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Properties of mitochondria treated with 1-chloro-2,4-dinitrobenzene*

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A role for GSH in mitochondria has so far not been established [1–3]. The substance may, however, be necessary for maintaining the sulphydryl groups of some mitochondrial enzymes [3, 4]. Such effects of GSH depend on its oxidizability to the disulphide form GSSG, and it should thus be effectively removed as a metabolically active substance by *S*-substitution. Specific blockage is theoretically feasible because GSH can react with various xenobiotics under catalysis by glutathione transferases [5]. CDNB is a favoured substrate for these enzymes which are now known also to be present in mitochondria [6]. This report describes the effect of CDNB on mitochondrial GSH and on the oxidative capacity of these depleted particles towards several substrates.

Materials and methods

[^{14}C]-pyruvate, [^{14}C]-NEM and [^{86}Rb]-rubidium chloride were purchased from Amersham International Ltd. (Amersham, U.K.) and dihydrolipoate from Sigma Ltd. (London, U.K.). The buffer used throughout was 0.125 M KCl containing 25 mM Tris HCl, pH 7.2 and 0.1 mM EDTA. Rat liver mitochondria were obtained as described previously [7] and used within 3 hr of preparation. Proteins were assayed by a biuret method [8], mitochondrial NPSH by the DTNB method [7, 9] and GSH by reaction with [^{14}C]-NEM followed by electrophoretic separation [1] then elution of the adduct with 1.5% acetic acid prior to counting. Pyruvate dehydrogenase was assayed by the release of [^{14}C]-CO_2 from 1-[^{14}C]-pyruvate [10]. The pyruvate dehydrogenase activity was maximized by preincubating mitochondria in buffer containing CCCP (0.5 μM) for 10 min. Lipoate dehydrogenase was assayed with 0.1 ml sonicated mitochondrial supsension added to 1 ml buffer containing lipoamide (100 nmoles), NADH (1 μmole) and CDNB (100 nmoles). Thiol formed was measured 10 min later with DTNB. Membrane potential ($\Delta\psi$) was determined with ^{86}Rb and valinomycin [11].

Results and discussion

When mitochondria are incubated with CDNB their concentration of GSH rapidly falls as shown by sedimentation and subsequent specific assay. The amount of GSH lost depends on the incubation time (since it slowly diffuses into the suspension medium [1]) and on the amount of CDNB

* Abbreviations used: BCNU, bischloronitrosourea; CCCP, carbonylcyanide-*m*-phenylhydrazone; CDNB, 1-chloro-2,4-dinitrobenzene; DTNB, 5,5-dithiobis (2-nitrobenzoic acid; GSH, glutathione; NEM, *N*-ethylmaleimide; NPSH, non-protein thiols.

used. Maximal loss due to the inhibitor is attained at an initial CDNB concentration of 55–60 μM and an exposure to the reagent at 30° of 2 min (Fig. 1). Under these conditions there is negligible loss of GSH from control suspensions and CDNB does not induce any mitochondrial swelling as measured by the turbidity method [12]. Moreover, the adduct formed is largely retained within the mitochondrial pellet as shown by direct spectrophotometric assay [5]. A small but significant amount of GSH (10%) remains at the above CDNB concentration but is unaffected by raising the concentration further. This may indicate some sequestration of GSH within the mitochondrial matrix. This loss of GSH is greater than reported using as depleting agent BCNU [13] or phorone [14]. Total NPSH has been concurrently assayed and it is clear that the increment contributed by thiols other than GSH (about 20%) is not significantly affected by CDNB (Fig. 1). This NPSH is presumably contributed chiefly by coenzyme A and perhaps by some dihydrolipoate. This interpretation is confirmed by incubating 4 mg mitochondrial protein in 1 ml buffer containing 1 mg lubrol with mixtures of 200 nmoles of CDNB and 200 nmoles of various NPSH. GSH is severely depleted (above 85%) but other thiols, in particular dihydrolipoate, cysteine and coenzyme A are little affected (below 8%). The depletion of GSH does not occur in the absence of either CDNB or the mitochondria. Treatment of mitochondria for 2–6 min with CDNB does not impair their respiratory control and they give normal P/O ratios with succinate (not shown) and 3-hydroxybutyrate. Their membrane potential, measured with ^{86}Rb and valinomycin [11] is also unaffected. When untreated mitochondria are uncoupled, the addition of CDNB does not affect, 2 min later, the oxidation of succinate or glutamate but inhibits to some extent that of hydroxybutyrate, isocitrate, ketoglutarate and pyruvate (Table 1). Mitochondria depleted of GSH have also been prepared in bulk from homogenates by adding CDNB (to give 100 μM) to the medium used for the second washing. After 2 min they are sedimented then rewashed in normal medium before making a concentrated suspension. The following concentrations (nmoles/mg of protein) of GSH and NPSH have been measured (mean of two preparations): untreated, GSH 5.7 ± 0.1; NPSH 7.2 ± 0.2: CDNB-treated, GSH 0.65 ± 0.1; NPSH 2.3 ± 0.05. These CDNB-treated mitochondria when compared with untreated mitochondria are also well coupled and oxidize succinate normally. They show more inhibition of ketoacid oxidation and much more

