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Hydroperoxides can modulate the redox state of pyridine nucleotides and the calcium balance in rat liver mitochondria

(glutathione peroxidase/selenium/calcium release)

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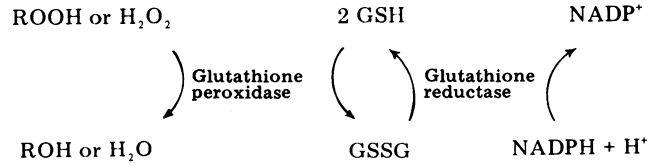
ABSTRACT **When rats are fed a selenium-deficient diet, the glutathione peroxidase activity in liver mitochondria decreases within 5 weeks to 0–6% of that of control animals fed on a diet supplemented with 0.5 ppm of selenium as sodium selenite. Analysis of the temperature dependence of energy-linked Ca^{2+} uptake by means of Arrhenius plots reveals two breaks (at around 11°C and 24°C) in mitochondria isolated from selenium-supplemented animals, whereas in selenium-deficient rats the break at 11°C is absent. Ca^{2+}-loaded mitochondria of selenium-supplemented rats—i.e., with active glutathione peroxidase in the matrix—lose Ca^{2+} rapidly, with a concomitant oxidation of endogenous NAD(P)H, when exposed to *t*-butyl hydroperoxide or H_2O_2. In contrast, in selenium deficiency, *t*-butyl hydroperoxide and H_2O_2 induce neither a release of Ca^{2+} nor an oxidation of NAD(P)H. The peroxide-induced oxidation of NAD(P)H is reversible in the presence of succinate when no Ca^{2+} has been taken up. When Ca^{2+} has previously been accumulated, however, the oxidation of NAD(P)H is irreversible. Enzymatic analysis of mitochondrial pyridine nucleotides reveals that the peroxide-induced oxidation of NAD(P)H in Ca^{2+}-loaded mitochondria leads to a loss of NAD^+ and $NADP^+$. It is proposed that the redox state of mitochondrial pyridine nucleotides can be or is in part controlled by glutathione peroxidase and glutathione reductase and is a factor in the balance of Ca^{2+} between mitochondria and medium.**

In rat liver, hydroperoxides can be reduced by glutathione peroxidase [glutathione (GSH):H_2O_2 oxidoreductase, EC 1.11.1.9]. Glutathione reductase [NAD(P)H: oxidized-glutathione (GSSG) oxidoreductase, EC 1.6.4.2.] regenerates GSH from GSSG at the expense of NADPH, according to (ROOH = organic hydroperoxide):



The enzymes are located in the cytosol and mitochondrial matrix space (1). Two kinds of glutathione peroxidase are known. They differ in the requirement for selenium, substrate specificity, and subcellular distribution (2, 3). The selenium-dependent glutathione peroxidase shows high activity towards organic hydroperoxides and H_2O_2 as substrates, whereas the selenium-independent enzyme is mainly active towards organic hydroperoxides and has only little activity towards H_2O_2. Evidence has been presented that liver mitochondria generate H_2O_2 and O_2^- (4–6). The formation of lipid hydroperoxides, measured as increased malondialdehyde production or increased O_2 consumption, is enhanced when mitochondria are exposed to oxidative stress (7, 8).

A fundamental property of mitochondria is the ability to accumulate Ca^{2+} ions in the matrix space (9). It is now generally accepted that Ca^{2+} uptake is an electrogenic process that is driven by the electrical component of the total proton motive force (10), generated either by an active respiratory chain or ATP hydrolysis. By lowering the potential—e.g., by uncouplers—a release of the accumulated Ca^{2+} is observed, which is due to the reversal of the uptake pathway (11). However, it has recently been shown that the release of Ca^{2+} from mitochondria normally occurs via a different pathway than the uptake. In some types of mitochondria, this pathway has been identified as an obligatory Na^+/Ca^{2+} exchange (12, 13). In liver, the separate release pathway has not been identified, although conclusive evidence for its existence has been presented (14). Presently, the regulation mechanisms of the Ca^{2+} influx and efflux systems are not well understood. It is clear, however, that their regulation is mandatory, because many cytosolic enzyme activities are modulated by Ca^{2+} (15).

