of chloroform–methanol 2:1, and the absorbances at 215 and 233 nm were measured against a solvent blank. The ratio $absorbance_{233}/absorbance_{215}$ was calculated and corrected for the background value of fresh, washed lipid and for the presence of $CuCl_2$.

RESULTS

Removal of untrapped glucose

Two peaks of [2-^{14}C]glucose counts emerged from the Sephadex G-25 column. The turbidity associated with the liposome fraction was found only in the first peak. Most of the untrapped glucose emerged later. The first 3 ml of the turbid solution, which was later utilized in diffusion rate measurements, was virtually free from untrapped label. The dispersed lipid, which had been layered on the column at a concentration of 30–45 μmoles/ml, emerged at a concentration of 3–8 μmoles/ml for untreated samples and 2–3 μmoles/ml for peroxidized samples. However, both peroxidized and control lipids trapped the same quantity of glucose per micromole of lipid (735 ± 194 nmoles of glucose for untreated samples and 713 ± 232 nmoles for peroxidized samples).

Glucose permeability and unsaturation indices: unperoxidized lipids

Table 1 lists the results of experiments using unperoxidized lipids washed with 0.05 M KCl before use. These lipids were highly unsaturated (UI ≃ 115) and had glucose release rates of about 1.5%/hr. When only 5 mM $CuCl_2$ was added, essentially the same results were obtained. If only 0.60% hydrogen peroxide was present, no substantial change in unsaturation or glucose efflux was observed.

Glucose permeability and unsaturation indices: peroxidized lipids

Lipids peroxidized under various conditions and washed with 0.05 M KCl gave the results listed in Table 2. Significant increases in the release rates were observed with exposure to increasing concentrations of hydrogen peroxide (compare samples d and e, and f, g, and h). In general, the unsaturation index of the residual lipids also decreased with increasing peroxide content of the incubation mixture, but increased glucose efflux was

TABLE 2. Glucose efflux rates and unsaturation indices for liposomes prepared from washed peroxidized erythrocyte lipids

Sample	Treatment[a]	Average Rate	Initial Rate	Unsaturation Index
d	0.30% H_2O_2, Cu,[b] 5 hr	3.7 ± 0.23	3.7 ± 0.15	86.2 ± 6.9[c]
e	0.60% H_2O_2, Cu, 5 hr	7.0 ± 0.02	7.3 ± 0.32	61.1 ± 7.5
f	0.15% H_2O_2, Cu, 24 hr	3.1 ± 0.08	2.6 ± 0.26	106.7 ± 16.6[c]
g	0.30% H_2O_2, Cu, 24 hr	3.5 ± 0.79	3.9 ± 0.70	118.4 ± 17.3
h	0.60% H_2O_2, Cu, 24 hr	4.4 ± 0.99	4.9 ± 0.90	77.4 ± 9.2
i	0.60% H_2O_2, Cu, 1% BHT, 5 hr	2.0 ± 0.31	2.3 ± 0.20	112.0 ± 7.0
j	0.60% H_2O_2, Cu, 1% BHT, 24 hr	1.9 ± 0.16	2.2 ± 0.20	110.0 ± 9.0

[a] See footnotes to Table 1 for explanation of format and units. The results were obtained from six experiments for samples d–g and eight experiments for sample h. All lipids were washed with 0.05 M KCl before they were used to make liposomes.
[b] $CuCl_2$.
[c] Fatty acid analyses were performed on only five of the six samples in groups d and f.

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TABLE 3. Glucose efflux rates and unsaturation indices for liposomes prepared from unwashed peroxidized erythrocyte lipids[a]

Sample	Treatment	Average Rate	Initial Rate	Unsaturation Index
k	0.15% H_2O_2, Cu^{2+}, 24 hr	9.1 ± 0.51	11.2 ± 0.5	103 ± 7.0[b]
l	0.45% H_2O_2, Cu^{2+}, 24 hr	9.7 ± 0.30	10.2 ± 0.30	61.4 ± 5.4[b]
m	0.15% H_2O_2, Cu^{2+}, 1% BHT, 24 hr	2.2 ± 0.04	2.5 ± 0.06	104 ± 6.7
n	0.45% H_2O_2, Cu^{2+}, 1% BHT, 24 hr	2.3 ± 0.05	2.7 ± 0.10	100 ± 7.1

See footnotes to Table 1 for explanation of format and units.
[a] These results were obtained from six experiments for all samples. Lipids were used directly without washing at the lipid extraction stage for the formation of liposomes.
[b] Fatty acid analyses were performed on only five of the six samples in groups k and l.

apparently not solely contingent upon this change. Thus, for samples f and g, moderate increases in glucose efflux were observed (control values of 1.3–2.2, Table 1) even though in this series of experiments the unsaturation index did not differ from that of untreated cells.

