Effects of Carbon Tetrachloride on Rat Liver Plasmalemmal Calcium Adenosine Triphosphatase

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Calcium adenosine triphosphatase (Ca-ATPase) activity in rat liver plasma membrane fractions prepared by zonal centrifugation was studied for sensitivity to carbon tetrachloride (CCl₄). Levels of Ca-ATPase activity in such membrane fractions from animals given CCl₄ by gastric intubation were no different from those in plasmalemmal fractions obtained from control rats. When the fractions were incubated *in vitro*, however, this enzyme activity was inhibited by the presence of CCl₄ in a dose-dependent manner. Moreover, inhibition of Ca-ATPase could be reversed by the removal of CCl₄. These results would explain the observed increase in hepatic calcium content following the administration of CCl₄ if the Ca-ATPase were capable of actively extruding calcium and if this extrusion mechanism proved sensitive to CCl₄ present on or about the hepatocytes. (Am J Pathol 90:145–158, 1978)

ADMINISTERING CARBON TETRACHLORIDE (CCL₄) to rats results in a number of metabolic alterations, eg, increased lipid deposition,[1] a reduced rate of amino acid utilization,[2] a decrease in membranous phospholipid exchange,[3] modified mitochondrial function, and a depression in cellular levels of adenosine triphosphate (ATP).[4-6] The relationship of these changes to each other and to cell survival or death is not clear. Exposure to CCl₄ also results in an early and later sustained increase in hepatic calcium content. Adsorption of this divalent metal occurs in two stages: a) reversible influx which reaches a maximum 1 hour after CCl₄ administration (this condition resolves within 3 hours and is not associated with changes in magnesium, sodium, and potassium levels) and b) a later influx of calcium that continues to rise until approximately 30 hours after administration of the haloalkane.[6-8] Some investigators[5,6,9] have postulated that this late calcium influx reflects a passive accumulation of the ion in dead cells, but, heretofore, the early calcium inflow has been unexplained. The present experiments were designed to explore the potential modification of divalent-metal–activated adenosine triphosphatase (ATPase) during the early phase of CCl₄ intoxication and to suggest a role for this system in calcium homeostasis.

Materials and Methods

Animal Manipulation

Pathogen-free male Sprague-Dawley rats (Charles River Breeding Labs, Inc., Wilmington, Mass, or Tyler Co., Inc., Redmond, Wash.), weighing 150 to 250 g, were maintained in our animal quarters for 1 week before use in these experiments. They were given Purina lab chow and water *ad libitum* until late afternoon (5 PM) of the day before experimentation. During the subsequent period of fasting, they were allowed only water.

CCl₄ was administered by stomach tube without anesthesia. A 50% solution of CCl₄ in mineral oil in a volume equivalent to 0.25 ml CCl₄/100 g body weight was given to the treated animals. Control animals received an equal volume of mineral oil. Following intubation, only water was permitted. The animals were killed by cervical dislocation at time intervals indicated in the Text-figure legends. For each experiment, we used 6 to 8 rats, and the livers were pooled for isolation of plasma membranes. Each experiment was repeated at least twice and, more regularly, three or four times.

Preparation of Plasma Membranes

Plasma membranes were prepared by a modification of Evans' procedure.[10] The livers were perfused *in situ* via the thoracic aorta with ice-cold 0.9% NaCl until they were pale (blood-free). The organs were then removed, weighed in tared beakers with 5 mM NaHCO₃, and homogenized in 8 volumes of this same solution with a Dounce glass-pestled homogenizer. Four passes of the loose pestle were followed by four passes of the tighter ball. The resultant brei was passed through a nylon filter (400 mesh) and centrifuged at 1000 × g for 10 minutes at 0 to 4 C. The pellet was subsequently resuspended in ice-cold 5 mM NaHCO₃ and layered onto a discontinuous 6 to 54% sucrose gradient in 5 mM NaHCO₃ as described by Evans.[10] Zonal centrifugation was performed at 5 C with a type-"A-XII" rotor in an International PR 2 centrifuge for 60 minutes at 4000 rpm.

