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I. Toxicity to Isolated Hepatocytes Caused by the Intracellular Calcium Indicator, Quin 2

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ABSTRACT

To determine whether incubation for several hours with intracellular Ca^{++} indicators caused toxicity to freshly isolated hepatocytes from rats, cells were incubated under 95% O_2-5% CO_2 in medium containing 2 mM Ca^{++} and the acetoxymethyl (AM) esters of Quin 2, Indo 1, Fluo 3, 5,5′-Dimethyl BAPTA, 4,4′-Difluoro BAPTA or Fura 2 for up to 5 hr. Quin 2-AM and Indo 1-AM (2.5 μM) induced lipid peroxidation in the cells after 1 or 3 hr of treatment, respectively. Additional experiments with Quin 2-AM (25 μM) revealed that it also caused lactate dehydrogenase leakage, cell blebbing and vitamin E loss in cells, but did not affect reduced glutathione or intracellular Ca^{++} content. The ability of Quin 2-AM to cause toxicity was dependent on the amount of Quin 2 which was present in the cell. Ca^{++} appeared to be involved in the mechanism of Quin 2-AM toxicity, for modulation of the extracellular Ca^{++} concentration partially inhibited lipid peroxidation, vitamin E loss, cell blebbing and lactate dehydrogenase leakage.

In the past few years, several compounds have been developed for use as indicators of cytosolic Ca^{++}. These include the fluorescent indicators Quin 2, Fura 2, Indo 1 or Fluo 3, as well as other nonfluorescent analogs of BAPTA such as 4,4′-DF BAPTA. Intracellular loading of these agents may be accomplished by microinjection or treatment of the cells with the membrane-permeant AM esters (Tsien, 1981; Cork *et al.*, 1989). After entering the cells, the AM ester of the indicator is hydrolyzed to its active form. Each AM group hydrolyzed from the ester also gives rise to two protons, one acetate and one formaldehyde molecule. Although some of these hydrolysis products are known toxi cants (Temcharoen and Thilly, 1983; Bolt, 1987), with the exception of Quin 2-AM, none of the aforementioned calcium indicators have caused adverse effects in cells. Because Quin 2 has a high affinity for Ca^{++}, it can inhibit cell responses which are associated with an increase in intracellular Ca^{++}, such as platelet aggregation and macrophage phagocytosis (Rao *et al.*, 1986; McNeil *et al.*, 1986). Other than these effects which have been associated with buffering of Ca^{++} transients, few adverse effects associated with Quin 2-AM use have been documented. In 1983, Hesketh and co-workers showed that concentrations of Quin-AM used for measurement of intracellular concentrations of free Ca^{++} stimulated DNA synthesis, phosphatidylinositol metabolism, lactate production and ATP loss in mouse lymphocytes (Hesketh *et al.*, 1983). To date, effects such as these have not been observed in other cell types after treatment with this agent.

When utilizing an AM ester as a Ca^{++} indicator, investigators commonly incubate cells with micromolar concentrations of the compound from 30 min to 1 hr, whereafter the cells are washed and treated with various agents of interest. Fluorescence of the cells is then measured for a period of time not greater than 1 hr. Therefore, the cells are in contact with the compound for a period of 2 hr or less. When attempting to measure changes in intracellular Ca^{++} which may occur after treatment of hepatocytes with a toxicant for up to a period of 5 hr, we noted that certain Ca^{++} indicators, in particular Quin 2-AM, induce untoward side effects in a time and concentration-dependent manner. The characterization of the toxic effects, as well as the role of Ca^{++} in their manifestation is the focus of this investigation.

Methods

Preparation and treatment of hepatocytes. Parenchymal cells were isolated from the livers of male Sprague-Dawley rats (180–200 g) as described previously (Fariss *et al.*, 1985a). After isolation, they were resuspended in modified Fisher's medium (Fariss *et al.*, 1985a), counted and tested for viability by the ability to exclude trypan blue. Initial viability of cells was > 90% in all experiments.

For most experiments, cells were then resuspended at a concentration of 2×10^6/ml in medium containing 2 mM Ca^{++}. In some instances,

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ABBREVIATIONS: DF, difluoro; AM, acetoxymethyl; DM, dimethyl; LDH, lactate dehydrogenase; GSH, reduced glutathione; MDA, malondialdehyde; DBH, dibutylphthalate; PCA, perchloric acid; HBSS, Hank's balanced salt solution; ANOVA, analysis of variance.

cells were resuspended in medium which contained either 0 or 10 mM Ca^{++}. Aliquots of cell suspension (20 ml) were then placed in 125-ml culture flasks and were treated with the AM esters of 4,4′-DF BAPTA, 5,5′-DM BAPTA, Fluo 3, Indo 1, Fura 2 or Quin 2, with the potassium salt of Quin 2 (Molecular Probes, Eugene, OR) or with the respective vehicles at concentrations listed in the figure legends. Flasks were then placed in an incubator (37°C) and were rotated slowly under an atmosphere of humidified 95% O_2-5% CO_2 for 5 hr. Aliquots of cell suspension were taken from each flask before and at various times during the incubation period for analysis of LDH release, lipid peroxidation, intracellular Ca^{++}, vitamin E or GSH content and, in some cases, dye uptake as described below.

Biochemical analyses. Lipid peroxidation was assessed by assaying an aliquot (0.25 ml) of cell suspension for MDA formation (Stacey and Klaassen, 1981). Before analysis of LDH release and intracellular Ca^{++}, vitamin E or GSH content, separation of live from dead cells was desired. This was achieved by centrifuging an aliquot of cell suspension (0.5 ml) through DBH and into either 36% buffered Percoll or 10% PCA as described previously (Fariss *et al.*, 1985a). LDH activity in the medium above the DBH was measured using a Sigma assay kit. It is expressed as percentage of maximal LDH release elicited by treatment of cells with Triton X-100 (0.04%). The concentration of Ca^{++} in the PCA extract was measured by atomic absorption spectrometry as described previously (Fariss *et al.*, 1985a). The content of GSH in the PCA extract was determined by the high-performance liquid chromatography method of Reed *et al.* (1980). Vitamin E content of cells which pelleted into the Percoll was assessed by reversed phase high-performance liquid chromatography with fluorescence detection (Fariss *et al.*, 1985b). Both intracellular GSH and vitamin E concentrations were corrected for the number of viable hepatocytes which came through the DBH as determined by the DNA content of the PCA precipitate (Erwin *et al.*, 1981) and are expressed per 10^6 cells.