In some preliminary experiments (data not shown), it was observed that the presence of the antioxidant BHT (1% concentration) in the incubation mixture had no effect on liposome formation rate or glucose efflux. However, as expected, BHT in this concentration prevented all of the reduction in unsaturation indices, and at least 90% of the increased glucose permeability in these samples (compare samples e and h vs. samples i and j).

Liposomes formed from peroxidized lipids not washed with 0.05 M KCl, but used directly (Table 3), had considerably greater glucose permeability than the washed samples (compare sample k, Table 3 with sample f, Table 2). This occurred in spite of the fact that these lipids had unsaturation indices comparable to those of the washed lipids. As before, 1% BHT prevented more than 90% of this increased permeability (samples m and n as compared with samples k and l in Table 3).

Further experiments, not presented in detail here, in which up to 1% hydrogen peroxide was used, produced lipid samples with unsaturation indices as low as 12; not only were the samples devoid of polyunsaturated fatty acids, such as linolenic and arachidonic acids, but they also had very low contents of oleic acid. These lipids formed a slightly turbid suspension in 0.15 M glucose and they were not eluted from the Sephadex G-25 column, apparently because stable liposomes had not been formed.

Table 4 shows the fatty acid compositions for all untreated lipids and for those incubated with 5 mM $CuCl_2$ and 0.60% peroxide for 24 hr (washed with KCl). In the peroxidized samples, standard deviations about the mean mole percentages and unsaturation indices are high; this reflects the fact that a wide variation in peroxidative destruction of polyunsaturated fatty acids occurred under even a single set of conditions. The major cause of decreased unsaturation in all the peroxidized samples is the loss of arachidonic acid (20:4), a phenomenon that has been observed before (27). When minor components are taken into account, the fatty acid composition of untreated lipids agrees well with the data of Dodge and Phillips (24).

Phospholipid analyses

Because the correlation between glucose release and lipid unsaturation was inconclusive, the effect of peroxidation on distribution of phospholipids in the lipids before liposome formation (Table 5) was determined by TLC. Washed, untreated lipids contained little or no lysolecithin and had a composition comparable to that reported by previous investigators (24). Samples treated with 0.60% hydrogen peroxide and 5 mM $CuCl_2$ were poorly resolved with TLC and exhibited streaking when they were not previously washed with 0.05 M KCl. These lipids contained greater proportions of lipid phosphorus in the phosphatidylinositol + phosphatidylserine and the lysolecithin zones, and less in the phosphatidylethanolamine zone, than untreated lipids. When the peroxidized sam-

TABLE 4. Fatty acid compositions of untreated and peroxidized erythrocyte lipids

	Mole Percentage of Total Fatty Acids	
Fatty Acid	Untreated Lipids	Peroxidized Lipids
14:1[a]	1.23 ± 0.95	0.24 ± 0.21
15:0	0.32 ± 0.16	0.28 ± 0.27
16:0	27.2 ± 2.07	37.8 ± 4.41
16:1	2.08 ± 1.63	1.19 ± 0.41
18:0	19.4 ± 1.56	23.5 ± 2.08
18:1	17.0 ± 1.46	16.4 ± 3.88
18:2	11.9 ± 0.63	10.7 ± 2.18
18:3	1.7 ± 1.50	0.70 ± 0.64
20:0	0.24 ± 0.26	0.16 ± 0.06
20:2	0.46 ± 0.48	0.38 ± 0.24
20:4	16.3 ± 0.91	8.18 ± 2.06
22:0	2.13 ± 0.91	0.59 ± 0.30
Unsaturation index[b]	115.6 ± 6.46	74 ± 10.3

Fatty acids from untreated and peroxidized (5 mM $CuCl_2$, 0.60% H_2O_2, 24 hr, with 0.05 M KCl wash) lipids were analyzed as described in Materials and Methods. These results were obtained from 16 experiments with untreated lipids and 6 experiments with peroxidized lipids.
[a] Number of carbon atoms: number of double bonds.
[b] See footnote *b* to Table 1.

