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RED CELL AGING AND ACTIVE CALCIUM TRANSPORT

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Abstract — The authors have investigated the relationships between the active calcium transport across the human red blood cell (RBC) membrane and the RBC aging processes in vivo and in vitro. For the study of this biological system, the authors have determined the active calcium uptake by inside-out membrane vesicles obtained from selected RBC populations. This model provided an optimal way to assess the biochemical and functional responses of the human cell to the oxidative stimulus triggered by the cellular aging processes. The activity of the calcium pump is indeed strictly correlated to the oxidative damage suffered by the RBC, being higher in the aged RBC. It appears that the main controller of the active calcium transport is the age-dependent protein inhibitor of the calcium pump.

Key Words: active calcium transport, inside-out vesicles, red cell density, oxidative damage, red cell aging, calcium homeostasis

RED CELL AGING

THERE IS strong evidence that the human red blood cell (RBC) is an appropriate model to study the mechanisms underlying the cellular and tissutal aging. First, the anatomy and the physiology of the human RBC are relatively simple in comparison to normal tissue cells because of the lack of intracellular compartments. In addition, no protein biosynthesis, nor turnover, occurs for the lack of nuclei and of genetic material, thus extensive repair from lesions and replacement of cellular components are prevented and the age-induced oxidative damage suffered by the cell is permanent. Finally, the environment in which the RBC lives, that is, the plasma, is relatively stable and thus the level of the stressing factors is quite constant over time.

The life span of the human RBC is approximately 120 days (Eadie and Brown, 1953), an equilibrium set point between the production of new cells by the bone marrow and their removal from the circulation by the spleen. In humans, the spleen removes selectively the oldest RBC only (Jandl and Cooper,1983; Mentzer and Clark, 1983), in contrast to other animal species such as mice, in which the RBC removal is randomized (Clark, 1988). As a consequence, the RBC survival in humans in the absence of hemorrhages follows a normal distribution (Shemin and Rittenberg, 1946) and the average life span of the RBC in the circulation is constant. It is, however, likely that the life span can be altered at varying activities of the stressing factors in the plasma.

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RBC OXIDATIVE DAMAGE

During its life, the RBC undergoes a noteworthy stress, as it results from the following considerations. If it is assumed that the average time required for a RBC to perform a complete lung-to-lung cycle is 30 sec, then it can be calculated that a single RBC undergoes $2 \times 60 \times 24 \times 120 = 345\,600$ cycle during its life. In each cycle, a RBC undergoes an oxygenation/deoxygenation transition, is exposed to atmospheric oxygen in the lungs, and must elongate several times its diameter to pass through narrow capillaries. If it is assumed that 20.9 ml oxygen are carried per ml of RBC, and that half of the carried oxygen is released to the tissues in each cycle, then it can be calculated that during its life span a single RBC carries a volume of oxygen of more than 3 000 000 times its own volume. This stress necessarily leads to several biochemical and morphological changes. The most striking alterations are the loss of membrane fragments (Linderkamp and Meiselman, 1982) and the progressive dehydration that lead the RBC to shrink as a function of the time spent in the circulation (Piomelli *et al.*, 1967). However, the hemoglobin content remains constant and therefore the RBC aging is well reflected by the increase of the cell hemoglobin concentration (Danon and Marikovsky, 1964). This feature is the easiest parameter to separate the RBC according to age and to define the mean age of a RBC suspension. Other features are the impairment of the activity of several enzymes as well as the decrease of the energetic level (Seaman *et al.*, 1980). The latter has an important consequence when dealing with the main function of the RBC, that is the oxygen transport. Indeed, low energetic levels imply low concentrations of 2,3-diphosphoglycerate (2,3-DPG) (Haidas *et al.*, 1971), that is an important cofactor of the hemoglobin oxygenation (Benesch and Benesch, 1967). This in turn decreases the oxygen affinity of the RBC. It was, in fact, recently observed that the p50, that is, the oxygen tension at which half the hemoglobin is saturated with oxygen and a useful index of the blood oxygen affinity, is severely diminished in the aged RBC (Schmidt *et al.*, 1987), implying that the oxygen transport function by the RBC is impaired (Samaja, 1988).