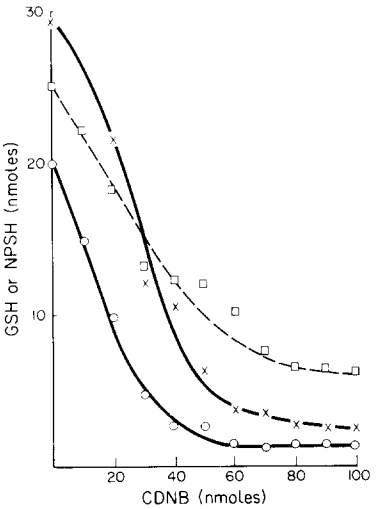

Fig. 1. Effect of CDNB on GSH and NPSH concentrations. Mitochondria (0.1 ml; 4 mg protein) were added to 1.2 ml buffer at 30° containing 3-hydroxybutyrate (10 μmoles) and CDNB as specified. After 2 min (x) or 5 min (o) incubation, the pellet was sedimented by centrifugation for 1 min in an Eppendorf centrifuge then triturated with 2.4% perchloric acid (0.5 ml). NPSH (□, dotted line) was assayed with DTNB and GSH (x, o) with [^{14}C]-NEM (see Methods).

Table 1. Oxygen uptake by mitochondria before and after treatment with CDNB

Substrate	Oxygen uptake: Untreated (nmoles/mg)	Oxygen uptake: 2 min after CDNB (% untreated)	Pretreated with CDNB (% untreated)
Hydroxybutyrate:			
coupled (state 4)	20 ± 2	62	70 ± 8
+ ADP (state 3)	102 ± 30	110	75 ± 6
uncoupled	69 ± 5	64 ± 24	73 ± 15
Uncoupled:			
succinate	121 ± 5	100	102 ± 2
pyruvate	36 ± 1.5	41 ± 25	41 ± 5
ketoglutarate	12 ± 5	70 ± 25	40 ± 30
isocitrate	38.5 ± 10	60 ± 13	13 ± 4
glutamate	12.5 ± 0	100 ± 0	—

CDNB pretreated mitochondria were made from 20% liver homogenates: after the first deposition, the pellet was washed once then resuspended in the original volume of medium containing 100 μM CDNB initially at 20°. 2 min later it was resedimented at 8000 *g* and washed again by resuspension in fresh medium. Untreated mitochondria were similarly prepared from another portion of the same homogenate. (For GSH and NPSH content of these mitochondria see text.) 0.1 ml mitochondrial suspension (4–4.5 mg protein) was incubated in the chamber of an oxygen electrode at 30° with 2 ml of buffer. For coupled measurements, 3-hydroxybutyrate (10 μmoles), phosphate (0.25 μmole) were present and, where indicated, CDNB (200 nmoles injected from a 10 mM ethanolic solution). Oxygen uptake was measured after 2 min then ADP (1 μmole) added and the new rate determined. For uncoupled measurements, 0.5 μM CCCP was added and when oxygen consumption ceased (about 5 min) the substrate (10 μmoles) was added with or without CDNB and uptake determined for 2 min.

of isocitrate oxidation than untreated mitochondria pre-incubated 2 min directly with CDNB. Some direct reaction with sulphydryl groups of the enzyme protein is thus a possible explanation. The inhibition of ketoacid oxidation could be due to autoxidation of coenzyme A after removal of GSH. Direct assay of pyruvate dehydrogenase activity of lysates where coenzyme A is present in excess [12] gives some inhibition with CDNB (63 ± 6% of untreated values are obtained). This inhibition is not due to an effect on the dihydrolipoate component of the complex since its direct assay (81 ± 11% of untreated value) is little affected by CDNB. Inhibition of the oxidation of isocitrate could be due to the sensitivity of isocitrate dehydrogenase to lipid hydroperoxides [15, 16] since a recognised role of GSH is to prevent their formation [17]. In conclusion, mitochondria treated with CDNB are depleted of most of their normal content of GSH. Nevertheless, in this condition they remain well coupled and maintain their membrane potential. They oxidize succinate, glutamate and 3-hydroxybutyrate at near normal rates but isocitrate and ketoacid dehydrogenations are partially inhibited. GSH depleted mitochondria obtained as described may be suitable for studying further the specific effects of GSH on mitochondrial functions.

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