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TABLE 5. Phospholipid analyses of erythrocyte lipids

Phospholipid Fraction	Mole Percentage of Lipid Phosphorus: Untreated Lipids	Washed Peroxidized Lipids	Unwashed Peroxidized Lipids
PE	28.2 ± 1.36	24.4 ± 1.46	21.3 ± 2.63
PI + PS	13.9 ± 2.24	16.1 ± 2.50	16.9 ± 1.50
PC	29.9 ± 0.68	30.0 ± 0.67	29.1 ± 0.94
SM	25.6 ± 2.17	25.4 ± 1.59	25.6 ± 1.38
LPC	1.5 ± 1.67	4.2 ± 1.16	6.9 ± 1.27

Lipids were peroxidized (5 mM $CuCl_2$, 0.60% H_2O_2, 24 hr) and then washed with 0.05 M KCl or left unwashed before analysis. PE is phosphatidylethanolamine; PI + PS, phosphatidylinositol plus phosphatidylserine; PC, phosphatidylcholine; SM, sphingomyelin; and LPC, lysophosphatidylcholine (lysolecithin). The values shown represent lipid phosphorus found in the zones of the TLC plate corresponding to each of the phospholipids. Some of the material found in the PC zones of the peroxidized samples may represent LPE or other degenerated lipids.

ples were washed, the streaking on the TLC plates was lessened. Even after washing, elevated lysolecithin and decreased phosphatidylethanolamine fractions were observed.

Estimation of the extent of peroxidation

Table 6 gives the values of absorbance$_{233}$/absorbance$_{215}$ for several samples of peroxidized lipids. This ratio is a measurement of conjugated diene content and hence reflects the extent of peroxidation (26). The values at 5 hr indicate that copper is necessary for initial diene accumulation. By the end of 24 hr, however, the concentration of conjugated dienes decreased in those samples containing only hydrogen peroxide. Washing the samples with 0.05 M KCl in some cases increased the value of the ratio.

TABLE 6. Conjugated diene content of liposomes prepared from erythrocyte lipids

Sample	Treatment	Absorbance$_{233}$/Absorbance$_{215}$: 5 hr, Unwashed	24 hr, Unwashed	24 hr, Washed
1	0.6% H_2O_2, Cu^{2+}	0.448[a]		
2	0.6% H_2O_2, Cu^{2+}	0.433	0.241	0.274
3	0.6% H_2O_2	0.036	0.290	0.301
4	0.6% H_2O_2	0.044	0.300	0.366
5	None	0.006	0.009	0.008

Conjugated diene content was estimated by the method of Klein (26) as described in Materials and Methods. At the start of the experiment, all samples were freshly washed and arbitrarily assigned values of 0.00. Liposomes were prepared and incubated as indicated under Treatment. Corrected values are then given for the unwashed samples at the end of 5 and 24 hr and also after a final aqueous KCl wash of the lipid extract at 24 hr for the ratio absorbance$_{233}$/absorbance$_{215}$.

[a] Sample 1 was run for 5 hr only.

Effect of lysolecithin incorporated into liposomes

Table 7 shows the effect of adding different amounts of lysolecithin to the lipids used for liposome formation. Since possible differences in lysolecithin (LPC) concentration might occur due to partition between the aqueous preparation media and the liposomes, LPC levels determined in the purified liposomes as well as those initially added are reported. The final levels obtained in the liposomes themselves (3.1–7.1% LPC) cover the maximum range of LPC found in the previous experiments (viz. LPC zones in Table 5). As can be seen, at final LPC concentrations above 5.2% there is a slight augmentation of initial glucose efflux rates, and average efflux rates may increase at even lower LPC levels. However, these efflux rates are considerably lower than those found for unwashed peroxidized lipids (viz. samples k and l).

Effect of peroxidation of preformed liposomes

The data in Table 8 show that the peroxidation of preformed liposomes produces the same general increase in glucose permeability that was observed in liposomes made from peroxidized lipids. These experiments are not entirely analogous to the previous experiments, so rate comparisons are probably not appropriate. (Glucose concentrations, and hence gradients, were considerably lower by the time that the samples could be studied.) However, peroxidation, as evidenced by a drop in the unsaturation index, was again associated with an increase in permeability, and this effect could be prevented by BHT.