The gradient was separated while the rotor was spinning by pumping 60% sucrose into the outer zone; 5- to 10-ml fractions were collected and analyzed as follows: Quantitative assays of 5'-nucleotides, glucose-6-phosphatase, and acid phosphatase (AcPase) were accomplished as described by Evans,[10] except that 50 mM paranitrophenylphosphate or 50 mM β-glycerophosphate was employed as a substrate in the AcPase assay. In all cases, the liberation of phosphate was linear over incubation periods of 15 to 60 minutes. Succinate dehydrogenase (SDH) activity was measured as previously described,[11] and results of the enzyme assays were converted to specific activity utilizing protein concentrations as determined by the Lowry technique.[12] Assay of the sucrose gradient concentration was performed with index-of-refraction measurements obtained with a Bausch and Lomb refractometer at room temperature. Standards of sucrose concentrations were prepared in 5 mM NaHCO₃.

Electron Microscopy

Aliquots of gradient fractions were fixed for 2 hours at 0 C in 2% osmium tetroxide in 0.2 M cacodylate buffer, pH 7.4. The suspensions were centrifuged at 1000 × g and the resulting pellets were dehydrated in alcohols and embedded in Epon epoxy resin with propylene oxide as an intermediate solvent. For electron microscopic observation, the pellets were sectioned parallel to the axis of rotation. Sections 200 to 500 Å in width were stained with uranyl acetate and lead hydroxide and examined in an AEI 6B electron microscope.

ATPase Assays

Plasma membrane fractions were pooled and assayed directly, stored at 0 C, or repelleted at 105,000 × g for 1 hour in a Beckman 60 Ti rotor. The pellets were resuspended in 5 mM NaHCO₃, and protein concentrations were then assayed.

Calcium adenosine triphosphatase (Ca-ATPase) assays were conducted with incubation solutions containing 50 mM Tris-H_2SO_4 buffer (pH 7.8), 1 mM ouabain, 5 mM Tris-ATP (pH 7.8), and varying concentrations of $CaCl_2$ and/or CCl_4 as described in *Results*.* We measured Ca+Mg-ATPase activities in solutions of the above composition but also containing 5 mM $MgSO_4$. The addition of up to 1 mM Na-EDTA to these solutions did not affect the results except in the shifting of the curves along the $CaCl_2$ axes by degrees proportional to the EDTA concentrations. The liberated phosphate levels were determined as previously described.[14]
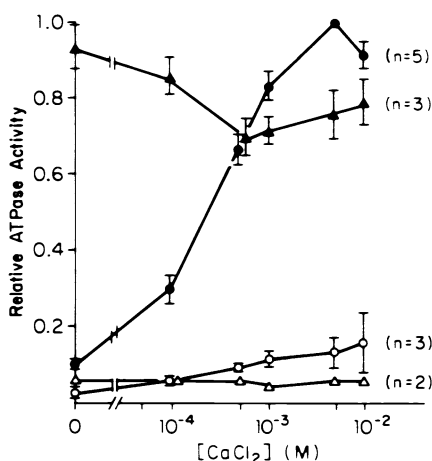
Results

Results of zonal centrifugation (1000 × *g*) of the crude hepatic plasma-lemmal preparations obtained from control animals are recorded in Text-figure 1. We·observed four distinct bands that separated after centrifugation at 4000 rpm for 1 hour. The innermost band contained high levels of all the enzymes tested and consisted of loaded material which had not migrated into the gradient (Fractions 0 through 2, Text-figure 1). Electron microscopy confirmed this assumption (Figure 1). In contrast, the second band was comprised of mitochondria, as indicated by succinate dehydrogenase activity (Text-figure 1) and confirmed by electron microscopy (Fractions 5 through 12 in Text-figure 1 and Figure 2). (Monoamine oxidase activity was distributed with that of SDH but was omitted from Text-figure 1 for clarity.) Mitochondrial architecture had been preserved, and we were able to identify some cristal space swelling. Scattered among these organelles were single-membrane–limited bodies with dense inner

