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THE REDUCTION OF DITHIOBIS(2-NITROBENZOATE) BY RAT LIVER MITOCHONDRIA

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Abstract—5,5′-Dithiobis(2-nitrobenzoate) (DTNB) is reduced in mitochondrial suspensions to 5-mercapto-2-nitrobenzoate (MNB) by 3-hydroxybutyrate and isocitrate. Although most of the MNB produced is found in the suspension medium, there is also some within the particles. The amount of MNB found in these fractions varies with the DTNB concentration used and is much lower if mitochondrial glutathione (GSH) is depleted with 1-chloro-2,4-dinitrobenzene. If hydroxybutyrate is present, the reduction of DTNB is increased by ATP and oligomycin. The pellet contains only a little MNB and GSH but these are considerably elevated by antimycin and rotenone as well as by ATP and oligomycin. If isocitrate is present, the reduction of DTNB is greatly stimulated by valinomycin, triethyltin and, to a lesser extent, oligomycin. MNB in the pellet falls and GSH concentrations are unchanged. The results suggest that with hydroxybutyrate (an NAD reducing substrate), the rate of reduction of DTNB is limited by the rate of regeneration of GSH while with isocitrate (an NADP reducing substrate) it is limited by the rate of export of MNB from the matrix.

DTNB†, an aromatic disulphide, reacts by thiol-disulphide exchange with SH groups to give stoichiometric amounts of the corresponding thiol, MNB [1]. The reagent has been used to titrate SH groups of mitochondria when, under suitable conditions, it gives values [2] similar to those obtained by other thiol assay methods, e.g. by amperometric titration with silver ions [3].

DTNB has however been considered [4] not to be able to penetrate the inner membrane of mitochondria because the GSH concentration in mitochondria treated with it does not fall. However, DTNB is much used in a cyclical method for assaying micro amounts of GSH in extracts in the presence of added glutathione reductase and NADPH [5, 6]. Since these components are all present in the mitochondrial matrix [7], any DTNB penetrating there would be reduced to MNB. However, if the rate of this penetration were less than the rate of regeneration of GSH from the GSSG thus formed then the observed level of mitochondrial GSH would not fall.

In this paper, evidence is presented to suggest that there is indeed a GSH dependent reduction of DTNB to MNB and that transport of this disulphide into the matrix may be the rate limiting step in its reduction.

MATERIALS AND METHODS

Rat liver mitochondria were prepared as previously described [8] and stored for use within 3 hr as a concentrated suspension (40–50 mg protein/ml) in 0.25 M mannitol containing 1 mM EGTA and 25 mM Tris–HCl, pH 7.2.

Incubation procedure. After preincubating, for 1 min at 30° ± 0.5°, suspensions of mitochondria (0.1 ml; 4–5 mg protein) in 0.5 ml buffer plus [^{14}C]-sucrose (0.1 μCi), fluorocitrate (10 nmoles) and substrate (hydroxybutyrate or isocitrate) (10 μmoles), DTNB as indicated was added in 0.5 ml buffer and the incubation continued. To terminate the reaction, the pellet was rapidly sedimented using an Eppendorf high speed microfuge. The suspension medium was poured into 0.2 ml 12% perchloric acid and the pellet washed by resuspension for 1 min in 1 ml ice-cold mannitol-MOPS then redeposited. The washed pellet was stirred with 0.5 ml 2.4% perchloric acid and recentrifuged to obtain the protein-free pellet extract.

Assays. MNB was assayed on the acid suspension medium (0.1–0.5 ml made up to 0.5 ml with 2.4% perchloric acid) or pellet extract (0.5 ml) by adding 0.5 M Tris–HCl, pH 7.8 containing sufficient sodium hydroxide to neutralise the perchloric acid then measuring at once the extinction at 412 nm [1]. MNB (generated from GSH and DTNB), when added in amounts up to 1 mM to mitochondrial suspensions, was recovered quantitatively by this technique after incubation for 30 min at 30°. Non-protein thiol was assayed on the mitochondrial extract after the MNB assay by adding DTNB (1 μmole in 20 μl) and measuring after 2 min the subsequent increase at 412 nm (less that due to DTNB alone). Protein assays and the preparation of sonicates were as previously described [9]. The extent of contamination of the washed pellet extract with the original suspension medium was assayed by measuring its ^{14}C content after including [^{14}C]-sucrose in the incubation. Values found were below 1%.

