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USE OF AN OXIDASE ELECTRODE TO DETERMINE FACTORS AFFECTING THE *IN VITRO* PRODUCTION OF HYDROGEN PEROXIDE BY EHRLICH CELLS AND 1-CHLORO-2,4-DINITROBENZENE

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Abstract—A hydrogen peroxide (H_2O_2) electrode was used to continuously monitor H_2O_2 concentrations *in vitro* in a system designed to determine some of the factors which influence cellular H_2O_2 production and reduction. 1-Chloro-2,4-dinitrobenzene (CDNB), a clinical and analytical agent, was found to alter electron transport in Ehrlich ascites tumor cells (EATC), causing formation of H_2O_2. H_2O_2 accumulation in the cell suspension began after an initial lag period required for glutathione (GSH) depletion. *N*-Ethylmaleimide (NEM), which removes thiols, shortened the lag period but did not alter the rate at which H_2O_2 accumulated. H_2O_2 accumulation was influenced by glucose and drug concentrations and glucose was important in determining the total amount of H_2O_2 that eventually accumulated. The metabolic reduction of exogenously added H_2O_2 in the absence of CDNB was stimulated by glucose, unaffected by azide (a catalase inhibitor), and inhibited by NEM. The data show that glutathione peroxidase is chiefly responsible for inactivating intracellularly produced H_2O_2 and that at least three mechanisms may operate to remove glutathione in CDNB-treated cells. The findings imply that artificially low levels of GSH may be found when CDNB is used as an analytical reagent in whole cells and that topical use of CDNB may pose a genetic health hazard.

Various types of nitro-compounds (e.g. nitroimidazoles, nitrofurans and nitrobenzenes) are used clinically as antibiotics, radiosensitizers, and topical ointments. The safety of these drugs with regard to their mutagenicity and that of their metabolites is of wide current interest.

For example, the nitrobenzene derivative 1-chloro-2,4-dinitrobenzene (CDNB) has been used clinically to induce hair growth in patients with the spot-balding condition alopecia areata [1, 2] and to investigate areas of topical immunotherapy and sensitivity [3–5]. It is known to be toxic to cells *in vitro* and, under certain conditions, will inhibit cellular oxygen utilization and sensitize cells to radiation [6]. It has also been shown to mutate *Salmonella typhimurium* without prior metabolic activation by liver enzymes and to deplete reduced glutathione (GSH) and oxidized glutathione (GSSG) when applied topically to rat skin [7]. CDNB is also used analytically as a substrate for GSH-transferase (EC 2.5.1.18) [8]. CDNB, like 1-fluoro-2,4-dinitrobenzene (FDNB) [9], probably lowers the GSH content of *S. typhimurium* due to the action of glutathione-*S*-transferase [8, 9]. This possibility was of interest to us because previous studies with 4-nitroquinoline-1-oxide demonstrated an important relationship between transferase levels and the production of potentially hazardous oxygen-reactive drug intermediates [10].

In previous work, hydrogen peroxide (H_2O_2) production was demonstrated by the measurement of oxygen evolution ($2H_2O_2 \rightarrow O_2 + 2H_2O$) [11–13] induced by the addition of exogenous catalase (H_2O_2:H_2O oxidoreductase; EC 1.11.1.6). The recent development of an H_2O_2-sensing electrode ("oxidase electrode") [14] and oxidase meter now allows for the continuous monitoring of H_2O_2 concentrations in test solutions. We investigated the ability of this electrode to measure CDNB-stimulated H_2O_2 formation in cell suspensions, to determine the effects of glucose and of thiol removal by *N*-ethylmaleimide (NEM), and to measure the ability of EATC to reduce exogenously added H_2O_2 under various metabolic conditions.

MATERIALS AND METHODS

Hydrogen peroxide concentrations were measured by a Yellow Springs Instruments model 25 oxidase meter fitted with a model 2510 oxidase electrode. The electrode was covered with a single layer of the collagen membrane supplied with the electrode.

A Heathkit IR-18M chart recorder fitted with an external 23:1 RC voltage-divider circuit gave a 1:1 correspondence between the meter reading and the chart reading.

