Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 4641–4645, July 1986
Biochemistry

Exogenous glutathione protects intestinal epithelial cells from oxidative injury

(basolateral membrane/sodium-dependent transport/menadione/peroxides)

LAWRENCE H. LASH*, TORY M. HAGEN, AND DEAN P. JONES†

Department of Biochemistry, Emory University School of Medicine, Atlanta, GA 30322

Communicated by Ronald W. Estabrook, February 28, 1986

ABSTRACT **Exogenous GSH provided rat small-intestinal epithelial cells with significant protection against injury induced by *t*-butyl hydroperoxide or menadione. This protection was found to be dependent upon uptake of intact GSH. Uptake of GSH occurred by a Na^+-dependent electrogenic system found in the basolateral membrane. Thus, rat small-intestinal epithelial cells can utilize plasma GSH to support intracellular detoxication systems that function in protection against chemically induced injury.**

Drug-metabolizing and oxidation–reduction systems in the epithelium of the small intestine represent a first line of defense against ingested xenobiotics and toxins. Glutathione (GSH) is an important determinant of the protection against chemical injury, by serving as a substrate for glutathione transferases (RX:glutathione R-transferase, EC 2.5.1.18) and glutathione peroxidase (glutathione:hydrogen peroxide oxidoreductase, EC 1.11.1.9). Glutathione transferases catalyze the reaction of GSH with electrophilic compounds to form nontoxic conjugates (1), and glutathione peroxidase utilizes GSH as a reductant to reduce toxic peroxides (2, 3). Depletion of GSH potentiates injury from both types of processes (4, 5), and stimulation of processes that support maintenance of intracellular GSH protects against injury (6, 7).

The liver releases GSH at a substantial rate (8) and contributes to maintenance of circulating GSH in the plasma (9). In principle, transport systems in extrahepatic cells could allow uptake of this GSH to protect against chemical injury. Although of obvious toxicological importance, it has not been determined whether exogenous GSH can protect against toxicity in small-intestinal cells, or whether an uptake system, such as has been found in kidney basolateral membrane (10, 11), is also present in small intestine. We therefore addressed these issues with isolated rat intestinal cells and purified plasma membrane vesicles. The results show that exogenous GSH provides substantial protection against oxidative injury by *t*-butyl hydroperoxide or menadione due to the function of a Na^+-dependent GSH uptake system which is present in the basolateral membrane of the epithelial cells.

EXPERIMENTAL PROCEDURES

GSH, phenylmethylsulfonyl fluoride, valinomycin, 1-fluoro-2,4-dinitrobenzene, Percoll, collagenase (type I), and hyaluronidase were purchased from Sigma. Nitrocellulose filters (0.45-μm pore size) were purchased from Gelman. [*glycine*-2-^{3}H]GSH (1.1 Ci/mmol, 1 Ci = 37 GBq) was purchased from New England Nuclear. The purity of the radiolabeled GSH was routinely assessed by derivatizing it with iodoacetic acid and 1-fluoro-2,4-dinitrobenzene, followed by HPLC analysis (see below). Greater than 92% of the ^{3}H counts were eluted in a single peak that coincided with that of authentic GSH. AT-125 [L-(α*S*,5*S*)-α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid] was a gift from D. J. Reed (Oregon State University). All other chemicals were of reagent grade and were purchased locally.

Male white rats (Sprague–Dawley-derived, barrier-reared outbred albino, King Animal Laboratories, Oregon, WI; 200–300 g) were anesthetized with diethyl ether and killed by cutting through the diaphragm. A 40-cm segment of the jejunum was removed from each of two rats, the lumens were washed with ice-cold 0.9% (wt/vol) NaCl, and epithelial cells were obtained by gently scraping the villous mucosa. The epithelial scrapings were immediately placed in 250 mM sucrose containing 10 mM Tris/Hepes (pH 7.6) and 0.1 mM phenylmethylsulfonyl fluoride to inhibit proteolysis. The brush-border and basolateral regions of the plasma membrane were prepared according to the Percoll density-gradient centrifugation method of Scalera *et al.* (12). The two regions of the plasma membrane were identified and contamination with other cellular organelles was assessed by use of marker enzymes as described (13). For use in transport studies, fractions from the Percoll gradient corresponding to the basolateral region of the plasma membrane were pooled and concentrated as described (14). Protein concentration was determined by the method of Bradford (15) with the dye-reagent concentrate from Bio-Rad and bovine serum albumin as standard.