RBC DENSITY PROFILES

Alterations of the RBC mean age or the level of the stressing factors in the circulation are not uncommon. If it is assumed that: (1) the RBC emerges from the bone marrow as an homogeneous population, (2) the RBC are uniformly stressed by the factors present in the circulation, (3) the stressing degree is proportional to the strength of the stressing factors, and (4) the morphological changes derived by the stress are accompanied by functional changes, then the determination of the average "age" of the RBC population is an expression either of the actual life span of the RBC, or of the degree of the circulatory stress. This approach was used to assess some hematological diseases with respect to the RBC morphology (Nakashima *et al.*, 1973). Additional evidence for this is provided by the determination of the percent RBC that are lighter than 1.099 g/ml on a standard Percoll solution in several classes of subjects (Fig. 1). It appears that the RBC from adult menstruating women are lighter than the RBC from males and from nonmenstruating women. This finding emphasizes that the alterations in the average RBC density and presumably the average RBC "age" is a rather common phenomenon. Thus, investigations on the functional properties of the lightest and heaviest RBC should allow a deep insight into the mechanism of the RBC senescence.

RBC AGING AND THE HOMEOSTASIS OF CALCIUM

An important functional consequence that is expected from the biochemical and morpholog-

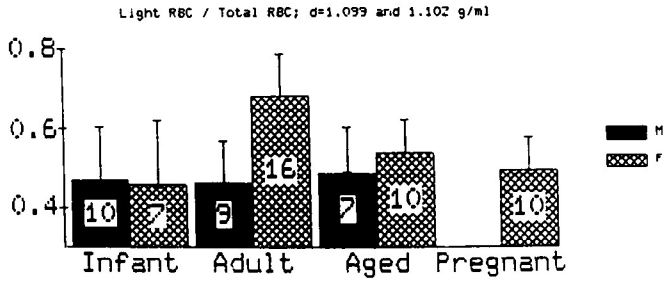

FIG. 1. The subjects are normal volunteers in the 2–12, 25–35, and 62–85 age groups, labelled as infants, adult, and aged, respectively. Freshly drawn blood (0.05 mL) was carefully layered over 0.15 mL isotonic solution at d = 1.099 or d = 1.102 g/mL containing Percoll. The tubes were quickly centrifuged at room temperature (2 min) with an Eppendorf centrifuge mod.5414. At the end of the centrifugation, the lightest and the heaviest RBC fractions were recovered and total hemoglobin was measured by a standard Drabkin's method. Data (means ± *SD*) are expressed as the ratio light RBC/total RBC, which is representative of the average "age" of the RBC in the subject.

ical changes induced by aging concerns the homeostasis of ions, particularly calcium. It was indeed shown that high cytosolic free calcium concentrations potentiate the oxidative injury, and represent an important step in the biochemical pathways that lead to cell death (Schanne *et al.*, 1979; Jewell *et al.*, 1982; Thor *et al.*, 1984). While it is uncertain whether the accumulation of calcium in the cytosol is a cause that triggers the events associated to the cellular aging, or is a consequence of the same events following the cell metabolic depletion, it appears that the active calcium pump of the RBC is primarily involved (Borle, 1981). The active calcium pump in the RBC is indeed composed by: (1) the enzyme (Ca-ATPase), (2) the protein inhibitor of the enzyme, and (3) the protein stimulator of the calcium pump, that is, calmodulin (Carafoli, 1987). The experiments described herein were performed to assess whether the active calcium pump is altered as a function of the cell aging, and to determine which component of the calcium pump is the primary target of the cellular senescence mechanism.

Literature in this field is rather controversial, mainly because of heterogeneity in methods and materials. Some investigators (Danon, 1973; Luthra and Kim, 1979) have found that Ca-ATPase is inactivated by aging because of the high sensitivity of this enzyme to activated oxygen and to glutathione depletion (Shalev *et al.*, 1985; Hebbel *et al.*, 1986), two major phenomena associated to the RBC senescence. Other investigators (Gopinath and Vincenzi, 1979) have not found appreciable differences in Ca-ATPase activity between the fresh RBC and the RBC that have been stored at cold temperatures for several weeks (outdated RBC), but Ca-ATPase from the outdated RBC appeared to be more susceptible to calmodulin activation than Ca-ATPase from the fresh RBC. Still other investigators (Cohen *et al.*, 1976; Leclerc *et al.*, 1987) have found that Ca-ATPase is more active in the densest RBC, but with a great variability approaching nonsignificativity.