Intracellular Quin 2, Indo 1 or Fluo 3 measurement. At various time points, a 1-ml aliquot of cells (2×10^6) treated with Quin 2-AM, salt or vehicle was collected, washed and resuspended in 2 ml of HBSS containing 2 mM Ca^{++}. Cells were then lysed with Triton X-100 (0.02%) and spun in a centrifuge. The fluorescence of the supernatant from vehicle-, Quin 2-AM-, or salt-treated cells at excitation and emission wavelengths of 339 and 492 nm, respectively, was then determined using a Perkin Elmer model LS5 fluorescence spectrometer. Fluorescence associated with the supernatant of vehicle-treated cells was subtracted from that of Ouin 2-treated cells before determining the intracellular Quin 2 content. The value obtained was compared to those of standards prepared by adding various amounts of Quin 2 salt to 2 ml of HBSS containing 2 mM Ca^{++}. Similar experiments were performed to determine the extent of Indo 1 (excitation 355, emission 490 nm) or Fluo 3 (excitation 488, emission 530 nm) loading after treatment of cells with the respective esters. The final concentration of Ca^{++} indicator within the cells was then calculated after correcting for the intracellular water content of 2×10^6 cells (6.4 μl) (Fariss *et al.*, 1985a).

Preparation of hepatocytes for scanning electron microscopy. At various time points, 1-ml aliquots of cell suspension (2×10^6 cells) were collected from some flasks and were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 24 hr at 4°C. After fixation, the cells were washed 2 times with 0.2 M sodium cacodylate buffer (pH 7.4). An aliquot of each cell suspension was added to membrane holders (Nucleopore Corp., Pleasanton, CA) equipped with a 13-mm diameter silver membrane (SPI Supplies, West Chester, PA) with a pore size of 0.8 μm. The cells were settled onto the membrane surface by gently aspirating the excess fluid. The silver membranes were removed from the filtration assemblies and were dehydrated through a graded series of ethanol (10, 30, 50, 70, 80, 95 and 100% × 3). The ethanol was replaced gradually with Freon TF ($ClCF_3$-CCl_2F), and the cells were dried with Freon 13 ($CClF_3$) in a critical point drier (Balzers, Hudson, NH). The silver membranes were mounted on aluminum specimen mounts and coated with a 20-nm layer of gold with an Edwards Sputter Coater (Edwards High Vacuum, Grand Island, NY). The hepatocytes were viewed with an AMRAY 1000 (AMRAY, Inc., Santa Clara, CA) scanning electron microscope at an accelerating voltage of 10 kV. Images were recorded on 55 P/N film (Polaroid, Cambridge, MA).

Statistical analysis. Data were analyzed using an ANOVA program from SYSTAT (Evanston, IL) and compared using a least-significant difference test (Steel and Torrie, 1980). Where variances were nonhomogeneous, log or square root transformations were performed before ANOVA. The criterion for significance was $P < .05$. Unless otherwise noted in figure or table legends, data are presented as mean ± S.E.

Results

When treated with the cytosolic Ca^{++} chelators Quin 2-AM and Indo 1-AM (2.5 μM), cells incubated in medium containing 2 mM Ca^{++} underwent lipid peroxidation as evidenced by an increased amount of MDA formation (fig. 1A). The increase was significantly different from control after 1 or 3 hr of incubation with Quin 2-AM or Indo 1-AM, respectively. Although there was a trend toward an increase in lipid peroxidation at early time points in cells treated with 5,5′-DM BAPTA-AM, values were not significantly different from control after 3 hr. By contrast, neither 2.5 μM Fluo 3-AM, Fura 2-AM or 4,4′-DF BAPTA-AM (data not shown) induced lipid peroxidation during the duration of the experiment. Furthermore, at the concentration used (2.5 μM), Indo 1-AM and Quin 2-AM also accelerated LDH leakage from the cells (Fig. 1B). However, by 5 hr of incubation, LDH leakage from cells treated with these agents was not significantly different from control.

As seen in figure 2A, the effect of Quin 2-AM on lipid peroxidation in the cells was dose-dependent. Cells treated with 10 or 25 μM Quin 2-AM underwent almost twice as much lipid peroxidation by 5 hr as those treated with 2.5 μM. In addition, at this time point, more LDH was released by cells treated with 10 or 25 μM than by cells treated with 2.5 μM Quin 2-AM or vehicle (fig. 2B). Because the effect of Quin 2-AM on lipid peroxidation was marked, the effect of the agent on other indicators of oxidative stress was determined. As shown in figure 2C, Quin 2-AM caused a severe dose- and time-dependent depletion of vitamin E. In contrast, Quin 2-AM did not have much of an effect on GSH content (fig. 2D).

To determine whether the presence of the ester moiety was required for Quin 2-AM to cause toxicity to hepatocytes, the effect of treatment with 25 μM of the potassium salt of Quin 2 on lipid peroxidation and LDH release was assessed. As seen in figure 3A, although MDA formation was elevated slightly after incubation for 5 hr with the Quin 2 salt, approximately 5 times greater MDA was released after treatment with the Quin 2-AM ester for the same length of time. Furthermore, whereas the ester caused an increase in LDH release from the cells, the potassium salt did not (fig. 3B).