Recent experiments by Lehninger *et al.* (16) indicate that the redox steady state of the mitochondrial pyridine nucleotides might be involved in regulating the Ca^{2+} balance between liver mitochondria and the medium. Oxidation of NAD(P)H by oxaloacetate induced a release, whereas a rereduction of $NAD(P)^+$ by β-hydroxybutyrate promoted reuptake and retention of Ca^{2+}. Another conceivable factor in the regulation of the influx and efflux of Ca^{2+} in mitochondria is the fluidity of the membrane. That the fluidity, as modified, for example, by unsaturated fatty acids, may influence the translocation of solutes is a logical assumption, which has already been demonstrated in some cases (17). Evidence has been provided that peroxidation can also modify the fluidity state of the membrane (18).

Glutathione peroxidase could represent a link between the two regulating factors outlined above, and in the work described in the present article the metabolism of H_2O_2 and organic hydroperoxides has been investigated by using mitochondria from selenium-supplemented and selenium-deficient animals.

Our experiments clearly demonstrate alterations in the temperature dependence of Ca^{2+} uptake by liver mitochondria under conditions in which the selenium-dependent glutathione peroxidase is absent; hydroperoxides are therefore expected to accumulate in the membrane. They also show a hydroperoxide-induced oxidation of the mitochondrial pyridine nucleotides concomitant with efflux of previously accumulated Ca^{2+} into the medium. Oxidation of NAD(P)H and release of Ca^{2+} upon

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Abbreviations: GSH, glutathione (reduced form); GSSG, glutathione (oxidized form).

addition of hydroperoxide are observed only in mitochondria isolated from selenium-supplemented animals, which have a normal level of glutathione peroxidase, but not in mitochondria isolated from selenium-deficient animals. These findings indicate that hydroperoxides, as metabolized by glutathione peroxidase, are an important factor in the maintenance of the appropriate balance of Ca^{2+} between mitochondria and the cytosol in the liver and possibly in other pyridine nucleotide-dependent metabolic processes.

MATERIALS AND METHODS

Animals and Diet. Weanling male Wistar rats were fed on a Torula yeast-based, selenium-deficient diet or on the same diet supplemented with 0.5 ppm of selenium as sodium selenite (controls) for 5–7 weeks (19). The diet was purchased from ICN Pharmaceuticals, Inc., Cleveland, OH and had the following composition: 30% Torula yeast, 59% sucrose, 5% tocopherol-stripped lard, 5% salt mix HMW, 100 international units of vitamin E, plus 1% ICN vitamin diet fortification mixture omitting vitamin E.

Chemicals. Rotenone and bovine serum albumin (fraction V) were obtained from Sigma, arsenazo III was from Fluka AG, Buchs, Switzerland, and murexide was from E. Merck AG, Darmstadt, Germany. All other reagents were commercial products of the highest available grade of purity.

Preparation of Mitochondria and Mitoplasts. Liver mitochondria of fasted rats were prepared as described (20) in 210 mM mannitol/70 mM sucrose/10 mM Tris·HCl, pH 7.4/1 mM EDTA as isolation medium. The mitochondria were washed twice in this medium without EDTA. Mitoplasts were prepared according to ref. 21. Protein was determined by the Biuret method with bovine serum albumin as standard.

Assays. Mitochondrial glutathione peroxidase activity was measured at 25°C in Triton extracts of mitochondria as described (22), except that NADPH was 50 μM instead of 0.25 mM because NADPH at higher concentration is inhibitory in the glutathione peroxidase assay (23). The temperature dependence of the Ca^{2+} uptake by mitochondria was measured in a medium containing 210 mM mannitol, 70 mM sucrose, 10 mM Tris·HCl (pH 7.4), 3 μM rotenone, 0.5 mg of bovine serum albumin per ml, 50 μM arsenazo III, 5 mM succinate, and 1.5 mg of mitochondrial protein. Total volume was 2 ml. The reaction was started by addition of 50 μM $CaCl_2$ and followed by monitoring the absorption changes of arsenazo III at 675–685 nm in an Aminco DW-2 spectrophotometer. The simultaneous measurement of the redox level of pyridine nucleotides and Ca^{2+} flux was carried out in a quadruple-wavelength spectrophotometer (designed and constructed in the workshop of The Johnson Research Foundation, Philadelphia, PA). The reaction medium contained 210 mM mannitol, 70 mM sucrose, 2 mM Hepes (pH 7.4), 3 μM rotenone, 100 μM murexide, 5 mM succinate, and 3.5–4.0 mg of mitochondrial or mitoplast protein. Further additions were as indicated in Figs. 2 and 3. Total volume was 2 ml and the reaction temperature 25°C. The redox level of pyridine nucleotides was followed at the wavelength pair 345–375 nm, and Ca^{2+} fluxes—i.e., absorption changes of murexide—were followed at 507–540 nm.

