Determinants of MTT reduction in rat hepatocytes

Perminder Dhanjal and Jeffrey R. Fry

The determinants of reduction of the dye MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) in rat hepatocytes have been investigated. NADH, NADPH, and succinate were substrates for MTT reduction in rat liver homogenate, activity being greatest with NADH and least with succinate. Similar results were obtained with sub-mitochondrial particles isolated from rat liver. NAD(P)H-dependent reduction of MTT was also detected in rat liver microsomes and cytosol. Rotenone, at a concentration that inhibited NAD(P)H-dependent MTT reduction in sub-mitochondrial particles, did not inhibit MTT reduction in rat hepatocytes. Malonate, at a concentration that inhibited succinate-dependent MTT reduction in liver homogenate, did not inhibit MTT reduction in rat hepatocytes. Incubation of rat hepatocytes with ethanol or lactate (increase NADH levels), dicoumarol (inhibitor of DT-diaphorase), aminopyrine or hexobarbitone (substrates for the NADPH-requiring cytochrome P450-dependent microsomal monooxygenase) led to significant increases in the level of cellular MTT reduction. From these data, it is concluded that extra-mitochondrial NAD(P)H is the principal reductant for MTT reduction in rat hepatocytes, with mitochondrial dehydrogenase activity being only a minor contributor. It is also possible that cellular generation of superoxide (as might be expected on redox cycling of endogenous quinones following inhibition of DT diaphorase by dicoumarol) may be another source of MTT reduction. Caution should be exercised in ascribing an alteration in the level of cellular MTT reduction to a change in mitochondrial performance in the absence of corroborating evidence.

Keywords: *MTT reduction, rat liver, hepatocytes, NAD(P)H, succinate, mitochondria.*

Introduction

Reduction of the tetrazolium dye MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; thiasolyl blue) is commonly used to measure cell viability and number in a variety of *in vitro* situations (Mosmann 1983). Slater *et al.* (1963) used MTT reduction as a measure of succinate dehydrogenase activity in rat liver mitochondria, and this report has been used by some workers to infer that MTT reduction in intact cells is a measure of mitochondrial dehydrogenase activity (Bonnefoi 1992, Yamashoji *et al.* 1992). Indeed, some authors have stated that changes in the level of MTT reduction in advance of gross cellular damage on exposure to toxic agents is indicative of a primary action of the toxic agent to the mitochondria (Bonnefoi 1992).

Set against this, a number of workers have advocated caution in the use of the MTT assay as a measure of cell number in light of numerous metabolic influences on the level of MTT (Thayer 1990, Vistica *et al.* 1991, Schiller *et al.* 1992, Berridge and Tan 1993, Burdon *et al.* 1993). Furthermore, reduction of tetrazolium dyes is widely used in biochemistry and histochemistry as a detection method for a variety of mitochondrial and non-mitochondrial dehydrogenase activities (Chayen *et al.* 1973, Abdallah and Biellmann 1980, Munujos *et al.* 1993). Recently, MTT reduction has been used as a measure of the alteration of redox balance in PC12 cells following exposure to amyloid protein (Shearman *et al.* 1994).

In light of these divergent views on what the MTT assay actually measures, and our interest in the use of isolated hepatocytes in studying mechanisms of xenobiotic-mediated toxicity, we have investigated the mechanisms of MTT reduction in rat liver homogenates and in rat hepatocytes.

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MATERIALS AND METHODS

Chemicals

MTT and all substrates, inhibitors and reagents for cell isolation and incubation were obtained from Sigma Chemical Company, Poole, Dorset, UK.

Animals

Male Wistar rats (170–200 g) were obtained from the University of Nottingham Medical School Animal Unit. They were housed at a constant room temperature of 22°C, with a 12-h light–dark cycle. Animals had free access to standard laboratory diet and water at all times.

Preparation of homogenate and tissue fractions

Liver homogenate was prepared by polytron homogenization in 0.25 M sucrose. For preparation of the microsomal and cytosol fractions, this homogenate was centrifuged at 10000 *g* for 20 min to sediment the mitochondria and tissue debris, and the supernatant centrifuged at 100000 *g* for 1 h. The supernatant from this step was used as the cytosol fraction. The pellet was resuspended in Tris/sucrose/EDTA buffer (20 mM/0.25 M/5.4 mM; pH 7.4) and recentrifuged at 100000 *g* for 1 h. The pellet was finally resuspended in Tris/sucrose/EDTA buffer; this represented the microsomal fraction. Liver sub-mitochondrial particles were isolated as described by Cain and Skilleter (1987). All tissue fractions were stored at –80°C until use.