Because, theoretically, the AM ester of the Ca^{++} chelators would be expected to enter the cells, whereas the potassium salt would not, the relationship between the amount of chelator taken up by the cells and the extent to which it induced lipid peroxidation was assessed. As seen in table 1, Indo 1-AM and Quin 2-AM, which caused lipid peroxidation, gained access to the cytosol. Interestingly, a similar dose-dependent relationship existed between Quin 2-AM uptake as did lipid peroxidation and vitamin E loss; that is, treatment with 10 μM Quin 2-AM induced a similar amount of dye uptake, lipid peroxidation and vitamin E loss as did treatment with 25 μM. Furthermore, the intracellular concentration of Quin 2, lipid peroxidation, and vitamin E loss induced by 2.5 μM Quin 2-AM were less than

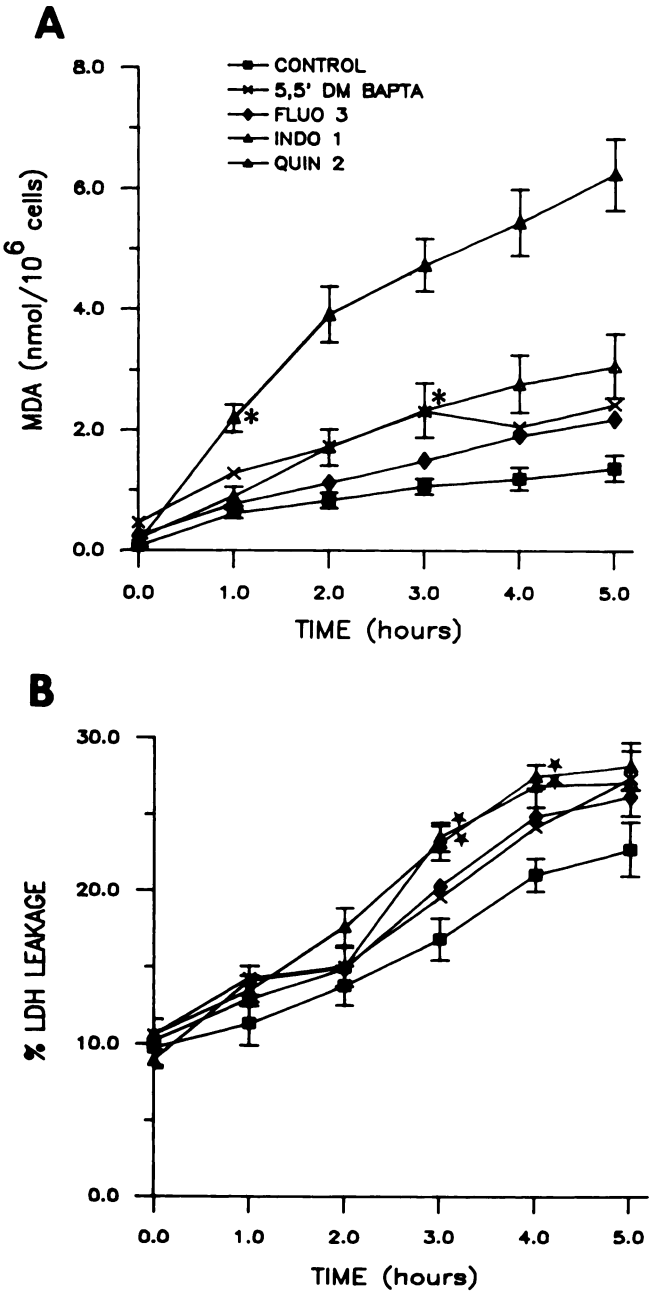

Fig. 1. Effect of various intracellular Ca^{++} chelators on (A) MDA formation and (B) LDH release by isolated hepatocytes. Cells were treated with 2.5 μM Quin 2-AM, Indo 1-AM, 5,5'-DM BAPTA-AM, Fluo 3-AM, 4,4'-DF BAPTA-AM, Fura 2-AM or dimethylsulfoxide vehicle and incubated as described under "Methods." Data for 4,4'-DF BAPTA-AM and Fura 2-AM are omitted because they are similar to that of Fluo 3-AM; $n = 3$ or greater for each group. *Significantly different from control at this and subsequent time points; ★significantly different from control at only these time points.

that caused by 10 or 25 μM. Another interesting observation is that the Quin 2 salt, which only slightly increased peroxidation after 5 hr of incubation, was either not found inside the cells at all or only at this time. However, the ability of the compound to penetrate the plasma membrane was not the only prerequisite for the induction of toxicity, for Fluo 3, which did not cause lipid peroxidation, also gained access to the cytosol.

Inasmuch as results of others suggest that treatment of cells with Quin 2-AM can cause an influx of extracellular Ca^{++} (Hesketh *et al.*, 1983: Tsien *et al.*, 1982), the effect of manipulation of extracellular Ca^{++} on toxicity induced by Quin 2-AM was explored. As shown in figure 4, either omitting Ca^{++} from or increasing the Ca^{++} concentration (10 mM) of the medium retarded the lipid peroxidation and vitamin E loss caused by Quin 2-AM (25 μM). Although omission of Ca^{++} from the medium also inhibited LDH leakage caused by Quin 2-AM, increasing Ca^{++} to 5 or 10 mM had no effect on LDH leakage.

As well as causing biochemical changes in cells, treatment of cells with Quin 2-AM also induced morphologic changes (fig. 5). In contrast to cells suspended for 4 hr in medium containing 2 mM Ca^{++} and vehicle (fig. 5A), cells treated with 2 mM Ca^{++} and Quin 2-AM (25 μM) for this length of time exhibited large blebs in membranes (fig. 5b). In addition to causing an increase in the size of membrane blebs, Quin 2-AM also caused an increase in the number of cells with blebs (table 2). In agreement with the biochemical data, omission of Ca^{++} from the medium inhibited both the increased frequency and size of blebs caused by Quin 2-AM (fig. 5B; table 2). By contrast, increasing the concentration of Ca^{++} in the medum to 10 mM did not protect cells from blebbing caused by Quin 2-AM (table 2).

To determine whether Quin 2-AM caused toxicity by allowing increased entry of extracellular Ca^{++} into the cells, the amount of intracellular Ca^{++} which was in the cells after treatment with Quin 2-AM (25 μM) and 2 mM Ca^{++} for various amounts of time was measured by atomic absorption. As seen in figure 6, treatment with Quin 2-AM did not cause an increase in intracellular Ca^{++}. Interestingly, there is a trend toward a decrease in intracellular Ca^{++} in cells treated with Quin 2-AM; however, the results are not significantly different from control at any time point.

