Pergamon

Biochemical Pharmacology, Vol. 49, No. 2, pp. 147–155, 1995.
Elsevier Science Ltd
Printed in Great Britain.
0006-2952/95 $9.50 + 0.00

0006-2952(94)00463-3

UNIMPAIRED METABOLISM OF PYRIDINE DINUCLEOTIDES AND ADENYLATES IN CHINESE HAMSTER OVARY CELLS DURING OXIDATIVE STRESS ELICITED BY CYTOTOXIC DOSES OF COPPER-PUTRESCINE-PYRIDINE

ARNO NAGELE*

Strahlenbiologisches Institut, Ludwig-Maximilians-Universität, München, Germany

(*Received* 1 *April* 1994; *accepted* 3 *October* 1994)

Abstract—Copper-putrescine-pyridine (Cu-PuPy) effectively dismutates superoxide but is also known to produce H_2O_2 in a redox cycle with glutathione. The treatment of Chinese hamster ovary (CHO) cells with 0.2 mM Cu-PuPy reduced clonogenic survival to 10^{-3} in 50 min and caused significant oxidation and depletion of glutathione and continuous accumulation of protein-glutathione mixed disulfides. Remarkably, other important functional parameters of cell metabolism were not impaired: adenylate pool size, adenylate energy charge and the redox ratios of NADP(H) and NAD(H) remained constant. Moreover, within 200 min the pool size of NADP(H) increased linearly by a factor of four at the expense of the NAD(H) pool, resulting in an 8-fold increase in the ratio of NADPH to glutathione disulfide. Also, Cu-PuPy led to a dose-dependent, persistent inactivation of glutathione reductase, which could be reversed by copper chelators. In contrast to Cu-PuPy, glucose oxidase-generated H_2O_2 induced oxidation and loss of pyridine dinucleotides, depletion of the adenylate pool and deterioration of the energy charge. Oxidation and depletion of bulk glutathione were comparable to a Cu-PuPy treatment, but formation of protein-glutathione mixed disulfides was significantly less pronounced and reversible. The data indicate that the critical factor in Cu-PuPy cytotoxicity is not its function as catalyst of glutathione oxidation and H_2O_2 generation, but essentially its disruption of antioxidative cellular defence by inactivation of glutathione reductase. The data further suggest that Cu-PuPy inhibits ADP-ribosylation. This would explain why pyridine dinucleotide and adenylate pools are unaffected, and may be an essential prerequisite for the observation that cells, albeit sublethally damaged and denuded of their antioxidative defence, may be rescued by extending Cu-PuPy treatment.

Key words: hydrogen peroxide toxicity; copper toxicity; glutathione reductase; poly(ADP-ribose) polymerase; NAD; NADP

Superoxide dismutating copper complexes are of considerable pharmacological interest. A variety of effects *in vitro* and *in vivo* have been described, including reactivities of clearly cytoprotective (like anti-inflammatory) and cytotoxic (like anti-neoplastic) quality [for review see Ref. 1].

Cu-PuPy† is a remarkably stable [2] and membrane permeable [3] copper complex, which dismutates O_2^- (superoxide) in a similar two-step reaction and at a comparable rate [4] as described for Cu_2Zn_2-superoxide dismutase (EC 1.15.1.1). As demonstrated by Steinkühler *et al.* [5], the important cellular antioxidant glutathione competes with O_2^- for the reduction of Cu-PuPy. The reaction consumes O_2 and yields GSSG and H_2O_2 in a stoichiometry indicating that Cu-PuPy enters a redox cycle with glutathione and oxygen. Neither NADH nor NADPH is able to reduce the complex. In an *in vitro* study, the higher sensitivity to Cu-PuPy of human erythroleukemia cells in comparison with human peripheral lymphocytes was attributed to their lower content of GSH and lesser antioxidant enzyme activities [3]. The hypothesis of a preferential toxicity of Cu-PuPy towards tumour cells was supported by an *in vivo* rat carcinosarcoma tumour model where a significant increase in host survival was observed in animals administered i.v. 5×6.7 μmol/kg Cu-PuPy [6].

Our recent investigation revealed a time-dependent interaction of toxic and protective effects during Cu-PuPy treatment of CHO cells [7]. At Cu-PuPy concentrations above 0.1 mM we observed a marked increase of toxicity and glutathione oxidation. The time course of clonogenic survival was unusual and characterized by a gain in survival when treatments were continued (see Fig. 1). From mechanistic restrictions imposed by the shape of the survival curve as well as from the observation that addition of toxic H_2O_2 doses did not enhance the toxicity of Cu-PuPy treatment, we concluded that cell death was delayed and that a transiently accumulating pool of H_2O_2-derived free radical species was amenable

* Present address: Strahlenbiologisches Institut, Ludwig-Maximilians-Universität, Schillerstrasse 42, 80336 München, Germany. Tel. (049) 89 5996 807; FAX (049) 89 5996 840.

† Abbreviations: apo-PuPy, *N*,*N*′-bis(2-pyridylmethylene)-1,4-butanediamine; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; Cu-PuPy, *N*,*N*′-bis(2-pyridylmethylene)-1,4-butanediamine (*N*,*N*′,*N*″,*N*‴)-Cu(II)-diperchlorate; GR, glutathione reductase; GSH, reduced glutathione; GSSG, glutathione disulfide; ProtSSG, protein-glutathione mixed disulfide.

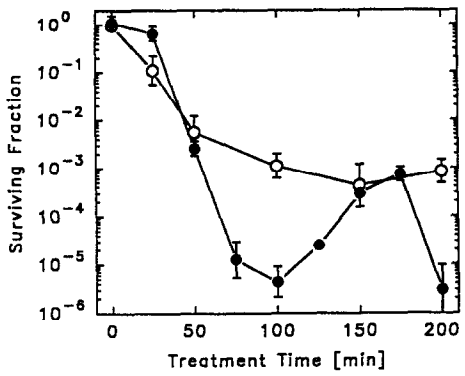

Fig. 1. Effect of treatments with 0.2 mM Cu-PuPy (●) or 0.2 U/mL glucose oxidase (○) on clonogenic cell survival. Shown are typical curves of three experiments per treatment. Error bars represent standard errors from three to six replicate colony counts of single experiments.

to inactivation by Cu-PuPy, presumably by virtue of its superoxide dismutating activity.