TEXT-FIGURE 1—Distribution of hepatic plasmalemmal enzymic activities in the A-XII rotor following centrifugation. ○ = SDH; ● = 5'-AMPase; ▲ = glucose-6-phosphatase (G-6-Pase) and acid *p*-nitrophenylphosphatase (acPNPase); △ = Lowry protein values. Units: SDH, scale value × 10^5; 5'-AMPase, G-6-Pase, and acPNPase, × 100. The Lowry scale is shown at left. Plasma membranes accumulated at a sucrose density of 1.17 g ml. Fraction 18.
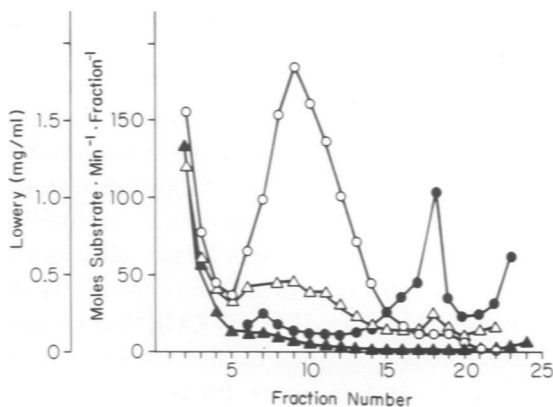
* The solubility of CCl_4 in H_2O is 1.3 μl ml or 5.2 mM. Solubility in the lipid-containing plasmalemmal suspensions was much greater. Incubations were accomplished in a total of 2 ml, previously equilibrated with the indicated quantities of CCl_4 and with minimal gas volumes, in screw-top vials. No undissolved CCl_4 was noted in any experiment. On the basis of direct chemical assay[1] and radioactive-labeling experiments,[13] the amount of CCl_4 in the liver at 1.5 hours with this dosage is 5 to 15 μl. In the *in vitro* experiments, 5 to 25 μl of CCl_4 was added to the plasma membrane fractions; this level is the same as 10 to 50 μl liver equivalent, in keeping with the observed *in vivo* concentrations.

TEXT-FIGURE 2—CCl$_4$ inhibition of liver Ca-ATPase and Ca+Mg-ATPase activities in the plasma membrane fraction. Increasing concentrations of CaCl$_2$ in the absence (●) of MgSO$_4$ (5 mM) and in its presence (▲) resulted in increased and decreased levels of ATPase activity, respectively. The addition of 25 μl CCl$_4$ 2.0 ml incubation solution inhibited both activities (○ in the absence of MgSO$_4$ and △ in its presence). The unit relative activity corresponds to a specific activity of 53.2 μmoles P$_i$ mg protein hour. The values shown are the means ± SEM, with the number of experiments in parentheses.

matrices, presumably microbodies. (Unpublished results intimated that urate oxidase activity was also concentrated in this band.) As demonstrated by 5'-adenosine monophosphatase (5'-AMPase) activity (Text-figure 1) and by electron microscopic examination (Figure 3), the plasma membrane fragments were concentrated in the third band from the hub (Fraction 18, Text-figure 1). Again, the outermost band contained high levels of all enzymatic activities, and electron microscopy disclosed that this band consisted of cellular debris (Fractions 21 and up). The activity of this material in respect to ATP in the presence and absence of divalent metal and CCl$_4$ was measured.

Text-figure 2 indicates that plasmalemmal Ca-ATPase activity increased with rising concentrations of CaCl$_2$ in the incubation medium and reached a peak at 5 mM of the compound (closed circles, Text-figure 2). On the contrary, Ca+Mg-ATPase activity was maximal in the presence of Mg^{2+} alone, and increasing concentrations of CaCl$_2$ were inhibitory. Both Ca-ATPase and Ca+Mg-ATPase activities were repressed by 5 to 25 μl CCl$_4$/2 ml solution during incubation (Text-figures 2 through 6). This is equivalent to 10 to 50 μl of CCl$_4$ liver (open circles, Text-figure 2).