Hepatocyte isolation and incubation

Hepatocytes were isolated by lobe perfusion with collagenase and purified by Percoll centrifugation as described previously (Hammond *et al.* 1995). Cells were suspended in Williams' E medium containing 10% (v/v) newborn calf serum and plated at 10^6 cells per well into tissue culture-grade six-well plates (Falcon). The plates were incubated at 36°C in an atmosphere of 5% CO_2 in air for 2 h to allow the cells to attach before use.

Assays

Microsomal cytochrome P450 was assayed by the method of Omura and Sato (1964), whilst homogenate P450 was assayed by the method of Joly *et al.* (1975)

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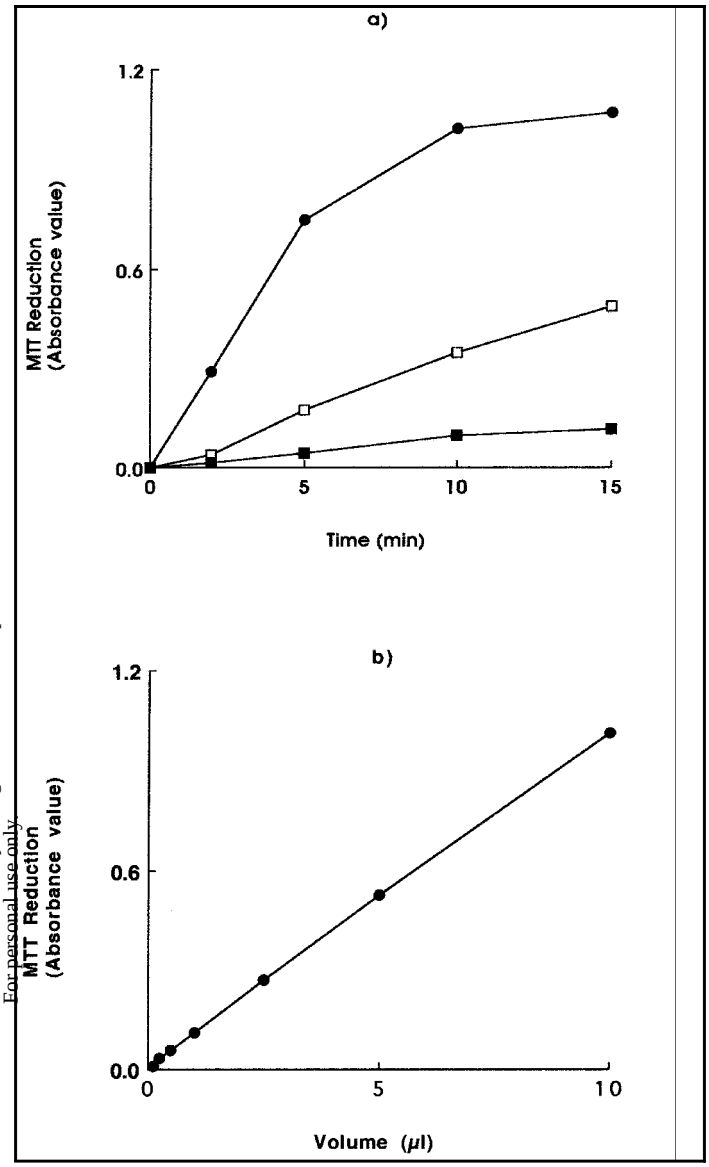

Figure 1. Influence of incubation time and homogenate volume on MTT reduction in liver homogenate. (a) Rat liver homogenate (10 μl ml^{-1} incubation) was incubated at 37°C with 1 mM NADH (●), 1 mM NADPH (□), or 20 mM succinate (■) in the presence of MTT for various times, and the MTT reduction measured. (b) Rat liver homogenate (0–12.5 μl ml^{-1} incubation) was incubated at 37°C with 1 mM NADH in the presence of MTT for 5 min, and the MTT reduction measured. Values are mean of five determinations.

