Accelerated Phospholipid Degradation and Associated Membrane Dysfunction in Irreversible, Ischemic Liver Cell Injury*

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Interruption of the blood supply to rat liver produces a progressive loss of phospholipids from the ischemic cells. Whole homogenates and post-mitochondrial supernatants from livers ischemic for 3 h showed a 40% and 55% decrease in phospholipids, respectively. Phosphatidylcholine and phosphatidylethanolamine were predominantly affected without accumulation of either lysophosphatidylcholine or lysophosphatidylethanolamine. Pretreatment of the animals with chlorpromazine prevents the liver cell death produced by as much as 3 h of ischemia and prevents the loss of phospholipids from both the whole homogenates and post-mitochondrial supernatants. This phospholipid depletion is entirely accountable by an accelerated rate of degradation with a half-life of 2 to 4 h for ischemic as compared to 24 h for control microsomal membrane phospholipids. Microsomes prepared from the lipid-depleted post-mitochondrial supernatants exhibit considerable alterations in their structure and function with inhibition of glucose-6-phosphatase and calcium pump activities and a 25- to 50-fold increase in their passive permeability to Ca²⁺. Pretreatment with chlorpromazine prevents this increased Ca²⁺ permeability and addition of phosphatidylcholine to the ischemic microsomes restores a normal Ca²⁺ permeability. Treatment of control liver microsomes with Naja naja phospholipase A₂ produces a similar spectrum of abnormalities with increased phospholipids, inhibition of glucose-6-phosphatase and calcium pump activities, and increased Ca²⁺ permeability. These changes could not be attributed to accumulation of the lysophospholipids accompanying the loss of phospholipids. Electron micrographs of freeze-fractured ischemic microsomes showed fewer intramembranous particles, bare membrane regions devoid of particles, and areas containing aggregations of intramembranous particles. It is suggested that the loss of phospholipid from the microsomal membranes results from the activation of endogenous, membrane-bound phospholipases as a result of increased cell Ca²⁺ shown previously to accompany liver cell ischemia. Chlorpromazine, an inhibitor of Ca²⁺ fluxes and phospholipases, would act by preventing the increased Ca²⁺ or by directly inhibiting the activation of membrane-bound phospholipases. Accelerated phospholipid degradation and its resultant membrane dysfunction are proposed as the critical alteration that produces irreversible liver cell injury and ultimately cell death in ischemia.

Ischemia, the interruption of blood supply to a tissue, is an important cause of cell injury and has been, therefore, the subject of a great deal of investigation. Despite this there is still considerable uncertainty as to the specific sequence of biochemical events leading to the irreversible injury and subsequent death of ischemic cells. In large part this stems from the difficulties in assessing causal significance as ischemic cells rapidly manifest a great many biochemical alterations. In addition there are inherent difficulties in analyzing many systems in which irreversible injury may relate more to a lack of perfusion upon reflow than to any specific biochemical alteration developing during the ischemic period itself.

Rat liver offers certain definite advantages in this respect. Liver cells are susceptible to ischemia, and the cell injury is initially reversible and then irreversible (1, 2). Second, irreversible injury can be produced by periods of ischemia that do not prevent prompt reperfusion (3). There is not a so-called "no-reflow" (4, 5) phenomenon. And third, liver cells can be protected from the effects of otherwise irreversible periods of ischemia by pretreatment with chlorpromazine (3). This action of chlorpromazine is not attributable to any change in the rate or extent to which liver cells become ischemic or to the perfusion patterns following release of the obstruction. Chlorpromazine must act on some component(s) of the reaction of the cells to the ischemia itself. Using these features criteria for assessing the causal relationship of any biochemical abnormality to irreversible liver cell injury can be developed. Any biochemical change that has the same dose-response relationship to the duration of ischemia as the cell death itself and which is affected by chlorpromazine pretreatment would be a potential causal factor.

Return of the blood flow to irreversibly injured liver cells produces large increases in the total cell and mitochondrial Ca²⁺ contents (3). Chlorpromazine prevents this (3). There is increasing evidence that accumulation of Ca²⁺ may be the ultimate mediator of ischemic cell death (6), and in order to determine the basis for the changed Ca²⁺ metabolism in irreversibly injured liver cells, we began a study of the effects of ischemia on liver cell membranes.

Membranes play a central role in liver cell Ca²⁺ homeostasis both as a permeability barrier to the passive diffusion into the cell down a very steep concentration gradient and as the site of active efflux from the cell against this same gradient. We have reported (2) microsomal membrane alterations that develop in association with the onset of irreversible cellular damage. The pattern of these changes suggested that alterations in membrane phospholipids could be responsible. In the present paper we have examined the effect of ischemia on rat liver phospholipid metabolism and have observed that ischemia induces an acceleration of the rate of degradation of phospholipids that is prevented by chlorpromazine. The relationship between this disturbed phospholipid metabolism...
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**Materials and Methods**

Female Wistar rats (Charleo River Bredco) weighing 140 to 165 g were fasted 18 h overnight prior to use. To induce liver ischemia an abdominal midline incision was made under light ether anesthesia and the portal vein and hepatic artery blood supply clamped (Thomas, 27 mm, No. 3873-S60) to the left lateral and median lobes. Sham-operated animals were used as controls. All animals were killed by decapitation. The preparation of liver microsomes and assay of glucose-6-phosphate and calcium pump activities were as previously described (2). Protein was determined by the method of Lowry et al. (7).