For the determination of the various forms of mitochondrial pyridine nucleotides under different experimental conditions, mitochondria (4–6 mg of protein per ml) were incubated at 25°C for 4 min in 210 mM mannitol/70 mM sucrose/10 mM Tris·HCl, pH 7.4/3 μM rotenone/5 mM succinate/±200 μM $CaCl_2$. *t*-Butylhydroperoxide (1 mM) was added (where indicated) and after an additional 4 min, the pyridine nucleotides were extracted and analyzed (24).

Table 1. Glutathione peroxidase activity in liver mitochondria isolated from selenium-supplemented and selenium-deficient rats

Substrate	Selenium supplemented	Selenium deficient
t-But·OOH	104.3	6.1
H_2O_2	96.5	*

Values are given in nmol of NADPH oxidized per min per mg of protein. *t*-But·OOH = *t*-butyl hydroperoxide.
* Below detection limit.

RESULTS

Glutathione Peroxidase Activity in Liver Mitochondria of Selenium-Deficient and Selenium-Supplemented Rats. Table 1 shows the mitochondrial glutathione peroxidase activity in selenium-deficient and control rats with both *t*-butyl hydroperoxide and H_2O_2 as substrates. In selenium-deficient mitochondria no enzyme activity was detected with H_2O_2, and only about 5–6% of that of controls was detected with *t*-butyl hydroperoxide as substrate. This result demonstrates that the selenium-dependent glutathione peroxidase is totally lacking and that the selenium-independent glutathione peroxidase is almost completely absent in liver mitochondria of selenium-deficient rats.

Temperature Dependence of the Ca^{2+} Uptake in Liver Mitochondria of Selenium-Deficient and Selenium-Supplemented Rats. The possibility that hydroperoxides might influence the fluidity of the inner mitochondrial membrane (see *Introduction*) prompted the investigation of the temperature dependence of the process of mitochondrial Ca^{2+} uptake in normal and selenium-deficient animals.

In an Arrhenius plot (Fig. 1) of the temperature dependence of the rate of energy-linked Ca^{2+} uptake in liver mitochondria of selenium-supplemented animals, two breaks were observed; the first was at ≈11°C and the second was at ≈24°C. Mitochondria of selenium-deficient animals, however, showed only the discontinuity at 24°C.

Oxidation of NAD(P)H and Ca^{2+} Release in Mitochondria. When Ca^{2+} uptake in mitochondria containing active glutathione peroxidase was promoted by succinate, a small shift in the redox state of the pyridine nucleotides was observed, as shown in Fig. 2 *left*. After addition of a small amount of *t*-butyl hydroperoxide, the nucleotides became further oxidized and the accumulated Ca^{2+} leaked into the medium. Mitochondria of selenium-depleted animals, with their very low activity of glutathione peroxidase (see Table 1), accumulated Ca^{2+} in a manner similar to mitochondria from control animals (Fig. 2 *right*). Externally added organic hydroperoxide, however, failed to induce the oxidation of NAD(P)H and the release of Ca^{2+}. It seems, therefore, that *t*-butyl hydroperoxide cannot be reduced in the absence of glutathione peroxidase by GSH and NADPH via glutathione reductase. Therefore, the pyridine nucleotides remain in their reduced form, which apparently is a necessary condition for the retention of the accumulated Ca^{2+}.