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† Abbreviations used: CDNB, 1-chloro-2,4-dinitrobenzene; DTNB,5,5′-dithiobis-(2-nitrobenzoate); GSH, reduced glutathione; GSSG, oxidised glutathione; hydroxybutyrate, DL-3-hydroxybutyrate; isocitrate, DL-isocitrate; MNB, 5-mercapto-2-nitrobenzoate; SH, sulphydryl group.

RESULTS

DTNB (1 mM) was added to mitochondria suspended at 30° in an isotonic buffer with or without hydroxybutyrate or isocitrate present and, at various times, samples were rapidly centrifuged and the concentration of MNB formed assayed on the deproteinised suspension medium. Without any added substrate, MNB reaches a fairly constant value attained after 15–20 min (e.g. 60 nmoles MNB/mg mitochondrial protein). With added hydroxybutyrate, the same pattern is observed but the final value is considerably higher (e.g. 85 nmoles/mg protein). With added isocitrate, however, the rate continues to increase until, after 30 min, the MNB formed is 2–3 times more than the maximum obtained without substrate (Fig. 1A).

Sonicate obtained from washed mitochondria has no significant capacity to reduce DTNB at the expense of added hydroxybutyrate or isocitrate. Likewise, detergents (lubrol, triton) completely inhibit the reaction.

MNB is also present in the subsequently sedimented and washed mitochondrial pellet as shown by assay after deproteinising with perchloric acid and allowing for residual supernatant contamination. When no substrate is present during the incubation, only a small amount of MNB is found. With hydroxybutyrate present, the pellet contains a larger amount at first but with further incubation this falls

