Tetrazolium dyes as tools in cell biology: New insights into their cellular reduction

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Tetrazolium dyes as tools in cell biology: New insights into their cellular reduction

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Abstract. Tetrazolium salts have become some of the most widely used tools in cell biology for measuring the metabolic activity of cells ranging from mammalian to microbial origin. With mammalian cells, fractionation studies indicate that the reduced pyridine nucleotide cofactor, NADH, is responsible for most MTT reduction and this is supported by studies with whole cells. MTT reduction is associated not only with mitochondria, but also with the cytoplasm and with non-mitochondrial membranes including the endosome/lysosome compartment and the plasma membrane. The net positive charge on tetrazolium salts like MTT and NBT appears to be the predominant factor involved in their cellular uptake via the plasma membrane potential. However, second generation tetrazolium dyes that form water-soluble formazans and require an intermediate electron acceptor for reduction (XTT, WST-1 and to some extent, MTS), are characterised by a net negative charge and are therefore largely cell-impermeable. Considerable evidence indicates that their reduction occurs at the cell surface, or at the level of the plasma membrane via trans-plasma membrane electron transport. The implications of these new findings are discussed in terms of the use of tetrazolium dyes as indicators of cell metabolism and their applications in cell biology.

Keywords: tetrazolium salts, NBT, MTT, XTT, MTS, WST-1, cell reduction, NADH, plasma membrane electron transport, superoxide, mitochondria, microorganism.

Abbreviations

MTT	2-(4,5-dimethyl-2-thiazolyl)-3,5-diphenyl-2H-tetrazolium bromide
XTT	sodium 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)-carbonyl]-2H-tetrazolium inner salt
MTS	5-[3-(carboxymethoxy)phenyl]-3-(4,5-dimethyl-2-thiazolyl)-2-(4-sulfo-phenyl)-2H-tetrazolium inner salt;
WST-1	sodium 5-(2,4-disulfophenyl)-2-(4-iodophenyl)-3-(4-nitrophenyl)-2H-tetrazolium inner salt
INT	2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium chloride
TTC	2,3,5-triphenyl-2H-tetrazolium chloride
IEA	intermediate electron acceptor
PMS	5-methyl-phenazinium methyl sulfate
mPMS	1-methoxy-5-methyl-phenazinium methyl sulfate
TTFA	thenoyltrifluoroacetone
SOD	superoxide dismutase

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Introduction

The reduction of tetrazolium salts from colourless or weakly coloured, aqueous solutions to brightly coloured derivatives known as formazans, has been the basis of their use as vital dyes in redox histochemistry and in biochemical applications for more than half a century [1–4]. Whereas most histological applications have involved ditetrazolium salts such as nitroblue tetrazolium (NBT) that form insoluble formazans, most cell-based applications have favoured monotetrazolium salts, the most widely used being MTT. Because MTT also forms an insoluble formazan it has usually been applied in endpoint assays. Other monotetrazolium salts such as XTT, MTS and more recently WST-1, are used in conjunction with intermediate electron acceptors (IEAs) that facilitate dye reduction. They form soluble formazans and consequently can be used in real time assays. The vast majority of cellular applications of tetrazolium dyes involve microplate assays that measure cell proliferation where it is assumed that dye reduction will be proportional to the number of viable cells in exponential growth phase. Although this is usually a good approximation for defined growth conditions with a particular cell type averaged across the cell cycle, problems often arise when growth conditions are non-ideal or when growth-modifying agents are used. In these situations, dye reduction will be dependent not only on cell type and number, but also on the site of action of the compound, the tetrazolium salt used and its subcellular site of reduction. A critical review of the use of tetrazolium assays to measure cell growth and function, a decade ago now [5], summarised thinking at that time about the mechanisms of bioreduction and discussed limitations surrounding the use of these microculture assays. It was suggested that these assays measure the integrated pyridine nucleotide redox status of cells.

This review will focus primarily on new knowledge about cellular reduction of the most commonly used monotetrazolium salts with particular emphasis on understanding their site of reduction and applications in cell biology. Although it is generally assumed that tetrazolium salt reduction is intracellular and related to energy metabolism, most reduction appears to be non-mitochondrial, and several tetrazolium salts are now known to be reduced extracellularly by electron transport across the plasma membrane. These unexpected findings prompt a re-evaluation of the way we consider and use tetrazolium dyes in cell-based applications.

Tetrazolium salts

The unique chemical and biological properties of tetrazolium salts that have led to their widespread application in histochemistry, cell biology, biochemistry and biotechnology depend on the positively charged quaternary tetrazole ring core containing four nitrogen atoms. This central structure is surrounded by three aromatic groups that usually involve phenyl moieties (Fig. 1). Following mild