Due to the contamination of the mitochondrial preparation with peroxisomal catalase (25), the H_2O_2-induced oxidation of NAD(P)H in intact mitochondria could not be observed. Outer membrane-free mitoplasts obtained by digitonin treatment of mitochondria, however, showed a markedly decreased catalase activity. Mitoplasts prepared either from selenium-deficient or selenium-supplemented rats took up and retained Ca^{2+} when energized by succinate. In the case of control mitoplasts, addition of H_2O_2 resulted in oxidation of NAD(P)H and in concomitant release of Ca^{2+}. In mitoplasts from selenium-deficient animals, however, no response of pyridine nucleotides and Ca^{2+}

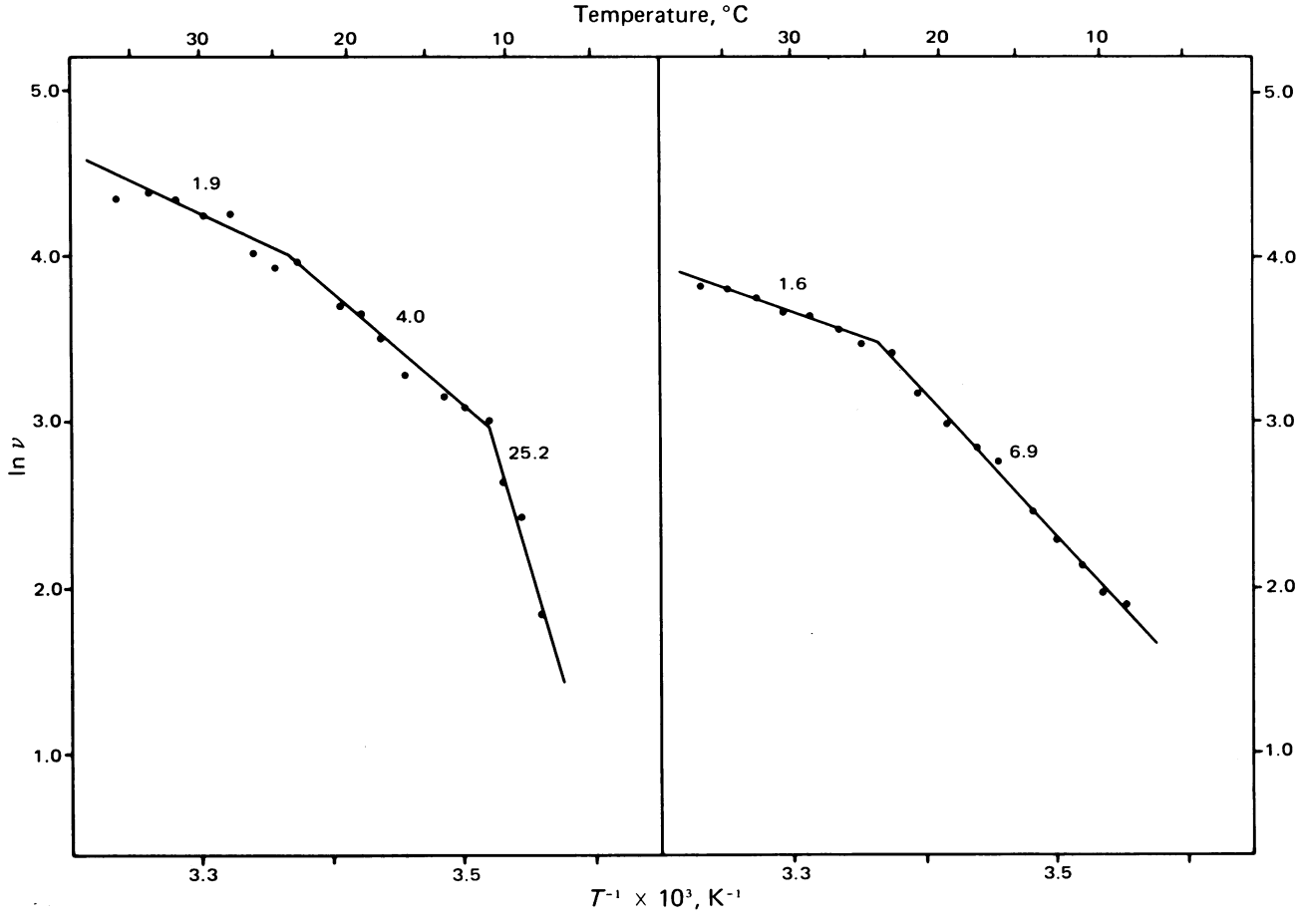

FIG. 1. Arrhenius plot of the Ca^{2+} uptake in liver mitochondria of selenium-supplemented (*Left*) and selenium-deficient (*Right*) rats. Numbers in the figures indicate activation energies in kcal/mol. v, Initial rate of Ca^{2+} uptake (nmol of Ca^{2+} per min per mg of protein).

to external H_2O_2 was detected (results not shown). Mitochondrial glutathione peroxidase can, therefore, react with externally added organic hydroperoxides and H_2O_2, but in the latter case the interaction of H_2O_2 with contaminating catalase must be prevented.