All reactions in which H_2O_2 was measured were performed in an Bolab BB438 (40-ml capacity) water-jacketed reaction vessel at 37° on a magnetic stirrer. Phosphate-buffered saline ("buffer") was made from 0.9% sodium chloride buffered at pH 7.2 to 7.4 by 0.05 M mono- and dibasic potassium phosphates. Buffer and cells were added to achieve a final density of 10^7/ml in a volume of 10 ml. Three

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minutes was usually sufficient for this mixture to equilibrate with the electrode and for the current to stabilize. Microliter quantities of other reagents were added and the reactions monitored.

All measurements were taken in nanoamps (nA) of electrode current and converted to H_2O_2 concentrations. Calibration of the instrument for each experiment was accomplished by adding aliquots of standard 0.1% H_2O_2 to the reaction medium. Linear regression analysis was used to determine the slope (μM H_2O_2/nA electrode current) by the least squares method. R^2 values were always > 0.99, indicating nearly perfect linearity between chart readings and H_2O_2 concentrations. The slope varied somewhat from day to day but the mean slope of all calibrations was approximately 0.4 μM/nA.

Reagents were purchased from various commercial sources, were used without further purification, and were dissolved at 0.1 M in dimethylsulfoxide. Glucose was dissolved at 1 or 0.1 M in water and NEM at 0.1 M in buffer.

Spectral changes related to CDNB metabolism were monitored at 340 nm [8, 10, 15] with an Aminco DW-2 double beam spectrophotometer.

The Ehrlich ascites tumor cells (EATC) used were inoculated interperitoneally into female CF_1 mice at 10^6 cells/animal and were collected after 6–8 days. The EATC were then washed in 50 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES)-buffered 0.9% saline and resuspended in 20 mM HEPES-buffered 0.9% saline at 1–2 $\times$ 10^8 cells/ml. Cells were stored on ice until use.

The following procedure was adopted to minimize background current and carry-over from run to run. The electrode and reaction vessel were prepared for each run by first rinsing them with deionized water and then immersing the electrode in deionized water with stirring. When the electrode current dropped to zero, the water was discarded and replaced with a measured amount of buffer. The current was allowed to stabilize 2–3 min before proceeding. This current level was the baseline and was electrically offset to read zero.

A typical measurement. The sequence of events in a typical experiment is represented in Fig. 1. After the baseline was established, preliminary additions (such as glucose and NEM) were made. (Little or no electrode response was generated by these additions.) EATC were then added to bring the final volume to 10 ml and the cell concentration to 10^7/ml. The cells alone were sufficient to induce a catalase-insensitive 10–30 nA response which stabilized after about 2 min. This period also allowed the drugs, added previously, ample time to interact with the cells. When CDNB was added, the initial formation of H_2O_2 caused a change in current which was monitored for several minutes. Glucose and drug concentrations could be adjusted so that substrate depletion (viz. glucose, which is important for both the production of H_2O_2 from oxygen-reactive radicals and the reduction of same) allowed H_2O_2 concentrations to remain within the measurement range of the electrode at the completion of the reaction. Without such adjustments the H_2O_2 concentrations went above the accuracy limit of the electrode (approximately 40 μM H_2O_2). Catalase addition could then reduce the accumulated H_2O_2 to oxygen and water, thus indicating any change in the background electrode current. In most cases involving CDNB metabolism, there was a return to a baseline nearly equal to that recorded before the addition of CDNB. The rate (maximum velocity) of H_2O_2 accumulation was determined from the maximum slope of the H_2O_2 concentration curve obtained several seconds after addition of drug (see Fig. 1).