**Phospholipid Determinations**—Livers were homogenized in 4 volumes of 100 mM KCl, 30 mM histidine/imidazole buffer, pH 6.6, and determined by modification of the method of Folch was removed for measurement of total phospholipid and 10 ml centrifuged for 20 min at 11,000 rpm in an SW 40 rotor of a Beckman L2-65B ultracentrifuge. The post-mitochondrial supernatant was aspirated and the pellet resuspended in 10 ml of buffer. Phospholipids were extracted from aliquots of the original homogenate, PMS(1), and pellet fractions by method of Bligh and Dyer (8). Each aliquot was kept at 4°C for 15 min on ice. One milliliter of buffer and 1 ml of chloroform were added, the samples mixed and centrifuged in a tabletop centrifuge for 2 min. The top layer was discarded and the lower phase removed and washed three times with chloroform. The dried lipids were dissolved in 0.5 ml of perchloric acid (Aristar) for 15 min on a Kjeldahl rack. Phosphate was measured on the digest by the method of Hoskins and Popat (9). For isolation of the individual phospholipid species the extracted, dried phospholipids were resuspended in chloroform/methanol (1:1), stirred on Silica Gel 60, 0.25-mm precoated TLC plates (Merck), and chromatographed in a solvent mixture of chloroform/methanol/acetic acid/water (65:25:2:4) (10). The lipids were visualized in an L, vapor, scraped, and eluted two times with 4 ml of methanol. The extracts were dried, digested, and assayed as above.

**Measurement of Phospholipid Degradation**—The rate of degradation of phospholipids in postmitochondrial supernatants prepared as discussed above was determined by modification of the method of Omura et al. (11). Rates were given 10 pCi of [1,14C]glycerol (New England Nuclear) by intraperitoneal injection. Control animals were killed at 24, 48, and 72 h. Ischemia was induced in another group of animals after 48 h with and without chlorpromazine pretreatment (30 mg/kg intraperitoneal 15 min before surgery) and killed after 1, 2, and 2 h of ischemia. Phospholipids were extracted from approximately 100 mg of (protein) PMS and chromatographed as above. Spots were scraped and counted directly in 10 ml of Aquasol (New England Nuclear).

**Measurement of Permeability Properties of Ischemic Microsomes**—The permeability of microsomal membranes to Ca++ was determined by modification of the method of Lata et al. (12). Approximately 0.2 mg of (protein) microsomes prepared as previously described (2) were incubated for 18 h at 4°C with concentrations of CaCl₂ from 10⁻⁶ to 10⁻³ M each containing 1.5 × 10⁶ cpm of [⁴⁰CaCl₂ (New England Nuclear) in 100 mM KCl, 10 mM histidine/midazole buffer, pH 7.4, in a total volume of 75 μl. Calcium influx was measured by counting the amount of the incubate into 1 ml of Ca⁺⁺ free buffer at 0.45 μl Millipore filter, washed with 3 ml of buffer, and counted in 10 ml of Aquasol. The “zero” time Ca⁺⁺ content of the microsomes was obtained by diluting an aliquot of the original incubate and immediately filtering it through a Millipore filter. Measurement of the permeability to Na⁺, choline, and sucrose was essentially the same with substitution of NaCl (New England Nuclear) (1.125 × 10⁶ cpm/75 μl), [³H]choline (New England Nuclear) (5.0 × 10⁶ cpm/75 μl), and unlabeled NaCl, choline chloride, or sucrose, respectively. In separate experiments 25 μl of a soybean lecithin (Sigma Chemical Co.) dispersion (10 mg/ml) was added to control and ischemic microsomes during the 18-h incubations. The ratio of phospholipid as lecithin to phospholipid as microsomes was 2:1. The dispersions were prepared by incubation at room temperature for 60 min in a sonicating water bath (Branson Ultrasoniics Corp.). There was no change in the purity of the added lecithin following sonication as revealed by thin layer chromatography.

**Exogenous Phospholipase Digestion of Liver Microsomes**—Approximately 15 mg of (protein) control liver microsomes were incubated in a total volume of 2.0 ml containing 100 mM KCl, 10 mM histidine/imidazole buffer, pH 7.4, 100 μg of bovine serum albumin, and 20 μg of Naja naja phospholipase A₁ (1,000 units/mg, Sigma Chemical Corp.) at 37°C for 20 min. Phospholipid hydrolysis was terminated by addition of 1 ml of 10 mM EGTA and the microsomes resolated by centrifugation. Measurement of phospholipid content and assay of glucose-6-phosphatase, calcium pump, and Ca⁺⁺ permeability were performed as above.