When no Ca^{2+} had been accumulated in the matrix, the hydroperoxide-induced oxidation of pyridine nucleotides was reversible (Fig. 3). In contrast, when Ca^{2+} had been taken up (see Fig. 2), oxidation of NAD(P)H occurred, but the rereduction of $NAD(P)^+$ was prevented. It appears, therefore, that nucleotides in the oxidized form are no longer available for the enzymatic reduction when Ca^{2+} is present inside mitochondria. Experiments performed with arsenazo III instead of murexide gave results similar to those reported in Figs. 2 and 3, except that Ca^{2+} translocation and NAD(P)H redox level could not be monitored simultaneously because Ca^{2+} causes large changes of the spectral properties of arsenazo III in the UV region.

Determination of the Amount and Redox State of Mitochondrial Pyridine Nucleotides after Addition of Peroxide and Ca^{2+}. Table 2 shows the results obtained by enzymatic

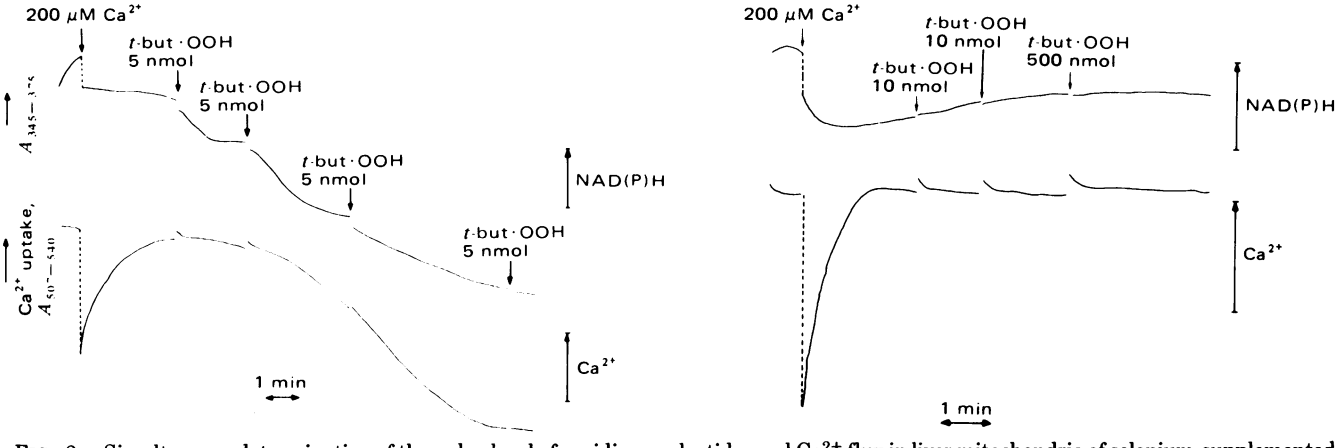

FIG. 2. Simultaneous determination of the redox level of pyridine nucleotides and Ca^{2+} flux in liver mitochondria of selenium-supplemented (*Left*) and selenium-deficient (*Right*) rats. *t*-But·OOH, *t*-butyl hydroperoxide. Curves: upper, NAD(P)H (3 nmol) trace; lower, Ca^{2+} (200 nmol) trace.

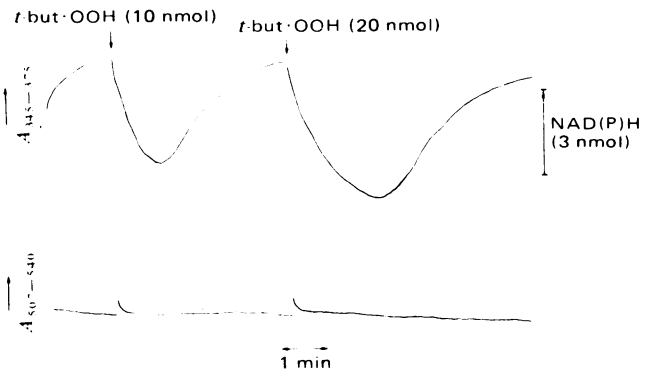

FIG. 3. Reversible titration of pyridine nucleotides by *t*-butyl hydroperoxide (*t*-but·OOH) in rat liver mitochondria from selenium-supplemented animals in the absence of Ca^{2+}. Other conditions were as in Fig. 2. Curves: upper, NAD(P)H trace; lower, murexide trace (without Ca^{2+}). The sensitivity of the recorder for the wavelength couple 507–540 nm was the same as that shown in Fig. 2.