To investigate the properties of the calcium pump as a function of the cellular aging, two experimental models were selected: in the former, the authors have considered the RBC aged in vitro outside its natural environment by comparing the freshly drawn RBC versus the outdated RBC; in the latter, the authors have considered the in vivo aging of the RBC in the presence of the natural stressing factors of plasma by comparing the lightest and the heaviest RBC separated by a Percoll density gradient (Samaja *et al.*, 1989).

INSIDE-OUT RED CELL MEMBRANE VESICLES

The technique for preparing inside-out red cell membrane vesicles (IORCMV) (Steck *et al.*, 1970) is an optimal model to study the active calcium transport by the RBC. Indeed, IORCMV do not require any calcium preload because the reversed orientation of the membrane exposes the enzyme and the pump to the medium; thus, the integrity of the membrane is preserved and there is no need to expose the cell to rather unphysiological conditions. In addition, the necessity of using radioactive calcium is overcome by the introduction of probes such as Arsenazo III, that are highly sensitive to calcium concentration changes (Steck *et al.*, 1970). If appropriately used, signals satisfactorily proportional to the changes in the concentration of calcium can be obtained, with little or no interference from divalent ions such as magnesium and pH (Scarpa and Brinley, 1978). Finally, the presence of only two compartments in the RBC allows the extrapolation of the information gained from the IORCMV to the living cell condition.

The IORCMV were prepared as described (Rubinacci *et al.*, 1986) by washing the RBC suspension with isotonic saline, and hemolyzing the cells by hypoosmotic shock (5 mM phosphate at pH 8). The resulting ghosts were repeatedly washed with the same buffer, and were incubated overnight in 0.5 mM phosphate at pH 8. Vesiculation was initiated by passage through a 27G needle, and the percent of inside-out vesicles was estimated by the acetylcholinesterase accessibility assay (Steck *et al.*, 1970).

THE ACTIVE CALCIUM UPTAKE IN THE AGING RBC

The calcium uptake by IORCMV was monitored by the absorbance change recorded by a dual-wavelength spectrophotometer operating at 675–685 nm to offset the interferences due to the turbidity of the suspension, to magnesium and to pH. The active calcium uptake was initiated by adding 0.5 mM ATP to the incubation medium containing the vesicles (3 to 4 mg/ml total protein), 0.02 mM $CaCl_2$, 1 mM $MgCl_2$, 0.1 M KCl, 5 mM sodium azide, and 0.1 mM Arsenazo III, at 37 °C. At appropriate times, 350 I.U. calmodulin was added to the mixture to obtain maximal calcium uptake. Data are expressed as μmoles calcium uptaken by the IORCMV per min per mg protein, and corrected for the inside-out vesicles percent.

Table 1 and Fig. 2 (Samaja *et al.*, 1989) show that the active calcium transport across the plasma membrane of the human RBC increases with the cell aging. The increase is more significant for the in vitro aged RBC than for the in vivo aged RBC ($p < 0.0005$, and $p < 0.08$, respectively). Excess exogenous calmodulin increases the calcium uptake of the IORCMV by similar factors regardless of the RBC type. Consequently, the difference in aged versus nonaged RBC is still present under the maximal activation condition, although at a lower significance level.

As explained above, there are at least three components of the calcium pump that are involved in the regulation of calcium homeostasis and that can be altered by the RBC senescence, that is the enzyme, calmodulin, and the inhibitor of the pump. To assess which of the components is primarily involved in the accumulation of calcium in the cytosol during the RBC aging processes, the authors have obtained IORCMV preparations free of both the endogenous calmodulin and the inhibitor. This was accomplished by repeatedly washing the ghosts in the presence of 1 mM EDTA before the vesiculation (Au and Lee, 1984). All EDTA was removed by further washing in EDTA-free buffer. In separate experiments, it was shown that high-ionic strength solutions are not a necessary requisite for this. The calcium uptake by such IORCMV