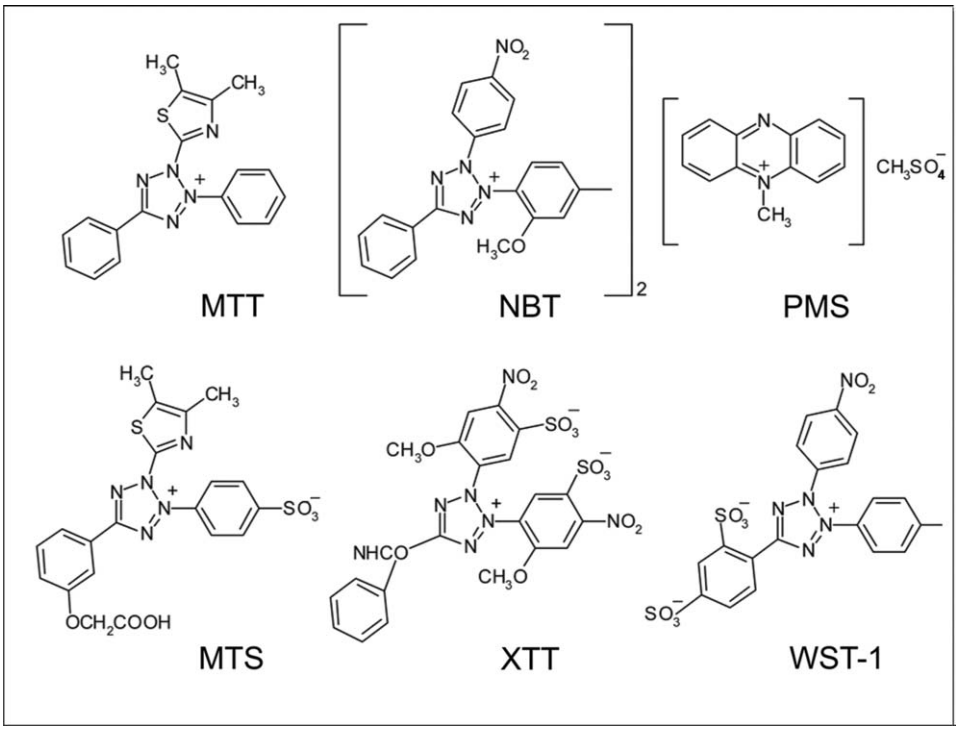

Fig. 1. Chemical structures of selected tetrazolium salts and of the intermediate electron acceptor, phenazine methosulfate (PMS).

reduction, tetrazolium structures transform from colourless or weakly coloured salts into brightly coloured formazan products by disruption of the tetrazole ring. The prototype compound, triphenyl tetrazolium chloride (TTC), first synthesized more that a century ago [6,7], has been modified in many ways over the years by adding nitro, iodo and methoxy groups to the phenyl rings. In the case of MTT, one of the phenyl groups is replaced with an alternative thiazolyl ring structure while CTC has a nitrile group replacing the phenyl ring in position 5 of the tetrazole core. These modifications have resulted in compounds with a range of different properties that have been applied both qualitatively and quantitatively in an impressive variety of biological measuring systems. Ditetrazolium salts such as neotetrazolium (NT), NBT and tetranitroblue tetrazolium (TNBT) are used widely in histological applications. Their success in these areas has been largely associated with strong binding of their formazans to tissue proteins, thus minimising diffusion artefacts [4]. The monotetrazolium salts, iodonitrotetrazolium (INT) and MTT, an iodinated dimethylthiazolyl derivative, were found to be less useful in histochemistry. This has been attributed to their greater lipophilicity and to their ability to form large needle-like crystals that with MTT, were dispersed throughout the tissue [8]. Nevertheless,

these monotetazolium salts have been used effectively to explore mitochondrial electron transport where tissue localisation was less important.

Cellular reduction of tetrazolium salts

The use of tetrazolium salts in cell biology initially favoured compounds that were both water-soluble and lipophilic, but in retrospect it is likely that the net positive charge on these molecules was the primary factor responsible for their successful application in cell biology. This net positive charge would have facilitated cellular uptake via the plasma membrane potential.

Tetrazolium dyes that form insoluble formazans

Although the ditetrazolium salt, NBT, is most commonly associated with histological applications, it is also widely used in cellular applications, particularly those involving superoxide production [9–11]. Attempts to develop microplate assays using NBT [12,13] were hampered by insolubility of the formazan product. This led to the use of monotetrazolium salts that more readily enter cells, are reduced by NAD(P)H-dependent oxidoreductases and dehydrogenases of metabolically active cells, and produce formazans that can be more efficiently solubilised. Thus, in 1983 Mosmann [14] developed a colorimetric MTT microplate assay for measuring cell proliferation and cytotoxicity, and this simple assay, and modifications of it, are now used extensively in cell biology laboratories around the world. In addition to MTT, other monotetrazolium dyes that form insoluble formazans, including INT and CTC, have been used as vital dyes and as indicators of cellular redox activity [15–17] (see later discussion).

Tetrazolium dyes that form water-soluble formazans

The need to solubilize MTT formazan crystals prior to spectrophotometric analysis in a microplate reader, and the inherent endpoint nature of the assay limited some applications. This led to the development of tetrazolium analogues with their phenyl moieties decorated with negatively charged sulfonate groups, *e.g.*, XTT, a negatively charged inner salt [18,19] and MTS, a weakly acidic inner salt closely related to MTT [20,21] (see Fig. 1 for chemical structures). These modifications resulted in the production of soluble formazans that equilibrated in the culture medium without the need for solubilization procedures. In general, such modifications were associated with the need to employ an IEA to facilitate cellular dye reduction. However, the increased negative charge on these molecules would also have reduced their ability to move across cell membranes [5]. This raises the possibility that cellular reduction mediated by IEAs may be extracellular and involve trans-plasma membrane electron transport, a universal redox regulatory system linking intracellular metabolism with extracellular

electron acceptors. The plasma membrane redox system involves a number of different electron transport pathways that reduce cell-impermeable indicator dyes such as ferricyanide, ferricytochrome *c*, dichloroindophenol and certain tetrazolium salts [22,23].