analysis of the different forms of mitochondrial pyridine nucleotides after incubation under different experimental conditions. It can be seen that in the presence of succinate the accumulation of Ca^{2+} by mitochondria caused a decrease of endogenous NADPH and NADH as also observed spectrophotometrically (see Fig. 2). Furthermore, *t*-butyl hydroperoxide oxidized NADPH as well as NADH, and in the absence of Ca^{2+} these nucleotides were detectable as $NADP^+$ and NAD^+. However, when the oxidation of NAD(P)H by *t*-butyl hydroperoxide was induced in the presence of intramitochondrial Ca^{2+}, only about 30% of $NADP^+$ and about 50% of NAD^+ appeared free in the matrix space which can be extracted and analyzed. Neither $NADP^+$ nor NAD^+ were detected in the extramitochondrial medium.

DISCUSSION

Reactive oxygen species like hydrogen peroxide and superoxide radicals are physiological reactants in cellular metabolism (25). They are known to be produced in mitochondria and to contribute to the formation of lipid peroxides in mitochondrial membranes. The role of peroxides in mitochondria can be investigated by modulating the selenium status of the animal, because the only enzyme known to reduce both hydrogen peroxide and organic hydroperoxides in mitochondria, glutathione peroxidase, requires selenium for its activity. By maintaining rats on a diet deficient in selenium or supplemented with 0.5 ppm of selenium as sodium selenite (control animals), the activity of liver mitochondrial glutathione peroxidase can be decreased to 0% and 5–6% of controls, using hydrogen peroxide and *t*-butyl hydroperoxide, respectively, as substrates (Table 1).

Table 2. Analysis of mitochondrial pyridine nucleotides after incubation under different experimental conditions

Nucleotide	$-Ca^{2+}$		$+Ca^{2+}$	
	−*t*-but·OOH	+*t*-but·OOH	−*t*-but·OOH	+*t*-but·OOH
NADPH	2.21	0.74	1.70	0.37
NADH	1.02	0.55	0.87	0.23
$NADP^+$	0.10	1.20	0.13	0.18
NAD^+	0.30	0.83	0.39	0.37
NADPH + $NADP^+$	2.31	1.94	1.83	0.55
NADH + NAD^+	1.32	1.38	1.26	0.60

Values are given in nmol per mg of protein. Data shown in this table were obtained with mitochondria isolated from selenium-supplemented animals. Identical results were also obtained with chow-fed animals. *t*-But·OOH, *t*-butyl hydroperoxide.

When the temperature dependence of the mitochondrial Ca^{2+} uptake was measured and analyzed in Arrhenius plots, mitochondria isolated from selenium-supplemented animals showed two breaks at 11°C and 25°C, confirming the recent report of Smith (26). In mitochondria isolated from the livers of selenium-deficient animals, however, the break around 11°C was absent (Fig. 1). Caution is always necessary in interpreting Arrhenius plots, and it is in principle possible that the disappearance of the break at around 11°C reflects a direct modification of a protein molecule (carrier?) involved in the uptake of Ca^{2+}. It is very likely, however, that the finding reflects a modification of the fluidity of the immediate surroundings of the Ca^{2+} uptake system. We found the same breaks in Arrhenius plots when Ca^{2+} uptake by mitochondria in the presence of rotenone, antimycin A, and oligomycin was induced by valinomycin (unpublished results). This result strongly suggests that Ca^{2+} uptake is indeed influenced by membrane fluidity.