TABLE 1. ACTIVE CALCIUM UPTAKE IN IORCMV FROM THE LIGHT AND DENSE RBC FRACTIONS

	Light RBC		*Dense RBC*	
Exp	*Basal*	*Activated*	*Basal*	*Activated*
1	1.0	3.7	1.8	4.9
2	1.2	3.2	1.6	5.4
3	0.9	2.7	1.0	3.0
Mean	1.03	3.20	1.47	4.43
SD	0.15	0.50	0.42	1.27

Data are expressed as μmol/min/mg protein, and are corrected for the percentage of inside-out vesicles. The differences between light and dense RBC are significant at the $p = 0.08$ level for any of the described cases.

is the same for the aged and the nonaged RBC (Fig. 2, right panel), and lower than that of the respective nonwashed IORCMV. This indicates that the factor responsible for the increased uptake of calcium in the senescent RBC is either calmodulin or the inhibitor of the pump. The observation that the maximally activated uptake of calcium by such IORCMV is the same for both the aged and the nonaged RBC indicates, although not being a proof, that the major role in this process can be attributed to the inhibitor rather than to calmodulin.

Further evidence that the protein inhibitor of the calcium pump is the major cause responsible

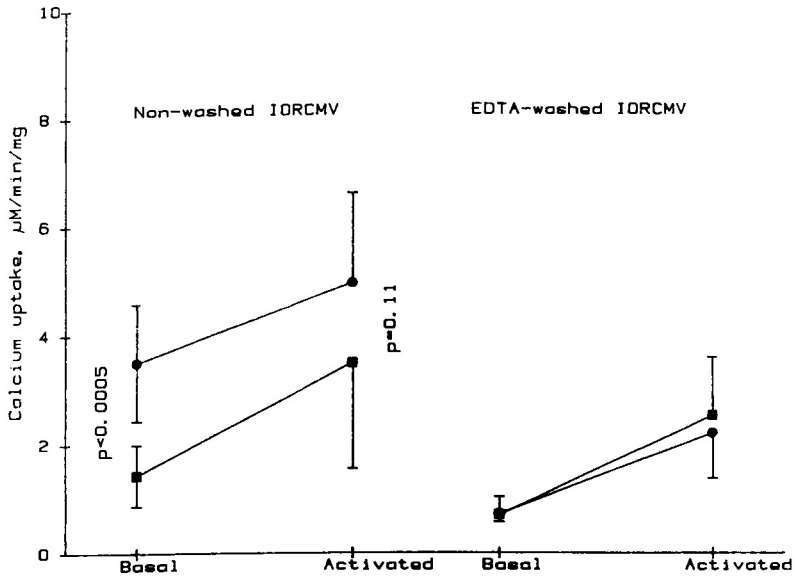

FIG. 2. Calcium uptake (μM/min/mg protein, corrected for the percentage of IORCMV) in fresh (■) and outdated (●) RBC. The left and the right panels report data from nonwashed vesicles, and of vesicles free of calmodulin and inhibitor, respectively. The vertical bars are SD, and the significativity level (unpaired Student's *t*-test) is indicated.

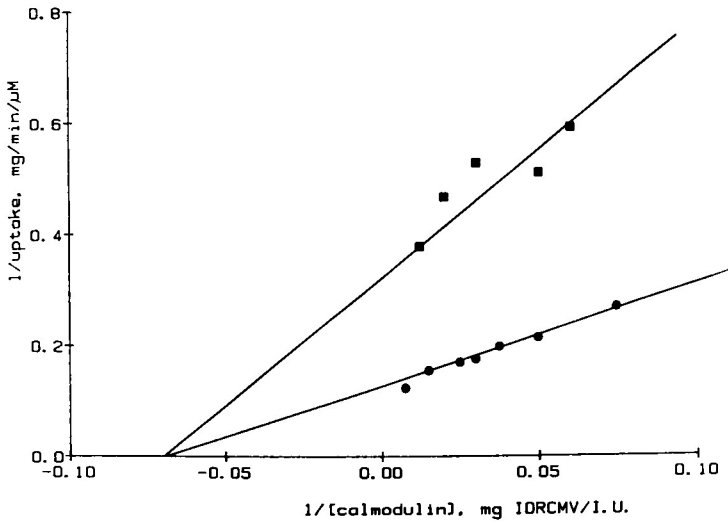

FIG. 3. Double reciprocal plot for the activation of the calcium uptake by calmodulin. Symbols are as in Fig. 2. The approximate K_m value (reciprocal of the intercept on the *x*-axis) is 12 I.U./mg IORCMV.