More recently, a new generation of Water Soluble Tetrazolium salts has been developed of which WST-1 is the prototype [24,25]. WST-1, a negatively charged disulfonated inner salt containing an iodine residue, is more stable in the presence of mPMS, its obligatory IEA, than XTT and MTS. This led to WST-1 being marketed as a convenient single reagent Cell Proliferation kit containing mPMS. Unexpectedly, WST-1 was shown to be reduced extracellularly to its soluble formazan, by electron transport across the plasma membrane of dividing cells [26–28], contradicting the earlier assumption that it was reduced by succinate dehydrogenase in the mitochondria of metabolically active cells. In addition, by dismantling the kit, we were able to show that WST-1 could be used in the absence of an IEA to measure superoxide production by professional phagocytic cells [29], an application made possible by its extracellular site of reduction. Several other tetrazolium salts have also been developed in the WST series, perhaps the most useful being WST-8 [30] which appears to have very similar cellular reduction properties to WST-1 and is being marketed independently as Cell Counting Kit-8 (CCK-8) containing mPMS.

The role of membrane potential in cellular reduction of tetrazolium salts

With MTT and most tetrazolium salts used in histological applications, the positive charge on the tetrazole ring would act to facilitate transfer across the plasma membrane of viable cells via the membrane potential (ψ_{PM} −30 to −60 mV, negative inside), and if not reduced in the cytoplasm, across the mitochondrial inner membrane (ψ_M −150 to −170 mV, negative inside) (see Fig. 2). In this context, MTT has recently been used in conjunction with rhodamine B to measure mitochondrial membrane potential [31]. In this assay, the MTT-formazan that is generated in mitochondria acts as a fluorescence quencher for rhodamine that distributes across membranes of viable cells according to membrane potential. By way of analogy, triphenylphosphonium salts, with a positively charged quarternary phosphorous atom, are both soluble and rapidly concentrated across the plasma membrane of viable cells by 5–10-fold, and across the mitochondrial membrane by a further 20–50-fold [32].

The positive charge on the tetrazole core of MTS is counterbalanced by a negatively charged sulfonate group on one phenyl ring, generating an ‘‘inner salt’’. When considered together with a weakly acidic carboxymethoxy group on a second phenyl ring, MTS would not be expected to readily enter viable cells via the membrane potential. However, its lipophilic properties may counter the weak negative charge resulting in a limited ability to cross the plasma membrane.

XTT and WST-1 both contain two sulfonate groups giving them a net negative charge that would exclude them from cells. The reduction rates of MTT, MTS,

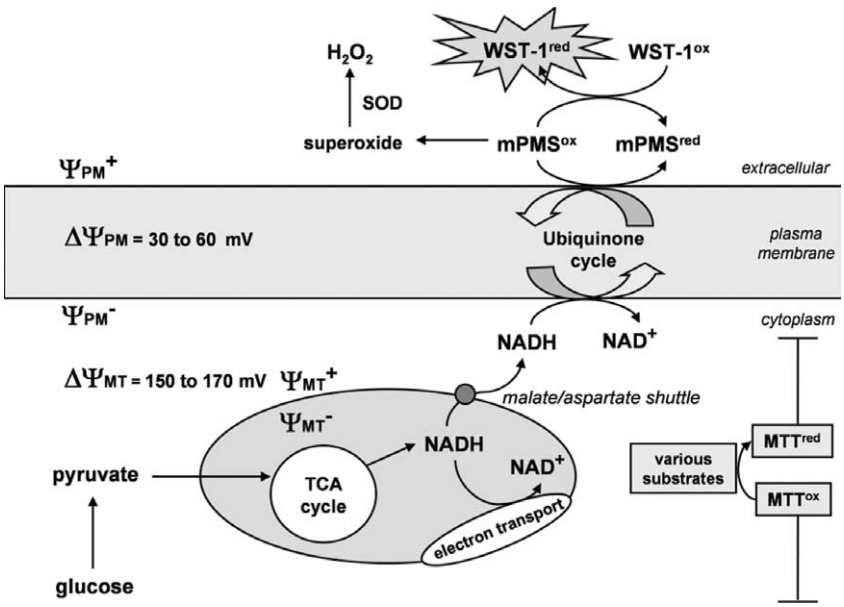

Fig. 2. Schematic representation of the proposed mechanisms of cellular reduction of MTT and WST-1. Whereas MTT is reduced by a variety of intracellular reductants, most notably NADH, WST-1 is reduced by trans-plasma membrane electron transport via the electron carrier, 1-methoxyPMS, in which case the cellular reductant is NADH derived mainly from the mitochondrial TCA cycle. The plasma membrane potential, which is proposed to be the major cellular determinant of tetrazolium dye uptake is also depicted.

XTT and WST-1 by viable cells in the absence of mPMS (see Fig. 3), are entirely consistent with the net charge on these molecules and the plasma membrane potential being the principal factors that determine their reduction rates, *i.e.*, MTT > MTS > XTT = WST-1.