The electrode continuously measures extracellular H_2O_2 concentrations. This measurement represents the net H_2O_2 accumulation in the reaction mixture, i.e. the total (gross) amount of H_2O_2 produced less that which is reduced before entering the extracel-

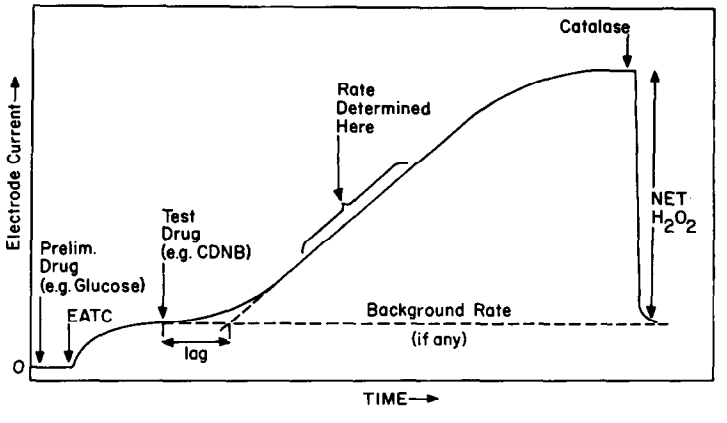

Fig. 1. A generalized scheme demonstrating electrode current levels (measured from 0 to 100 nA) during a typical experimental run. The baseline was established when the electrode current stabilized in phosphate-buffered saline. Preliminary metabolism-modifying drugs (e.g. glucose and NEM) were added immediately before the EATC so that these drugs might interact with the EATC during the period it takes for the cells to establish their baseline. CDNB was then added and H_2O_2 began to accumulate in the reaction mixture following a lag period. If a substrate was depleted before the electrode current reached 100 nA (the point above which the electrode response was non-linear), a plateau was reached and catalase addition allowed the ascertainment of total accumulated H_2O_2 and any increase in the EATC-induced baseline. The entire sequence took about 10 min.

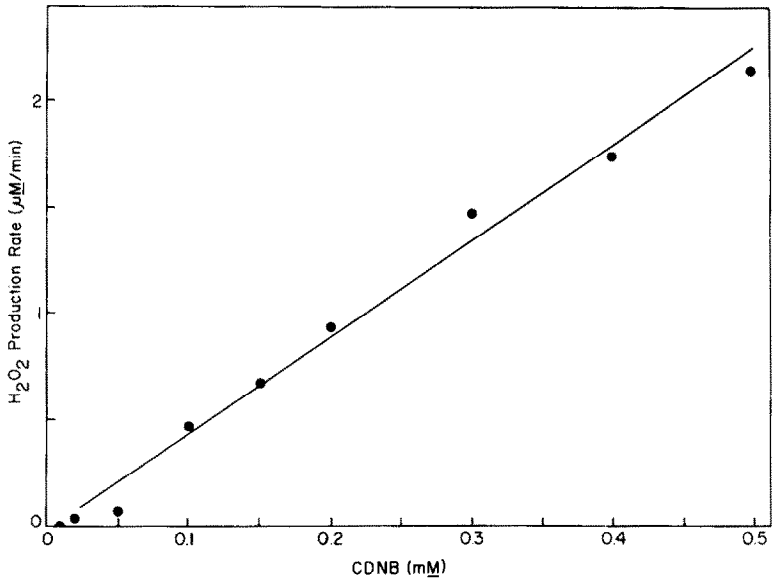

Fig. 2. Dependence of rate of H_2O_2 accumulation on CDNB concentration. All points were taken at 37° in buffer containing 1 mM glucose and 10^7 EATC/ml in a total of 10 ml.

lular pool. In all of the experiments, a background electrode current (see Fig. 1) was induced by some undetermined substance present in the cell preparation. The fact that the total current dropped to this background level when catalase was added suggested that this substance was not altered during drug metabolism. However, it was found in preliminary experiments that the substance reacted with either exogenously added or metabolically produced H_2O_2 if horseradish peroxidase (donor:H_2O_2 oxidase: EC 1.11.1.7) was added to the reaction mixture. This treatment almost completely removed the background and did not interfere with subsequent H_2O_2 production.

RESULTS

Effects of drug concentration. The dependency of H_2O_2 accumulation on drug concentration was determined. Concentrations of CDNB below 0.05 mM induced no electrode-measurable H_2O_2. However, at CDNB concentrations above that, the rate of H_2O_2 formation was linear up to at least 0.5 mM (Fig. 2).