**Electron Microscopy of Normal and Ischemic Microsomes**—Microsomal pellets were fixed at room temperature in a 0.1 M phosphate buffer, pH 7.2, containing 2.5% glutaraldehyde (Ladd Research Industries, Burlington, Vt.). After fixation the small blocks of tissue (1/16 mm³) were washed in two 15-min changes of the same buffer and infiltrated with 25% glycerol in the 0.1 M phosphate buffer for 1/2 h at 4°C. Small pieces were mounted on cardboard discs, rapidly frozen in liquid Prosn (Allied Chemical Corp.), cooled with liquid N₂, and stored in liquid N₂ until used. Freeze-fracturing was performed with a Balzers device (model BA 360, Balzers High Vacuum Corp.). The specimen temperature was maintained at −103°C at the time of fracture and replication. The platinum-carbon replicas were cleaned with household bleach and, after washing in distilled H₂O, picked up on Formvar-coated grids. All replicas were examined in a Philips EM 300 operating at 80 kV. Images were recorded on Kodak Electron Image Plates.

**Results**

**Phospholipid Metabolism in Ischemic Liver Tissue**—After ischemic periods of increasing duration, the affected lobes were removed, weighed, and homogenized, and the homogenate fractionated into a postmitochondrial supernatant (microsomes and cytosol) and a pellet containing tissue debris, unbroken cells, nuclei, mitochondria, and plasma membranes. Table 1 shows that at all times the total protein content of the whole homogenates and of the two subcellular fractions was not altered, while there was a slight increase in the weights of the ischemic tissue. Recovery of total protein was 90 to 100%. In sharp contrast, Fig. 1 shows that the phospholipid content of whole homogenates decreased noticeably with increasing duration of ischemia. With 3 h of ischemia there was a 40 to 50% loss of total phospholipids. Fig. 2 shows a similar loss from the PMS fractions isolated from the same homogenates. Table 1 shows the total amount of phospholipid in each of the two fractions and in the original homogenates. Recovery was 80 to 90% in each case, and there is no indication of redistributing the lipids from the PMS to the pellets fractions. Table I also shows the weight, total protein, and phospholipid contents of liver tissue pretreated with chlorpromazine and made ischemic for 3 h. Table II summarizes these data on the effect of chlorpromazine pretreatment. The data in Tables I and II clearly indicate that there is virtually complete protection from the depletion of phospholipids as a result of chlorpromazine pretreatment.

In order to assess the extent of the loss of each major species of phospholipid, these lipids isolated from whole liver homogenates and postmitochondrial supernatants from sham-operated and ischemic livers and livers made ischemic for 3 h were fractionated by thin layer chromatography. Table III shows that each of the major species with possible exception of phosphatidylserine or inositol is reduced. Loss, however, of phosphatidylcholine and phosphatidylethanolamine accounts for the major loss of phospholipid. The nature of the phosphatidylcholine-containing material that remains at the origin in the isoelectric cases is not clear. That it is not lysophosphatidylcholine or lysophosphatidylethanolamine was demonstrated by the chromatography of purified standards under these same conditions. Ischemia, then produces a progressive loss of phospholipids from liver cells that affects predominantly phosphatidylcholine.
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Table 1

<table>
<thead>
<tr>
<th>Weight of ischemic lobes (g)</th>
<th>0 (sham)</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>Chlorpromazine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight of ischemic lobes (g)</td>
<td>3.83 ± 0.15</td>
<td>3.73 ± 0.26</td>
<td>4.17 ± 0.45</td>
<td>4.53 ± 0.55</td>
<td>4.44 ± 0.44</td>
</tr>
<tr>
<td>Protein content (mg)</td>
<td>712.6 ± 39.7</td>
<td>658.9 ± 36.2</td>
<td>757.7 ± 48.0</td>
<td>766.9 ± 84.4</td>
<td>704.5 ± 88.4</td>
</tr>
<tr>
<td>a. Whole homogenate</td>
<td>342.2 ± 24.4</td>
<td>296.9 ± 7.7</td>
<td>339.7 ± 5.5</td>
<td>346.6 ± 38.9</td>
<td>356.1 ± 57.7</td>
</tr>
<tr>
<td>c. PMS</td>
<td>766.29 ± 32.9</td>
<td>371.0 ± 36.0</td>
<td>413.3 ± 64.8</td>
<td>378.4 ± 73.1</td>
<td>305.5 ± 92.7</td>
</tr>
<tr>
<td>Phospholipid content (µmol PO₄⁻⁻)</td>
<td>143.4 ± 9.6</td>
<td>109.0 ± 11.1</td>
<td>115.7 ± 7.9</td>
<td>100.1 ± 10.2</td>
<td>95.2 ± 19.1</td>
</tr>
<tr>
<td>a. Whole homogenate</td>
<td>87.0 ± 4.6</td>
<td>61.1 ± 5.6</td>
<td>63.8 ± 5.5</td>
<td>59.2 ± 6.6</td>
<td>67.6 ± 13.9</td>
</tr>
<tr>
<td>c. PMS</td>
<td>43.59 ± 3.6</td>
<td>34.0 ± 6.1</td>
<td>30.8 ± 4.3</td>
<td>30.5 ± 6.1</td>
<td>24.2 ± 8.8</td>
</tr>
</tbody>
</table>

* Chlorpromazine (20 mg/kg) given 30 min before induction of ischemia for 3 h.