Zonal centrifugation of the material prepared from rats treated with CCl$_4$ 2, 4, and 6 hours previously resulted in enzyme and organelle distribution identical to that derived from control-animal preparations. Compare Text-figures 1 and 3; note that the exact coincidence of fraction positions is related to sample size and individual gradient formation, but the density levels of position are identical. In both instances, plasma membranes were collected at a sucrose density of 1.17 g/ml. Also, the activation curve of the plasmalemmal Ca-ATPase activity remained unaf-

A Comparison of the Migration Patterns of Normal and Malignant Cells in Two Assay Systems

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The migration patterns of normal mouse embryo fibroblast (MEF) cells and mouse fibrosarcoma (FS) cells were compared in two assay systems. The two assay systems used were the modified Boyden chamber (micropore membrane) assay and the agarose drop explant assay. In both assays the major population of MEF cells exhibited a greater rate of migration than the major population of FS cells. However, a small subpopulation of FS cells which had a much greater rate of migration than the major population of either MEF or FS cells was detected in the agarose drop assay. A number of drugs which are known to inhibit the migration of leukocytes were tested against the MEF and FS cells. Concentrations were found that inhibited the major population of both groups by greater than 90%. However, at concentrations which inhibited the migration of the major population of FS cells by greater than 90%, a small group of fast-moving cells was still detected. Although the fast-moving cells were relatively resistant to treatment with the various drugs, this group was sensitive to a factor in serum. When normal human serum was used in place of fetal calf serum, the migration of the major population of FS cells was inhibited very little but movement of the fast-moving population was completely eliminated. We speculate that the small subgroup of fast-moving cells may be responsible for the invasive nature of the FS cells. (Am J Pathol 90:159–172, 1978)

RECENT STUDIES IN THIS LABORATORY have indicated that cells from several different tumors are capable of chemotactic responses to a cleavage product of the fifth component of complement.[1-3] The Walker carcinosarcoma cell, the murine mastocytoma cell, and the Novikoff hepatoma cell were used in this work. In extending these studies to other cell types, including a methylcholanthrene-induced fibrosarcoma of mice and normal mouse embryo fibroblasts, we observed significant differences between the migratory behavior of the normal and neoplastic cells in two different *in vitro* assay systems. This report describes the two assay systems used and the migratory behavior of the two cell types.

Materials and Methods

Tumor Cells

The fibrosarcoma cells (FS) used in this study were obtained from a tumor in C57 bl/6 mice; the tumor was induced with 3-methylcholanthrene. The tumor cells were grown in
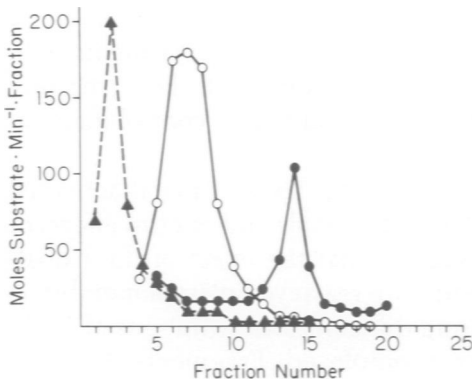
TEXT-FIGURE 3—Distribution of hepatic enzymic activities in the A-XII rotor following centrifugation of material prepared from plasma membranes of CCl₄-treated rats. ○ = SDH: ● = 5'-AMPase: ▲ = G-6-Pase and acPNPase. The scale is the same as in Text-figure 1. Because of variations in sample size, the enzymic peaks appear shifted toward the origin when compared with those in Text-figure 1. However, the sucrose density of the plasma membrane fraction was still 1.17 g ml. Two other experiments resulted in plasma membrane peaks in Fractions 17 and 18, but the sucrose density was the same in all of these preparations.