Effects of glucose. Previous work demonstrated

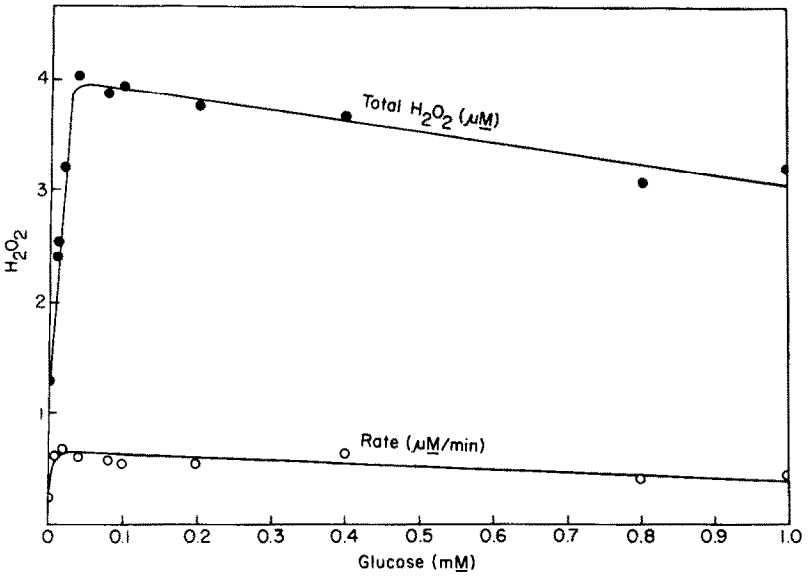

Fig. 3. Effect of glucose concentration on both the rate of CDNB-induced H_2O_2 accumulation and the total amount of H_2O_2 accumulated in the reaction mixture at the attainment of maximum steady-state concentrations. CDNB was present at 0.2 mM for all points. Both curves peak at 0.04 mM glucose.

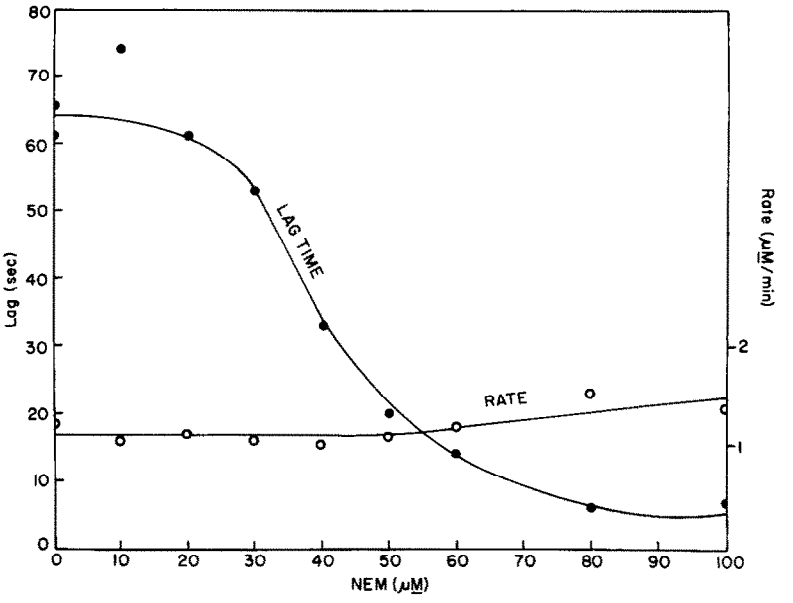

Fig. 4. Effects of NEM concentration on lag time and rate of CDNB-induced H_2O_2 production. CDNB was present at 0.2 mM and glucose at 1 mM. The rate was little affected but the lag was reduced from 64 sec at 0 μM NEM to about 5 sec at 100 μM.

that glucose is important in activating nitro-compounds to oxygen-reactive intermediates [10, 12, 13]. To determine if glucose has a similar effect on H_2O_2 accumulation and the total amount of H_2O_2 that eventually accumulates, glucose concentration was varied while the CDNB concentration remained fixed. It can be seen in Fig. 3 that a glucose concentration as low as 0.01 mM significantly enhanced both the rate of H_2O_2 formation and the total amount of H_2O_2 that accumulated in the reaction mixture. This marked effect of glucose was found for virtually all H_2O_2-inducing drugs tested (unpublished data). It is concluded that glucose is a strong promoter of H_2O_2 production.