DISCUSSION

The glucose efflux observed in these experiments for unperoxidized red cell lipids was comparable to that reported previously (3, 4). In most cases, the average rate over a 5-hr period was approximately equal to the initial

TABLE 7. Glucose efflux rates for liposomes prepared from fresh unperoxidized erythrocyte lipids: effect of lysolecithin (LPC) added to lipid extracts prior to formation of liposomes

Sample	LPC Added	LPC Determined	Average Rate	Initial Rate
	%	%		
o	None	1.7	2.1	1.5
p	2	3.4	2.3	1.4
q	4	5.2	2.7	2.7
r	6	7.1	3.0	3.4

Quadruplicate aliquots of liposomes were prepared from fresh erythrocyte lipids with added LPC. Three samples were used for determination of rates, and the averages are reported. The remaining aliquot was used to determine by TLC (23) and lipid phosphorus (22) the actual LPC content of the liposomes used in the efflux studies.

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TABLE 8. Glucose efflux rates and unsaturation indices for liposomes prepared from fresh erythrocyte lipids subsequently treated with H_2O_2 and $CuCl_2$

Sample	Treatment	Average Rate	Initial Rate	Unsaturation Index
o	0.15% H_2O_2, Cu^{2+}, 1 hr	7.6 ± 2.0	7.8 ± 1.6	86 ± 8.0
p	0.15% H_2O_2, Cu^{2+}, 5 hr	7.9 ± 1.7	8.0 ± 0.8	81 ± 5.0
q	0.15% H_2O_2, Cu^{2+} + BHT, 5 hr	2.7 ± 0.09	3.1 ± 0.10	111 ± 7.0
r	No additives, 5 hr	2.5 ± 0.08	2.7 ± 0.8	116 ± 8.0

See footnotes to Table 1 for explanation of format and units. These results were obtained from six experiments for all samples. Liposomes containing [^{14}C]glucose were prepared from fresh RBC lipids, treated with various agents, and then purified on Sephadex G-25 prior to glucose permeability measurements and lipid analyses.

rate. Because glucose efflux slowed substantially after 3 hr, this finding may have been caused by small residual amounts of untrapped glucose in the liposome preparations. The fact that dialysate counts do not extrapolate to zero at the start of the experiment supports this possibility. The lipid dispersion was initially highly concentrated (45 μmoles/ml) but was diluted to customary levels on the Sephadex G-25 column. In addition, the efficiency of glucose trapping was comparable to that in other studies (4). Some anomalous results remain however, where samples peroxidized for 24 hr have lower efflux rates than those treated for only 5 hr (e.g., samples e vs. h). We have no explanation for this phenomenon other than to speculate that surface-active peroxidation products may have either leached out of the liposomes or broken down further to innocuous derivatives with time.

It is apparent that $CuCl_2$ together with hydrogen peroxide was required for peroxidation to occur under our conditions, even though $CuCl_2$ in the high concentrations used in these experiments has been shown to inhibit autoxidation (28). We have no explanation for this copper effect and can only suggest that possible contamination of the copper with traces of iron may have rendered it a more effective peroxidant in these studies.

Previous investigations have suggested that permeability decreases when fatty acid saturation increases in liposomes from both synthetic lipid mixtures (4, 5) and from lipids of biological origin (3, 4, 17). However, in the current studies, peroxidation of red cell lipids was associated with both an increase in relative lipid saturation and an increase in glucose permeability. Similar results have been observed with cation flux through liposomes made from purified phospholipids (17). It should be noted that this system is entirely different from in vivo systems using whole cells, which may have intact membrane repair mechanisms operative (29). Indeed, in these studies we have examined the ability of a pure lipid system to form stable lipid structures and to prevent the egress of trapped solutes from such structures. These liposome models are not direct models of the whole cell, in which protein moieties would be expected to modify the type and extent of peroxidative damage.

Since peroxidation itself, and not necessarily a decrease in the unsaturation index, appears to be required for the increased efflux, some factors beyond changes in saturation are needed to explain these results. Furthermore, glucose leakage was increased when the lipid extracts of the peroxidized samples were not treated with aqueous washes prior to liposome formation. Accordingly, these factors are apparently partially removed with aqueous salt solutions. In addition, the difficulty of forming liposomes from peroxidized lipids suggests that these structures may not be completely stable. (It should be noted, however, that the ability of the peroxidized liposomes to trap as much glucose per mole of lipid as the fresh liposomes argues that both types are similarly constructed.) For these reasons, surface-active agents must be considered as possible causes for the increased glucose efflux found for peroxidized lipids.