Correlation With *In Vivo* Observations of CCl₄ Intoxication

Hepatic accumulation of CCl₄ is maximal approximately 1.0 to 1.5 hours after administration and then falls to control levels. In the blood, CCl₄ concentration reaches peak level 1 hour after administration.[11] Liver calcium content also becomes maximal at 1 hour after administration of the agent.[2,15] This temporal correlation between hepatic concentrations of CCl₄ and calcium has prompted a number of investigations of the effects of CCl₄ on several liver organelles which are capable of accumulating calcium, eg, mitochondria, microsomes, and lysosomes.[4,16-18] Whereas these structures may be important to the regulation of intracellular ionic calcium concentration, they are incapable of direct responsibility for the transmembrane calcium influx essential to the increase in intracellular calcium content.
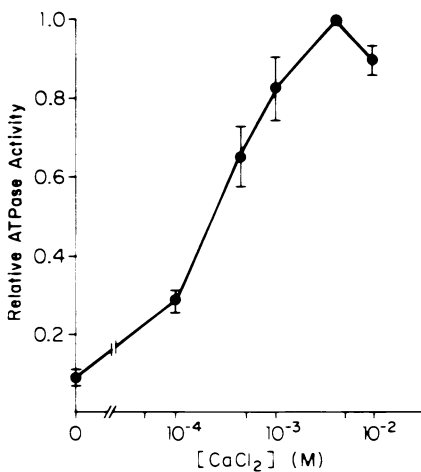

TEXT-FIGURE 4—Calcium activation curve of the Ca-ATPase activity in plasma membrane fragments prepared from the livers of CCl₄-treated rats. The values given are the means ± SEM for three replicate experiments. The unit activity corresponds to a specific activity of 59.4 μmoles P_i mg protein hour. Compare this activation with that in Text-figure 2.

TEXT-FIGURE 5—Reversal of CCl₄ inhibition of Ca-ATPase activity. Hepatic plasmalemmal preparations were preincubated for 15 (or 30) minutes with the various indicated concentrations of CCl₄ at 37 C. The sample was divided into equal portions. One half (●) was maintained at 37 C. while the second half (○) was kept at 37 C and bubbled with air for 30 minutes. Both preparations were then assayed for Ca-ATPase activity. See text for further details. The values given are the means from two experiments. The unit ATPase activity was 46.4 μmoles Pᵢ/mg protein/hour.
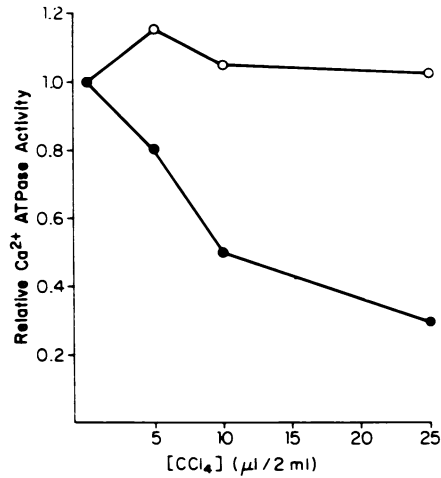
Earlier workers have also examined the effects of CCl₄ on the electrical properties of the membrane surface as well as on some of its enzymes. Alterations in Na+K-ATPase and Mg-ATPase activities have been described [3,19] and changes in 5'-nucleotidase activity or lack of them have been recorded,[3,19] but the Ca-ATPase activity was not examined; these studies have not concerned the early period of reversible metal-ion change. Wands et al[5] assessed the effects of CCl₄ on membrane potential and discovered a progressive depolarization beginning approximately 4 hours after administration and reaching minimal values (of polarization) at 24 hours. Obviously, this contradicts the theory that a generalized