The present work focuses on prooxidative aspects of this copper complex at the cytotoxic concentration of 0.2 mM. The basic experimental conditions are similar to those used in our previous study. Since Cu-PuPy catalyses formation of H_2O_2 by direct oxidation of glutathione, it is to be anticipated that the stress on the glutathione regenerating system is much greater than that caused purely by treatment with H_2O_2. Therefore, the effects on the cellular status of pyridine dinucleotide redox systems, which are intimately associated with the glutathione redox system, were investigated. Also of interest was the status of adenylates, another essential functional parameter of cell metabolism, which is known to be sensitive to oxidative stress by H_2O_2. In parallel experiments Cu-PuPy-induced effects were compared to those elicited by H_2O_2. Due to the short half-life of 5 min in the experimental system used [7], H_2O_2 was not added as a bolus but continuously generated enzymatically by glucose/glucose oxidase.

MATERIALS AND METHODS

Chemicals. Cu-PuPy and the copper-free ligand apo-PuPy were synthesized as described [1, 7]. Glucose oxidase (EC 1.1.3.4) type II-S from *Aspergillus niger*, GSH, GSSG, D(-)penicillamine, 1,10-phenanthroline, histidine and reagents for the determination of glutathione were obtained from Sigma Chemie (Deisenhofen, Germany). Pyridine dinucleotides and adenylates were from Boehringer (Mannheim, Germany). Dulbecco's PBS, McCoy's 5A medium, serum, trypsin and antibiotics were purchased from Gibco BRL (Eggenstein, Germany). All other chemicals were obtained from Merck (Darmstadt, Germany). Tissue grade polystyrene culture flasks were purchased from Greiner (Frickenhausen, Germany).

Subculture and treatment of cells. CHO cells were grown in McCoy's 5A medium, supplemented with 10% (v/v) newborn calf and 5% (v/v) fetal calf serum, 50 mg/L penicillin, 50 mg/L streptomycin, and 100 mg/L neomycin sulfate. Cells were maintained at 37° in a 5% CO_2 atmosphere and subcultured every 2 or 3 days. For subculture, exponential cells were detached with 0.25% trypsin (without EDTA) and appropriately diluted with fresh growth medium. Routine control of growth parameters yielded population doubling times of 13–14 hr and plating efficiencies of 80–95%. For experiments, confluent cell layers were trypsinized and seeded at densities of 1×10^6 cells/mL in T_{25} flasks (25 cm^2 growth area, 3 mL). After 2 hr in the incubator, where cells were firmly attached, treatment was started by replacing the medium with treatment medium (37°) and returning the flasks to the incubator. All additions were sterilized by filtration through a 0.2 μm cellulose acetate filter (Schleicher & Schuell FP030) which had been primed by prior filtration of 1 mL growth medium. The treatment medium had been prepared immediately before by adding appropriate amounts of Cu-PuPy stock solution (20 mM in water) or glucose oxidase stock solution (20 U/mL medium).

Determination of glucose. The medium of glucose oxidase treated cells was boiled for 1 min to inactivate glucose oxidase and assayed for glucose with a commercial kit [Glucose(HK), Sigma Chemie, Deisenhofen, Germany].

Determination of glutathione. Cells were processed for the measurement of GSH, GSSG and ProtSSG as described [7]. Derivatization, separation by HPLC and simultaneous analysis of GSH and GSSG was performed as described by Fariss and Reed [8].

Determination of pyridine coenzymes and adenine nucleotides. Cell layers were washed with PBS and extracted by addition of 200 μL 0.25 M KOH, followed by the addition of 200 μL water after 3 min. The extract was centrifuged in an Ultrafree-MC 10,000 NMWL Filter Unit (Millipore, Bedford, MA, U.S.A.) at 5000 *g* for 7 min. The filtrate was adjusted to pH 6.5 by addition of 0.1 part of 1 M KH_2PO_4. All these manipulations were performed at 0–4°. Ten microlitres of the sample were injected onto a 3 μm Supelcosil LC-18 column and analysed for NAD, NADH, NADP, NADPH, ATP, ADP and AMP by ion-pair reversed-phase HPLC as described by Stocchi *et al.* [9].

Assay of GR (EC 1.6.4.2). The monolayers (3×10^6 cells) were washed with PBS and lysed by addition of 300 μL distilled water. The flasks were then rapidly frozen to −20°, the thawed lysate collected, sonicated and centrifuged to remove insoluble debris. The activity of GR was essentially determined as described by Goldberg and Spooner [10], except that EDTA was usually omitted and the enzyme reaction was started by addition of GSSG. One hundred microlitres of the lysate were added to 1425 μL of substrate solution (25°). The final assay contained KH_2PO_4 (100 mM, pH 7.2), NADPH (0.17 mM) and GSSG (2.2 mM). The decrease in absorption at 340 nm was registered for 5 min in a Zeiss DMR 10 spectrophotometer.

Protein determination. In order to compensate for cell lysis during toxic treatments, any quoted cellular concentrations are based on the total cell protein content. This was determined by the Lowry

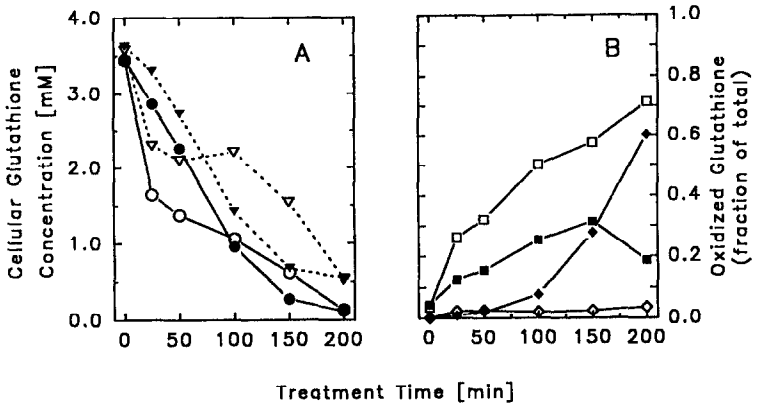

Fig. 2. Time course of cellular glutathione status during treatments with 0.2 mM Cu-PuPy (closed symbols) or 0.2 U/mL glucose oxidase (open symbols). (A) GSH (●, ○), total glutathione (GSH + 2GSSG + ProtSSG; ▼, ▽). (B) Fraction of GSH equivalents present as GSSG (■, □) or as ProtSSG (◇, ◆). Results of typical experiments are shown, with a maximal variation of 12 and 15% between experiments.

procedure [11], using bovine serum albumin as standard. The protein content of untreated cells was 0.11 ± 0.01 mg/10^6 cells (N = 32). A mean volume of 1.0×10^{-12} L/cell, determined by Coulter Counter measurements, was taken as solvent volume for the calculation of volume concentrations.