for the increased activity of the calcium uptake in the aged RBC is in Fig. 3. The activation by exogenous calmodulin was here performed by several subsaturating steps rather than by a single step. Data were reported in a double reciprocal plot. It appears that although the slopes of the two lines are different, they intersect at a point along the abscissa, indicating that the inhibitor has two different levels of activity in the two preparations, and that the inhibition is noncompetitive, as an expression of nonreversible degradative phenomena induced by aging and by the oxidative stress in general. It is noteworthy that the same pattern was observed when the purified inhibitor was added to a solution containing Ca-ATPase (Lee and Au, 1983).

CONCLUSIONS

The biochemical mechanism underlying the aging process in the tissue cell is an expression of a still elusive system, the full resolution of which is presently unfeasible. The human RBC is a simple model that allows extrapolating data to the tissue cell. Investigations into the alterations of the properties of the active calcium pump induced by the cellular aging provide useful information to understand the accumulation of calcium in the cytosol, a phenomenon that is recognized as an important key in the pathway leading to the cell senescence and death. In this work, the authors have reported data that emphasize the role of the calcium pump in the aging human RBC. It appears that the inhibitor of the calcium pump is a main controller of the homeostasis of calcium in the senescent cell. Whether this is the primary cause that triggers the events associated to the cell senescence, or an early consequence of the cell senescence itself, remains a matter for further investigations. This study, however, stresses the importance of investigating selected RBC populations in order to emphasize phenomena that are masked when studying the blood in toto. It is likely that the investigations into the properties of the light and dense RBC can provide useful information when studying the processes that lead to cellular aging and death in other more complex cell systems.

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REFERENCES

AU, K.S. and LEE, K.S. A protein modulator of erythrocyte membrane (Ca-Mg)-ATPase inhibitor protein. *Biochim. Biophys. Acta* **784,** 108–115,1984.

BENESCH, R. and BENESCH, R.E. The effect of organic phosphates from the human erythrocyte on the allosteric properties of hemoglobin. *Biochem. Biophys. Res. Commun.* **26,** 162–167, 1967.

BORLE, A.B. Control, modulation and regulation of cell calcium. *Rev. Physiol. Biochem. Pharmacol.* **90,** 14–122, 1981.

CARAFOLI, E. Intracellular calcium homeostasis. *Annu. Rev. Biochem.* **56,** 395–433, 1987.

CLARK, M.R. Senescence of red blood cells: Progress and problems. *Physiol. Rev.* **68,** 503–554, 1988.

COHEN, N.S., EKHOLM, J.E., LUTHRA, M.G., and HANAHAN, D.J. Biochemical characterization of density separated human erythrocytes. *Biochim. Biophys. Acta* **419,** 229–242, 1976.

DANON, D. and MARKOVSKY, Y. Determination of density distribution of red cell population. *J. Lab. Clin. Med.* **64,** 668–674, 1964.

DANON, D. Aging of the erythrocyte. In: *CRC Handbook of Cell Biology of Aging,* pp. 317–340, CRC Press, Boca Raton, FL, 1983.

EADIE, G.S. and BROWN, I.W. Red blood cell survival studies. *Blood* **8,** 1110–1136, 1953.

GOPINATH, R.M. and VINCENZI, F.F. (Ca-Mg)-ATPase activity of sickle cell membrane: Decreased activation by red cell cytoplasmic activator. *Am. J. Hematol.* **7,** 303–312, 1979.

HAIDAS, S., LABIE, D., and KAPLAN, J.-C. 2,3-diphosphoglycerate content nad oxygen affinity as a function of red cell age in normal individuals. *Blood* **38,** 463–467, 1971.

HEBBEL, R.P., SHALEV, O., FOKER, W., and RANK, B.H. Inhibition of erythrocyte Ca-ATPase by activated oxygen through thiol- and lipid-dependent mechanisms. *Biochim. Biophys. Acta* **862,** 8–16, 1986.

JANDL, J.H. and COOPER, R.A. Hereditary spherocytosis. In: *Hematology,* Williams, W.J., Beutler, E., Erslev, A.J., and Lichtmann, M.A. (Editors), p. 547, McGraw-Hill, New York, 1983.