Conjugated dienes in the phospholipids do not seem to be responsible for this behavior, because washing, which reduced the permeability of the liposomes, did not necessarily decrease the absorbance$_{233}$/absorbance$_{215}$ value of their lipids. Furthermore, the low 24-hr values suggested that the conjugated diene stage is merely an initial step in lipid peroxidation; these species may break down in some manner to form the final active agent that caused high glucose efflux in both the 5- and 24-hr samples. The increased value of this ratio for samples containing only peroxide (Table 6, samples 5 and 6) is consistent with this hypothesis in that at 24 hr the unaccelerated peroxidation process is still in the conjugated diene stage and the agent responsible for increased glucose efflux has not yet been formed.

The TLC data indicate that peroxidation increases the proportions of lipid phosphorus found in the phosphatidylinositol + phosphatidylserine and, in particular, lysolecithin zones. This might suggest that lysophosphatides are being formed that, perhaps by their detergent properties (11, 30), would cause a partial disruption of the liposome structure.

Dodge and Phillips, however, have cautioned that this interpretation may be deceptively simple (27). It is likely that we are also observing lipoperoxides formed from the

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phosphatides, which migrate more slowly than the parent compounds on the TLC plate because of their more hydrophilic nature. Indeed, Table 5 does not suggest that lysolecithin per se is being formed at the expense of phosphatidylcholine. It is more likely that the material found in the lysolecithin zone throughout may consist of peroxidized phospholipids (phosphatidylethanolamine, in particular). With increased polarity, these derivatives would be more surface active than the original lipids; these properties could overwhelm the tendency towards impermeability caused by the increased percentage of saturated fatty acids and could thus be responsible for the high efflux found with unwashed peroxidized samples. Moreover, the amphiphilic nature of both these products and lysophosphatides would facilitate their loss after the 0.05 M KCl aqueous washes, as suggested by the data in Table 5. Glucose loss from the resulting liposomes would be less than from unwashed samples but greater than from unperoxidized ones, as confirmed by the data shown in Tables 1–3.

The data in Table 7 directly confirm that lysolecithin, at levels up to the maximal amount that might have been generated in the previous studies, has only a moderate effect on the glucose permeability of liposomes prepared from fresh erythrocyte lipid extract. This moderate effect of LPC implies that, though LPC may contribute to the permeability excess noted in samples i, k, and l, it cannot be solely responsible for the extent of the observed increase in glucose efflux.

The data in Table 8 show that peroxidation of preformed liposomes also results in increases in glucose permeability. This suggests that lipids in a bilayer array are not protected from the peroxidative changes we observed for dissolved lipids. This, in turn, suggests that the processes we have observed in the subsequent behavior of such peroxidized lipids may have some relevance to intact membrane systems. It should be noted, however, that these experiments are not precisely analogous to the solubilized lipid peroxidation studies: it was not possible to wash these vesicles in the same way as the lipid extracts were washed. Hence, the possibility of retained peroxidation products in these lipid structures may explain the high rates of glucose efflux noted in samples o and p.

One additional explanation for the increased glucose permeability seen in all of these studies is suggested by previous observations that peroxidation may convert polyunsaturated fatty acids to short acyl fragments (31). Previous work by DeGier, Mandersloot, and van Deenen (5) has shown that liposomes containing short fatty acids are significantly more permeable than those with long-chain fatty acids.

Finally, it should be noted that all of the agents that may have contributed to the apparent increase in glucose efflux from these liposomes may also have contributed to the well-known difficulty of preparing liposomes from peroxidized lipids in high yield, and they may be responsible for producing incompletely sealed liposomes (which appear to leak) in the current studies. However, the experiments on the preformed liposomes suggest that this is not the major explanation for our findings; and although we do not feel that we can directly extrapolate from these purely lipid studies to erythrocyte membrane structure, we do feel that it is likely that there is some considerable influence of lipid oxidation on the ability of the membrane to maintain an analogous sealed structure.

In summary, a membrane model system prepared from dispersed human erythrocyte lipids previously treated with $CuCl_2$ and hydrogen peroxide shows markedly increased glucose permeability in comparison with untreated lipids. This may be due to the generation of amphiphilic peroxidation products, lysophosphatides, or short-chain acyl fragments. All of these degradation products would tend to disrupt lipid lamellae (5, 30). The exact nature of the deleterious peroxidation products is as yet unknown, and further clarification of this phenomenon requires their isolation and characterization. The use of this model with lipid extracts from vitamin E-deficient red blood cells from patients with hemolytic anemia (29) will be of interest.

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