TEXT-FIGURE 6—Reversal of CCl₄ inhibition of Ca-ATPase activity in rat liver plasmalemmal preparations at different calcium concentrations following preincubation with varying concentrations of the haloalkane. The broken and unbroken lines represent the results with and without aeration, respectively. The experimental details are given in Text-figure 5. Solid circles represent the preparations maintained at 37 C without aeration; open circles represent preparations bubbled to drive off the CCl₄. The unit relative activity corresponds to a specific activity of 53.4 μmoles Pᵢ/mg protein/hour in this experiment. Similar results were obtained in one other duplicate experiment.
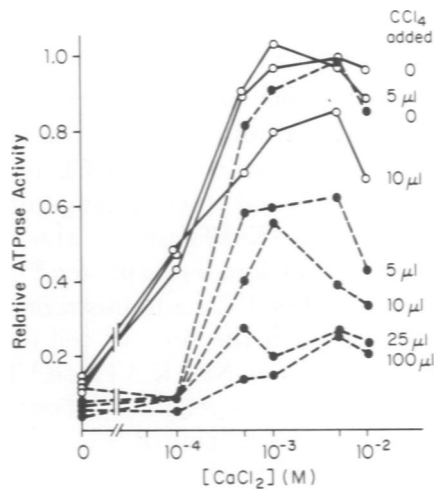
increase in membrane permeability is responsible for the calcium influx
which we noted 1 hour after administering the agent.

Our present results would explain the increase observed in hepatic
calcium content following CCl_4 administration *if* the plasmalemmal Ca-
ATPase could actively transport calcium out of the cells. We have estab-
lished that this particular Ca-ATPase is sensitive to CCl_4, as evidenced by
our *in vitro* findings. The increasing concentrations of CCl_4 in the blood-
stream and in the liver would inhibit the active efflux of calcium by
repressing Ca-ATPase, leading to an unbalanced, passive calcium influx
and accumulation within the cells—a situation consistent with the per-
ceived stability of other hepatic metals at the same time. Since this effect
of CCl_4 on liver Ca-ATPase is presumably completely reversible when
CCl_4 is cleared from the blood and the liver, the inhibition reverts, and
the accumulated calcium is extruded from the cells. Data supporting this
reversibility of Ca-ATPase inhibition are presented in Text-figures 5 and
6. The observations of Van Rossum [20] reveal that active calcium extrusion
at 37 C following its accumulation in the liver is kinetically consistent with
this concept. Furthermore, because of hepatic accumulation of the ele-
ment 1 hour after CCl_4 administration (when its level in the blood is
maximal) and because the majority of this calcium has been extruded by
1.5 hours after CCl_4 administration (when the level of the haloalkane is
maximal in the liver), we must also infer that the plasmalemmal Ca-
ATPase is sensitive to extracellular blood levels of CCl_4 and insensitive to
intracellular stores of the agent. This may be related to differences in
compartmentalization of the toxin, as well as a differentiation of this
membrane effect and other components of cell injury.

The Role of Na+K-ATPase

Two factors of our interpretation which conflict with the previous
literature warrant mention. The first concerns the role of Na+K-ATPase
in the hepatic response to CCl_4 administration. Data from *in vitro* experi-
ments by several other investigators indicate that CCl_4 additionally inhib-
its liver Na+K-ATPase,[3,18] which should logically result in decreased
cellular potassium levels *in vivo*.[20] Such a decrease is demonstrable only at
24 hours after CCl_4 administration [14] and not earlier.[2] This latter period
coincides with the time of cell depolarization [5] and the time of *in vivo*
effect of CCl_4 on Na+K-ATPase.[3] Thus, the concordance of these observa-
tions supports the reliability of our results and implies that CCl_4 inhibition
of hepatic plasmalemmal Na+K-ATPase is probably of lesser significance
during the early phase of calcium administration and plays a role late in

the injury when loss of selective permeability may be related to loss of cellular homeostasis.

Ca+Mg-ATPase: A Transport Enzyme?

The second point of contention is that hepatic Ca-ATPase has likewise been discounted as a transport enzyme on the basis that it apparently does not involve a phosphorylated intermediate,[21] yet it is thought to be responsible for the active transport of calcium in other tissues.[22] Both our observations and those of Chambaut et al [21] indicate that the rat liver plasma membrane possesses such an enzyme activity: Ca-ATPase exists. Van Rossum [20] declares that an active calcium efflux takes place in rat liver slices and that this transport is not of the calcium/sodium-exchange variety. The molecular mechanism of calcium transport in rat liver therefore requires further investigation, but a role for the Ca-ATPase still must be considered.