Intestinal epithelial cells were prepared by the method of Grafström *et al.* (16), using collagenase and hyaluronidase to digest connective tissue. Cell viability and concentration were estimated with 0.16% (wt/vol) trypan blue in a hemacytometer. Cell viability was typically 90–95% and yield was $\approx 50 \times 10^6$ cells per intestine.

Uptake of radiolabeled GSH in membrane vesicles was measured by filtration at room temperature under anaerobic conditions or in the presence of 5 mM dithiothreitol, as described (11). Vesicles were preincubated with 0.25 mM AT-125 to inhibit GSH catabolism (17, 18). Membrane potential values are K^+-diffusion potentials obtained by preincubating vesicles with the K^+ ionophore valinomycin (20 μg/ml) and subsequently measuring uptake in the presence of various intravesicular/extravesicular ratios of K^+ (11). Composition of intra- and extravesicular solutions is detailed in the legend to Fig. 2.

Uptake of GSH by isolated intestinal cells, suspended in Krebs–Henseleit buffer (118 mM NaCl/4.7 mM KCl/1.3 mM $CaCl_2$/1.2 mM K_2HPO_4/1.2 mM $MgSO_4$/25 mM $NaHCO_3$,

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: GSH, glutathione; GSSG, glutathione disulfide; AT-125, L-(α*S*,5*S*)-α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid.

*Present address: Department of Pharmacology, The University of Rochester School of Medicine, Rochester, NY 14642.
†To whom reprint requests should be addressed at: Department of Biochemistry, Emory University School of Medicine, Woodruff Memorial Building, Atlanta, GA 30322.

pH 7.4) supplemented with 25 mM Hepes, 5 mM glucose, 25 units of heparin per ml, and 500 units of penicillin per ml, was measured at 37°C on a shaking water bath by incubating 5×10^5 cells per ml with 1 mM GSH. In some experiments, cells were pretreated with 0.25 mM AT-125 and/or 0.2 mM buthionine sulfoximine to inhibit GSH degradation (17, 18) and synthesis (19), respectively. After incubation, 0.5-ml aliquots were layered on 1 ml of 10% (vol/vol) Percoll in Krebs–Henseleit buffer and were centrifuged for 1 min in a Fisher microcentrifuge. Acid extracts of the cell pellet were derivatized with iodoacetic acid and 1-fluoro-2,4-dinitrobenzene for analysis by HPLC by the method of Reed *et al.* (20) as modified (13). Derivatives of GSH and its metabolites were separated on a 10-μ Ultrasil-NH_2 column (4.6 mm × 25 cm; Beckman).

RESULTS

To test whether exogenous GSH can be utilized by enterocytes to protect against chemically induced injury, we employed isolated intestinal cells that were treated with either *t*-butyl hydroperoxide or menadione. Toxicity due to each of these compounds has been extensively studied with isolated hepatocytes and is known to involve thiol oxidation (21, 22). Studies of the time-dependence of enterocyte viability with various concentrations of *t*-butyl hydroperoxide or menadione showed that enterocytes are slightly more sensitive to these agents than are hepatocytes (data not shown). Either 50 μM *t*-butyl hydroperoxide or 25 μM menadione gave >50% loss of cell viability within 2 hr, whereas control viabilities were essentially unchanged (Fig. 1). These conditions were used to test whether exogenous GSH could afford protection against oxidative injury.