Statistics. Unless otherwise indicated, means with their standard errors are given in the text or graphs. Independent time course experiments were not averaged in order to retain time resolution. Instead, typical curves are shown and the maximal deviation that was observed between replicate points is given.

RESULTS

In this study, the effects of treatments with 0.2 mM Cu-PuPy are compared to those obtained by exposure to H_2O_2 generated by glucose oxidase from glucose and O_2 present in the medium. One unit (U) is defined as the amount of enzyme required to convert 1 μmol of β-D-glucose to D-gluconic acid and H_2O_2 per minute at pH 5.1 and 35°. A nominal enzyme activity of 0.2 U/mL was used in all glucose oxidase treatments. To test actual enzyme activity under the specific treatment conditions (i.e. 37°, 5% CO_2, pH 7.4, 10^6 cells/mL, serum-containing medium), an analysis of the glucose consumption in the medium was performed. A least squares fit of three independent experiments revealed an actual initial activity of 0.08 ± 0.02 U/mL, which decreased exponentially with a half-life of 61 ± 14 min. Thus, of the 14.7 ± 0.3 μmol/mL glucose initially present

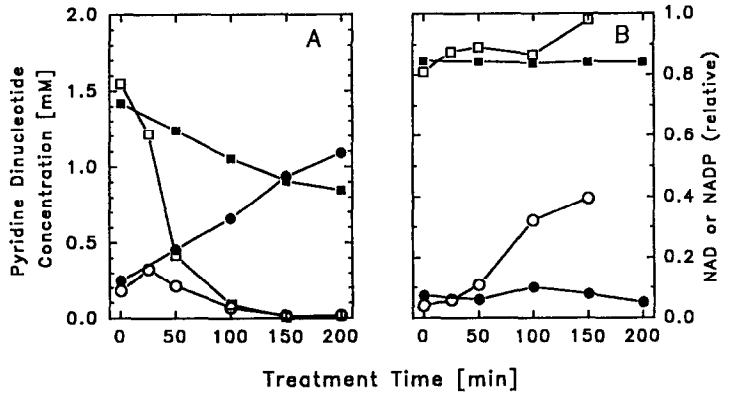

Fig. 3. Time course of cellular pyridine dinucleotides during treatments with 0.2 mM Cu-PuPy (closed symbols) or 0.2 U/mL glucose oxidase (open symbols). (A) Pyridine dinucleotide pool size, with NAD + NADH (■, □) and NADP + NADPH (●, ○). (B) Relative oxidation of NAD(H) (NAD/[NAD + NADH], ■, □) or NADP(H) (NADP/[NADP + NADPH], ●, ○). The deviations between two glucose oxidase treatments were less than 6% (absolute concentrations) and 10% [relative oxidation of NADP(H)]. A second Cu-PuPy treatment gave essentially similar results during the first 100 min, but resulted in a slight decrease in NADP(H) from 0.72 to 0.61 mM within the following 100 min and in an overall decrease in total pyridine dinucleotides by 30%. But similar to the experiment shown, the redox ratio of pyridine dinucleotides was not affected.

in the medium, a total of 6.1 ± 0.5 μmol/mL was finally consumed (or H_2O_2 produced) within 200 min. Control experiments of the effect of glucose oxidase on cell survival and on pyridine nucleotides and adenylates yielded essentially the same results when performed in medium containing 45 μmol/mL glucose (not shown), indicating that glucose consumption was not a limiting factor.

Effects on clonogenic survival

The nominal enzyme activity of 0.2 U/mL glucose oxidase was chosen for all reference treatments, since its survival reduction after 50 min was similar to that produced by 0.2 mM Cu-PuPy. As Fig. 1 shows, this choice is quite arbitrary due to the qualitatively different time courses of the two treatments. The glucose oxidase survival curve progressively flattens, which is in accordance with the decreasing activity of glucose oxidase. As discussed in detail in our previous paper [7], the survival curve of the Cu-PuPy treatment implies that not all cells are killed acutely, but that the loss of clonogenicity is delayed in at least a fraction of cells. No such inferences can be drawn from the glucose oxidase survival curve.

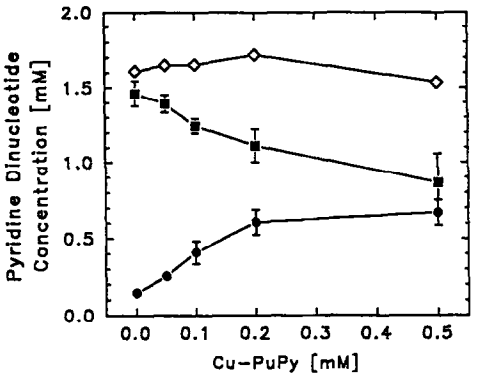

Fig. 4. Dependence of pyridine dinucleotide pool sizes at 50 min on Cu-PuPy concentration. Data points of NAD + NADH (■) or NADP + NADPH (●) are the mean and standard errors (bars) from two or three experiments. For each experiment, the 50 min values were estimated by a second degree polynomial least squares fit to the data from 0 to 100 min. These values were added to give total pyridine dinucleotide content (◇).

Effects on cellular glutathione

Both Cu-PuPy and glucose oxidase led to significant oxidation and depletion of cellular glutathione. Depletion by glucose oxidase was more pronounced at the beginning of the treatment, but by 200 min both treatments resulted in the same loss of bulk glutathione (Fig. 2A). As can be deduced from Fig. 3B, overall glutathione oxidation reaches the similar level of 75–79% at 200 min in either treatment, but there is a remarkable qualitative difference in the concentration of oxidized glutathione species. Thus, ProtSSG were increased 50 times by Cu-PuPy within 200 min (from 5.5 ± 1.3 μM to 260 ± 35 μM), comprising 60% of the total glutathione. The content of total oxidized glutathione (2 GSSG + ProtSSG) was nearly constant (440 ± 20 μM) in the time period of 50–200 min, indicating that Cu-PuPy-induced glutathione oxidation is essentially irreversible. During glucose oxidase treatments, however, the main oxidized glutathione species was GSSG (71%) and the maximal ProtSSG content, observed at 50–100 min (53 ± 5 μM), decreased to 19 ± 5 μM at 200 min. Reversibility of glucose oxidase-induced glutathione oxidation is further confirmed by the fact that the content of total oxidized glutathione, after a rise from 150 ± 20 to 1150 ± 20 μM (100 min), decreased to 390 ± 40 μM (200 min).