Effects of NEM. EATC are known to contain 35 nmoles of non-protein thiols (viz. GSH) per 10^7 cells [10]. The possibility that the removal of these intracellular thiols would enhance H_2O_2 production was explored by the addition of NEM to the reaction mixture immediately before the EATC were added. This gave ample time (2–5 min) for thiol depletion (see Fig. 5C).

It was found in several experiments that NEM concentrations below 0.1 mM, an amount which completely removes thiols [10], did not greatly increase the rate of H_2O_2 accumulation (Fig. 4) as they do for nitro-compounds such as misonidazole [1-(2-hydroxy-3'-methoxypropyl)-2-nitroimidazole] [13]. However, NEM did decrease the lag period (Fig. 4) between CDNB addition and the point where the linear portion of the H_2O_2 concentration curve extrapolated back across the current level at which the CDNB was added (see Fig. 1).

CDNB (0.2 mM) alone was measured to deplete intracellular thiols by 78 per cent in 1 min, 85 per cent in 10 min, 99 per cent in 20 min, and 100 per cent in 30 min (B. Jacobson, unpublished data).

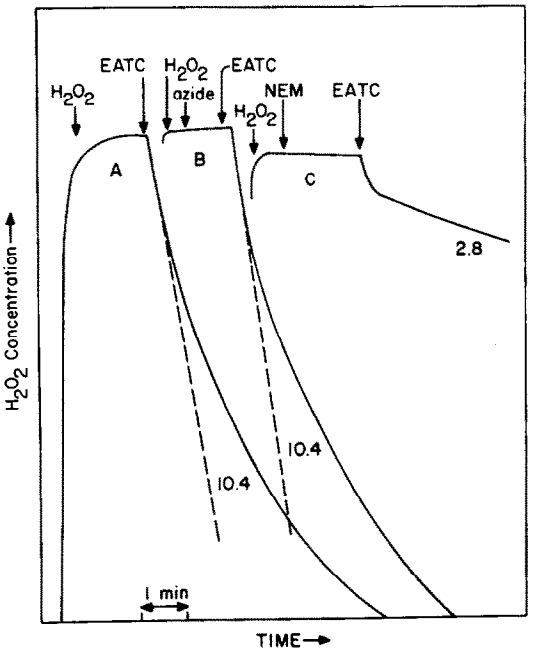

Fig. 5. Metabolic reduction of exogenously added H_2O_2 by EATC under various conditions. Graph A shows that 10^7 EATC/ml could reduce H_2O_2 at the rate of 10.4 μM/min. Graph B demonstrates that the addition of 2 mM sodium azide had almost no effect on H_2O_2 reduction. Graph C indicates that, when 0.5 mM NEM was added to the reaction mixture to remove GSH, the rate of reduction decreased dramatically to 2.8 μM/min. All runs were in the absence of glucose. Rates for A and B were determined immediately after EATC addition; the rate for C was determined on the linear portion of the curve after the 15-sec NEM reaction time. Reactions were performed at 37° at pH 7.2 to 7.4.

Table 1. Rates of metabolic reduction of exogenously added H_2O_2 by EATC under various metabolic conditions*

Experiment A		Experiment B	
Additions	Rate (μM/min)	Additions	Rate (μM/min)
1. H_2O_2	10.8	H_2O_2	10.8
2. H_2O_2	6.9	H_2O_2	6.9
3. Azide + H_2O_2	6.1	H_2O_2	4.8
4. Glucose + H_2O_2	19.0	Glucose + H_2O_2	17.3
5. H_2O_2	19.0	H_2O_2	18.6
6. NEM + H_2O_2	1.3	H_2O_2	18.6

* Two cell suspensions (10^8 EATC in 10 ml buffer) were sequentially treated six times with 30 μM H_2O_2 and reduction rates (μM/min) were determined. Sodium azide, glucose, and NEM (2, 1, and 0.1 mM final concentrations respectively) were added before certain H_2O_2 additions as indicated. Experiment B served as a control for A. See text for details.