Properties of tetrazolium salt reduction by viable cells

Key properties of tetrazolium dye reduction in cell-based assays are summarised graphically in Fig. 3 where MTT, XTT, MTS and WST-1 reduction by Jurkat cells are compared with cytochrome *c* reduction. In the absence of mPMS, cellular reduction of WST-1, XTT and cytochrome *c* are minimal. In contrast, MTS gave a weak signal while MTT was more strongly reduced. SOD did not affect MTS or MTT reduction in the absence of mPMS. These results are consistent with WST-1 and XTT being unable to enter cells due to their net negative charge, with MTT and to a much lesser extent, MTS being reduced intracellularly. When mPMS (20 μM) was added, both WST-1 and cytochrome *c* reduction were facilitated and this reduction was 90% SOD-sensitive indicating an extracellular mechanism involving extracellular superoxide. The quantitative

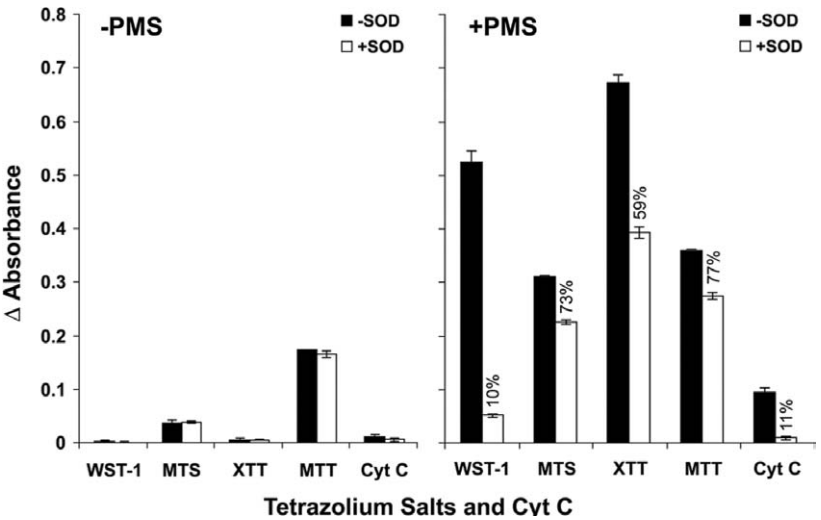

Fig. 3. Comparison of cellular tetrazolium dye reduction in the presence and absence of mPMS and SOD. Human T-lymphoblastic Jurkat cells (2–3 × 10^4 per microplate well) were incubated for 1 h with WST-1 (400 μg/ml), MTS (313 μg/ml), XTT (313 μg/ml), MTT (500 μg/ml) or ferricytochrome *c* (80 μM) in the presence and absence of mPMS (20 μM) and SOD (20 μg/ml). Absorbance was measured in a microplate reader at 450 nm for WST-1, MTS and XTT, 570nm for MTT and 550 nm for cytochrome *c*. SOD inhibition is presented as % control. Results are presented as the mean of duplicate determinations ± standard error.

difference between WST-1 and cytochrome *c* reduction can be accounted for in part by differences in their molar extinction coefficients (WST-1, 37 × $10^3 M^{-1}cm^{-1}$ at 438 nm; cytochrome *c*, 21.1 × $10^3 M^{-1}cm^{-1}$ at 550 nm). The high background absorbance with cytochrome c also makes it less useful in these assays. In contrast, MTS and to a lesser extent, MTT reduction was enhanced by mPMS but only about 25% of reduction was sensitive to SOD. Interestingly, XTT reduction was strongly promoted by mPMS and this reduction was 40% inhibited by SOD.

Taken together with molecular charge considerations, these results show that WST-1 is reduced extracellularly, most likely by electron transport across the plasma membrane from intracellular NADH to WST-1 via mPMS. Involvement of extracellular superoxide indicates one electron transfer to mPMS to generate a radical, with some transfer of electrons to oxygen to form superoxide which would be efficiently removed by SOD ($K_{cat} = 1.6 \times 10^9$ $M^{-1}s^{-1}$) [33]. mPMS radicals which we have shown to accumulate in cell culture supernatants over 30 min (Davies M and Berridge MV, unpublished results) would then be responsible for reducing WST-1 via a radical intermediate. Direct involvement of superoxide in WST-1/mPMS reduction is inconsistent with oxygen inhibition, and a 3–5-fold increase in WST-1 reduction under anoxic conditions [34]. These results suggest indirect involvement of superoxide in WST-1/mPMS reduction with oxygen and mPMS competing for reducing electrons from the

plasma membrane electron transport system, or alternatively oxygen and WST-1 competing for reducing electrons from mPMS radicals. A similar indirect involvement of superoxide in cytochrome *c* and INT reduction has been noted previously [35,36].

Cellular uptake of MTT via the plasma membrane potential and subsequent reduction by intracellular NAD(P)H-oxidoreductases readily explains the MTT results. Contrary to this view, Liu *et al.* [37] have argued that MTT is membrane-impermeable when incorporated into large unilamellar liposomes, and that MTT is therefore taken up by cells via endocytosis. However, synthetic liposomes would not exhibit a membrane potential and therefore are not analogous to the plasma membrane of living cells. The view that MTT readily enters viable cells via the plasma membrane potential and is reduced intracellularly is supported by imaging studies with HepG2 cells [38,39]. Furthermore, as previously discussed, MTT has been used in conjunction with rhodamine B to measure mitochondrial membrane potential [31], an application that is explained by fluorescence quenching by MTT-formazan generated in mitochondria.