Discussion

Although few side effects have been documented with the use of the fluorescent, intracellular Ca^{++} indicators, incubation of hepatocytes with Quin 2-AM was found to severely deplete vitamin E content, induce lipid peroxidation and cause loss of plasma membrane integrity as characterized by blebbing and LDH leakage. In contrast, recent reports suggest that chelators such as Quin 2-AM can protect cells against toxicity of certain agents (Cantoni *et al.*, 1989; Dhanbhoora and Babson, 1990; Boobis *et al.*, 1990). In these and other studies, concentrations of Quin 2-AM used to detect or chelate intracellular Ca^{++} are greater than or equal to the concentration of Quin 2-AM which we used. Therefore, use of a higher concentration than others can be ruled out as a possible explanation for our results. As our results indicate, Quin 2-AM has a much more marked effect on vitamin E loss, cell blebbing and lipid peroxidation than on LDH leakage. To our knowledge, the effect of Quin 2-AM on indices of "oxidative stress" such as vitamin E loss and MDA formation has not been studied previously. The fact that LDH leakage was not greater than control until 3 hr of incubation is consistent with the reported lack of irreversible toxicity of this agent.

Although the mechanism for Quin 2-AM toxicity cannot be discerned from the present study, the results suggest that Ca^{++} may play a role in its development. The fact that omission of Ca^{++} from the medium partially inhibits vitamin E loss and lipid peroxidation suggests that Quin 2-AM may promote toxicity by enhancing the uptake of Ca^{++} from the extracellular

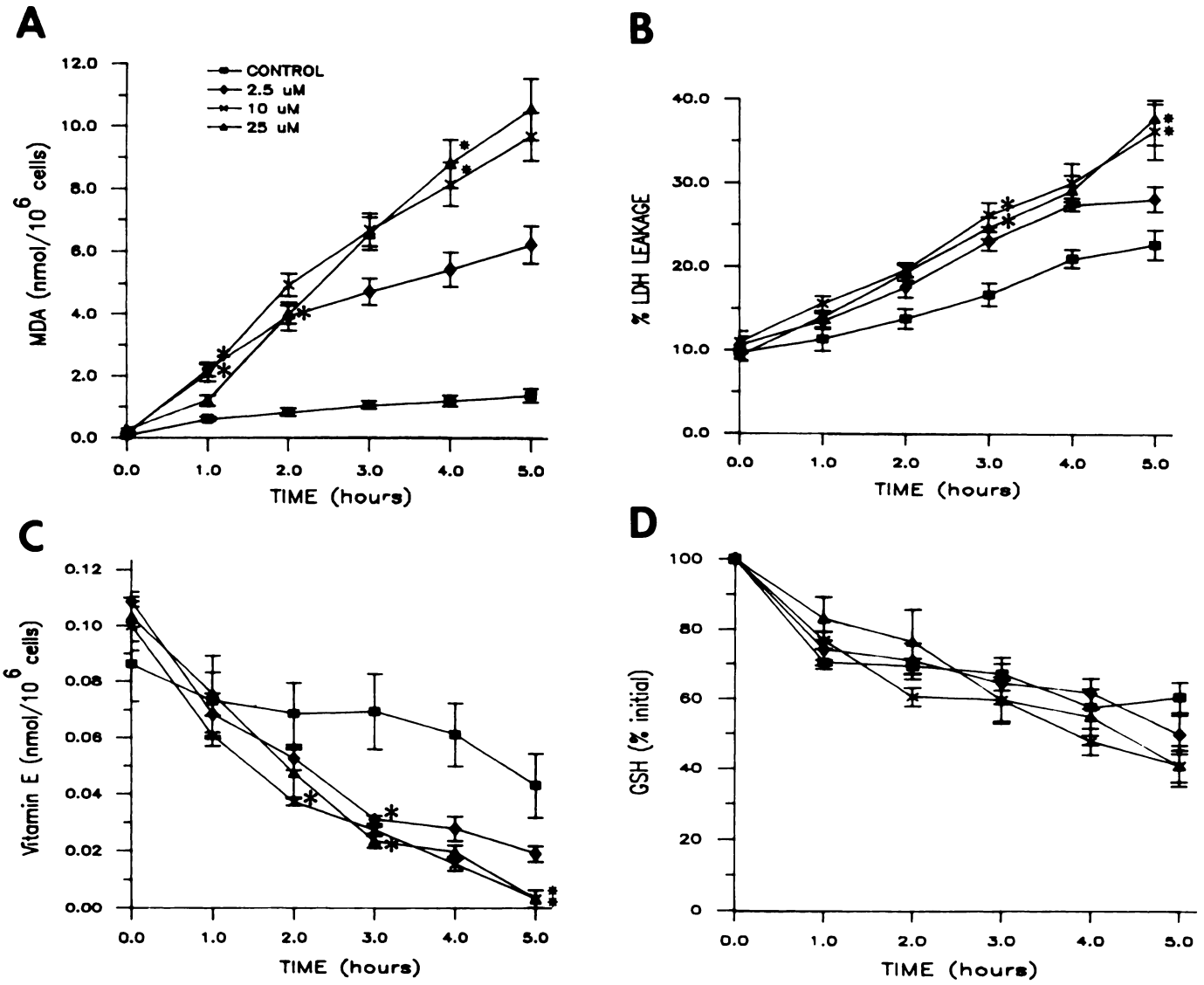

Fig. 2. Effect of various concentrations of Quin 2-AM on (A) MDA formation, (B) LDH release, (C) vitamin E content and (D) GSH content in isolated hepatocytes. Cells were treated with 2.5, 10 or 25 μM Quin 2-AM or vehicle and incubated as described under "Methods"; $n = 3$ or greater for each group. *Significantly different from control at this and subsequent time points; ⁎significantly different from 2.5 μM at this and subsequent time points.

medium. Indeed, an increase in intracellular Ca^{++} after treatment with Quin 2-AM has been noted by other investigators (Hesketh *et al.*, 1983; Tsien *et al.*, 1982). However, analysis of total cell Ca^{++} in our hepatocytes revealed that intracellular Ca^{++} in cells treated with Quin 2-AM was equal to that of control. These data suggest that, in our system, Quin 2-AM did not promote toxicity by causing an influx of Ca^{++} from the extracellular medium. In further support of this conclusion, increasing the Ca^{++} concentration in the medium did not promote, but rather protected cells against lipid peroxidation and vitamin E loss induced by Quin 2-AM. Whether the protective effect of increasing the extracellular Ca^{++} concentration is due to an inhibition of the process which initiates lipid peroxidation and/or *via* a promotion of vitamin E production is not known. However, it should he noted that increasing the extracellular concentration of Ca^{++} from 2 to 10 mM also promoted vitamin E retention in control cells (data not shown). Because extracellular Ca^{++} omission promotes, rather than inhibits, vitamin E loss from vehicle-treated hepatocytes (data not shown), it is clear that this is not the mechanism whereby Ca^{++} omission protects cells from toxicity caused by Quin 2-AM. Clearly, additional studies need to be performed to assess the role of Ca^{++} in toxicity due to this agent.