Effects on pyridine dinucleotides

Treatments with Cu-PuPy and glucose oxidase had strikingly different effects on the pool size (Fig. 3A) and the oxidation (Fig. 3B) of cellular pyridine dinucleotides. Cu-PuPy induced a 4-fold increase of NADP(H) within 200 min accompanied by an equimolar decrease of NAD(H), but did not affect the redox ratios of either NAD/NADH (5.19 ± 0.05) or NADP/NADPH (0.072 ± 0.007). In marked contrast, glucose oxidase significantly increased both NAD/NADH (40 ± 9) and NADP/NADPH (0.71 ± 0.09) within 150 min, and led to total depletion of NAD(H) and NADP(H) within the time course of the experiment.

With all Cu-PuPy concentrations tested (0–0.5 mM), the increase in NADP(H) was essentially linear for at least 100 min. Also, the rate of change of the NADP(H) or NAD(H) pool sizes was closely dependent on the Cu-PuPy dose. Oxidation of both pools was only observed with 0.5 mM Cu-PuPy in excess of 100 min. Likewise, the association of an increase in NADP(H) with an equimolar decrease in NAD(H) was observed over the entire concentration range tested (Fig. 4), and is indicative of an interconversion of NAD to NADP.

Effects on adenylates and adenylate energy charge

Cellular concentrations of ATP, ADP and AMP were not affected and the energy charge remained remarkably constant (0.89 ± 0.01) for at least 150 min of exposure with 0.2 mM Cu-PuPy. Glucose oxidase treatments, on the other hand, were characterized by a complete loss of cellular adenylates and a decrease in the energy charge to a value of 0.43 ± 0.07 within 150 min (Fig. 5). At higher doses, Cu-PuPy also induced deterioration of the adenylate status. Thus, the curves obtained with 0.5 mM Cu-PuPy were nearly congruent to the curves obtained with glucose oxidase (not shown).

Effects on the activity of GR

As the high degree of glutathione oxidation implies, 0.2 mM Cu-PuPy induces severe oxidative stress in cells. On the other hand, the redox ratio of NADP(H) remained unchanged and the concentration of NADP(H) even increased considerably. This resulted in an increasing ratio of NADPH/GSSG from 2.7 ± 0.5 to 20.3 ± 0.5 within 200 min. Together with the fact of continuing glutathione oxidation, this indicates that the utilization of

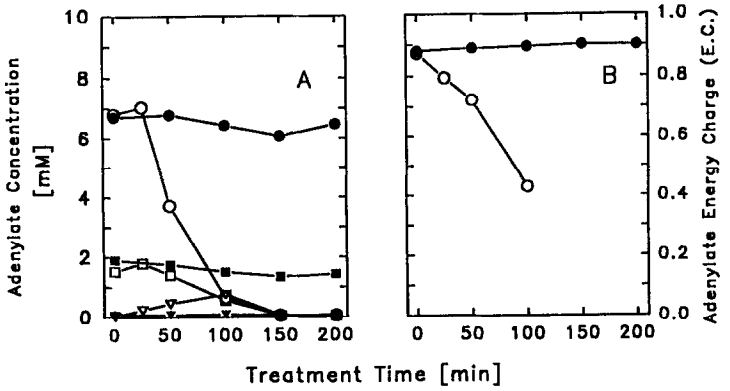

Fig. 5. Time course of cellular adenylates during treatments with 0.2 mM Cu-PuPy (closed symbols) or 0.2 U/mL glucose oxidase (open symbols). Single experiments. Maximal concentration differences in two replications amounted to 20% at 200 min. (A) Intracellular concentrations of ATP (●, ○), ADP (■, □) and AMP (▼, ▽). (B) Adenylate energy charge (E.C. = [ATP + ½ADP]/[ATP + ADP + AMP]) of the same treatments with Cu-PuPy (●) or glucose oxidase (○).

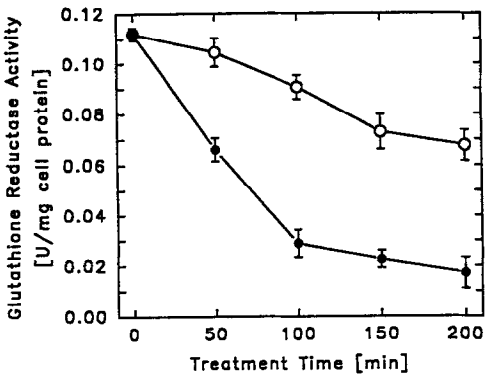

Fig. 6. Time course of glutathione reductase inhibition by treatments with 0.2 mM Cu-PuPy (●) or 0.2 U/mL glucose oxidase (○). Single experiment. Shown are means and standard errors (bars) or three independent replications.

NADPH for the reduction of GSSG is prevented and points to an inactivation of GR. Measurements of GR activity in lysates of Cu-PuPy treated cells clearly confirmed this presumption (Fig. 6). Glucose oxidase treatments also inactivated GR, though by a factor of four slower than Cu-PuPy, and the onset of inactivation appeared to be delayed. Also in contrast to Cu-PuPy, glucose oxidase led to a continuous decrease in the NADPH/GSSG ratio to a value of 0.023 ± 0.08 within 150 min.

To gain insight into the mechanism of GR inactivation by Cu-PuPy, the composition of the standard assay as described in Materials and Methods was modified (Table 1). Thus, inclusion of EDTA or FAD, a prosthetic cofactor of GR, slightly increased GR activities, but their effect was not specific to Cu-PuPy treated cells and did not correlate with the degree (not shown) of enzyme inactivation. Tests with the lysates of untreated cells suggest that Cu-PuPy inactivates only the reduced form of the enzyme. Thus, incubation of lysates for 50 min at 37° with 0.2 or (not shown) 0.5 mM Cu-PuPy did not inhibit GR when the assay was started with NADPH. Similarly, inclusion of 0.05 mM Cu-PuPy in the assay mixture had no effect when started with NADPH, but inhibition developed within 30 sec when the reaction was started with GSSG, i.e. when the enzyme was exposed to Cu-PuPy in the presence of NADPH.