For measurement of cellular MTT reduction, cells were exposed to MTT in medium at 5 mg ml^{-1} for 25 min, after which the medium was removed, propan-2-ol (1 ml per well) added to each well to dissolve the formazan crystals, followed by measurement of absorbance of a 200 μl sample in a plate-reader at a wavelength pair of 570/655 nm. The measurement of MTT reduction in tissue fractions was adapted from the method of Hansen *et al.* (1989). Tissue fraction (typically 10 μl), diluted in Tris/sucrose/EDTA buffer (to a total volume of 800 μl), was mixed with 100 μl of substrate solution, and the reaction started by addition of 100 μl of MTT solution (5 mg ml^{-1}). After incubation at 37°C, the reaction was terminated by addition of 100 μl of SDS/DMF buffer (10%/45%, adjusted to pH 7.4 with glacial acetic acid) to dissolve the formazan crystals, followed by measurement as described above.

Data presentation

Values are reported as the mean (±SEM in tables) of at least three separate experiments, each representing the mean of triplicate determinations, for the homogenate/fraction studies, and of six culture dishes for the cell studies. The data presented in the tables demonstrate there to be only a small variation in values within a single experiment. Similar findings were noted with the results presented in the figures, and, accordingly, error bars were omitted from the graphs for clarity. Statistical analysis was performed by paired *t*-test or ANOVA followed by Dunnett's test as appropriate.

Results

Homogenate experiments

MTT was reduced to a formazan when incubated with rat liver homogenate in the presence of succinate, NADH, or NADPH, at the concentrations used by Berridge and Tan (1993) (20 mM, 1 mM, and 1 mM respectively). This response was dependent on incubation time (Figure 1(a)) and homogenate volume (exemplified with NADH as substrate, Figure 1(b)). Incubation of homogenate in the absence of any exogenously-added substrate yielded a constant absorbance throughout the incubation period. This value was subtracted to produce the test values illustrated. From these studies, an incubation time of 5 min and a tissue fraction volume of 10 μl per ml incubation volume (equivalent to 5 mg tissue per ml) were chosen for further studies. Essentially identical time- and volume-responses were observed in the homogenate fractions (data not shown).

MTT reduction was also dependent on substrate concentration (see Figure 2 for data on NADH and NADPH). From these data approx. K_m values of 0.10 mM for NADH and 1.0 mM for NADPH were calculated. Accordingly, for further studies substrate concentrations of 800 μM NADH, 10 mM NADPH (both based on present study) and 20 mM succinate (based on data of Berridge and Tan (1993) and Munujos *et al.* (1993)) were used to ensure saturation of the respective pathways.

Under these optimal conditions, NADH was the most effective substrate for MTT reduction in liver homogenate, followed by NADPH with only poor reduction with succinate as substrate (Table 1). As expected, the difference between NADH and NADPH was exaggerated when equimolar concentrations (800 μM) were used (see Figure 1). The oxidized forms of NADH and NADPH were not substrates for MTT reduction (data not shown).

Attempts were made to identify the subcellular site(s) of this MTT reduction. In the first experiment, liver homogenate was fractionated into microsomal and cytosol fractions. Microsomal recovery was assessed by measurement of the cytochrome P450 content of homogenate and microsomes. This value for microsomal recovery (54% of homogenate P450 recovered in microsomes, similar to published values (Joly *et al.* 1975)) was used to factor the results for microsomal MTT reduction to allow for the loss of endoplasmic reticulum during preparation of the microsomal fraction. The results are presented in Table 1. No succinate-dependent MTT reduction could be detected in the microsomal and

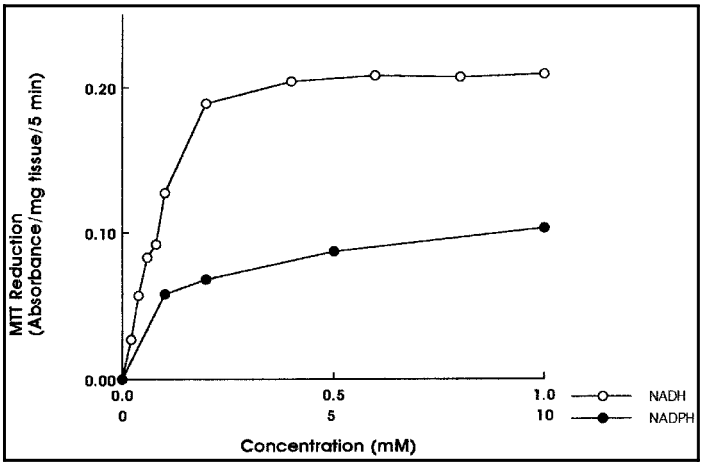

Figure 2. Influence of NADH and NADPH concentration on MTT reduction in rat liver homogenate. Homogenate (80 μl ml^{-1} incubation) was incubated at 37°C with 0–1 mM NADH (○) or 0–10 mM NADPH (●), in the presence of MTT for 5 min, and the MTT reduction measured. Values are mean of five determinations.