** All values are the mean ± S.D. of separate determinations on three animals.

From these data a half-life of 24 h for the total phospholipid was calculated. This compares closely to a value of approximately 29 h reported previously (11) for rat liver microsomal membrane total phospholipids. Since all of the phospholipid in a PMS is sedimented at 100,000 × g for 2 h (data not shown), these results indicate that phospholipid degradation can be measured in these supernatants as well as in the microsomal membranes obtained from them by centrifugation. Fig. 3 also shows the effect of ischemia on the degradation of PMS phospholipids. Liver cells labeled 48 h earlier with [³⁸C]glycerol were made ischemic for ½, 1, and 2 h, respectively (open circles). Ischemia produced a very rapid loss of specific radioactivity from the total phospholipids and from phosphatidylcholine and ethanolamine. In ischemic cells the half-life
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Prevention by chlorpromazine of phospholipid depletion induced by ischemia

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Treatment Phospholipid content</th>
<th>µmol PO₄⁻³/µg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Sham operated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>a. Whole homogenate</td>
<td>0.200 ± 0.010</td>
<td></td>
</tr>
<tr>
<td>b. PMS</td>
<td>0.120 ± 0.001</td>
<td></td>
</tr>
<tr>
<td>2. 3-h ischemia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>a. Whole homogenate</td>
<td>0.122 ± 0.016</td>
<td></td>
</tr>
<tr>
<td>b. PMS</td>
<td>0.037 ± 0.010</td>
<td></td>
</tr>
<tr>
<td>3. Chlorpromazine-3-h ischemia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>a. Whole homogenate</td>
<td>0.197 ± 0.032</td>
<td></td>
</tr>
<tr>
<td>b. PMS</td>
<td>0.100 ± 0.010</td>
<td></td>
</tr>
</tbody>
</table>

All values are the mean ± S.D. of separate determinations on three animals.

of these phospholipids is only 2 to 4 h. This agrees closely with the actual amount of phospholipid depletion in Figs. 1 and 2 that was determined by direct measurement. This would indicate that accelerated degradation is the major factor accounting for the phospholipid depletion associated with liver cell ischemia. Fig. 3 also shows the effect of chlorpromazine pretreatment on the rate of degradation of PMS phospholipids. The specific radioactivity after 2 h of ischemia to the chlorpromazine-pretreated animals is illustrated by the X's. The values for the total phospholipid, phosphatidylethanolamine, and ethanolamine fell on the control line indicating that chlorpromazine prevents the accelerated rate of degradation associated with ischemia. This agrees with the protection shown by the direct measurements of the phospholipid contents in Tables I and II.

Microsomal Membrane Function in Ischemic Liver Cells—The accelerated degradation of phospholipids produced by ischemia is associated with considerable evidence of membrane dysfunction. Periods of ischemia that result in irreversible liver cell injury produce loss of microsomal membrane calcium pump and glucose-6-phosphatase activities (2). When the blood supply is returned to liver cells irreversibly injured by ischemia, there is rapid accumulation of Ca²⁺ (3). Since these cells normally maintain a considerable gradient of Ca²⁺ across their plasma membranes, the simplest explanation of this accumulation is a breakdown of the permeability barrier to Ca²⁺. It became important, therefore, to examine the permeability properties of our model membranes at a time when their phospholipid content had been significantly depleted by periods of irreversible ischemia.

Fig. 4 compares the Ca²⁺ permeability of microsomal membranes from sham-operated and 2-h ischemic livers. The rate of Ca²⁺ efflux from microsomal vesicles preloaded with Ca²⁺ concentrations from 10⁻⁶ to 10⁻³ M was determined and the log of this efflux plotted against the log of the Ca²⁺ concentration.

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TABLE III

<table>
<thead>
<tr>
<th>Phospholipid</th>
<th>Sham homogenate</th>
<th>Sham PMS</th>
<th>Ischemic homogenate</th>
<th>Ischemic PMS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Phosphatidylethanolamine</td>
<td>0.069 (32)</td>
<td>0.026 (31)</td>
<td>0.029 (22)</td>
<td>0.012 (25)</td>
</tr>
<tr>
<td>2. Phosphatidylcholine</td>
<td>0.079 (43)</td>
<td>0.041 (48)</td>
<td>0.039 (30)</td>
<td>0.018 (30)</td>
</tr>
<tr>
<td>3. Phosphatidyserine and phosphatidylinositol</td>
<td>0.019 (10)</td>
<td>0.007 (8)</td>
<td>0.022 (16)</td>
<td>0.0065 (13)</td>
</tr>
<tr>
<td>4. Sphingomyelin</td>
<td>0.027 (15)</td>
<td>0.011 (13)</td>
<td>0.022 (16)</td>
<td>0.0074 (15)</td>
</tr>
<tr>
<td>5. Origin</td>
<td>0.189</td>
<td>0.085</td>
<td>0.132</td>
<td>0.0486</td>
</tr>
</tbody>
</table>

* All values are the average of two separate determinations.