Incubation of cells with 1 mM GSH for 20 min prior to addition of either *t*-butyl hydroperoxide or menadione provided marked protection against loss of cell viability (Fig. 1). To test whether this protection was due to supply of amino acid precursors from the hydrolysis of GSH and resynthesis of intracellular GSH, cells were incubated with 1 mM concentrations of glutamate, cysteine, and glycine. Only a small amount of protection was seen under these conditions (Fig. 1), indicating that intracellular synthesis of GSH could not account for the protection seen. Substitution of 0.5 mM cystine for cysteine similarly only provided a small amount of protection (data not shown). Furthermore, treatment of cells with AT-125 (to inhibit GSH breakdown) and buthionine sulfoximine (to inhibit GSH resynthesis) did not alter the ability of exogenous GSH to protect cells from loss of viability due to *t*-butyl hydroperoxide or menadione. The results of these two types of experiments clearly show that the protection is due to intact GSH and does not involve hydrolysis and resynthesis.

Preincubation of cells with 0.5 mM glutathione disulfide (GSSG) prior to addition of 50 μM *t*-butyl hydroperoxide provided the same degree of protection against loss of cell viability as was observed when cells were preincubated with 1 mM GSH, indicating that GSSG is reduced intracellularly to exert protection. Dithiothreitol (1 mM) fully protected against *t*-butyl hydroperoxide cytotoxicity, suggesting that toxicity is associated with thiol oxidation that results from hydroperoxide metabolism; however, 1 mM cysteine did not provide protection.

To test whether intact GSH is transported into enterocytes, cells were pretreated with AT-125 to inhibit γ-glutamyltransferase, and GSH content was measured as a function of time during incubation with exogenous GSH. In the absence of exogenous GSH, intracellular GSH concentration declined over 60 min from 1.3 to 0.5 nmol per 10^6 cells (Fig. 2), indicating a slow utilization of GSH or release from the cells. In the presence of 1 mM extracellular GSH, intracellular GSH content increased to 9.7 ± 3.6 nmol per 10^6 cells (equivalent to ≈7.7 mM, assuming 1.25 μl per 10^6 cells) after 60 min, showing that GSH is transported against a concentration gradient into these cells. To exclude the possibility of degradation due to residual γ-glutamyltransferase activity, uptake of the constituent amino acids, and resynthesis of