Mediators of tetrazolium dye reduction (intermediate electron acceptors)

In early histochemical applications, the intermediate electron carrier, PMS, was used in conjunction with tetrazolium salts to localise sites of NAD(P)H production [40]. 1-methoxyPMS (mPMS) was later introduced by Hisada and Yagi [41] as a photochemically stable electron mediator with greater efficiency and lower background in some applications. It is worth noting that mPMS was also favoured for extra-mitochondrial assays because it failed to penetrate the mitochondrial membrane [42]. With viable cells, the use of mPMS (20 μM optimum concentration) has been associated with the development of second generation tetrazolium salts like XTT, MTS and WST-1 that produce soluble formazans [18–21,24]. The ability of mPMS to facilitate tetrazolium dye reduction is associated most strongly with those dyes that are excluded from the cell (XTT, WST-1 and to some extent MTS) and also with the reduction of cytochrome *c* which is also cell-impermeable (see Fig. 3). In contrast, cellular reduction of MTT, which readily enters the cell, is much less affected by mPMS [27]. Taken together, these results suggest that mPMS mediates tetrazolium salt reduction by picking up electrons at the cell surface, or at a site in the plasma membrane that is readily accessible, to form a radical intermediate that then reduces the dye by two single electron reduction events. The fact that a small percentage of cellular MTT reduction is extracellular [43] and SOD-sensitive [26,27] and that SOD inhibition increases to about 25% in the presence of mPMS argues that a small amount of MTT is reduced at the cell surface by electron transport across the plasma membrane, and that mPMS can increase the efficiency of MTT reduction by this route. We have observed that mPMS results in a rapid 5–6-fold increase in oxygen consumption by HL60ρ^o and HeLaS3ρ^o cells that are devoid of mitochondrial DNA and therefore incapable of

mitochondrial respiration. A similar effect was seen in wild type cells in the absence and presence of inhibitors of mitochondrial respiration and these effects were completely abrogated by 2mM WST-1. These results are consistent with oxygen and WST-1 competing for electrons from mPMS radicals (Herst PM and Berridge MV, unpublished results). Interestingly, the soluble ubiquinone analogue, Q1, was also found to mediate WST-1 reduction with low efficiency, and this reduction was SOD-sensitive [44].

Although this discussion has focused primarily on those electron carriers that have been most widely used to facilitate tetrazolium due reduction by cells, a number of other mediators of dye reduction have been used including Medola's Blue, Methylene Blue and menadione. In general their use has been limited in cell studies (for detailed discussion of the use of exogenous IEAs, see Stoward [4]). Nevertheless, Medola's Blue has been applied as the most efficient IEA in facilitating CTC reduction [17,45], and Goodwin *et al.* [46] used menadione as an IEA to support MTS reduction, in which case MTS-formazan production was exclusively mediated by DT-diaphorase.

Cofactor requirement for tetrazolium dye reduction

In the 1960s and 70s, tetrazolium salts were widely used to study the mitochondrial respiratory chain and, based on inhibitor studies, the main sites of NBT and MTT reduction were shown to be Complex I and Complex II respectively [4,47]. It is not surprising therefore that cellular reduction of MTT came to be associated with the flavin-containing enzyme, succinate dehydrogenase (SDH), and that mitochondria became established as the main cellular sites of tetrazolium salt reduction. Little attention was paid to other potential non-mitochondrial sites of cellular MTT reduction such as NAD(P)H-dependent oxidoreductases like NQO1 and cytochrome P450. Nevertheless, non-mitochondrial pyridine nucleotide-dependent enzymes, some requiring an intermediate electron acceptor, were known to be involved in the reduction of tetrazolium dyes as well as other terminal electron acceptors [4]. Studies by Vistica *et al.* [48] indicated that cellular reduction of MTT was related to intracellular NAD(P)H concentration. Later subcellular fractionation studies showed that most cellular MTT reduction could be accounted for by non-mitochondrial reduction via reduced pyridine nucleotides, and that succinate accounted for less than 10% of the dye-reducing potential of the cell [49]. Involvement of NAD(P)H as the major electron donor in MTT reduction is supported by inhibitor studies which showed that the succinate dehydrogenase inhibitor, TTFA, has little effect on cellular MTT reduction and that in the short term, MTT reduction was resistant to and in some cases stimulated by inhibitors of mitochondrial electron transport including cyanide, azide and rotenone [27,49,50]. These results are consistent with an NADH sparing effect in the absence of active mitochondrial electron transport. In contrast, MTT reduction was acutely sensitive to cytochalasin B [50] and 2-deoxyglucose which inhibit

glucose uptake through plasma membrane glucose transporters, and to inhibitors of glycolysis such as iodoacetamide (Tan and Berridge, unpublished results).

Cellular sites of tetrazolium dye reduction

Many oxidoreductase enzymes are capable of catalysing electron transfer from an electron donor to an acceptor tetrazolium salt. In many cases, particularly those that do not involve superoxide, an IEA such as PMS may be required to facilitate dye reduction or to enhance the rate of reduction. Although many cofactors and metabolites are potential donors of reducing electrons, NADH, NADPH, succinate and pyruvate have been the main focus of attention. The most commonly studied systems are the oxidoreductases of the mitochondrial electron transport chain, but numerous other cellular dehydrogenases, oxidases and peroxidases have been shown to reduce tetrazolium dyes biochemically.