JEWELL, S.A., BELLOMO, G., THOR, H., ORRENIUS, S., and SMITH, T.M. Bleb formation in hepatocytes during drug metabolism is caused by disturbances in thiol and calcium homeostasis. *Science* **217,** 1257–1259, 1982.

LECLERC, L., GIRARD, F., GALACTEROS, F., and POYART, C. The calmodulin-stimulated (Ca-Mg)-ATPase in hemoglobin S erythrocyte membranes: Effect of sickling and oxidative agents. *Biochim. Biophys. Acta* **897,** 33–40, 1987.

LEE, K.S. and AU, K.S. A protein inhibitor of erythrocyte membrane (Ca-Mg)-ATPase. *Biochim. Biophys. Acta* **742,** 54–62, 1983.

LINDERKAMP, O. and MEISELMAN, H.J. Geometric, osmotic and membrane mechanical properties of density-separated human red cells. *Blood* **59,** 1121–1127, 1982.

LUTHRA, M.G. and KIM, H.D. Influence of calcium and cytoplasmatic activator protein on various states of membrane Ca-Mg-ATPase of total and density separated human red cells. *Fed. Proc.* **38,** 745, 1979.

MENTZER, W.C. and CLARK, M.R. Disorders of erythrocyte cation permeability and water content associated with hemolytic anemia. In: *Pathological Membranes,* Nowotny, A. (Editor), pp. 79–118, Plenum, New York, 1983.

NAKASHIMA, K., SUSUMA, O., and MIWA, S. Red cell density in various blood disorders. *J. Lab. Clin. Med.* **82,** 297–302,1973.

PIOMELLI, S., LURINSKY, G., and WASSERMAN, L.R. The mechanism of red cell ageing. 1. Relationship between cell age and specific gravity evaluated by ultracentrifugation in a discontinuous density gradient. *J. Lab. Clin. Med.* **69,** 659–674, 1967.

RUBINACCI, A., FULLER, B., WUYTACK, F., and DELOECKER, W. Calcium transport and permeability in inside-out red cell membrane vesicles after freezing. *Cryobiology* **23,** 134–140, 1986.

SAMAJA, M. Prediction of the oxygenation of human organs at varying blood oxygen affinity in humans. *Respir. Physiol.* **72,** 211–218, 1988.

SAMAJA, M., RUBINACCI, A., DE PONTI, A., and PORTINARO, N. The effect of in vivo and in vitro cellular aging on the active calcium transport in human inside-out red cell membrane vesicles. *Biochem. Biophys. Res. Commun.,* in press.

SCARPA, A., BRINLEY, F.J., TIFFERT, T., and DUBYAK, G.R. Metallochromic indicators of ionized calcium. *Ann. NY Acad. Sci.* **307,** 86–112, 1978.

SCHANNE, F.A.X., KANE, A.B., YOUNG, E.E., and FARBER, J.L. Calcium dependence of toxic cell death: A final common pathway. *Science* **206,** 700–702, 1979.

SCHMIDT, W., BONING, D., and BRAUMANN, K.M. Red cell age effects on metabolism and oxygen affinity in humans. *Respir. Physiol.* **68,** 215–225, 1987.

SEAMAN, C., WYSS, S., and PIOMELLI, S. The decline in energetic metabolism with aging of the erythrocyte and its relationship to cell death. *Am. J. Hematol.* **8,** 31–42, 1980.

SHALEV, O., LAVI, V., HEBBEL, R.P., and EATON, J.W. Erythrocyte Ca-Mg-ATPase activity: increased sensitivity to oxidative stress in glucose-6-phosphate dehydrogenase deficiency. *Am. J. Hematol.* **19,** 131–136, 1985.

SHEMIN, D. and RITTENBERG, D. The life span of the human red blood cell. *J. Biol. Chem.* **166,** 627–636, 1946.

STECK, T.L., WEINSTEIN, R.S., STRAUSS, J. H., and WALLACH, D.F. Inside-out red cell membrane vesicles: Preparation and purification. *Science* **168,** 225–227, 1970.

THOR, H., HARTZELL, P., and ORRENIUS, S. Potentiation of oxidative cell injury in hepatocytes which have accumulated calcium. *J. Biol. Chem.* **259,** 6612–6615, 1984.