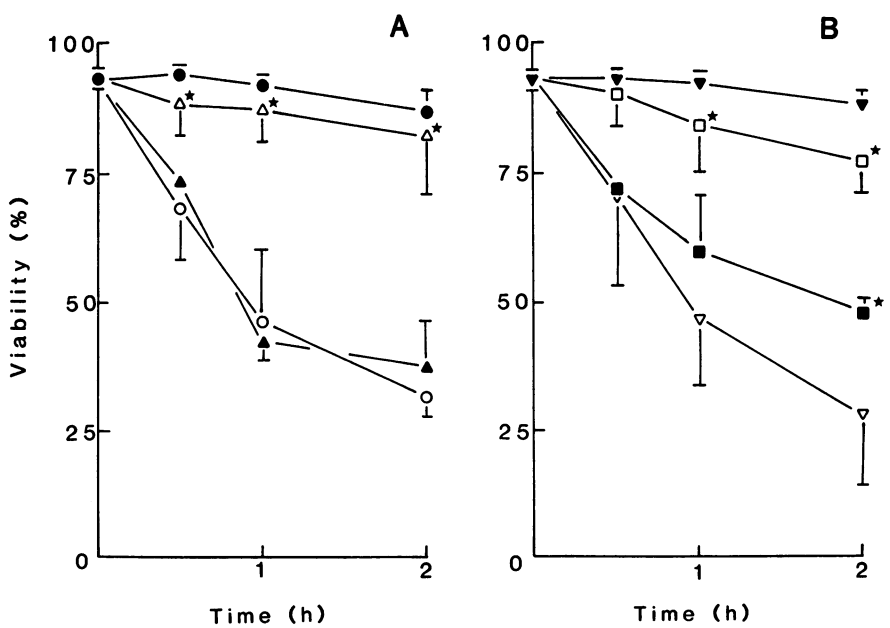

FIG. 1. Exogenous GSH protects against toxicity due to *t*-butyl hydroperoxide (*A*) and menadione (*B*) in suspensions of isolated rat small-intestinal cells. Cells (10^6 per ml) were incubated at 37°C on a shaking water bath under the conditions described below. Where indicated, cells were preincubated with 1 mM GSH for 20 min prior to the start of incubations. Cell viability was measured at the indicated times on a hemacytometer in the presence of 0.16% (wt/vol) trypan blue. (*A*) Control cells (●); cells + 50 μM *t*-butyl hydroperoxide (○); cells + 50 μM *t*-butyl hydroperoxide + 1 mM GSH (△); cells + 50 μM *t*-butyl hydroperoxide + 1 mM each glutamate, cysteine, and glycine (▲). (*B*) Cells + 1 mM each glutamate, cysteine, and glycine (▼); cells + 25 μM menadione (▽); cells + 25 μM menadione + 1 mM GSH (□); cells + 25 μM menadione + 1 mM each glutamate, cysteine, and glycine (■). Results are means ± SEM for cells from four animals. Stars indicate statistically significant difference ($P < 0.05$) between time pairs with and without GSH.

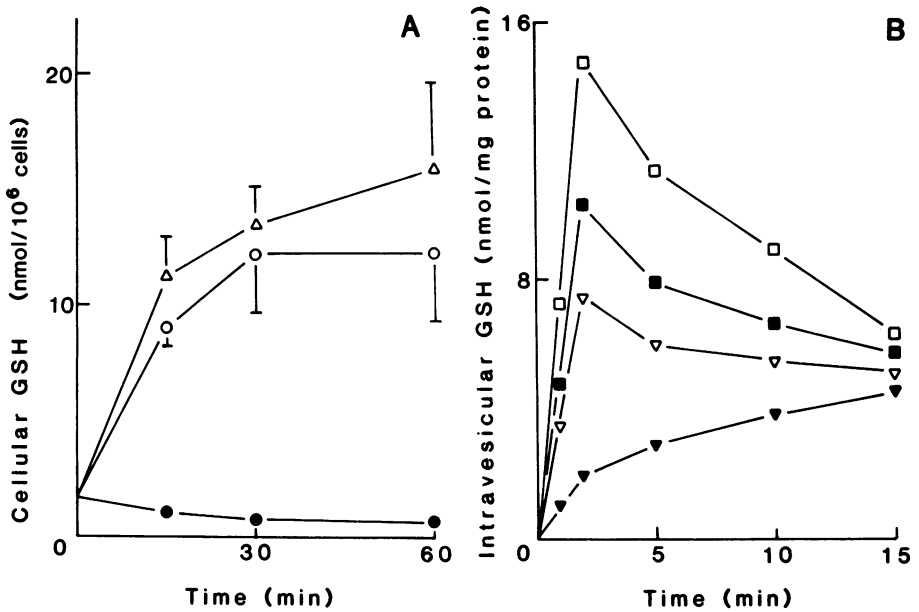

FIG. 2. Uptake of GSH by isolated intestinal cells (*A*) and intestinal basolateral membrane vesicles (*B*). (*A*) Cells (5×10^5 per ml), suspended in Krebs–Henseleit buffer, were pretreated with 0.25 mM AT-125 and were incubated at 37°C without added substrates (●) or with 1 mM GSH in the absence (○) or presence (△) of 0.2 mM buthionine sulfoximine. At the indicated times, 0.5-ml aliquots were centrifuged through 10% (vol/vol) Percoll, and GSH content in the cell pellet was measured by HPLC. Results are means ± SEM of five preparations. Protein content was 0.25 ± 0.01 mg per 10^6 cells ($n = 7$). (*B*) Uptake of 1 mM GSH was measured with radiolabeled GSH. The calculated Nernst K^+-diffusion potential was varied using valinomycin in the presence of various intra- to extravesicular KCl gradients. Under all conditions, solutions contained 10 mM Tris/Hepes, pH 7.6. Intravesicular solutions contained 100 mM sucrose, 50 mM KCl, and 100 mM TrisCl (adjusted to pH 7.6 with Tris base). For measurement of uptake at 0 mV in the absence of Na^+ (▽), extravesicular solutions contained 50 mM KCl, 100 mM sucrose, and 100 mM TrisCl (adjusted to pH 7.6 as above). For measurement of uptake in the presence of Na^+, extravesicular solutions contained 100 mM NaCl and KCl and TrisCl, respectively, as follows: 5 mM, 95 mM (−60 mV, □); 50 mM, 50 mM (0 mV, ■); 100 mM, 0 mM (+18 mV, ▽). Results are the means of three preparations.