In mitochondria of selenium-supplemented animals, addition of peroxides at low concentrations in the presence of succinate led to a reversible oxidation of pyridine nucleotides (Fig. 3). This oxidation of NAD(P)H, however, became irreversible when mitochondria had previously been loaded with Ca^{2+}. Under these conditions, peroxides caused an efflux of Ca^{2+} from mitochondria, concomitant with an irreversible oxidation of pyridine nucleotides (Fig. 2 *left*). Extraction and enzymatic analysis of the various forms of mitochondrial pyridine nucleotides after incubation with hydroperoxides ± Ca^{2+} revealed that the sum of NADPH plus $NADP^+$, and NADH plus NAD^+, remained unchanged when hydroperoxide was added in the absence of Ca^{2+} (Table 2). In the presence of Ca^{2+}, however, addition of hydroperoxide led to a decrease in the total nucleotide level, as determined by enzymatic analysis. It is interesting to note that Sies *et al.* (27) have reported selective oxidation of NADPH after addition of *t*-butyl hydroperoxide to intact hepatocytes. The oxidation of both NADH and NADPH in the experiments reported in the present paper probably reflects the activity of the energy-linked mitochondrial transhydrogenase (28).

The specificity of the effect caused by hydroperoxides is demonstrated in Fig. 2 *right*; hydroperoxides were added to mitochondria that had less than 6% of glutathione peroxidase activity compared with controls. In these mitochondria the hydroperoxides caused neither an oxidation of NAD(P)H nor an efflux of Ca^{2+}.

In control mitochondria, the observed oxidation of pyridine nucleotides preceded slightly the efflux of Ca^{2+} (Fig. 2 *left*). At the moment, it cannot be decided whether the oxidation of NAD(P)H is the cause of the Ca^{2+} efflux or whether it is an independent process. It has been suggested by Lehninger *et al.* (16) that pyridine nucleotides could function as positive or negative modulators of Ca^{2+} transport by binding to hypothetical Ca^{2+} influx and efflux carriers. Oxidized nucleotides would inhibit the influx-carrier and activate the efflux-carrier, whereas reduced nucleotides would have the opposite effect.

It has been reported that organic peroxides like *t*-butyl hydroperoxide cause a perturbation of the mitochondrial NAD(P)H/$NAD(P)^+$ *and* 2 GSH/GSSG redox steady state (22). In view of the study by Jocelyn (29) of hydroperoxide reduction and GSH oxidation in mitochondria, it seems unlikely that Ca^{2+} efflux under the conditions described in this paper was caused directly by the oxidation of GSH. Jocelyn reported that in the presence of succinate the addition of 0.35 mM *t*-butyl hydro-

peroxide caused only about 10% oxidation of mitochondrial GSH in 5 min. Thus, the small amounts of *t*-butyl hydroperoxide (5–10 μM) which induced the release of Ca^{2+} in the present experiments can be expected to leave the level of GSH in the presence of succinate virtually unchanged.

The hydroperoxide-induced disappearance of pyridine nucleotides in the presence of Ca^{2+} in mitochondria containing active glutathione peroxidase raises the question of whether Ca^{2+} forms membrane-bound complexes with $NAD(P)^+$, because $NAD(P)^+$ can neither be reduced inside the mitochondria (spectrophotometrical assay) nor reduced by alcohol dehydrogenase (specific for NAD^+) or glucose-6-phosphate dehydrogenase (specific for $NADP^+$) after extraction with perchloric acid. On the basis of fluorimetric and enzymatic studies, Vinogradov *et al.* (30) have reported some years ago the total disappearance of mitochondrial NAD(P)H and $NAD(P)^+$ after accumulation of large amounts of Ca^{2+}. They proposed the formation of a membrane-soluble NAD(P)H–Ca^{2+} complex having fluorescence properties different from those of uncomplexed NAD(P)H. Another possible explanation would be a Ca^{2+}-dependent destruction of mitochondrial pyridine nucleotides in the oxidized form.

One important aspect of the work described in this article is its possible relationship to the regulation of the Ca^{2+} homeostasis in liver cells. The relationship among the redox level of pyridine nucleotides, Ca^{2+}, the activity of oxidative enzymes, and the phosphorylation potential is rather complex (16). Ca^{2+} can be released from liver mitochondria by artificial (e.g., uncouplers), or natural (e.g., phospho*enol*pyruvate or prostaglandins) agents (31–33). Even in the cases in which the inducers of the release are "natural", however, they act either by collapsing the membrane potential (i.e., like uncouplers) (34) or only when added to the medium in unphysiological amounts. Because hydroperoxides are effective already at micromolar concentrations and because they are known to be physiological reactants, the peroxide-mediated process described in the present work seems particularly interesting and potentially important from the standpoint of physiological regulation.

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