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Fig. 3. The effect of ischemia on the rate of degradation of microsomal phospholipids. All animals were given an intraperitoneal injection of 40 µCi of [¹⁴C]glycerol at 0 h. The control animals (●) were killed at 24, 48, and 72 h and the specific radioactivity (cpm/mg of PMS protein) of the sum of each of the individual phospholipid activities (A) and of phosphatidylcholine (B) and phosphatidylethanolamine (C) determined as described under "Materials and Methods." Another group of labeled animals were made ischemic at 48 h for 1, 1, or 2 h (○). Total, phosphatidylcholine, and ethanolamine specific radioactivities were determined as for the control animals. A third group of animals were pretreated with chlorpromazine (20 mg/kg) 30 min before induction of liver ischemia for 2 h (×'s). All values are the average of separate determinations on two animals.
indicates that there is no change in the permeability of ischemic or [4C]sucrose did not change the permeability of either permeability is not a consequence of some action of Ca2+ on the ischemic membranes during the loading incubation. This was confirmed by the absence of any change in the lipid or result of the 18-h incubation with or without Ca2+.

Ca2+ upon reflow. Table IV shows that there is no increase in measure the Ca2+ permeability of microsomes isolated from ischemic membranes indicating that increased Ca2' production no change in glucose-6-phosphatase (15). There was no change in the protein content of our microsomes as a result of the 18-h incubation with or without Ca2+.

Liver cells made ischemic for only ½ h do not accumulate Ca2+ upon reflow. Table IV shows that there is no increase in the permeability to Ca2' of microsomal membranes isolated from ½-h ischemic livers. Another important control was to measure the Ca2+ permeability of microsomes isolated from chlorpromazine-pretreated livers. Chlorpromazine treatment alone (without any ischemia) did not change the Ca2+ permeability, and Table IV shows that chlorpromazine pretreatment prevents the increases in Ca2+ permeability associated with 2 h of ischemia.

It was argued at this point that if the change in Ca2+ permeability of ischemic microsomes is related to the depletion of phospholipids, then it should be possible to restore a normal Ca2+ permeability by adding back phospholipid to the depleted membranes. Microsomal membranes from 3 h ischemic livers were incubated for 18 h at 4°C with 4Ca2+ at 3.3 X 10-5 M and soybean lecithin micelles prepared as described under "Materials and Methods." Table V shows the rate of Ca2+ efflux from these microsomes as well as from the appropriate controls. Incubating the ischemic microsomes with soybean lecithin produced a 6.5-fold reduction in the rate of Ca2+ efflux to a value the same as sham-operated microsomes incubated with the same phospholipid. The calcium efflux from the sham microsomes was reduced only slightly (25% at best) by the incubation with the soybean lecithin. These results were obtained at a soybean lecithin to microsomal phospholipid ratio of 2:1. Some reconstitution of membrane Ca2+ permeability was obtained with this ratio as low as 1:4. This would suggest that the results cannot be explained by the fusion of the microsomes with the liposomes. That the lecithin micelles themselves did not contribute to the measured Ca2+ efflux other than by interaction with the microsomal membrane is also indicated by the fact that the zero time radioactivity of the control and ischemic microsomes was not significantly different when microsomes were incubated with or without phospholipid. Pure phospholipid vesicles are very impermeable to Ca2+ (13), and whatever 45CaCl2 may have been released with a time course considerably slower than that of the microsomal membrane vesicles. The fact, therefore, that the Ca2+ efflux of control membranes was reduced only slightly would imply that the phospholipid micelles were not contributing significantly to the measured efflux.

Phospholipids will restore the permeability of ischemic microsomal membrane to control values. An opposite result would be to increase the permeability of control microsomes by depleting them of phospholipids. This can be done with phospholipases. There have been several previous studies of the effects of treating rat liver microsomes with phospholipases (reviewed in Ref. 14). Our aim was to compare the pattern of functional impairment produced by such treatments with that produced by ischemia. Can phospholipid depletion account for all the functional changes associated with ischemic microsomal membrane injury? Normal microsomes were treated with N. naja phospholipase A2. Table VI shows the effect on the phospholipid content and on the three functional parameters altered by ischemia. A 30% depletion of phospholipids was associated with inhibition of calcium pump and glucose-6-phosphatase activities and an increase in the Ca2+ permeability. Contamination of the added phospholipase by proteases cannot account for these results. Treatment of rat liver microsomes with trypsin, pronase, or chymotrypsin sufficient to reduce the protein content by 40% produced no change in glucose-6-phosphatase (15). There was no change in the protein content of our microsomes as a result of the incubation with lipase preparation. Furthermore, a normal Ca2+ permeability was reconstituted by adding back phospholipids to the lipase-treated microsomes in a manner similar to that described in Table V. The pattern of change in the individual phospholipid species produced by the treatment with N. naja phospholipase A2 differed in two ways from that with ischemia, which may render comparison difficult. There

![Graph](http://www.jbc.org/)

**Fig. 4.** The Ca2+ permeability of microsomes prepared from sham-operated and 2-h ischemic livers. Microsomes were prepared from sham-operated (---) and 2-h ischemic livers (----) as described under "Materials and Methods." The microsomes were equilibrated with 4CaCl2 at the concentrations indicated by an 18-h incubation at 4°C in the presence of 100 mM KCl, 10 mM histidine/imidazole buffer, pH 7.4. The release of 4Ca2+ was measured by dilution into Ca2+-free buffer at 25°C as described under "Materials and Methods." The results are the mean ± S.D. of separate preparations from three animals.
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**FIG. 5.** The Na⁺, choline⁺, and sucrose permeability of microsomes prepared from sham-operated and 3-h ischemic livers. Microsomes were prepared from sham-operated (○) and 3-h ischemic livers (□) as described under "Materials and Methods." The microsomes were equilibrated with either [³²P]NaCl, [³H]choline chloride, or [¹⁴C]sucrose at different concentrations by an 18-h incubation at 4°C in the presence of 100 mM KCl, 10 mM histidine/imidazole buffer, pH 7.4. The release of either [³²P]Na, [³H]choline, or [¹⁴C]sucrose was measured by dilution into sodium, choline, or sucrose-free buffer at 25°C as described under "Materials and Methods."