Because some of the hydrolysis products of the AM esters are known toxicants, the possibility exists that the toxic effects of Quin 2-AM were mediated through one or more of these species. However, several lines of evidence suggest that this is not the case. First, Fluo 3-AM (2.5 μM), which was hydrolyzed to a similar extent as Quin 2-AM (2.5 μM), was not toxic to the cells. Furthermore, the potassium salt of Quin 2, which does not contain the AM group, caused lipid peroxidation after 5 hr of incubation with cells. Lastly, treatment of cells with a concentration of formaldehyde which is likely to be inside the cells after hydrolysis of 150 μM Quin 2-AM (500 μM), did not cause an increase in lipid peroxidation or LDH release above control (data not shown). These results suggest that the active form of Quin 2, not the hydrolysis products of the AM ester, is the toxic species. However, it is likely that presence of the AM

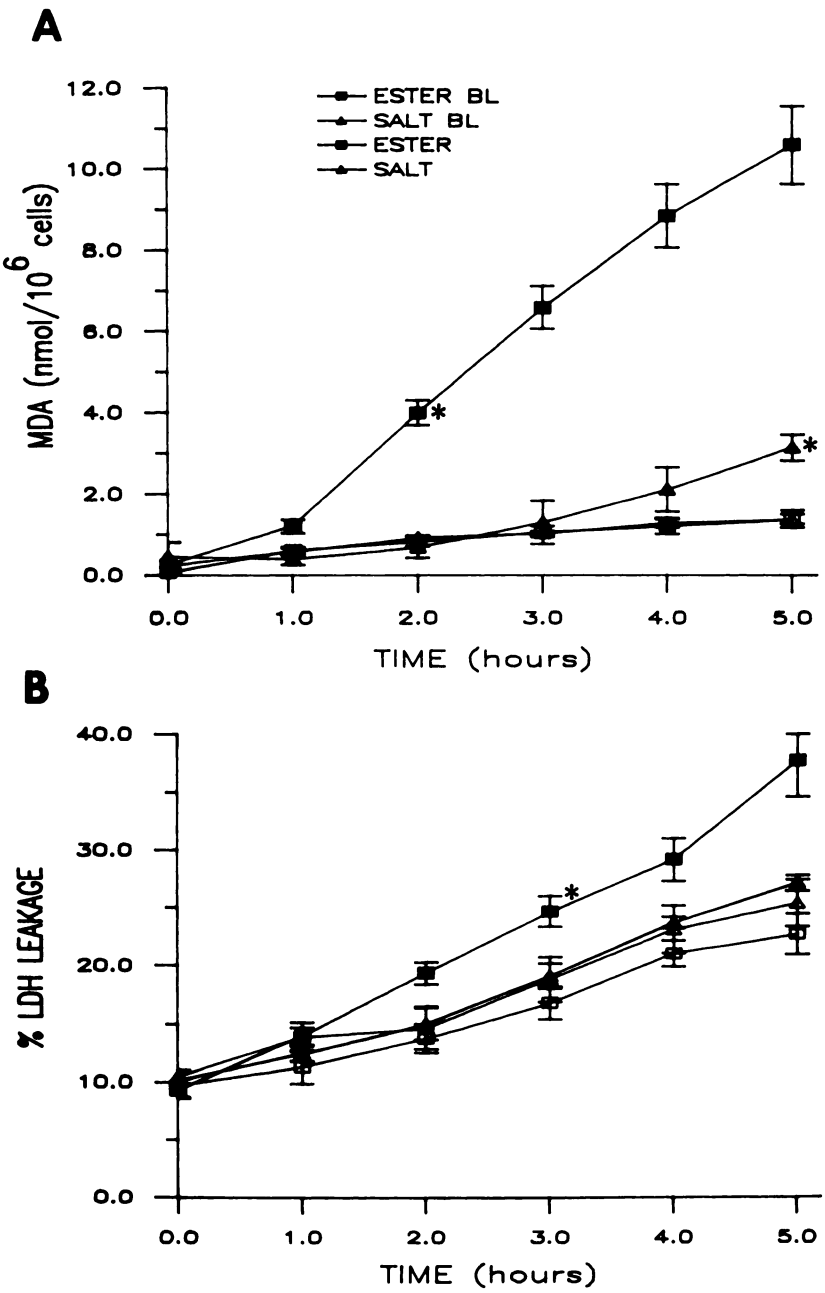

Fig. 3. Effect of treatment with Quin 2-AM or salt on (A) MDA formation and (B) LDH release from isolated hepatocytes. Cells were incubated with either 25 μM Quin 2-AM, Quin 2 potassium salt or the respective vehicles (BL) as described under "Methods"; $n = 3$ for each group. *Significantly different from control at this and subsequent time points.

TABLE 1

Concentration of fluorescent calcium indicator in cells

Hepatocytes (2×10^6/ml) were incubated in medium containing Quin 2-AM, Quin 2 salt, Indo 1-AM or Fluo 3-AM at concentrations listed and the amount of indicator (micromolar) present in the cytosol of the cells at various time points was determined as described under "Methods." Where $n > 3$, values expressed are means ± S.E. and where $n = 2$, values are expressed as means with ranges.

Treatment	Time (hr)		
	1	3	5
Quin 2-AM (2.5 μM)	0	16.3 ± 6.8	11.1 ± 9.9
Quin 2-AM (10 μM)	43.4 (25.9–60.8)	83.7 (64.4–103)	15.9 (15–16.7)
Quin 2-AM (25 μM)	152.2 ± 52.5	119.2 ± 20.9	45.5 ± 28.7
Quin 2 salt (25 μM)	0	0	24 (0–48)
Indo 1-AM (2.5 μM)	24.1 ± 3.3	16.7 ± 1.9	11.0 ± 2.3
Fluo 3-AM (2.5 μM)	13.5 ± 5.5	7.3 ± 1.4	4.2 ± 0.2

ester promotes toxicity by allowing for an increased amount of Quin 2 to enter the cells.