Non-enzymatic and enzymatic reduction of tetrazolium salts

With several tetrazolium salts including INT, MTS, XTT and WST-1, electron transfer reactions can occur in the absence of enzymes, providing a suitable reductant and an IEA is present [26,51–53]. With MTS, little non-enzymatic reduction was observed in the absence of PMS [53] and we have observed a similar dependence on mPMS with WST-1 (Tan AS and Berridge MV, unpublished results). The ability of MTS and WST-1 to be rapidly reduced by NAD(P)H in the presence of an IEA suggests that these tetrazolium dyes can be applied in simple microplate assays for NADH and NADPH measurement. We have established that WST-1/mPMS provides an accurate and sensitive microplate determination of NADH and NADPH and validated the results against literature values for pyridine nucleotides from rat liver. In general, NADH and NADPH are more efficient electron donors than succinate or glutathione [26], or the chemical reducing agents, dithiothreitol or mercaptoethanol [26,53]. In the absence of PMS, addition of crude cell fractions greatly enhanced the reduction of MTT when NADH, NADPH or succinate were used as electron donors. With XTT and WST-1 ''reagents'' that contain mPMS, complete reduction occurred with NADH and NADPH alone, and addition of cell fractions did not further enhance the signal. Surprisingly however, adding mitochondrial fractions inhibited NAD(P)H-dependent reduction of WST-1 and XTT which is consistent with efficient NAD(P)H utilisation by mitochondrial enzymes [26]. As mentioned above, succinate was an effective substrate for MTT reduction, particularly in the presence of mitochondrial fractions, consistent with a role for succinate dehydrogenase in MTT reduction. In contrast, XTT and WST-1 reagents gave weak signals with succinate when mitochondrial fractions were present indicating that mPMS may pick up electrons downstream of Complex II as suggested previously [4,54].

Subcellular localisation of tetrazolium dye reduction: Cell fractionation studies

Cell fractionation studies with bone marrow-derived murine 32D cells [49] and rat liver [26] have provided information on potential sites of MTT reduction. These studies also indicate that if WST-1 and XTT and their IEAs were to gain entry into the cell, they would be rapidly and non-specifically reduced by NADH which is present in most proliferating cells at millimolar concentrations. Using *in vitro* assays and optimum substrate concentrations, we have shown that NADH is the most favoured substrate for MTT reduction while succinate is least favoured accounting for less that 10% of the combined MTT-reducing potential in cell homogenates. These results, and others involving viable cells, are in direct conflict with the view still perpetuated in the literature today, that succinate dehydrogenase is responsible for most cellular MTT reduction, a view that led several groups to refer to the MTT assay as the succinate dehydrogenase inhibition (SDI) assay [55]. Nevertheless, succinate dehydrogenase is able to reduce MTT, and most succinate-reducing activity (77%) was found in the mitochondrial fractions [49]. The site of mitochondrial MTT reduction was between the amytal and azide-inhibitory sites and sensitivity of succinate-dependent MTT reduction to TTFA established mitochondrial Complex II as the site of reduction. The mitochondrial sites of reduction of several other tetrazolium salts have been discussed previously [4,56]. More recently, Rich *et al.* have shown that TTC is primarily reduced by Complex I in mitochondria, and that complete reduction to TTC-formazan only occurs under anaerobic conditions as the initial reduction product, presumably a TTC radical intermediate, is rapidly reoxidised by molecular oxygen [57].

Subcellular sites of tetrazolium dye reduction: Viable cell studies

Tetrazolium salts that form insoluble formazans

Subcellular fractionation studies indicate the potential of particular fractions to reduce tetrazolium dyes but do not show what actually happens in viable cells. An indication of the cellular site of reduction of various tetrazolium salts has been presented in Figs. 2 and 3 which show that MTT is primarily reduced intracellularly, while XTT and WST-1, and to some extent, MTS, are reduced at the cell surface. This is probably a result of their poor capacity to penetrate cells, and the ability of mPMS to pick up low potential electrons from cell surface oxidases that are coupled to intracellular NADH production by trans-plasma membrane electron transport [23,27,28,58]. The subcellular site of MTT reduction has been investigated in proliferating cells using a variety of metabolic inhibitors. An early indication that MTT reduction could be dissociated from DNA synthesis came from experiments with 32D cells where dibutyryl cyclic AMP stimulated MTT responses over 2.5 h while inhibiting ^{3}H-thymidine incorporation [50,59]. In other experiments, pretreating cells for 30 min with sodium azide or rotenone prior to adding MTT for 2 h stimulated or had little

effect on MTT reduction while severely compromising DNA synthesis. These results suggest a possible sparing effect of azide and rotenone on NADH utilisation by the mitochondrial electron transport chain and also that intracellular NADH production might be linked to MTT reduction [49,50]. This was further investigated with Jurkat cells where it was shown that inhibitors of glucose transport and glycolysis such as 2-deoxyglucose and iodoacetamide strongly inhibited MTT reduction [27].

In contrast, the succinate dehydrogenase inhibitor, TTFA, had no effect on MTT reduction, excluding succinate dehydrogenase as the primary site of MTT reduction in viable cells.

Using SOD and low molecular weight SOD mimetics and inhibitors, Burdon *et al*. [43] demonstrated that 20–30% of MTT reduction that occurred inside HeLa cells could be attributed to superoxide. In contrast, 80% of the MTT reduction that occurred extracellularly was SOD-sensitive.

Others have investigated the cellular site of MTT reduction using confocal imaging and concluded that most MTT-formazan deposits are not coincident with mitochondria but occur in the cytoplasm and in proximity to the plasma membrane under conditions where the plasma membrane remained intact as determined by the absence of nuclear propidium iodide staining [38,39]. The same group also investigated subcellular reduction of CTC, a fluorescent cyanotetrazolium salt with a similar net positive charge to MTT. In the absence of an electron carrier, CTC reduction by HepG2 cells occurred slowly and was associated with the plasma membrane. When Medola's Blue was used as an electron carrier, rapid CTC-formazan production was observed in plasma membrane regions but plasma membrane damage occurred and intracellular formazan deposition correlated with nuclear propidium iodide staining. Earlier studies using Ehrlich ascites tumour cells [16,17], had also flagged the plasma membrane as the site of CTC reduction and indicated a free radical mechanism of dye reduction. Although these results appear to be contrary to the general principle developed in this review, that positively charged tetrazolium salts accumulate inside cells via the plasma membrane potential, increased positive charge on the cyanotetrazole ring and consequent changes in reduction potential resulting from the electron-withdrawing cyanide group may have enhanced the ability of CTC to be reduced at the plasma membrane, particularly in the presence of Medola's Blue. In addition, altered charge distribution and reduced lipophilicity resulting from the loss of a phenyl group may have lowered the ability of the molecule to traverse the plasma membrane.