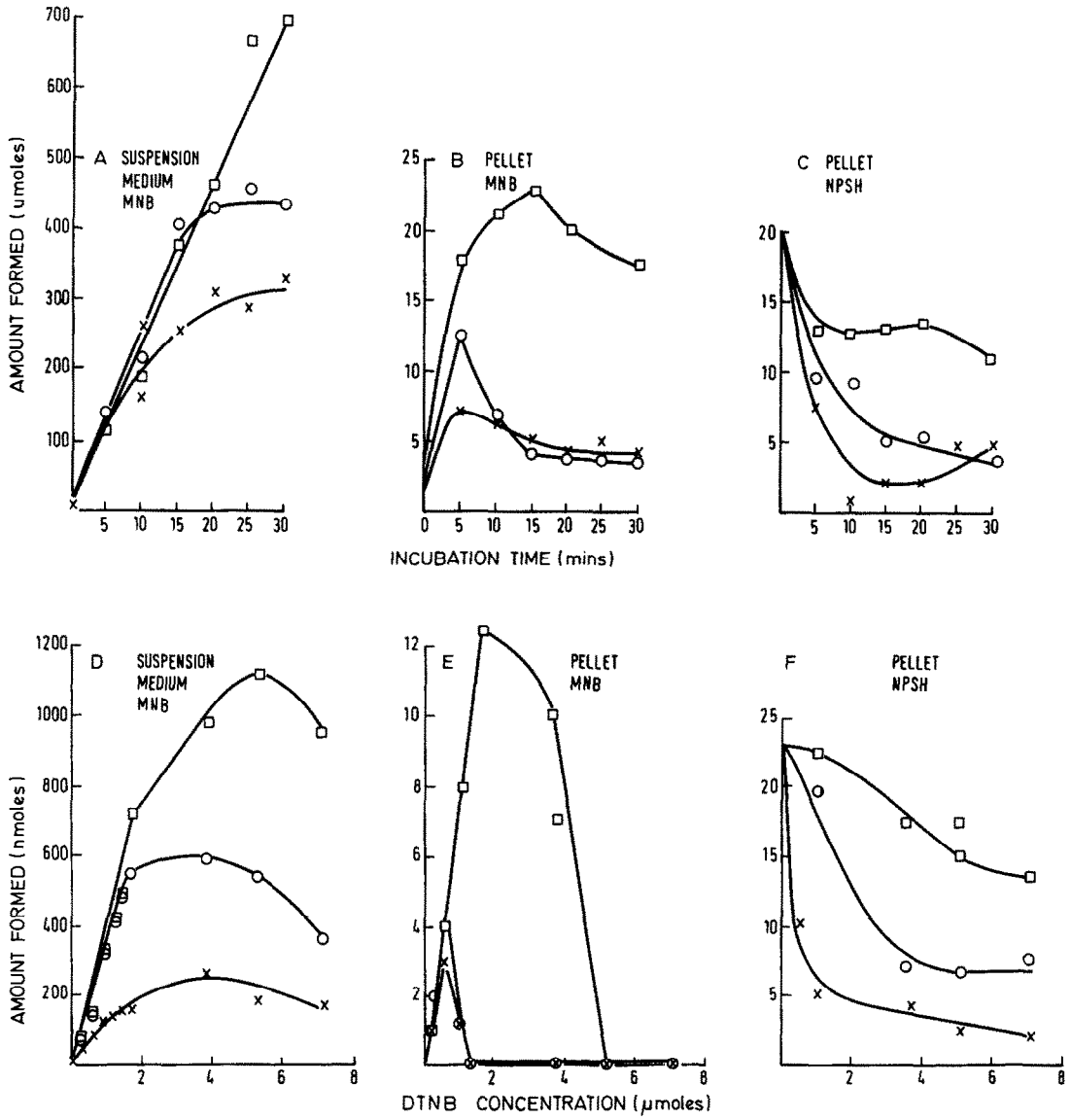

Fig. 1. MNB formation in the suspension medium (A, D) and the mitochondrial pellet (B.E.) and the corresponding nonprotein thiol content of the pellet (C, F) after incubating mitochondria in buffer containing DTNB either without substrate (×), with hydroxybutyrate (○) or with isocitrate (□). A–C show the effect of varying incubation time (DTNB, 1 μmole) and D–F the DTNB added (incubation time, 30 min).

to a low level. In contrast, with isocitrate the amount rapidly rises and is maintained at a much higher value (e.g. 4 times higher) (Fig. 1B).

The pellet content of non-protein thiol (chiefly GSH [10]) rapidly declines from 4–6 nmoles to 0.4 nmoles/mg protein as the incubation progresses if no substrate is present. This decline is slower if hydroxybutyrate is also added while with isocitrate it rapidly reaches a steady state value (e.g. 2.5 nmoles) which is largely maintained over the incubation period (Fig. 1C).

Evidence for the direct involvement of GSH in the reduction is given by the action of CDNB. This substance has been shown rapidly to deplete mitochondrial GSH [11] and with either hydroxybutyrate or isocitrate present it severely inhibits the production of MNB, the values found in the suspension medium and in the pellet being then of the same order as those found without substrate present (shown in Figs 2 and 3).

If the initial concentration of DTNB is increased from zero there are at first consequent increases in the amount of MNB found in the suspension medium (Fig. 1D) and in the pellet (Fig. 1E) when either substrate is added, but the values reach a maximum and then fall again at higher DTNB concentrations. Non-protein thiol falls continuously with increasing DTNB, but the fall is moderated by isocitrate and, less effectively, hydroxybutyrate (Fig. 1F). These findings suggest that the greater DTNB gradient forces more of it into the matrix until it overwhelms the enzymic mechanism for its reduction.

Known inhibitors of mitochondrial processes do not affect MNB production in the suspension medium or MNB and non-protein thiol in the pellet unless a substrate is added, in which case pronounced effects are obtained differing with the substrate used.

With hydroxybutyrate (Fig. 2), electron transport inhibitors (antimycin and rotenone) elevate pellet non-protein thiol and MNB far above their low control values (i.e. those found without inhibitor present) and also increase the MNB of the suspension medium. An uncoupler (pentachlorphenol), though it releases respiration in the presence of DTNB, shows no consistent effects on any of the parameters. A phosphorylation inhibitor (oligomycin) increases MNB in the suspension medium and both MNB and nonprotein thiol in the pellet substantially above the control values. Adding ADP or ATP (100 nmoles) itself affects the reduction of DTNB; MNB is increased both in the medium and in the pellet but non protein thiol is unchanged.

With isocitrate (Fig. 3), rotenone and antimycin also increase the already high values for pellet MNB and nonprotein thiol. However, in contrast to hydroxybutyrate, there are large increases in