In vivo or *in vitro*, lipid peroxidation can be generated directly by agents that are metabolized to molecules containing oxygen- or carbon-centered radicals (Kappus and Sies, 1981). Whether Quin 2-AM can be metabolized to a molecule containing an active oxygen specie(s) is currently unknown. However, a recent report by van der Zee and associates (1989) suggests that certain intracellular Ca^{++} indicators can be metabolized by prostaglandin H synthase to carbon-centered radicals. Inasmuch as prostaglandin synthase is present in our cells, it is possible that the lipid peroxidation caused by Quin 2-AM or Indo 1-AM was mediated *via* carbon-centered radicals formed by the metabolism of these compounds by this enzyme. However, because the degree to which various agents form the radical (5,5′-DM BAPTA ≥ 4,4′-DF BAPTA > Quin 2 ≥ Fura 2 >> Indo 1) (van der Zee *et al.*, 1989) does not correlate with the ability of

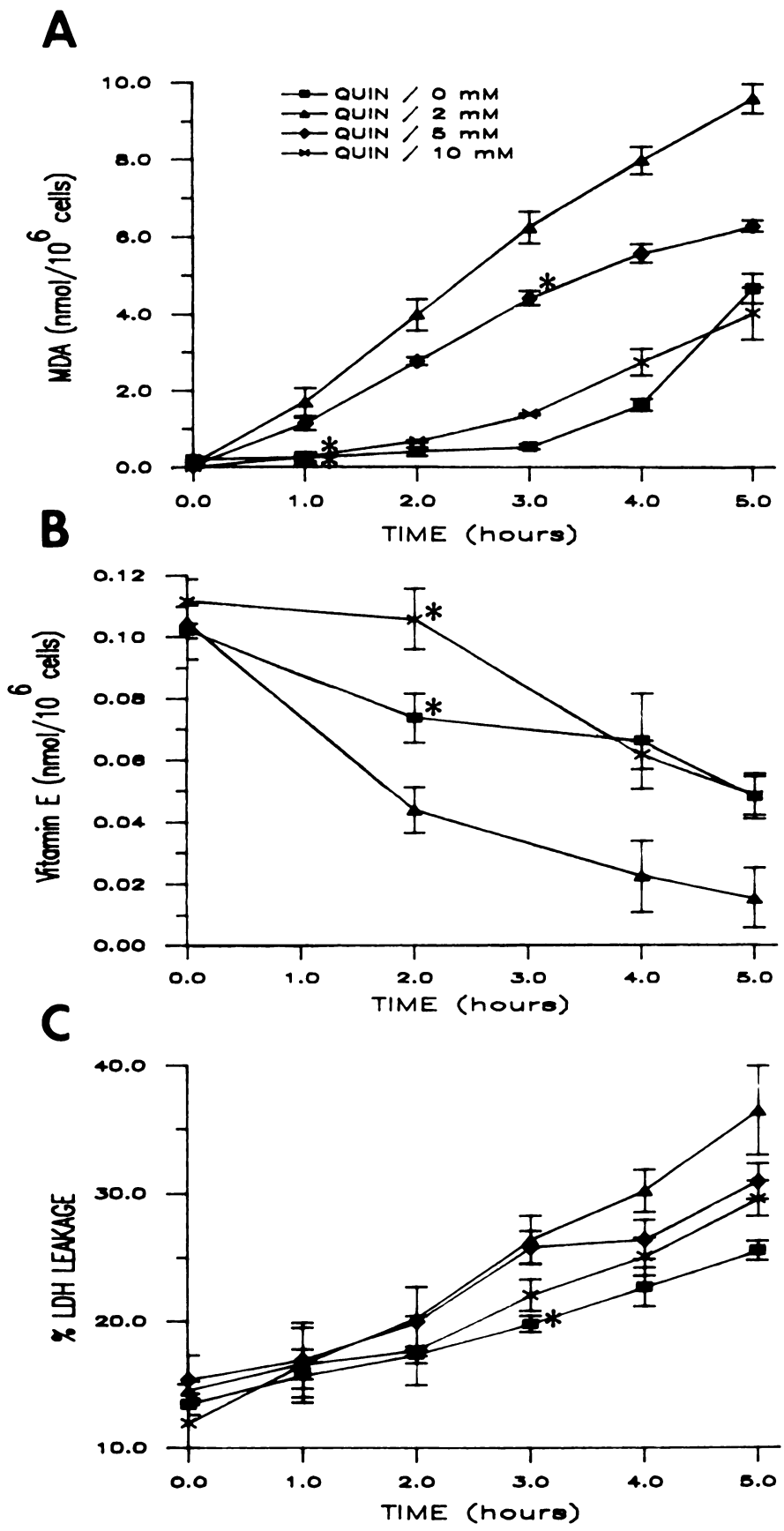

Fig. 4. Effect of varying the extracellular Ca^{++} concentration on Quin 2-AM-induced (A) MDA formation, (B) vitamin E loss and (C) LDH release from isolated hepatocytes. Cells suspended in medium containing either 0, 2, 5 or 10 mM Ca^{++} were treated with Quin 2-AM (25 μM) or vehicle and incubated as described under "Methods"; $n = 3$ or greater for each group. *significantly different from Quin/2 mM Ca^{++} at this and subsequent time points.