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**TABLE IV**
Calcium permeability of microsomes prepared from one-half hour and chlorpromazine-pretreated ischemic livers

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Calcium efflux from microsomes preloaded with Ca²⁺ – 1.0 × 10⁻⁶ M (nmol Ca²⁺/mg protein/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Sham-operated</td>
<td>0.206 ± 0.059</td>
</tr>
<tr>
<td>2. 1-h ischemia</td>
<td>0.437 ± 0.202</td>
</tr>
<tr>
<td>3. 2-h ischemia</td>
<td>1.125 ± 0.229</td>
</tr>
<tr>
<td>4. Chlorpromazine-pretreated 2-h ischemia</td>
<td>0.275 ± 0.053</td>
</tr>
</tbody>
</table>

*All values are the mean ± S.D. of separate determinations on three animals.

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**TABLE V**
Effect of added phospholipid on calcium permeability of ischemic microsomes

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Calcium efflux from microsomes preloaded with Ca²⁺ – 3.3 × 10⁻⁶ M (nmol Ca²⁺/mg protein/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minus phospholipid</td>
<td>0.145</td>
</tr>
<tr>
<td>Plus phospholipid</td>
<td>0.110</td>
</tr>
</tbody>
</table>

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**TABLE VI**
Effect of *N. naja* phospholipase A₂ on rat liver microsomal membranes

Ten units of *N. naja* phospholipase A₂ (1,000 units/mg) were incubated with 3.0 mg of (protein) microsomes in a final volume of 2.0 ml at 4°C for 20 min. The reaction was stopped by addition of 1.0 ml of 10 mM EGTA. The microsomes were reisolated by centrifugation and assayed.

<table>
<thead>
<tr>
<th>Function</th>
<th>Per cent change</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Total phospholipids</td>
<td>30% decrease</td>
</tr>
<tr>
<td>2. Glucose-6-phosphatase</td>
<td>42% decrease</td>
</tr>
<tr>
<td>3. Calcium pump activity</td>
<td>70% decrease</td>
</tr>
<tr>
<td>4. Calcium permeability</td>
<td>250% increase</td>
</tr>
</tbody>
</table>

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There was a greater loss of phosphatidylethanolamine than choline and, potentially more important, there was accumulation of lysophospholipids despite the presence of bovine serum albumin. Lysophospholipids are known to have effects on membrane structure and function, and it is conceivable that the inhibitions of calcium pump and glucose-6-phosphatase activities were related more to the accumulation of lysophospholipids than to the depletion of phospholipids per se. To test this possibility lysolecithin was added to the calcium pump and glucose-6-phosphatase assays. With lysolecithin concentrations equivalent to those calculated to be present as a result of the *N. naja* phospholipase treatment, there was no significant inhibition of either activity. With 3 times this concentration there was less than 10% inhibition of both activities. This indicates that the inhibitions of calcium pump and glucose-6-phosphatase activities produced by the phospholipase treatment are the result of the lipid depletion per se and not by the accumulation of lysophospholipids. It would seem, therefore, that removing phospholipids from normal rat liver microsomes produces a similar pattern of functional alterations as that associated with the in vivo depletion secondary to ischemia.

**The Structure of Ischemic Microsomes**—In attempting to account for how phospholipid depletion specifically produces a change in Ca²⁺ permeability knowledge of the structure of ischemic microsomes would be essential. In particular, since the Ca²⁺ permeability of phospholipid vesicles is influenced by the incorporation of membrane proteins (13), it would be important to know the distribution of the intramembranous proteins within the phospholipid bilayers of the microsomal membrane. Intramembranous proteins can be visualized as intramembranous particles and variations in the distribution of these particles in the lateral plane of the membrane appreciated by the technique of freeze-fracture electron microscopy. Fig. 6 compares the morphology as revealed by this technique of normal and ischemic liver microsomes. The structure of the normal microsomes is similar to that reported previously (16) and is characterized by concave particle-rich vesicles (P-face) and convex ones with a lower particle density (E-face). The membrane-associated particles in the P-faces of the microsomal vesicles are randomly distributed. The structure of the ischemic microsomes differs in at least three ways. There are fewer particles visible in the P-faces. In other areas of the P-faces there is aggregation of the intramembranous particles. The ischemic microsomes show significantly bare areas in the P-faces devoid of particles.
DISCUSSION