Evaluation of Mitochondrial Contamination

To ascertain that our observations were not based on mitochondrial ATPase, we measured mitochondrial contamination in the plasma membrane preparations by means of electron microscopy and calculation of the specific activity of succinate dehydrogenase in the mitochondrial fraction (the second band from the hub), SDH activity in the plasma membrane preparations themselves, and the maximal reported specific activity of mitochondrial Ca-ATPase (32 μmoles P_i/mg protein/hour).[23] (The above value pertains to mitochondria isolated from heart cells; the value for rat liver mitochondria is considerably less.[24]) The amount of mitochondrial contamination was approximately 6% in four preparations and 13% in one sample. We could detect no differences in enzymic properties among these preparations. In addition, electron micrographs of the plasma membrane preparations disclosed only rare profiles of this organelle. Finally, the calcium dependency of the plasmalemmal Ca-ATPase studied here is not the same as that of mitochondrial Ca-ATPase.[24] For these reasons, then, we conclude that mitochondrial contamination in our preparations was not the basis for our observations.

Conclusions

Considering all these data, the following points merit critical attention. A calcium-activated ATPase exists in the rat liver plasma membrane (as indicated by Chambaut et al [21] and the authors), but its role in calcium flux has been questioned.[21,22] Nonetheless, this enzyme is modified by the addition of CCl_4 and is reversibly inhibited by the haloalkane. The

temporal sequence of CCl₄ accumulation in the liver, the transient accumulation of calcium by liver cells, and the *in vitro* effect of CCl₄ on the ATPase suggest a relationship. If this is the basis for the observed biologic phenomena, then CCl₄ intervention is indicated. The relationship of these changes to the mechanisms involved in cell injury is not clear.