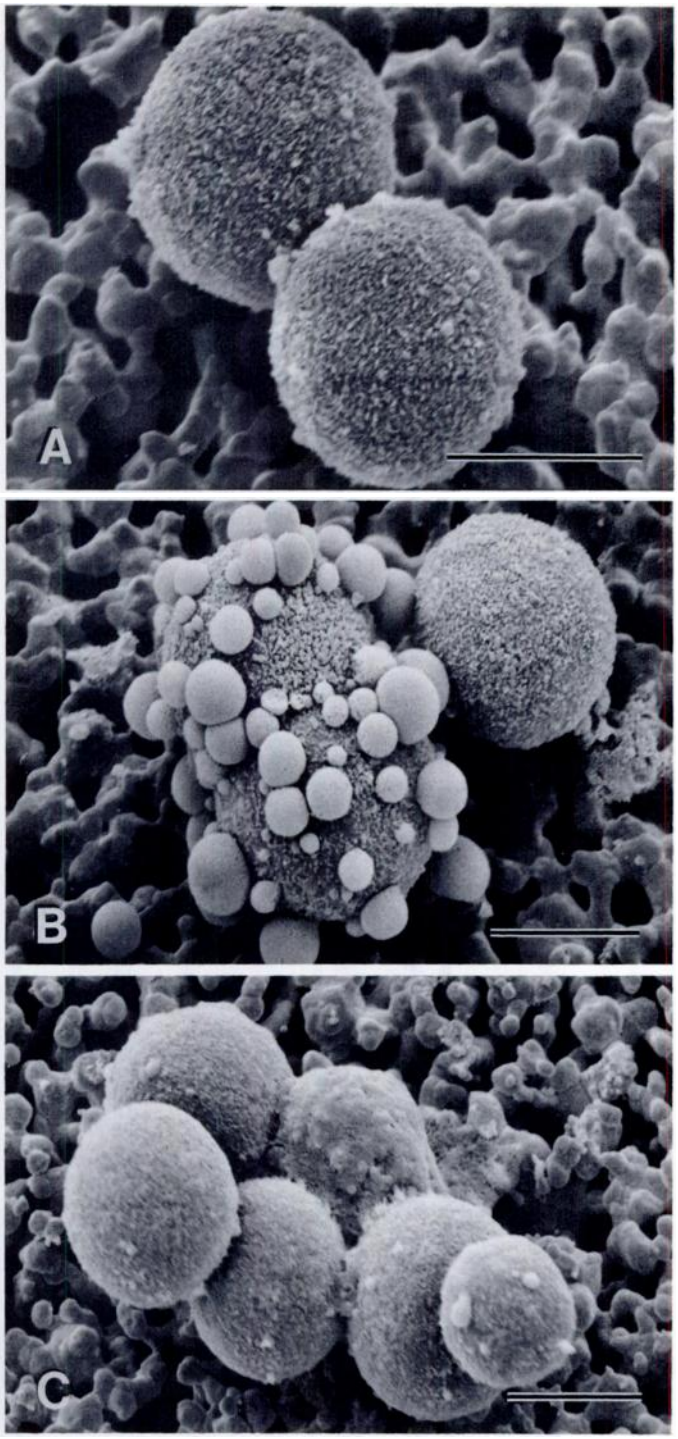

Fig. 5. Effect of extracellular Ca^{++} concentration on blebbing caused by Quin 2-AM. Cells treated as described in the legend to figure 4 were analyzed for blebbing by scanning electron microscopy. Photos taken are representative of the particular treatment group. A, cells 4 hr after treatment with 2 mM Ca^{++} and dimethylsulfoxide vehicle (×2700); B, cells 4 hr after treatment with 2 mM Ca^{++} and 25 μM Quin 2-AM (×2200); C, cells 4 hr after treatment with 0 mM Ca^{++} and 25 μM Quin 2-AM (×2200). Bar = 10 μm.

TABLE 2

Effect of Quin 2-AM and calcium on cell blebbing

Hepatocytes (2 × 10^6/ml) were incubated in medium containing 0, 2 or 10 mM Ca^{++} and either Quin 2-AM (25 μM) or vehicle and were fixed for examination by S.E.M. as described under "Methods." Values reported are based on examination of at least 100 cells/group.

Treatment		Cells with Blebs	Bleb Size
Calcium	Quin 2-AM		
mM		%	
0	+	10	Small
2	+	50	Medium-large
10	+	50	Small-medium
0	−	50	Large
2	−	20	Small
10	−	75	Small-medium

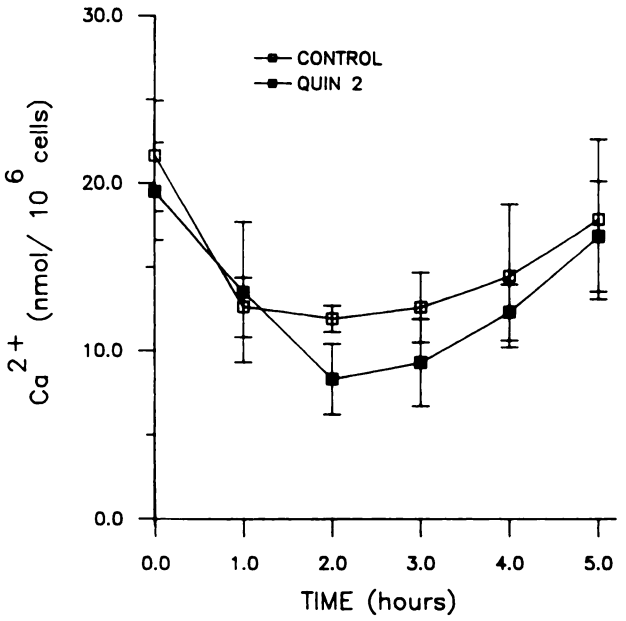

Fig. 6. Effect of Quin 2-AM on intracellular Ca^{++}. Cells were treated with Quin 2-AM (25 μM) or vehicle and were analyzed for intracellular Ca^{++} content by atomic absorption as described under "Methods"; $n = 5$ for each group.

the agent to induce lipid peroxidation (Quin 2 >> Indo 1 > 5,5′-DM BAPTA > Fluo 3 ≥ 4,4′-DF BAPTA), it appears that this is not the mechanism whereby the AM esters of the Ca^{++} indicators induce lipid peroxidation.

Because lipid hydroperoxides can be reduced by glutathione peroxidase (Horton and Fairhurst, 1987), it is curious why treatment of hepatocytes with Quin 2-AM induced lipid peroxidation without also causing GSH depletion. Furthermore, because it has been demonstrated that vitamin E helps maintain the intracellular GSH level during chemically induced injury (Reed *et al.*, 1987), it is peculiar that Quin 2-AM causes a substantial amount of vitamin E depletion without causing glutathione depletion. One possible explanation for these results is that treatment of cells with Quin 2-AM may cause MDA formation and vitamin E loss by stimulating arachidonic acid release from cells; not by stimulating lipid peroxidation. Results of experiments designed to address this question are described in the next article in this journal.