Investigation of the mechanism of MTT reduction by rat neuronal B12 cells [37] indicated that MTT reduction was associated with intracellular perinuclear vesicles including endosomes and lysosomes and that MTT-formazan crystals were transported to the cell surface by exocytosis. Although B12 cells and rat brain mitochondria could reduce MTT, reduction by B12 cells was resistant to mitochondrial inhibitors and stimulated by the uncoupler FCCP, results which are inconsistent with a predominantly mitochondrial mechanism of reduction.

Cell fractionation studies indicated similar specific activity (A_{570} [mg protein]$^{-1}$ h^{-1}) of MTT reduction by nuclear, mitochondrial, microsomal and cytosolic fractions when NADH was used as substrate and that with NADPH, cytosol had the greatest and mitochondria the least MTT reducing ability. Although these results differ quantitatively from those reported for 32D cells [49], they support the general view that the capacity for cellular MTT reduction is widely distributed throughout the cell, and is greater with NADH than with NADPH. The ability of cells to exocytose MTT formazan crystals occurred with all cells investigated including B12 and PC12 cells, primary cultures of rat cortical neurons, MDCK epithelial cells and L929 cells. In addition, we have confirmed that 32D cells exocytose MTT-formazan crystals over a 24 h period and that cell death results, probably as a result of the large formazan crystals perforating plasma membranes. Scanning laser confocal microscopy of B12 cells double-stained with MTT and subcellular organelle-specific dyes indicated that intracellular MTT-formazan did not colocalise with mitochondria, endoplasmic reticulum or Golgi apparatus, but partially colocalised with endosomes and lysosomes [37]. MTT reduction was inhibited by the flavin centre inhibitor, diphenyleneiodonium and the sulfhydryl inhibitors N-ethylmaleamide and iodoacetate that affect glycolysis. Surprisingly, the cell-impermeable sulfhydryl blocker, *p*-hydroxymercuribenzoate sulfonate, extensively inhibited MTT reduction by B12 cells, although the relatively high concentration used (50 μM) raises questions about whether these effects may be indirect as inhibition was not observed at 25 μM pCMBS with Jurkat cells [27]. Liu *et al*. also found that MTT was reduced by 143B ρ° cells that are deficient in mitochondrial respiration, although the rate was 40% that of wild type cells. Similar studies in our laboratory have shown that ρ° cells (143B, HL60, HeLa and P815) reduce MTT at rates comparable with wild type cells with ratios varying between 0.85 and 1.05, results that differ somewhat from those of Liu *et al*. for 143Bρ° cells, but support the view that non-mitochondrial MTT reduction makes a significant contribution to overall cellular reduction.

Tetrazolium salts that form water-soluble formazans

The earliest indication that second generation tetrazolium salts that form water-soluble formazans are reduced at the cell surface came from the unexpected discovery that reduction of WST-1 reagent, which contains mPMS, was extensively inhibited by low concentrations of SOD [26]. The observation that WST-1 was not reduced in the absence of mPMS indicates that the exponentially growing cells used in these experiments do not produce detectable amounts of superoxide. These results, when considered together with the fact that superoxide does not readily cross cell membranes [33], led to a model involving extracellular superoxide generation from mPMS radicals but little direct involvement in the pathway leading to WST-1 reduction (see Fig. 2). Although it is possible that mPMS could shuttle electrons across the plasma membrane to generate extracellular superoxide, numerous studies in our laboratory have excluded this

mechanism and indicated that mPMS, and consequently WST-1, are reduced by trans-plasma membrane electron transport [27–29,34,58].

In the presence of mPMS, XTT reduction was inhibited by 40–50% in the presence of SOD, and MTS by 7–45% depending on the cell type [27] (Fig. 3), indicating that various levels of extracellular superoxide are generated in these systems.

Sensitivity of WST-1/mPMS reduction to the vanilloid/ubiquinone redox inhibitors, capsaicin, resiniferatoxin and dihydrocapsaicin suggests that membrane ubiquinone redox cycling is involved in the generation of reducing electrons across the plasma membrane. The fact that inhibition of WST-1/mPMS reduction was similar in ρ^o cells that exhibit 2–3-fold greater dye reduction [28,34] argues against these effects being related to mitochondrial ubiquinone redox cycling. Furthermore, similar inhibition was not seen with ferricyanide reduction [60], which involves an alternative plasma membrane electron transport pathway [61]. As with MTT reduction, WST-1/mPMS reduction was sensitive to inhibitors of glucose uptake and glycolysis, the uncoupler and NQO1 inhibitor, dicoumarol, and stimulated by rotenone, cyanide and by the Complex II inhibitor, TTFA [27,58]. Stimulation of WST-1/mPMS reduction by inhibitors of the mitochondrial electron transport in wild type but not ρ^o cells indicates a sparing effect of these inhibitors on intracellular NADH levels. These results are in agreement with a 2–3-fold elevation of WST-1/mPMS reduction by ρ^o cells, and with a major role for NADH, produced by the mitochondrial TCA cycle in WST-1/mPMS reduction. Other studies have shown that mitochondrial NADH is linked to plasma membrane electron transport and WST-1/mPMS reduction via the malate/aspartate shuttle [44].