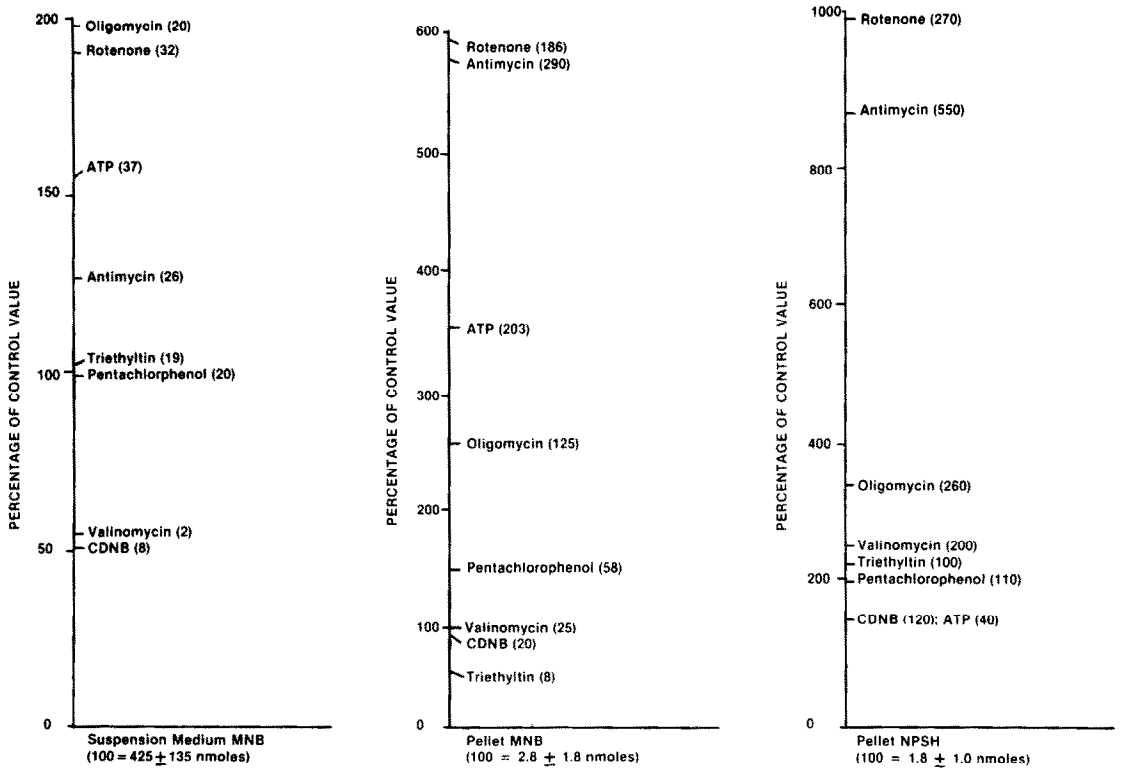

Fig. 2. The effect of added inhibitors on the MNB of the suspension medium and pellet and on the nonprotein thiol of the pellet after incubating for 30 min a mitochondrial suspension with DTNB and hydroxybutyrate. Results (as a percentage of the uninhibited control values) are the means of 3–6 assays each with a different batch of mitochondrial; standard deviations are in parenthesis. Amounts of inhibitor used: antimycin (2 nmoles), ATP (100 nmoles), CDNB (100 nmoles), oligomycin (2 μg), pentachlorphenol (3.5 nmoles), rotenone (2.5 nmoles), triethyltin sulphate (15 nmoles), valinomycin (1 nmole).