Liver ischemia produces a progressive depletion of cellular phospholipids secondary to an accelerated rate of degradation. This disturbance in phospholipid metabolism conforms to the two criteria outlined in the introduction that any biochemical change must obey in order to be a potentially causal factor in ischemic cell death. First, the time course of the phospholipid depletion parallels quite closely that of the loss of reversibility of ischemic liver cell injury. While ½ h of ischemia does produce a slight decrease in the phospholipid content of whole liver homogenates and of postmitochondrial supernatants (Figs. 1 and 2) the extent of this loss does not seem to be sufficient to produce much of a change in either the enzymatic

Fig. 6. Electron microscopy of freeze-fracture replicas of sham-operated or 2-h ischemic microsomes. Sham-operated (A) and 2-h ischemic microsomes (B) were prepared, fixed, infiltrated with glycerol, freeze-fractured, replicated, and prepared for electron microscopy as described under "Materials and Methods." Inner fracture faces are designated P and outer fracture faces E. × 60,000.
activity (2) or the Ca\(^{2+}\) permeability (Table IV) of isolated microsomal membranes. The liver cell injury associated with \(\frac{1}{2}\) h ischemia is completely reversible. In contrast, 2 to 3 h of ischemia produces much more significant phospholipid depletion which is now associated with considerable evidence of membrane dysfunction. The liver cell injury associated with 2 to 3 h of ischemia is not reversible. Second, the changes in phospholipid metabolism are prevented by pretreating the rats with chlorpromazine (Table II). Chlorpromazine prevents the liver cell death produced by as much as 3 h of ischemia (3).

This accelerated degradation of phospholipids is very likely the result of the activation of endogenous phospholipases. In liver cells there are two sources of potentially quite active phospholipases, the lysosome and the plasma and microsomal membranes themselves. Lysosomal phospholipases could be activated by a decreased cellular pH accompanying liver cell ischemia. While this remains a very distinct possibility, there was no change in the phospholipid content of mitochondria isolated from liver cells ischemic for as long as 3 h. This would tend to argue against the release of lysosomal enzymes with the random digestion of intracellular membranes as the explanation of our results. We tend to favor the activation of phospholipases in the endoplasmic reticulum and plasma membrane themselves. Virtually all membrane systems that have been isolated from liver cells have been shown to contain phospholipase activity (17), and there now appears to be some general agreement on the presence of an alkaline phospholipase A\(_1\) in rat liver microsomes (18, 19) and plasma membranes (19, 20). The latter organelle also seems to contain an additional phospholipase A\(_2\) activity (21, 22). Both enzymes require Ca\(^{2+}\) with optimal activity from 0.5 to 2.0 mM. Although the physiological role of these enzymes is not completely clear, they presumably function in the dynamic degradation of membrane phosphoglycerides. More relevant to our concern is the fact that the identification and preliminary characterization of these enzymes have indicated that liver cell microsomes and plasma membranes contain endogenous phospholipases that exist normally under conditions considerably less than optimal for their maximal activity because of low intracellular Ca\(^{2+}\) levels (23) and alkaline pH optima. We have shown previously that the total liver cell Ca\(^{2+}\) content increases with ischemia (3). Because of the reduced O\(_2\) tension, there is no accumulation of this increased Ca\(^{2+}\) in the mitochondria (3) and probably no sequestration in the endoplasmic reticulum (25), because of the low ATP levels. Most of the increased Ca\(^{2+}\) is, therefore, probably confined to the cytosol and should lead to activation of the phospholipases on the microsomes and plasma membranes. Incubation of normal liver microsomes in vitro with excess Ca\(^{2+}\) produces a progressive loss of phospholipid with a time course in hours similar to that with in vivo ischemia and associated with inhibition of glucose-6-phosphatase and calcium pump activities and an increased permeability to Ca\(^{2+}\). Omission of Ca\(^{2+}\) prevents these changes. Such an activation of endogenous phospholipases by Ca\(^{2+}\) would readily explain the prevention by chlorpromazine. Chlorpromazine is known to have multiple effects on membrane structure and function (24, 26, 27) and is a well established Ca\(^{2+}\) ion-blocking agent producing inhibition of Ca\(^{2+}\) fluxes in nerve, muscle, and epithelial cells (28-30). Pretreatment with chlorpromazine prevents the increases in liver cell Ca\(^{2+}\) associated with ischemia (3). In addition, chlorpromazine is a potent inhibitor of phospholipases including membrane-bound enzymes (31, 32). Incubation of normal microsomes with Ca\(^{2+}\) in the presence of chlorpromazine prevents the loss of phospholipids. 2

The phospholipid depletion produced by ischemia is not accompanied by an accumulation of lyso phospholipids (Table III) in contrast to treatment of normal rat liver microsomes with a known phospholipase. Lyso phospholipase activity in rat liver cells is found in the cytosol and bound to microsomal membranes (33-35). The relatively slow generation of lyso phospholipids as a consequence of the activation of endogenous, membrane-bound phospholipases in ischemic liver cells is different from their more rapid production in vitro at 4°C by N. naja phospholipase. The former situation presumably allows further decaying by the endogenous lyso phospholipases, while the latter conditions are not favorable for such a similar result.