Reductive metabolism of H_2O_2 by EATC. EATC have been reported to contain glutathione peroxidase (GSH:H_2O_2 oxidoreductase; EC 1.11.1.9) and to be relatively free of catalase [16]. To better interpret results demonstrating H_2O_2 accumulation, the ability of EATC to metabolically reduce exogenously added H_2O_2 was measured. Some of the measurements were in the presence and absence of glucose and inhibitors of glutathione peroxidase and catalase. Glutathione peroxidase catalyzes reaction 4 in Fig. 7.

Two types of experiments were performed. The first was the addition of EATC to buffer containing known quantities of H_2O_2 and inhibitors of known reduction reactions. Figure 5A illustrates how rapidly (10.4 μM/min) 10^7 EATC/ml in a 10-ml volume could reduce H_2O_2 in the absence of any metabolic modifiers. Figure 5B shows that 2 mM sodium azide (a respiration and catalase inhibitor) had little effect on the reduction rate (10.4 μM/min). As azide readily inactivates catalase (confirmed in this system using exogenous catalase), it is apparent that H_2O_2 reduction in EATC is not mediated by catalase.

The inclusion of 0.1 mM NEM in the reaction mixture (Fig. 5C), however, caused a dramatic decrease in the reduction rate to 2.8 μM/min. Neither the addition of 2 mM azide nor increased amounts of NEM slowed the reduction rate any further, indicating that the decrease in H_2O_2 may have been due to its reaction with protein thiols.

A separate pair of experiments (B serving as a control for A) was performed to determine the effects of repetitive additions of H_2O_2 to the same sample of cells (see Table 1). Glucose, azide, and/or NEM were added between some of the H_2O_2 additions to determine their effects on H_2O_2 reduction. Care was taken to inject H_2O_2 near the bottom of the reaction vessel. This way a transient, highly concentrated pool of H_2O_2 could not cross the electrode, causing dilution of that pool near the electrode to be artifactually interpreted as reduction. H_2O_2 added in this manner mixed uniformly within seconds and caused an immediate electrode response. Reduction was then monitored. The reduction of the small amount of H_2O_2 that occurred during mixing was not significant in the determination of reduction rates.

The results are summarized in Table 1. It can be seen that the ability of EATC to reduce H_2O_2 could be decreased by repetitive additions of H_2O_2. This decrease in the reduction rate was almost certainly due to depletion of glutathione, as will be further supported.

Experiment A shows that an addition of 2 mM sodium azide before the third H_2O_2 addition had no noticeable effect on the ability of EATC to reduce H_2O_2. The addition of glucose before the fourth addition of H_2O_2 in each experiment significantly increased the reduction rates, indication regeneration of the substrate. However, 0.1 mM NEM, added before the sixth H_2O_2 addition in experiment A, slowed the reduction drastically to only 1.3 μM/min.

The indications are that the levels of GSH are vitally important when using this system to monitor H_2O_2 production and reduction.

CDNB–GSH adduct formation. CDNB is a known substrate for glutathione-*S*-transferase. It displaces a chloride ion from CDNB, producing a glutathione adduct [8, 15, 17]. As seen in Fig. 6, the incubation

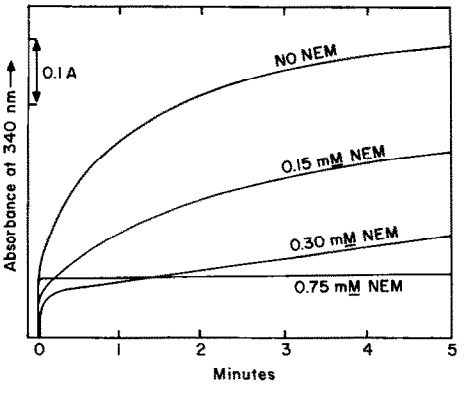

Fig. 6. Inhibition by NEM of the adduct formation between GSH and CDNB as monitored spectrophotometrically at 340 nm. Reactions were carried out at 37° in cuvettes containing 2×10^7 EATC and 2 ml buffer at pH 7.2, preincubated with the amount of NEM indicated, with 0.15 mM CDNB added at time zero. NEM did not affect the absorbance.

of EATC with CDNB resulted in the production of a substance which could be monitored spectrophotometrically at 340 nm. Both NEM (Fig. 6) and the glutathione oxidant "diamide" [diazenedicarboxylic acid bis(*N*,*N*-dimethylamide)] [18] were found to decrease the rate of formation of the substance (data not shown).