To test whether GR inactivation is due to binding of copper to the enzyme, known copper chelators were examined for their ability to reverse inactivation when added to the complete assay mixture. As shown in Table 1, all tested chelators had a significant effect. Apo-PuPy, the copper-free ligand of Cu-PuPy, phenanthroline and penicillamine, were able to completely revert GR inactivation. Table 1 also shows that the respective enzyme activities of untreated or Cu-PuPy treated cells were unchanged when lysates were measured after 20 hr at room temperature. In marked contrast, enzyme inactivation was reverted spontaneously when lysates were diluted in assay buffer and allowed to stand on ice for 5 hr.

No GR inactivation was observed in cells treated with 0.2 or 0.5 mM $CuSO_4$ for 50 or 100 min (not shown). When assays were started with GSSG, $CuSO_4$, like Cu-PuPy, inactivated GR in incubated lysates or immediately in the assay mixture.

DISCUSSION

The observed cellular effects of glucose oxidase treatments are typical for the response to hydroperoxide-induced oxidative stress [12–18], characterized by depletion of glutathione and oxidation of GSH to GSSG and ProtSSG, oxidation and loss of pyridine nucleotides, loss of adenylates and deterioration of energy charge (see Fig. 7). In contrast, the pattern of metabolic changes elicited by Cu-PuPy is very different to that induced by H_2O_2

Table 1. Effect of treatments with Cu-PuPy or $CuSO_4$ on the activity of GR

Assay conditions	GR activity (relative) Treatment of cells for 50 min with: No addition	0.2 mM Cu-PuPy
Immediate measurement of lysate, with additions to standard assay:		
none (reference)	1.00 ± 0.04 (8)*	0.66 ± 0.05 (8)
+EDTA (0.5 mM)	1.11 ± 0.05 (3)	0.71 ± 0.07 (6)
+FAD (5 μM)	1.12 ± 0.05 (3)	0.76 ± 0.03 (6)
+Cu-PuPy (50 μM)	0.17 ± 0.02 (3)	
+$CuSO_4$ (50 μM)	0.04 ± 0.01 (3)	
+apo-PuPy (1 mM)	1.31 ± 0.04 (3)	1.15 ± 0.04 (9)
+1,10-Phenanthroline (1 mM)	1.27 ± 0.04 (3)	1.10 ± 0.08 (3)
+D-Penicillamine (1 mM)	1.04 ± 0.05 (3)	1.05 ± 0.04 (3)
+L-Histidine (1 mM)	1.02 ± 0.04 (3)	0.83 ± 0.03 (3)
+GSH (1 mM)	0.98 ± 0.07 (3)	0.78 ± 0.04 (6)
Treatment of lysate for 50 min with 0.2 mM Cu-PuPy, start with NADPH	0.96 ± 0.05 (6)	
Measurement of lysate after 20 hr at room temperature	1.02 ± 0.05 (3)	0.60 ± 0.04 (3)
Measurement of lysate diluted in assay mixture after 5 hr at 0°	1.09 ± 0.05 (9)	0.90 ± 0.03 (9)

* Means ± standard deviations. In parentheses: number of independent assays, each with triplicate measurements.

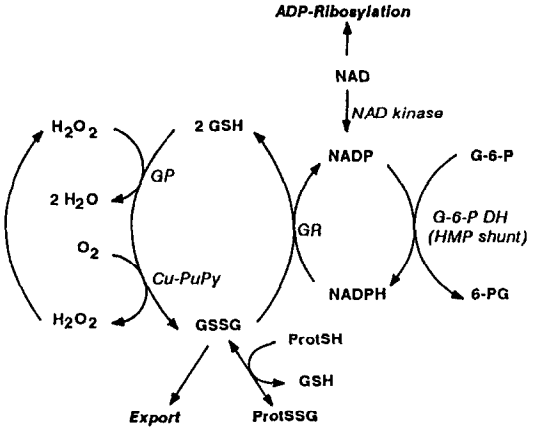

Fig. 7. Scheme of reactions associated with the detoxification of H_2O_2. GP, glutathione peroxidase; GR, glutathione reductase; G-6-P, glucose-6-phosphate; 6-PG, 6-phosphogluconate; G-6-P DH, G-6-P dehydrogenase.

or glucose oxidase. In essence, redox changes are confined to the glutathione system and are characterized by a much more pronounced formation of protSSG. Furthermore, the composition of the adenylate pool, and in consequence the energy charge, remains remarkably stable. Finally, no loss of total pyridine nucleotides is observed, but the decrease in NAD(H) is compensated by an increase in NADP(H). The data support the concept that in Cu-PuPy treatment metabolic pathways which are usually induced in response to oxidative stress by H_2O_2 become uncoupled from the glutathione redox system.

Inactivation of GR by Cu-PuPy

It is evident that the inactivation of GR plays an important role in this scenario. Although the activities measured in cell lysates do not necessarily reflect the true activities of GR in the intact cellular environment, the data suggest a significant and rather immediate *in situ* inactivation of the enzyme by Cu-PuPy treatment. The capability of copper chelators to markedly reverse this inactivation suggests that it is caused by binding of copper to the dithiol in the active center of the enzyme, similar to a mechanism proposed for $CuSO_4$ inactivation of purified yeast GR [19]. The inability of $CuSO_4$ to inactivate GR in intact cells is certainly due to insufficient permeation (by a factor of 50 slower than Cu-PuPy-mediated permeation of copper [7]), since in lysates $CuSO_4$ inactivated GR more effectively than the copper complex. Thus, the ability of Cu-PuPy to inactivate GR may largely be a consequence of its amphiphilic carrier function for copper. It is likely that in addition to GR other ligands with high affinity for copper, such as SH-containing enzymes or regulatory proteins, are similarly affected by Cu-PuPy, and that this may contribute to the observed metabolic effects.

Remarkably, inclusion of apo-PuPy or 1,10-phenanthroline in the assay also significantly increased the GR activity of untreated control cells. This might suggest that under physiological conditions a considerable fraction of cellular GR is inactivated. It is tempting to speculate that phenomena such as the increase in activity upon dilution of GR in phosphate buffer, an effect already described by others [20], as well as the observation that GR activity in whole cells or of the purified enzyme may double upon extended storage [21], are due to a similar inactivation of GR.