GSH, transport was also measured in the presence of 0.2 mM buthionine sulfoximine. This treatment did not inhibit the increase in cellular GSH (Fig. 2*A*), thus showing that it is the intact tripeptide which was transported into intestinal epithelial cells.

Intestinal epithelia and renal epithelia have many common transport systems, and renal epithelial cells contain a Na^+-dependent electrogenic uptake system in the basolateral region of the plasma membrane that functions in uptake of GSH (11). The possibility that a similar GSH transport system occurs in the intestinal epithelium was directly studied by measuring uptake of GSH in intestinal basolateral membrane vesicles in the presence or absence of Na^+ and at various membrane potentials (Fig. 2*B*). Results are given for studies in which uptake of radiolabeled GSH was measured, but assay by HPLC (20) showed that GSH was the species transported. With membrane potential clamped at 0 mV, the initial rate of GSH uptake was stimulated 4-fold by an inwardly directed gradient of 100 mM NaCl (5.22 ± 0.27 nmol/min per mg of protein) as compared to uptake in the absence of Na^+ (1.31 ± 0.05 nmol/min per mg of protein). In the presence of Na^+, the time course exhibited an overshoot, indicating transport of GSH against a concentration gradient. Hyperpolarization of the membrane potential to −60 mV in the presence of Na^+ increased the initial rate of uptake (7.36 ± 0.14 nmol/min per mg of protein), whereas depolarization of the membrane potential to +18 mV in the presence of Na^+ decreased the initial rate of uptake (4.37 ± 0.35 nmol/min per mg of protein) relative to uptake at 0 mV. In the absence of Na^+, variation of membrane potential had no significant effect on GSH transport, and, as found with renal basolateral membrane vesicles, transport was inhibited by other γ-glutamyl compounds and probenecid (data not shown). Therefore, an electrogenic Na^+/GSH cotransport system is present in intestinal basolateral membranes that has characteristics similar to the renal system. This system has appropriate localization and activity to provide transport of GSH from plasma into enterocytes.

To test whether uptake by this system is involved in protection against toxicity, incubations were performed as described for Fig. 1*B* with addition of inhibitors of Na^+-dependent GSH uptake. Additions of 10 mM ophthalmic acid and 0.1 mM probenecid to incubation mixtures with GSH and either *t*-butyl hydroperoxide or menadione eliminated the protective effect of GSH (data not shown). Thus, exogenous GSH must be taken up by the cell to protect against oxidative injury.

All of the above experiments were performed with a relatively high concentration of GSH to obviate problems related to decreases in GSH concentration during incubation. To determine whether a physiological concentration of GSH could also protect against oxidative injury, experiments were performed with cells pretreated with AT-125 and then treated with *t*-butyl hydroperoxide as described for Fig. 1, except that 20 μM GSH was substituted for 1 mM GSH. The results showed that this concentration provided the same extent of protection as seen with 1 mM GSH. Thus, exogenous GSH at a physiological plasma concentration provides enterocytes with protection against oxidative injury.

Direct distinction between uptake of GSH by the basolateral region and that by the brush-border region of the plasma membrane is not possible with isolated enterocytes. However, recent studies of isolated perfused intestinal segments show that addition of 0.1 mM probenecid to the vascular perfusate inhibits GSH transport, whereas addition to the lumenal fluid does not (T.M.H. and D.P.J., unpublished results). Consequently, we examined the ability of 0.1 mM probenecid to prevent the protection against cell death afforded by GSH. With cells pretreated with AT-125 and treated with *t*-butyl hydroperoxide as described above, probenecid almost completely prevented protection by 20 μM GSH but only partially (≈50%) prevented protection by

1 mM GSH. Thus, it appears that transport across the basolateral membrane is important in protection but may not be the sole system involved, especially at higher extracellular GSH concentrations.