cytosol fractions. This finding indicates a lack of succinic dehydrogenase in these fractions, which in turn indicates minimal contamination of these fractions with mitochondria. The bulk of NADH-dependent MTT reduction (approx. 80%) was recovered in the microsomal fraction, with approximately 9% in the cytosol. Only 32% of the NADPH-dependent MTT reduction was recovered in the microsomal fraction with 5% in the cytosol. It was assumed that the activities unaccounted for resided in the mitochondria, but more direct evidence for this was obtained from a study with sub-mitochondrial particles. The results (Table 2; absence of rotenone) indicated that the level of succinate-dependent MTT reduction in this preparation was similar to that measured in homogenate in the previous experiment, whilst NADH was a good substrate for MTT reduction, the activity with NADPH being intermediate.

Malonic acid produced a concentration-dependent inhibition of succinate-dependent MTT reduction in liver homogenate within the concentration range 0–10 mM, with little effect on NADH- or NADPH-dependent activity (Figure 3). Rotenone (10 μM) produced substantial (50% or greater) inhibition of NADH- and NADPH-dependent MTT reduction in sub-mitochondrial particles with no demonstrable effect on succinate-dependent activity (Table 2). Dicoumarol (10 μM) was without effect on NAD(P)H- or succinate-dependent MTT reduction in homogenate or microsomal fraction (data not shown).

Hepatocyte experiments

The level of MTT reduction was approx. 3.5-fold greater when hepatocytes were incubated in complex cell culture medium (Williams' E) when compared with Hank's/Hepes, a simple salt solution (Table 3). Omission of glucose from the simple salt solution was without effect on the level of MTT reduction. The following studies were performed with Hank's/Hepes without glucose.

Addition of 6 mM malonic acid was without effect on the level of MTT reduction. Also, rotenone (10 μM) did not diminish the level of MTT reduction, rather it produced a significant 19% increase (Table 4). Ethanol (20 mM) and lactate (10 mM) produced significant 2.5-fold increase in MTT reduction, whilst dicoumarol (10 mM) produced a highly-significant 4.9-fold increase in the level of reduction. In addition, the cytochrome P450 substrates aminopyrine (100 μM) and hexobarbitone (100 μM) produced significant 1.3- to 1.5-fold increases in the level of MTT reduction. The data in Table 4 also indicates that the level of MTT reduction in the control situation showed a 2.5-fold variation between experiments.

Discussion

Results presented in this paper have demonstrated that succinate, NADH and NADPH support MTT reduction in rat liver homogenate, the extent of which is greatest with NADH and least with succinate. These results are in agreement with those obtained with homogenates of cells of a bone-marrow derived cell line (Berridge and Tan 1993). Similar results were obtained with sub-mitochondrial particles. Collectively, these data indicate that succinate-mediated reduction of MTT, and, by implication, mitochondrial succinic dehydrogenase (SDH)-mediated reduction of MTT, is unlikely to be a major contributor to cellular MTT reduction. In support of this, malonate (a selective inhibitor of SDH in homogenate, Figure 3) exerted no inhibitory effect on MTT reduction in rat hepatocytes. Malonate was similarly ineffective in cells of the PC12 rat pheochromocytoma cell line (Hawtin *et al.* 1995).

The homogenate data point to the adenine nucleotides NADH and NADPH being the principal substrates for MTT reduction in rat hepatocytes. This conclusion is substantiated by the finding that ethanol and lactate, at concentrations documented to increase NADH levels (Reinke *et al.* 1982, Kowalski *et al.* 1992), increased the level of MTT reduction, and by the finding that two substrates of the microsomal cytochrome P450 monooxygenase system (aminopyrine and hexobarbitone), the metabolism of which leads to an increased flux of NADPH (Thurman and Kauffman 1980), also increased the level of MTT metabolism. Furthermore, Huet *et al.* (1992) have used MTT reduction as a measure of NADH-dependent dehydrogenase activity in intact cells.