As shown by López-Barea and co-workers [22, 23] for purified mouse and horse enzymes, GR is almost completely inactivated by its own electron donor NADPH in the absence of GSSG. This auto-inactivation and protection or reactivation by GSSG was thought to play an important regulatory role *in vivo*. It may become operative in response to the observed increase in the NADPH/GSSG ratio during

Cu-PuPy exposure and thus augment inactivation. On the other hand, such inactivation can be ruled out for glucose oxidase treatments, where the NADPH/GSSG ratio declines. The minor inactivation of GR observed here is possibly due to oxidation of the enzyme by H_2O_2 or derived reactive oxygen species.

Implications of GR inactivation for the antioxidative defence

Clearly the inactivation of GR curtails redox cycling of Cu-PuPy. At a first glance, one would expect it to be neutral in regard to cellular H_2O_2 concentrations, since Cu-PuPy-catalysed H_2O_2 formation would decrease to a similar extent as the capacity of glutathione peroxidase to reduce H_2O_2, due to loss of GSH as substrate (Fig. 7). But the specific kinetic properties of glutathione peroxidase predict that its activity rapidly declines when GSH levels fall below 10^{-4} M [24]. This enzyme was also shown to be irreversibly inactivated by hydroperoxides when incubated without GSH [25], similar effects being observed for superoxide dismutase and catalase. These findings predict that inactivation of GR by Cu-PuPy eventually triggers an irreversible autocatalytical process which denudes cells of their main antioxidative systems and that detrimental amounts of H_2O_2 will escape inactivation.

Enhanced formation of ProtSSG by Cu-PuPy

The immediate effect of GR inactivation is a decreasing GSH/GSSG ratio, and consequently the enhanced formation of ProtSSG in a thiol exchange reaction with GSSG. Under conditions of mild oxidative stress, the latter reaction is essentially reversible and considered to be protective, since it masks protein thiol groups and thereby prevents formation of intra- or intermolecular disulfide bridges that could lead to protein denaturation. Furthermore, formation of mixed disulfides regulates the activities of certain enzymes in a direction that increases cellular defense capacities against oxidative stress [for review see Refs. 26, 27].

In cells treated with glucose oxidase, the predominant species of oxidized glutathione was GSSG, and the ratio of ProtSSG/GSSG remained in the range of 0.07–0.18 throughout the time period investigated. In marked contrast, Cu-PuPy exposure led to an increase in this ratio to a value of 6.5 within 200 min. The data are compatible with the hypothesis that ProtSSG formation is enhanced and may become irreversible due to the more pronounced loss of GSH resulting from inactivation of GR. But this mechanism alone cannot account for the evident qualitative differences in the time course of glutathione oxidation. These imply that protein S-thiolation is not exclusively governed by a thiol exchange of ProtSH with GSSG and GSH, but suggest that Cu-PuPy treatments specifically shift the equilibrium towards protein oxidation. Thus, the known reactivity of the complex supports a mechanism in which Cu-PuPy catalyses the reaction GSH + ProtSH + $O_2 \rightarrow$ ProtSSG + H_2O_2, which proceeds through a protein radical intermediate. Also, if GR directly reduces certain ProtSSG as proposed by Bellomo *et al.* [28], its inactivation by Cu-PuPy would result in accumulation of ProtSSG.

Cu-PuPy induced increase of NADP(H)

The rise of the NADP(H) pool observed during Cu-PuPy exposure is probably an adaptive response to the oxidative stress perceived by the cells. A similar phenomenon was observed during exposure of cells to non-toxic doses of menadione and other redox cycling quinones [29–31]. An interconversion of NAD(H) to NADP(H) was first explicitly described in a study by Stubberfield and Cohen [31] and suggested to be mediated by NAD kinase (EC 2.7.1.23). This cytosolic enzyme is activated by Ca^{2+} via calmodulin [32]. The data presented here show that interconversion of NAD(H) to NADP(H) is triggered early during Cu-PuPy treatments and suggest that Cu-PuPy-induced inactivation of GR is causative of this phenomenon. This role of GR is corroborated by an uncommented figure in a study of Schraufstätter and colleagues [12], which displays a 2-fold increase within 50 min of (reduced) NADPH in $P388D_1$ macrophage-like cells during pure treatment with the GR inhibitor BCNU. Since redox ratios of pyridine dinucleotides remain unchanged during Cu-PuPy treatments, they are probably not involved in the activation process. This points to glutathione oxidation as sustained stimulus for the interconversion. Possibly, conversion of NAD to NADP is a common metabolic response to oxidative stress, which in most studies is concealed by a concurrent consumption of pyridine dinucleotides.

Preservation of total pyridine dinucleotide and adenylate pools

Many agents that cause DNA single strand breaks, including H_2O_2 and other hydroperoxides, stimulate ADP-ribosylation of various nuclear proteins. This process is catalysed by poly(ADP-ribose) polymerase (EC 2.4.2.30) and directly consumes NAD [15, 16]. There is evidence in the literature that under oxidative stress ADP-ribosylation is the main consumer of NAD and secondarily of ATP, and that their depletion is a major factor in a sequence leading to cell death. Thus, inhibitors of poly(ADP-ribose) polymerase not only prevented the decrease in NAD and ATP, but also the lysis of $P388D_1$ cells in the presence of H_2O_2 [16] or the lysis of cultured endothelial cells following treatment with the oxidant dihydroxyfumarate [33].

As noted by Schraufstätter *et al.* [12], the H_2O_2-induced loss of NADP(H) in $P388D_1$ cells was lower in cells that had been depleted of glutathione and was prevented in the presence of BCNU. Our preliminary experiments, which show that 3-aminobenzamide, an inhibitor of poly(ADP-ribose) polymerase, does not influence the survival of Cu-PuPy treated cells, are compatible with the assumption that Cu-PuPy inhibits ADP-ribosylation. Even by taking into account the observed interdependence of NAD(H) and NADP(H) and assuming unimpaired energy conversion during Cu-PuPy treatment, such an inhibition could furthermore account for the preservation of the entire pyridine dinucleotide and adenylate pools. The following finding lends further credit to this hypothesis and

offers a plausible mechanism for the role of Cu-PuPy. Pero *et al.* [34] have shown that the activity of poly(ADP-ribose) polymerase in cytoskeletons of human leukocytes is down regulated by GSSG, which suggests that the enzyme contains sensitive SH-groups. Since despite a more pronounced formation of GSSG, glucose oxidase treatments evidently did not inhibit ADP-ribosylation, GSSG itself is not a likely candidate for inhibition in intact cells. It is therefore suggested that the enzyme is inhibited by formation of a mixed disulfide, as a consequence of GR inhibition.