It can be estimated from Fig. 4 that the lag for 0.2 mM CDNB was 64 sec, and from Fig. 6 that about 70 per cent of the maximum adduct value was obtained at that time. This, supported by Jacobson's unpublished data showing that 0.2 mM CDNB causes 78 per cent thiol loss in 1 min suggests that about three-fourths of the thiol content of EATC must be depleted before CDNB-induced H_2O_2 accumulation commences.

DISCUSSION

The results demonstrate that the oxidase electrode and meter can be used effectively to continuously monitor drug-induced H_2O_2 production, metabolic reduction and factors influencing them.

It also shows that a number of reactions can influence H_2O_2 production and that GSH can be depleted in a number of ways. Depicted in Fig. 7 is a summary of the possible reactions involved in CDNB metabolism. A variety of nitrobenzenes (including CDNB), when activated by cells, yield oxygen-reactive nitro-radical anions [10, 19]. Flavoprotein enzymes, such as NADPH-cytochrome *c* reductase (EC 1.6.2.4), found predominantly in the microsomes, are known to have nitroreductase activity (reaction 1). Previous studies have indicated that these enzymes are responsible for most of the cellular activation of nitro-compounds [12, 20–22] that results in H_2O_2 production. The supply of reducing equivalents for nitro-reduction comes predominantly from the pentose cycle enzymes that reduce NADP to NADPH [12]. The enzyme-linked one-electron reduction of the nitro-compounds produces an oxygen-reactive nitro radical [12]. Reaction of the nitro-radical with oxygen (reaction 2) produces superoxide [12, 22] and the regeneration of the original nitro-compound. The oxygen radical may also react spontaneously with itself to produce H_2O_2 and possibly activated oxygen [12, 22]. It can also be enzymatically converted to produce H_2O_2 and oxygen by superoxide dismutase (reaction 3). In the presence of glutathione peroxidase, H_2O_2 is rapidly reduced by glutathione (reaction 4).

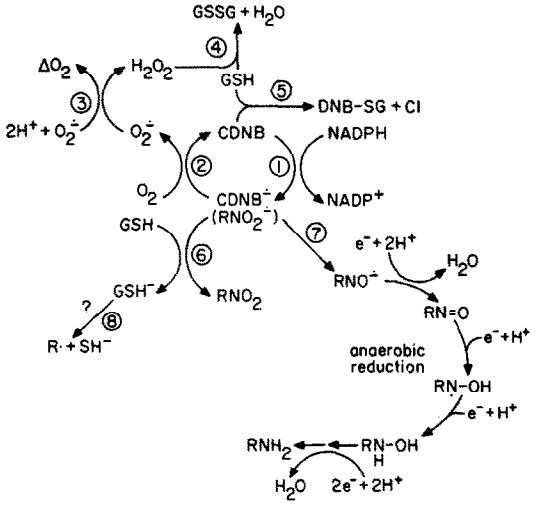

Fig. 7. A summary of reactions that may be involved in the metabolism of CDNB (and probably other nitro-compounds) by EATC. Details are discussed in the text. DNB-SG is a dinitrobenzene–glutathione adduct [6, 15, 17].

The results show that there is a lag period (see Fig. 1) before the onset of net H_2O_2 production. This lag period may be decreased markedly when cells are treated with NEM prior to CDNB exposure (Fig. 4). Removal of GSH by NEM would inhibit both the glutathione peroxidase and glutathione-*S*-transferase-mediated reactions (Fig. 7, reactions 4 and 5). In the presence of NEM, the lag period decreases but the net rate of H_2O_2 production is not affected. The lag may be partially accounted for by the reaction of nitro-radicals with GSH (reaction 6) instead of with oxygen to produce H_2O_2. This reaction is sufficiently fast ($10^4\ M^{-1}\ sec^{-1}$; E. M. Fielden, personal communication) to inhibit H_2O_2 production by reaction 2.