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[*Illustrations follow*]
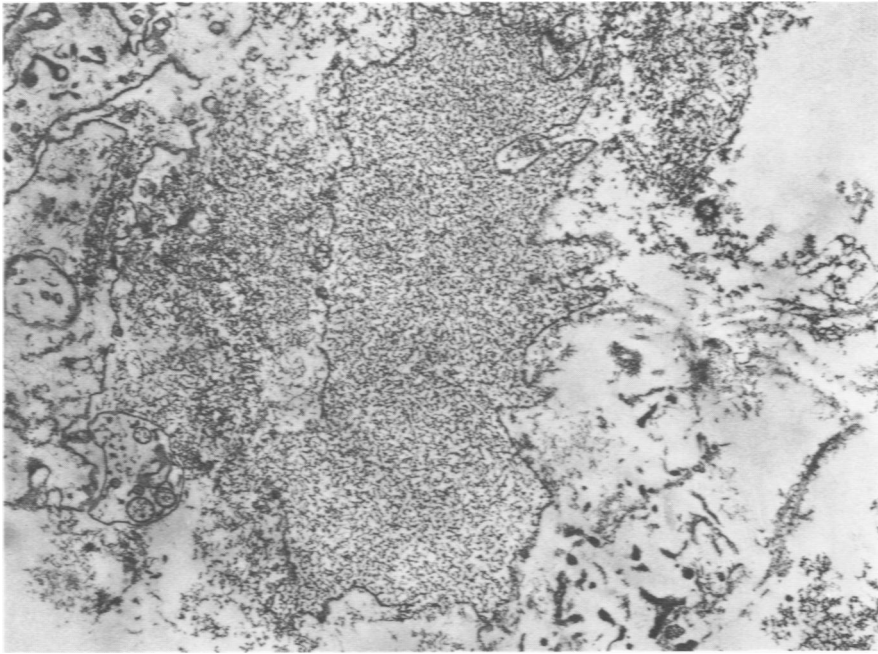
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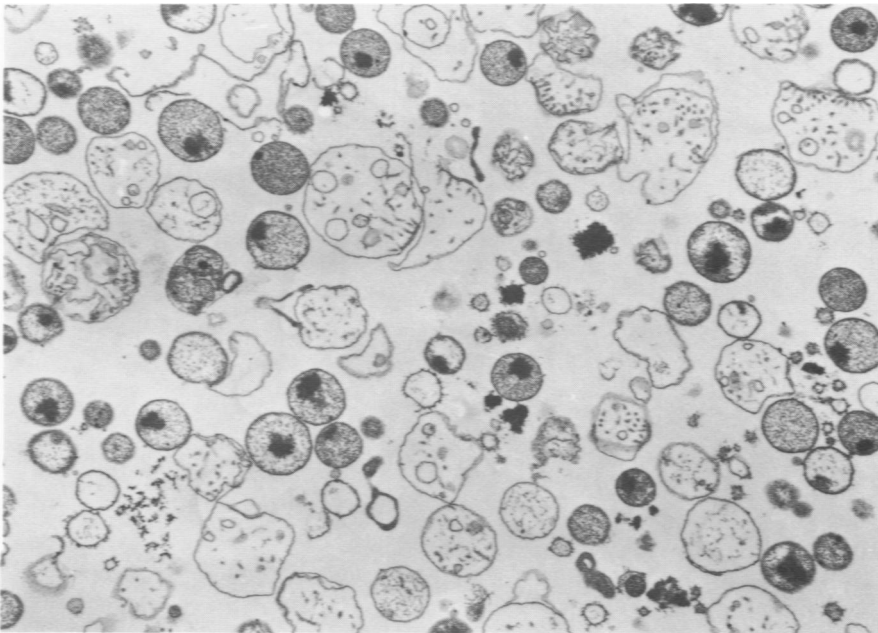
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Figure 1—This electron micrograph shows a representative inner band of material, with abundant and mixed enzymic activity. It consists of nuclei, cellular residua (including fragments of plasma membranes), and debris. (× 11,200)   Figure 2—This micrograph reflects the next distinct band and consists of mitochondria with swollen cristal spaces as well as microbodies with dense nucleoids. (× 11,200)
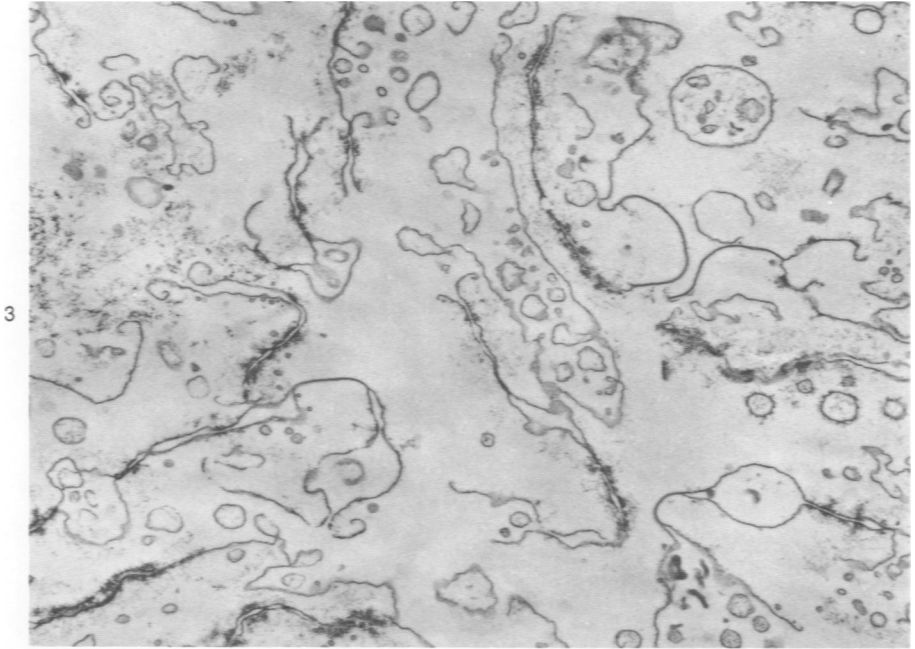
Figure 3—This micrograph represents the plasma membrane fractions. There are sheets of plasmalemma with and without desmosomes, rare profiles of rough endoplasmic reticulum, and occasional mitochondria (not shown in this picture). ($\times$ 18,000)