Cu-PuPy (0.2 mM) induces severe oxidative challenge and is highly toxic to cells. Taking into account the sparing effects due to the inactivation of GR and the inhibition of ADP-ribosylation, the sustained capacity of cells to maintain the observed high reduction potential of pyridine dinucleotides and the high energy charge is surprising. This implies that crucial metabolic functions remain intact for an appreciable period of time (200 min) and is in agreement with our previous conclusion, that irreversible loss of clonogenicity is delayed in Cu-PuPy treatments [7]. It is also compatible with the findings of Thies and Autor [33], who reported that cell lysis by the oxidant dihydroxyfumarate could still be prevented when the addition of 3-aminobenzamide was delayed by 2 hr.

Many clinically used antitumour agents have a prooxidant component of action. Thus, BCNU besides exhibiting alkylating properties, is also a potent inhibitor of GR. Also, redox cycling quinones impose considerable stress on glutathione reduction or, like menadione in high doses, inhibit GR [26]. A number of studies have suggested that tumours *in vivo* or tumour cells *in vitro* are often more sensitive towards oxidative stress [3, 35–39]. If these notions are true, redox cycling of Cu-PuPy alone would more severely challenge tumour cells than non-malignant cells. It is to be anticipated that any additional impairment of antioxidant capacity, such as the observed inactivation of GR by Cu-PuPy, could trigger a precipitous breakdown of cellular defences and finally lead to irreversible oxidation of biologically important molecules. Such effects would steepen the dose–response curve and provide a reasonable explanation for the marked threshold toxicity described in our previous paper [7]. In that study we proposed that tumour cells *in situ* may not profit from the putative antioxidative repair capacity of Cu-PuPy to the same extent as non-malignant cells in well-oxygenated host tissue, since this repair requires oxygen. Both effects would broaden the therapeutic margin of Cu-PuPy and could explain its antitumour activity.

In conclusion, the presented data show that Cu-PuPy effectively inactivates GR. In combination with its catalysis of GSH oxidation and H_2O_2 production this impairs any function of the glutathione redox system, leads to enhanced formation of ProtSSG, and presumably triggers a complete breakdown of the intrinsic antioxidative defence of cells. On the other hand, inactivation of GR by Cu-PuPy also has protective aspects. Evidence is presented that Cu-PuPy inhibits poly(ADP-ribose) polymerase and that it thereby prevents the usual consequences of H_2O_2-induced DNA damage, i.e. consumption of pyridine nucleotides and adenylates, and loss of energy charge. It is reasonable to assume that under these metabolic conditions any extrinsic improvement in the antioxidative status could rescue a considerable fraction of cells from loss of clonogenicity. This gives further credit to our previously proposed mechanism of initial damage and delayed protection by Cu-PuPy. Further experiments are needed to define the relative importance for cell protection of its capabilities to dismutate superoxide and prevent deterioration of critical metabolic functions. It also remains to be established to what extent the presented findings may be generalized to other copper compounds.

REFERENCES

1. Sorensen RJ, Pharmacological activities of SOD-like copper complexes. In: *Oxygen Radicals in Chemistry and Biology* (Eds. Bors W, Saran M and Tait D), pp. 821–830. Walter de Gruyter, Berlin, 1984.
2. Linss M and Weser U, Redox behaviour and stability of active center analogues of Cu_2Zn_2-superoxide dismutase. *Inorg Chim Acta* **138**: 163–166, 1987.
3. Steinkühler C, Mavelli I, Rossi L, Pedersen JZ, Melino G, Weser U and Rotilio G, Cytotoxicity of a low molecular weight Cu_2Zn_2-superoxide dismutase active center analog in human erythroleukemia cells. *Biochem Pharmacol* **39**: 1473–1479, 1990.
4. Felix K, Lengfelder E, Deters D and Weser U, Pulse radiolytically determined superoxide dismutase mimicking activity of copper-putrescine-pyridine, a dischiff base coordinated copper complex. *BioMetals* **6**: 11–15, 1993.
5. Steinkühler C, Pedersen JZ, Weser U and Rotilio G, Oxidative stress induced by a di-schiff base copper complex is both mediated and modulated by glutathione. *Biochem Pharmacol* **42**: 1821–1827, 1991.
6. Miesel R and Weser U, Anticarcinogenic reactivity of copper-dischiffbases with superoxide dismutase-like activity, *Free Rad Res Commun* **11**: 39–51, 1990.
7. Nagele A, Felix K and Lengfelder E, Induction of oxidative stress and protection against hydrogen peroxide-mediated cytotoxicity by the superoxide dismutase-mimetic complex copper-putrescine-pyridine. *Biochem Pharmacol* **47**: 555–562, 1994.
8. Fariss MW and Reed DJ, High-performance liquid chromatography of thiols and disulfides: dinitrophenol derivatives. In: *Sulfur and Sulfur Amino Acids, Methods in Enzymology* (Eds. Jakoby WB and Griffith OW), Vol. 143, pp. 101–109. Academic Press, Orlando, 1987.
9. Stocchi V, Cucchiarini L, Canestrari F, Piacentini MP and Fornaini G, A very fast ion-pair reversed HPLC method for the separation of the most significant nucleotides and their degradation products in human red blood cells. *Anal Biochem*, **167**: 181–190, 1987.
10. Goldberg DM and Spooner RJ, Glutathione reductase. In: *Methods of Enzymatic Analysis* 3rd Edn, (Ed. Bergmeyer HU) Vol III. pp. 258–265. Verlag Chemie, Weinheim, 1983.
11. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265–275, 1951.
12. Schraufstätter IU, Hinshaw DB, Hyslop PA, Spragg RG and Cochrane CG, Glutathione cycle activity and pyridine nucleotide levels in oxidant-induced injury of cells. *J Clin Invest* **76**: 1131–1139, 1985.
13. Spragg, RG, Hinshaw DB, Hyslop PA, Schraufstätter IU and Cochrane CG, Alterations in adenosine