It was also found that glucose markedly stimulated the rate at which H_2O_2 accumulated in the reaction mixture. Glucose enhanced a couple of competing reaction schemes to stimulate both the production and destruction of cellular H_2O_2 (Figs. 3 and 5). The amount of H_2O_2 accumulated at any given time depended, in part, on the rate at which it was produced (Fig. 7, reactions 1–3) and on the rate at which it was reduced by catalase ($2H_2O_2 \rightarrow 2H_2O + O_2$) and glutathione peroxidase (reaction 4).

The peroxidase reaction depended on the availability of GSH and that, in turn, depended on the glucose-stimulated reaction regenerating GSH from the GSSG formed by reaction 4 (GSSG + 2 NADPH $\rightarrow$ 2 GSH + 2 NADP). Glucose was also important for stimulating the initial reduction of the nitro-compound to a nitro-radical (reaction 1), thus initiating the production of H_2O_2. This was presumably due to the production of reducing equivalents for NADPH-cytochrome *c* reductase (EC 1.6.2.4) when nitro-compounds are used as electron acceptors. Our results suggest that, when excess CDNB is available, the metabolic generation of H_2O_2 eventually surpasses the ability of cellular GSH and GSH-peroxidase to reduce it. This is probably due, in part, to the glutathione-*S*-transferase-catalyzed conjugation of GSH with CDNB (Figs. 6 and 7, reaction 5) [8, 15, 17]. The lag period ended when endogenous GSH was removed by this reaction. The loss of the lag period was also seen with NEM-treated cells. Evidence for glutathione-*S*-transferase-catalyzed reaction of GSH with CDNB (reaction 5) was obtained spectrophotometrically by measuring the absorption of the adduct at 340 nm [8, 10, 15]. Prior treatment of cells with NEM prevented the formation of the adduct (Fig. 6).

There is also the possibility that nitro-radicals may react with glutathione (Fig. 7, reactions 6 and 8),

resulting in thiol loss as well as production of an organic radical [23].

The increased cellular oxygen consumption due to CDNB-stimulated H_2O_2 production may, under certain conditions, deplete the dissolved oxygen and cause the production of H_2O_2 to cease. Our system, however, was open to the air and well stirred, so lack of oxygen was not a factor during the measurement periods. Under metabolically produced hypoxia, the reduction of CDNB to the amine or hydroxylamine can probably be enhanced (reaction 7) [22], but we found no spectrophotometric evidence for this reduction in our aerobic whole-cell system (data not shown). Our conclusion that formation of oxygen-reactive nitro-radicals is probably the first step in aerobic reduction is in agreement with results found with purified enzymes [22] and cellular systems [12, 13, 19, 24] using other nitrocompounds. The depletion of glutathione and the production of radicals and/or H_2O_2 may thus help explain the mutagenicity of CDNB in *Salmonella* [7].

The findings suggest a number of areas that warrant further study. One, as mentioned above, is the possibility that CDNB mutagenicity of *Salmonella* may be due, in part, to radicals or H_2O_2. Hence, oxygen tension in the tests may be important factors in determining mutagenic potential.

It is also possible that the topical use of CDNB may deplete GSH levels in the area of application to a point where, if not otherwise controlled, cellularly produced H_2O_2 or the introduction of other potentially harmful agents could pose a genetic hazard. A preliminary report indicates that tumor cells are more sensitive to H_2O_2-induced lysis by granulocytes and macrophages when GSH is depleted by CDNB [25].

Lastly, the possibility that at least three mechanisms may operate for CDNB-stimulated thiol removal in whole cells (Fig. 7, reactions 4, 5, 6 and 8) may signify that artificially low levels of GSH may be found when CDNB is used as an analytical reagent in whole cells.

From a technical viewpoint, the use of the H_2O_2-sensing electrode in a novel manner—that of continuously monitoring both drug-stimulated H_2O_2 production and the metabolic reduction of H_2O_2 in whole-cell suspensions of EATC—was successful. In general, we found the electrode very useful for *in vitro* cellular studies of this type. Although CDNB was chosen for this study, preliminary work [26] indicated that 5-nitrofuraldehyde diacetate and menadione (vitamin K_3) are also good stimulators of H_2O_2 production in EATC.

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