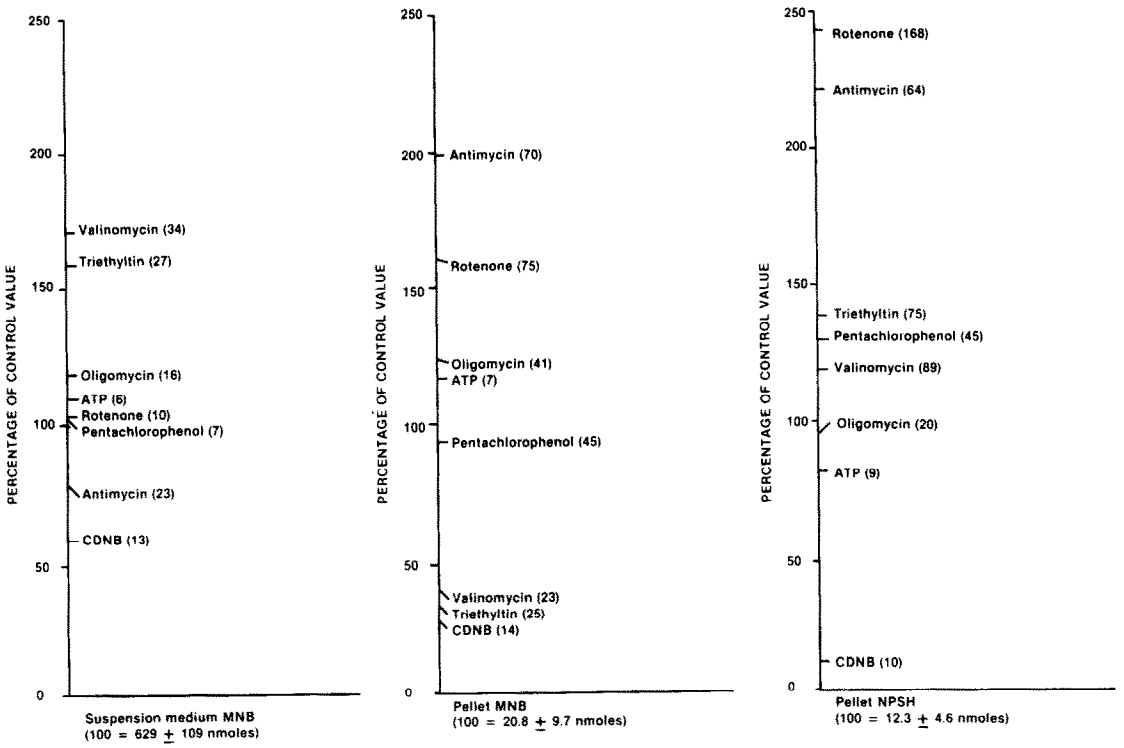

Fig. 3. As for Fig. 2 but with isocitrate replacing hydroxybutyrate.

medium MNB and large decreases in pellet MNB when a K^+ transporting agent (valinomycin) is added. This effect is not found if KCl in the medium is replaced by NaCl or NH_4Cl (not shown). A chloride transporter (triethyl tin [12]) also raises medium MNB and decreases pellet MNB but this is also effective in a medium in which chloride is replaced by mannitol.

DISCUSSION

The MNB produced by reduction of DTNB with rat liver mitochondria consists of two fractions, one formed by reaction with accessible SH groups and the other linked to the reduction of an added physiological substrate. The bulk of the MNB produced is found in the suspension medium but, with a substrate present, there is also an increment in the washed mitochondrial pellet suggesting that in this case reduction is preceeded by entry of DTNB into the mitochondrial matrix. The powerful inhibition obtained with the specific GSH depleting agent CDNB is further evidence of entry, for DTNB and GSH are known to react rapidly above pH 6 [5]. The pK for the SH group of the MNB formed by the reduction is a 4.4–4.5 and thus it is largely ionised [13, 14] in the matrix, but there is no evidence of a requirement for proton extrusion during its export since the reaction is unaffected by an uncoupler. Presumably therefore it escapes as the monoanion, $'OOC.C_6H_3NO_2$—SH, in exchange for the entry of DTNB dianion in the ratio of 2 to 1.

Of the two substrates used, isocitrate responds in a different way to inhibitors than hydroxybutyrate, supports a somewhat higher rate of reduction of DTNB and has much higher MNB and GSH concentrations in the corresponding pellets. These facts suggest that for hydroxybutyrate it is the rate of reduction of DTNB within the pellet which is rate limiting whereas for iscitrate it is rather the rate of export of the MNB formed. A ready explanation is that mitochondrial glutathione reductase, required to regenerate GSH from the GSSG produced with DTNB, is 13–14 times more active with NADPH than with NADH [7]. Whereas isocitrate can produce NADPH directly via the NADP-isocitrate dehydrogenase [7], hydroxybutyrate reduces NAD only. The increase with ATP or oligomycin of the hydroxybutyrate dependent reduction could be due to stimulation of the energy requiring transhydrogenase [15] allowing reduction of NADP by NADH perhaps by binding to and thus blocking the competing F_1-ATPase.

Rotenone and antimycin which substantially raise MNB and GSH values with either substrate may act by increasing the availability of NADPH for GSSG reduction by blocking its loss to oxygen as also noted for other GSH dependent reductions [16, 17].

The dramatic effects of valinomycin and triethyltin are attributable to facilitation of MNB transport from the matrix. Thus valinomycin may allow a more rapid exchange between incoming DTNB and outgoing MNB by compensating the charges of their anions with accompanying K^+ while triethyltin may promote thiol/hydroxyl exchange [12] since thiol tin derivatives have considerable lipophilicity as shown by, for example, extraction of cysteine into organic medium by trialkyltins [18].

The present work has shown that the maintenance of a high GSH level in the presence of DTNB

depends on the metabolic state of the mitochondria. Since they are constantly being exposed to endogenous thiol oxidants, e.g. oxygen derived radicals [19], this finding is relevant to the evidence that GSH, by maintaining critical SH groups [20], is a factor in preserving various membrane properties such as gross changes in appearance [21], permeability to Ca^{2+} [22], ADP binding [23] and activity of membrane located enzymes [20].

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