triphosphate and energy charge in cultured endothelial and P388D1 cells after oxidant injury. *J Clin Invest* **76**: 1471–1476, 1985.
14. Hyslop PA, Hinshaw DB, Halsey WA, Schraufstätter IU, Sauerheber RD, Spragg RG, Jackson JH and Cochrane CG, Mechanisms of oxidant-mediated cell injury. *J Biol Chem* **263**: 1665–1675, 1988.
15. Sims JL, Berger SJ and Berger NA, Poly(ADP-ribose) polymerase inhibitors preserve nicotinamide adenine dinucleotide and adenosine 5′-triphosphate pools in DNA-damaged cells: mechanism of stimulation of unscheduled DNA synthesis. *Biochemistry* **22**: 5188–5194, 1983.
16. Schraufstätter IU, Hyslop PA, Hinshaw DB, Spragg RG, Sklar LA and Cochrane CG, Hydrogen peroxide-induced injury of cells and its prevention by inhibitors of poly(ADP-ribose) polymerase. *Proc Natl Acad Sci USA* **83**: 4908–4612, 1986.
17. Cantoni O, Cattabeni F, Stocchi V, Meyn RE, Cerutti P and Murray D, Hydrogen peroxide insult in cultured mammalian cells: relationships between DNA single-strand breakage, poly(ADP-ribose) metabolism and cell killing. *Biochim Biophys Acta* **1014**: 1–7, 1989.
18. Yamamoto K and Farber JL, Mechanism of pyridine nucleotides in cultured rat hepatocytes intoxicated with *tert*-butyl hydroperoxide. *Biochem Phamacol* **43**: 1119–1126, 1992.
19. Rafter GW, Copper inhibition of glutathione reductase and its reversal with gold thiolates, thiol, and disulfide compounds. *Biochem Med* **27**: 381–391, 1982.
20. Spooner RJ, Delides A and Goldberg DM, Anomalous behaviour of glutathione reductase on dilution. *Clin Chem* **22**: 1005–1008, 1976.
21. D'Alessandri A, The stability of erythrocyte glutathione reductase. *Helv Med Acta* **35**: 118–123, 1969/1970.
22. López-Barea J and Lee C-Y, Mouse-liver glutathione reductase. Purification, kinetics, and regulation. *Eur J Biochem* **98**: 487–499, 1979.
23. García-Alfonso C, Martínez-Galisteo E, Llobell A, Bárena JA and López-Barea J, Regulation of horse-liver glutathione reductase. *Int J Biochem* **25**: 513–520, 1993.
24. Flohé L, Glutathione peroxidase: fact and fiction. In: *Oxygen Free Radicals and Tissue Damage. Ciba Foundation Symposium 65* (new series) (Ed. Fitzsimons DW), pp. 95–122. Excerpta Medica. Amsterdam, 1979.
25. Pigeolet E, Corbisier P, Houbion A, Lambert D, Michiels C, Raes M, Zachary M-D, Remacle J, Glutathione peroxidase, superoxide dismutase and catalase inactivation by peroxides and oxygen derived free radicals. *Mech Age Dev* **51**: 283–297, 1990.
26. Brigelius R, Mixed disulfides: biological functions and increase in oxidative stress. In: *Oxidative Stress* (Ed. Sies H), pp. 243–268, Academic Press, London, 1985.
27. Gilbert HF, Molecular and cellular aspects of thiol-disulfide exchange. *Adv Enzymol* **63**: 69–172, 1990.
28. Bellomo G, Mirabelli F, DiMonte D, Richelmi P, Thor H, Orrenius C and Orrenius S, Formation and reduction of glutathione-protein mixed disulfides during oxidative stress. *Biochem Pharmacol* **36**: 1313–1320, 1987.
29. Smith P, Alberts DW and Rush GF, Menadione-induced oxidative stress in hepatocytes isolated from fed and fasted rats: the role of NADPH-regenerating pathways. *Toxicol Appl Pharmacol* **89**: 190–201, 1987.
30. Kass GEM, Juedes MJ and Orrenius S, Cyclosporine A protects hepatocytes against prooxidant-induced cell killing. *Biochem Pharmacol* **44**: 1995–2003, 1992.
31. Stubberfield CR and Cohen GM, Interconversion of NAD(H) to NADP(H): a cellular response to quinone-induced oxidative stress in isolated hepatocytes. *Biochem Pharmacol* **38**: 2631–2637, 1989.
32. Andersson JM and Cormier MJ, Calcium-dependent regulation of NAD kinase. *Biochem Biophys Res Commun* **84**: 595–602, 1978.
33. Thies RL and Autor AP, Reactive oxygen injury to cultured pulmonary artery endothelial cells: mediation by poly(ADP-ribose) polymerase activation causing NAD depletion and altered energy balance. *Arch Biochem Biophys* **286**: 353–363, 1991.
34. Pero RW, Anderson MW, Doyle GA, Anna CH, Romagna F, Markowitz M and Bryngelsson C, Oxidative stress induces DNA damage and inhibits the repair of DNA lesions induced by *N*-acetoxy-2-acetylaminofluorene in human peripheral mononuclear leukocytes. *Cancer Res* **50**: 4619–4625, 1990.
35. Oberley LW, Superoxide dismutase and cancer: In: *Superoxide Dismutase* (Ed. Oberley LW), Vol II, pp. 127–165. CRC Press, Boca Raton, FL, 1982.
36. Mavelli I, Rotilio G, Ciriolo MR, Melino G and Sapora O, Antioxygenic enzymes as tumor markers: a critical reassessment of the respective roles of superoxide dismutase and glutathione peroxidase. In: *Human Tumor Markers* (Eds. Cimino F, Birkmayer S, Klavious P, Pimentel R and Salvatore F), pp. 883–888. Walter de Gruyter, Berlin, 1987.
37. Black SM and Wolf CR, The role of glutathione-dependent enzymes in drug resistance. *Pharmacol Ther* **51**: 139–154, 1991.
38. Meister A, Glutathione deficiency produced by inhibition of its synthesis, and its reversion: applications in research and therapy. *Pharmacol Ther* **51**: 155–194, 1991.
39. Galeotti T, Borrello S and Masotti L, Oxy-radical sources, scavenger systems and membrane damage in cancer cells. In: *Oxygen Radicals: Systemic Events and Disease Processes* (Eds. Das DK and Essman WB), pp. 129–148. Karger, Basel, 1990.