DISCUSSION

Epithelial cells have a characteristically rapid turnover rate that appears to be needed to protect against injury from exogenous physical, chemical, and infectious agents. Isolated intestinal epithelial cells are known to be susceptible to chemical injury, and the current studies show that under the conditions used, these cells are comparable to hepatocytes in their sensitivity to injury from menadione (4, 22). In the absence of extracellular GSH or amino acid precursors of the tripeptide, the intracellular GSH concentration decreased 60% during a 1-hr incubation (Fig. 2). This indicates that intestinal epithelial cells require a continuous supply of GSH, in the form of either the intact tripeptide or the precursors, to maintain intracellular GSH levels.

The current findings demonstrate that exogenous GSH protects cells from chemical injury. The inability of amino acid precursors of GSH to protect against *t*-butyl hydroperoxide or menadione suggests that intestinal cells have a relatively slow rate of GSH biosynthesis. The small amount of protection seen with cysteine was not due to its toxicity upon autooxidation, because cell viability did not decrease in the control incubation with cysteine (Fig. 1). Because of this, exogenous GSH may be particularly important in defense against chemical injury.

The significant protection against the cytotoxicity of *t*-butyl hydroperoxide or menadione by exogenous GSH, under conditions where both GSH synthesis and degradation were inhibited, provides evidence that added GSH is used directly to provide this protection. Moreover, the elimination of protection by inhibitors of GSH uptake shows that the presence of extracellular GSH is not sufficient for protection and that the reaction of extracellular GSH with *t*-butyl hydroperoxide or reactive metabolites formed from *t*-butyl hydroperoxide or menadione does not play a significant role in the mechanism of protection. Although the loss of cell viability may be due to other effects besides GSH depletion, the protection afforded by dithiothreitol indicates that thiol oxidation plays an important role in the toxicity.

The presence of a transport system was confirmed by measurement of uptake of intact [^{3}H]GSH by the isolated cells. Further examination of the properties of the GSH transport system in the basolateral membrane shows that the properties are the same as those reported for the renal system (10, 11). Previous studies have shown that GSH is transported out of liver at a substantial rate (8, 23–25). The renal and intestinal GSH transport systems have the correct subcellular localization to function *in vivo* to supply GSH to epithelial cells. The electrogenic nature and coupling to Na^+ uptake provide sufficient driving force to take up GSH from plasma at physiological plasma and cellular GSH concentrations (11). Thus, the GSH released from the liver may serve to support detoxification processes in epithelial cells.

A recent study employing intestinal brush-border membrane vesicles indicated that luminal GSH may also be transported as the intact tripeptide into intestinal cells (26). Thus, enterocytes may also be able to utilize ingested GSH as well as plasma GSH for maintenance of intracellular concentrations. The presence of transport systems on both poles of the enterocyte further suggests that transepithelial transport of GSH may occur, which we have recently confirmed (T.M.H. and D.P.J., unpublished data).

The relative importance of the two sources of GSH is dependent upon nutritional status. Under physiological conditions *in vivo*, intestinal cells are continuously exposed to plasma GSH. In the absence of luminal nutrients, such as during the post-absorptive state or in certain diseases, the importance of the plasma pool of GSH may be enhanced. Although exogenous GSH is equally accessible to the brush-border and basolateral membranes in isolated intestinal cells, the elimination of protection by probenecid suggests that the protection is dependent upon transport of GSH across the basolateral membrane, since this compound does not inhibit transport across the brush-border membrane (T.M.H. and D.P.J., unpublished data).

Another consideration involving the anatomy of the intestinal mucosa is the villus–crypt relationship. The cell preparation used in this study yields mostly villus tip cells (16). Whether or not GSH transport also occurs in crypt cells has not been investigated.

The agents used in the present study cause injury by oxidative mechanisms. Naturally occurring compounds that also can cause oxidative injury are common in human diets (27). Isolated intestinal cells are therefore a relevant system in which to investigate the role of GSH in protection against oxidative stress because the small-intestinal epithelium serves as a first line of defense against such ingested toxic chemicals. Furthermore, oxidative mechanisms have also been implicated in post-ischemic reoxygenation injury in the small intestine (28). Thus, supply of GSH to the intestine to protect against oxidative injury may be important under diverse physiological and pathological conditions and may be useful therapeutically.