The loss of phospholipids is accompanied by a number of specific structural and functional alterations in microosomal membranes isolated from the ischemic liver cells. One of the major objectives of the present study was to relate these changes in the microsomal membranes to the depletion of membrane phospholipids.

An increasing number of membrane-bound enzymes have been shown to require lipid for their correct functioning (reviewed in Ref. 36). This information has been obtained from studies of the effects of detergents, solvents, or phospholipases directly upon intact membranes or incidental to the solubilization and resolution of enzymes by these same agents. There is precedence for the dependence of both the active membrane transport of Ca\(^{2+}\) and membrane-associated glucose-6-phosphatase activity on phospholipids. Treatment of skeletal muscle microsomes with phospholipase C inhibits the adenosine triphosphatase activity and calcium transport in parallel with the hydrolysis of membrane phosphatidylcholine (37). This is analogous to the effect of ischemia on liver microsomal membrane calcium transport described above. Glucose 6-phosphatase is a complex, multifunctional microsomal enzyme that has long been known to be affected by treatment of microsomes with phospholipases (38-47). However, inhibition of rat liver microsomal calcium transport and an increased passive Ca\(^{2+}\) permeability have not previously been reported with phospholipase treatment of liver microsomes. That the loss of calcium transport and glucose-6-phosphatase activities produced by phospholipase A\(_2\) is not a consequence of the accumulation of lysospholipids rather than the loss of phospholipids per se was suggested by the inability to reproduce a similar degree of inhibition by adding lysolecithin to control microsomes. That the increased Ca\(^{2+}\) permeability is also a direct consequence of the loss of phospholipid is suggested by the ability to restore a normal Ca\(^{2+}\) permeability by adding phosphatidylcholine to ischemic microsomal membranes in vitro (Table V).

The structural basis for this increased Ca\(^{2+}\) permeability is of particular concern, since the repuffusion of irreversibly injured, ischemic liver cells is characterized by a rapid and marked increase in their Ca\(^{2+}\) content (3). Cells made ischemic for times that do not irreversibly injure exhibit no such changes. This inability to maintain a normal intracellular Ca\(^{2+}\) content may be the critical factor in the accelerated metabolic deterioration and death produced by repuffusion (6). Calcium ions are biologically very active. When present in excess they inhibit the function of mitochondria, alter the properties of cellular membranes and inhibit the activity of a variety of enzymes.

The increased Ca\(^{2+}\) permeability of ischemic liver cell microsomes is accompanied by several alterations in their structure as revealed by the technique of freeze-fracture electron microscopy (Fig. 6). There seems to be both a reduction in the number of intramembranous particles as well as a reor-
TABLE VII
Pathogenesis of ischemic liver cell death

1. Ischemia
2. Reduced O2 tension in liver cell’s environment
3. Reversible alteration in the permeability properties of plasma membrane
4. Influx of Ca2+ ions
5. Inability of mitochondria and microsomes to sequester excess calcium
6. Increased cytosolic free calcium concentration
7. Activation of endogenous, membrane-bound phospholipase
8. Progressive depletion of membrane phospholipids
9. Creation of calcium channels
10. Massive influx of calcium upon reflow
11. Inactivation of mitochondria, inhibition of enzymes, and denaturation of structural proteins
12. Cell death

organization in their distribution in the lateral plane characterized by aggregation. These two changes produce larger than normal bare areas of the membrane devoid of particles. It is unlikely that these bare areas are the site of increased Ca2+ permeability, since the principal barrier against the movement of Ca2+ across biological membranes seems to be provided by the membrane phospholipids (13, 48). It is much more likely that the structural basis of the increased Ca2+ permeability is related to altered lipid-protein interactions secondary to the depletion of membrane phospholipids. The permeability change may arise from a readjustment of the structure of the lipid phase in the environment of the protein or could represent a specific property of the aggregated proteins.

All of the above considerations would lead us to suggest the following tentative hypothesis for the sequence of cellular events associated with development of irreversible ischemic liver cell injury (Table VII). The initial cellular reaction to ischemia is probably an increase in the cell Ca2+ content as a result of some reactive change in the rate of Ca2+ influx and of a compromise in the energy-dependent ability to actively extrude Ca2+ from the cell. The changes producing this increased Ca2+ content probably occur very rapidly and are potentially reversible. However, the build up of Ca2+ is rather slow as a result of the limited quantities of Ca2+ available, because of the decreased blood flow. Eventually the elevated cytosolic Ca2+ activates the endogenous phospholipases bound to the microsomal and plasma membranes. Calcium channels are created in these membranes as a result of this phospholipid loss. Upon reperfusion of the liver cells there is a massive influx of Ca2+ through these channels driven by the large Ca2+ gradient across the plasma membranes. With the availability of O2, the increased Ca2+ is now taken up by the mitochondria. Inactivation of the mitochondria, inhibition of cellular enzymes, and disruption of cellular structural proteins are consequences of the rising liver cell Ca2+ content. A state of disequilibrium exists with progressive increases in total liver cell Ca2+ that is characteristic of irreversible ischemic injury and then cell death.

REFERENCES
Accelerated phospholipid degradation and associated membrane dysfunction in irreversible, ischemic liver cell injury.
K R Chien, J Abrams, A Serroni, J T Martin and J L Farber