The endoplasmic reticulum, mitochondria and cytosol were identified as possible sub-cellular sites of NAD(P)H-dependent MTT reduction. However, the lack of effect of rotenone, at a concentration that blocked NAD(P)H-dependent MTT reduction in sub-mitochondrial particles (Table 3), indicates that the bulk of NAD(P)H-dependent MTT reduction in hepatocytes occurs outside the mitochondria. This suggestion is consistent with the effects observed with lactate and ethanol, which generate NADH in the cytosol through lactic dehydrogenase and alcohol dehydrogenase respectively. Other authors, working with different cell types have arrived at similar conclusions. Thus, Loveland *et al.* (1992) demonstrated that respiratory-defective lymphocytes possessed levels of MTT reduction comparable to those measured in normal lymphocytes, whilst Hawtin *et al.* (1995) demonstrated that a variety of

Substrate (Concentration)	MTT reduction (absorbance units/mg tissue/5 min) Homogenate	Microsomes	Cytosol
NADH (800 μM)	0.189±0.001	0.147±0.002	0.018±0.001
NADPH (10 mM)	0.120±0.007	0.038±0.002	0.006±0.001
Succinate (20 mM)	0.014±0.000	ND	ND

Table 1. Substrate-mediated MTT reduction in rat liver homogenate and fractions.

The MTT reduction values were corrected for basal activity (no substrate). The microsomal data were corrected for microsomal recovery as indicated in 'Results'.
Values are mean±SEM of five determinations.
ND = not detected.

Substrate (Concentration)	MTT reduction (absorbance units/mg tissue/5 min) – Rotenone	+ Rotenone
None	0.001±0.000	0.001±0.000
NADH (800 μM)	0.132±0.009	0.030[a]±0.002
NADPH (10 mM)	0.022±0.001	0.011[a]±0.001
Succinate (20 mM)	0.011±0.001	0.011±0.001

Table 2. The influence of rotenone (10 μM) on MTT reduction in sub-mitochondrial particles isolated from rat liver.

Values are mean±SEM of three determinations.
[a] Where indicated, values are significantly different from (–Rotenone) value ($P < 0.001$; unpaired *t*-test).

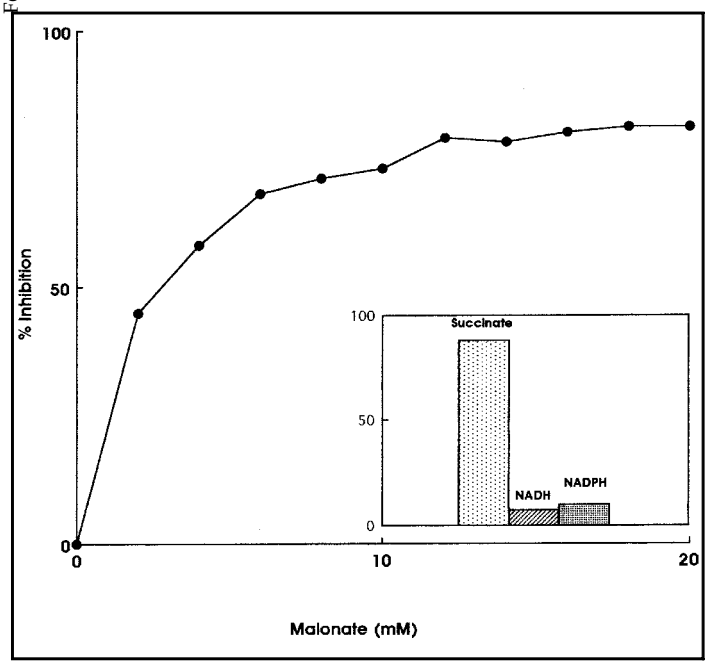

Figure 3. Influence of malonic acid on MTT reduction in rat liver homogenate. Homogenate (80 μl ml^{-1} incubation) was incubated at 37°C with 0–20 mM malonate in the presence of succinate (20 mM) and MTT for 5 min, and the MTT reduction measured. Inset shows the % inhibition of MTT reduction by 10 mM malonate with succinate (20 mM), NADH (800 μM) and NADPH (10 mM) as substrates. Values are mean of five determinations.

Medium	MTT reduction (absorbance/well) –Malonic acid	+ Malonic acid
Hank's/Hepes (no glucose)	0.125±0.005	0.118±0.011
Hank's/Hepes (+ 5.5 mM glucose)	0.126±0.010	0.135±0.016
Williams' E	0.433±0.045	0.492±0.054

Table 3. MTT reduction in rat hepatocytes incubated in different media in the absence and presence of malonic acid (6 mM).