Inasmuch as van der Zee and co-workers (1989) also showed that Quin 2 stimulates arachidonic acid metabolism *via* prostaglandin H synthase, the possibility exists that Quin 2-AM induces MDA formation in our model *via* this mechanism. However, it should be noted that phenol (25 μM), which stimulates prostaglandin synthase activity better than Quin 2 (van der Zee, *et al.*, 1989), does not stimulate MDA formation by isolated hepatocytes (data not shown). These results suggest

that Quin 2-AM did not induce MDA formation in hepatocytes by stimulating prostaglandin H synthase activity. Clearly, additional studies need to be performed to determine how Quin 2-AM causes MDA release and vitamin E loss from hepatocytes.

In conclusion, prolonged incubation of isolated hepatocytes with Quin 2-AM causes toxicity which is characterized by an increased amount of lipid peroxidation, LDH release, cell blebbing and vitamin E loss. As results of this investigation suggest, agents such as Quin 2-AM (or even Indo 1-AM) should not be used to monitor changes in intracellular Ca^{++} which may occur after treatment of hepatocytes with agents that can induce an "oxidative stress," particularly if incubation with Quin 2-AM over more than 1 hr is necessary. Although the mechanism for Quin 2-AM toxicity is unknown, our data suggest that Ca^{++} may be involved in its manifestation. The role of Ca^{++}, as well as the role of other species in development of this toxicity, is the focus of the next article.

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References

BOLT, H. M.: Experimental toxicology of formaldehyde. J. Cancer Res. Clin. Oncol. **113:** 305-309, 1987.

BOOBIS, A. R., SEDDON, G. E., NASSERI-SINA, P. AND DAVIES, D.: Evidence for a direct role of intracellular calcium in paracetamol toxicity. Biochem. Pharmacol. **39:** 1277-1281, 1990.

CANTONI, O., SESTILI, P., CATTABENI, F., BELLOMO, G., POU, S., COHEN, M. AND CERUTTI, P.: Calcium chelator Quin 2 prevents hydrogen peroxide-induced DNA breakage and cytotoxicity. Eur. J. Biochem. **182:** 209-212, 1989.

CORK, R. J., STRAUTMAN, A. F. AND ROBINSON, K. R.: Measuring cytoplasmic calcium: A review of three methods with emphasis on the practical aspects of their use. Biol. Bull. **176:** 25-30, 1989.

DHANBHOORA, C. M. AND BABSON, J. R.: Effects of ethacrynic acid on primary rat myocardial cell thiol status and intracellular Ca^{2+} homeostasis. Toxicologist **10:** 131, 1990.

ERWIN, B. G., STOSCHECK, G. M. AND FLORINI, J. R.: A rapid, fluorometric method for the estimation of DNA in cultured cells. Anal. Biochem. **110:** 291-294, 1981.

FARISS, M. W., BROWN, M. K., SCHMITZ, J. A. AND REED, O. J.: Mechanism of chemical-induced toxicity. 1. Use of a rapid centrifugation technique for the separation of viable and nonviable hepatocytes. Toxicol. Appl. Pharmacol. **79:** 283-295, 1985a.

FARISS, M. W., PASCOE, G. A. AND REED, D. J.: Vitamin E reversal of the effect of extracellular calcium on chemically induced toxicity in hepatocytes. Science (Wash. DC) **227:** 751-754, 1985b.

HESKETH, T. R., SMITH, G. A., MOORE, J. P., TAYLOR, M. V. AND METCALFE, J. C.: Free cytoplasmic calcium concentrations and the mitogenic stimulation of lymphocytes. J. Biol. Chem. **258:** 4876-4882, 1983.

HORTON, A. A. AND FAIRHURST, S.: Lipid peroxidation and mechanisms of toxicity. CRC Crit. Rev. Toxicol. **18:** 27-79, 1987.

KAPPUS, H. AND SIES, H.: Toxic drug effects associated with oxygen metabolism: Redox cycling and lipid peroxidation. Experentia (Basal) **37:** 1233-1241, 1981.

MCNEIL, P. L., SWANSON, J. A., WRIGHT, S. D., SILVERSTEIN, S. C. AND TAYLOR, D. L.: Fc-receptor-mediated phagocytosis occurs in macrophages without an increase in average $[Ca^{++}]_i$. J. Cell Biol. **101:** 1586-1592, 1986.

RAO, G. H., PELLER, J. D., SEMBA, C. P. AND WHITE, J. G.: Influence of the calcium-sensitive fluorophore, Quin 2, on platelet function. Blood **67:** 354-361, 1986.

REED, D. J., BABSON, J. R., BEATTY, P. W., BRODIE, A. E., ELLIS, W. W. AND POTTER, D. W.: High performance liquid chromatography analysis of nanomolar levels of glutathione, glutathione disulfide, and related thiols and disulfides. Anal. Biochem. **106:** 55-62, 1980.

REED, D. J., PASCOE, G. A. AND OLAFSDOTTIR, K.: Some aspects of cell defense mechanisms of glutathione and vitamin E during cell injury. Arch. Toxicol. **11:** (Suppl.) 34-38, 1987.

STACEY, N. H. AND KLAASSEN, C. D.: Copper toxicity in isolated rat hepatocytes. Toxicol. Appl. Pharmacol. **58:** 211-220, 1981.

STEEL, R. G. D. AND TORRIE, J. H.: Principles and Procedures of Statistics: A Biometrical Approach, 2nd ed., pp. 173-175, 188-189, McGraw-Hill, New York, 1980.

TEMCHAROEN, P. AND THILLY, W. G.: Toxic and mutagenic effects of formaldehyde in *Salmonella typhimurium*. Mutat. Res. **119:** 89-93, 1983.

TSIEN, R. Y.: A non-disruptive technique for loading calcium buffers and indicators into cells. Nature (Lond.) **290:** 527-528, 1981.

TSIEN, R. Y., POZZAN, T. AND RINK, T. J.: Calcium homeostasis in intact lymphocytes; cytoplasmic free calcium monitored with a new, intracellularly trapped fluorescent indicator. J. Cell Biol. **94:** 325-334, 1982.

VAN DER ZEE, J., MASON, R. P. AND ELING, T. E.: The oxidation of the calcium probe Quin 2 and its analogs by prostaglandin H synthase. Arch. Biochem. Biophys. **271:** 64-71, 1989.

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