In conclusion, the results of this study show that exogenous GSH can be used to protect intestinal cells against oxidative chemical injury. This suggests that plasma GSH can function as a source of epithelial cell GSH *in vivo* and indicates that therapeutic administration of GSH may be useful in protecting epithelial cells.

This study was supported by National Institutes of Health Grants HL30286 and GM36538.

1. Kaplowitz, N. (1980) *Am. J. Physiol.* **239,** G439–G444.
2. Wendel, A. (1980) in *Enzymatic Basis of Detoxication*, ed. Jakoby, W. B. (Academic, New York), Vol. 1, pp. 333–353.
3. Jones, D. P., Eklöw, L., Thor, H. & Orrenius, S. (1981) *Arch. Biochem. Biophys.* **210,** 505–516.
4. Thor, H., Smith, M. T., Hartzell, P., Bellomo, G., Jewell, S. A. & Orrenius, S. (1982) *J. Biol. Chem.* **257,** 12419–12425.
5. Wefers, H. & Sies, H. (1983) *Arch. Biochem. Biophys.* **224,** 568–578.
6. Bellomo, G., Jewell, S. A., Thor, H. & Orrenius, S. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 6842–6846.
7. Babson, J. R., Abell, N. S. & Reed, D. J. (1981) *Biochem. Pharmacol.* **30,** 2299–2304.
8. Sies, H. & Graf, P. (1985) *Biochem. J.* **226,** 545–549.
9. Lash, L. H. & Jones, D. P. (1985) *Arch. Biochem. Biophys.* **240,** 583–592.
10. Lash, L. H. & Jones, D. P. (1983) *Biochem. Biophys. Res. Commun.* **112,** 55–60.
11. Lash, L. H. & Jones, D. P. (1984) *J. Biol. Chem.* **259,** 14508–14514.
12. Scalera, V., Storelli, C., Storelli-Joss, C., Haase, W. & Murer, H. (1980) *Biochem. J.* **186,** 177–181.
13. Lash, L. H. & Jones, D. P. (1983) *Arch. Biochem. Biophys.* **225,** 344–352.
14. Lash, L. H. & Jones, D. P. (1984) *Am. J. Physiol.* **247,** G394–G401.
15. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254.
16. Grafström, R., Moldéus, P., Andersson, B. & Orrenius, S. (1979) *Med. Biol.* **57,** 287–293.
17. Reed, D. J., Ellis, W. W. & Meck, R. A. (1980) *Biochem. Biophys. Res. Commun.* **94,** 1273–1277.
18. Schasteen, C. S., Curthoys, N. P. & Reed, D. J. (1983) *Biochem. Biophys. Res. Commun.* **112,** 564–570.

19. Griffith, O. W. & Meister, A. (1979) *J. Biol. Chem.* **254,** 7558–7560.
20. Reed, D. J., Babson, J. R., Beatty, P. W., Brodie, A. E., Ellis, W. W. & Potter, D. W. (1980) *Anal. Biochem.* **106,** 55–62.
21. Di Monte, D., Ross, D., Bellomo, G., Eklöw, L. & Orrenius, S. (1984) *Arch. Biochem. Biophys.* **235,** 334–342.
22. Orrenius, S., Ormstad, K., Thor, H. & Jewell, S. (1983) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **42,** 3177–3188.
23. Bartoli, G. M. & Sies, H. (1978) *FEBS Lett.* **86,** 89–91.
24. Inoue, M., Kinne, R., Tran, T. & Arias, I. M. (1983) *Eur. J. Biochem.* **134,** 467–471.
25. Inoue, M., Kinne, R., Tran, T. & Arias, I. M. (1984) *Eur. J. Biochem.* **138,** 491–495.
26. Linder, M., DeBurlet, G. & Sudaka, P. (1984) *Biochem. Biophys. Res. Commun.* **123,** 929–936.
27. Ames, B. (1983) *Science* **221,** 1256–1264.
28. Granger, D. N., Rutili, G. & McCord, J. M. (1981) *Gastroenterology* **81,** 22–29.