Values are mean±SEM of six dishes.
Hepatocytes were incubated for 25 min in the appropriate medium containing MTT.

Experimental conditions	MTT reduction (absorbance/well)
1. Control	0.137±0.004
+ Rotenone (10 μM)	0.163[a]±0.004
2. Control	0.109±0.006
+ Ethanol (20 mM)	0.285[a]±0.026
+ Lactate (10 mM)	0.277[a]±0.022
3. Control	0.272±0.014
+ Dicoumarol (10 μM)	1.342[a]±0.040
4. Control	0.176±0.009
+ Aminopyrine (100 μM)	0.231±0.004
+ Hexobarbitone (100 μM)	0.257[a]±0.006

Table 4. Effect of various compounds on MTT reduction in rat hepatocytes.

Values are mean±SEM of six dishes.
Hepatocytes were incubated for 1 h in the absence (control) or presence of test agent, with MTT being added for the final 25 min.
[a] Where indicated, values are significantly different from control ($P < 0.01$ or less) by *t*-test or ANOVA/Dunnett as appropriate.

(including rotenone) were without effect on MTT reduction in PC12 cells.

Vistica *et al.* (1991) have reported that MTT reduction in human tumour cell lines varied with the medium used. This was found to be related to the level of glucose in the medium, which influenced the cellular concentrations of NADH and NADPH. This reinforces the view that MTT reduction is principally a measure of NAD(P)H redox status. We also determined that the level of cellular MTT reduction was influenced by the medium used (Table 4), the level obtained in Williams' E medium being approximately 3.5 times that measured in a simple salt solution (Hank's). However, the omission of glucose from the Hank's solution did not modify the level of MTT reduction. It is likely that the difference between tumour cells and hepatocytes in glucose dependence on MTT reduction is a consequence of the differing metabolic substrates available to drive NAD(P)H generation.

The enzymes(s) responsible for the NAD(P)H-dependent MTT reduction in rat hepatocytes remain to be established. Andrews *et al.* (1996) have reported that the NAD(P)H-dependent reduction of a related tetrazolium dye, Alamar Blue™, by rat liver fractions was inhibited by dicoumarol (30

μM), an inhibitor of DT-diaphorase (NAD(P):(quinone acceptor)oxidoreductase), but we were unable to detect any inhibition of MTT reduction by dicoumarol in rat liver fractions, and, paradoxically, produced a marked enhancement of reduction in intact cells. The reasons for this are currently unclear, although it is known that dicoumarol can inhibit a number of enzymes in addition to DT-diaphorase (Ross *et al.* 1993). Hawtin *et al.* (1995) similarly observed a lack of inhibitory effect of dicoumarol on MTT reduction in PC12 cells, although no enhancement was reported.

The plasma membrane has been demonstrated to contain an NADH-dependent transmembrane redox activity (Löw and Crane 1995), and it is possible that this subcellular organelle may be another site of cellular MTT reduction. Consistent with this suggestion was the finding of Hawtin *et al.* (1995) that an inhibitor of this transmembrane redox activity (actinomycin D) inhibited MTT reduction in PC12 cells.

It is well established that tetrazolium dyes related to MTT (nitroblue tetrazolium, iodonitro tetrazolium) can also be reduced on interaction with superoxide anion radical (Thayer 1990), and it has been reported that MTT can also be reduced by superoxide (Burdon *et al.* 1993). It is thus possible that cellular generation of superoxide may be another source of MTT reduction. The stimulatory effect of dicoumarol noted in the hepatocyte studies may be linked to superoxide generation, in that inhibition of DT-diaphorase may allow for an unopposed redox cycling of endogenous quinones with the consequent generation of superoxide. Reactive oxygen species, including superoxide, may also be generated by 'uncoupling' during metabolism of substrates of the microsomal cytochrome P450-dependent monooxygenase system (Bernhardt 1995), and it is possible that the results obtained with aminopyrine and hexobarbitone (Table 4) may also, in part, reflect this.

In conclusion, the results presented in this paper demonstrate that MTT reduction in rat hepatocytes is most likely a reflection of extra-mitochondrial NAD(P)H redox balance, but that the precise subcellular localization(s) of, and the involvement of superoxide in, this activity remain to be established. In a broader context, it also appears that the particular reaction(s) which determine cellular reduction of tetrazolium salts may vary with the cell type and the salt used. Caution should be exercised in ascribing an alteration in the level of cellular MTT reduction to a change in mitochondrial performance in the absence of corroborating evidence.

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