Recently, we have shown that WST-1/mPMS reduction by wild type or HL60ρ^o cells shows similar inhibitor characteristics to non-mitochondrial oxygen consumption at the cell surface suggesting that oxygen is a physiological electron acceptor for trans-plasma membrane electron transport [34]. Furthermore, mPMS and oxygen were shown to compete for reducing electrons from the plasma membrane electron transport system.

In summary, these results show that NADH produced in the mitochondrial TCA cycle is the primary reductant for extracellular WST-1 reduction via trans-plasma membrane electron transport in the presence of mPMS (see Fig. 2).

Cell proliferation and drug screening assays

The use of microplate tetrazolium assays to measure cell proliferation has increased exponentially since their introduction by Mosmann in 1983 [14]. Nevertheless, these assays do not actually measure the number of viable cells in a culture or their growth but rather, an integrated set of enzyme activities that are related in various ways to cell metabolism. They utilise the cofactor, NADH, and with MTT, other substrates like succinate and pyruvate may also contribute to their reduction. Depending on the particular dye chosen, reduction will be linked

in various ways to cofactor/substrate production, utilisation and compartmentalisation, and can be associated with the plasma membrane, intracellular membranes, organelles and the cytosol. Reduction can vary widely within and between cell populations depending on the cell growth conditions, whether the cells are in exponential growth phase and with the stage of the cell cycle. Many of these issues have been reviewed previously [5].

Given the complexities and uncertainties that surround cellular reduction of tetrazolium salts, the question could be asked as to why they have become so widely used in measuring cell proliferation and inhibition of cell proliferation. Apart from the more obvious attributes of the intense colouration of the formazans, the ease of use and ready application to relatively high throughput microplate-based assays, a major factor is that the integrated metabolic signal read by tetrazolium dyes with a particular cell type under defined growth conditions is a moderately robust measure of viable cells. This has been demonstrated on many occasions by the close correlation between viable cell numbers and the tetrazolium–formazan signal generated. In general, changes in growth conditions including growth factor, hormone and serum supplementation, and addition of cytotoxic and cytostatic drugs will alter the metabolic signal in a way that gives useful information about the effect of the particular compound or extract. Both acute effects (hours), and longer term effects (days) can be measured and these can differ considerably depending on the nature of the challenge. This has been most graphically demonstrated with the IL-3-dependent cell line, 32D, where the effects of various cytotoxic drugs and metabolic inhibitors on MTT reduction and ^{3}H-thymidine incorporation were determined in the presence and absence of IL-3 at 0.5 h, 4 h and 24 h [50]. In most situations, effects on MTT reduction and ^{3}H-thymidine incorporation diverged at early times but were similar at 24 h, cautioning that timing is a critical factor in interpreting the results of both commonly used readouts of cell proliferation.

Difficulties often arise with the need to compare the effects of drugs on different cell types such as the large-scale *in vitro* cancer drug-screening programme instituted by the National Cancer Institute in the early 1990s that now involves a panel of more than 60 tumour cell lines. In 1990, Rubinstein *et al.* [62] compared 197 compounds on 38 tumour lines representing seven tumour types using microplate assays based on MTT and the protein binding dye, sulforhodamine B (SRB). They concluded that although the assays performed similarly, the SRB assay had practical advantages for large-scale screening and this led to its subsequent adoption for routine *in vitro* antitumour drug screening. Previous investigations with XTT had indicated similar pitfalls to MTT [19] and the parameters affecting formazan production were outlined [48]. They showed that the kinetics of MTT–formazan production varied significantly among different cell lines as did the degree of saturability of the assay and the IC_{50} values obtained with adriamycin.

MTT-based assays have also been applied to predict cancer drug chemosensitivity and resistance [63–65]. The assays are highly predictive of drug

resistance, but chemosensitivity was dependent on the leukaemic cell type and the drug combination used. Hayon *et al*. [64] concluded that pre-treatment chemosensitivity assays on leukaemic cells from individual patients could be helpful in selecting the most effective drug treatment options.

Despite their limitations, tetrazolium dyes are widely used in anticancer drug research to investigate cytotoxic and cytostatic effects on cancer cell lines and tumour cells that are frequently associated with apoptosis. This large literature is outside the scope of this review.

Cell viability testing

The use of cell-permeable tetrazolium salts as vital dyes in seed testing was one of their earliest technological applications [1,66]. In this assay the ability of imbibed seeds to take up and reduce tetrazolium dyes like TTC and NBT is measured and these methodologies are still in use in some laboratories today [67]. These early cell viability tests laid the foundation for the current wide use of tetrazolium salts in cell biology where most applications depend on uptake by viable cells and intracellular reduction that is related to metabolic activity.

An MTT–formazan assay was developed for testing the viability of filarial worms [68], but it was subsequently observed that the assay was not suitable for L3 infective larvae as they did not reduce MTT to the same extent as healthy worms early in infection [69]. Mukherjee *et al*. also applied the MTT–formazan test to screen for antifilarial activity [70].

Cell viability testing, as opposed to measuring the metabolic activity of viable cells, requires evaluation at the level of single cells or discrete groups of cells, and this usually involves either tedious counting in a haemocytometer or the use of flow cytometry which can now be adapted to a microplate format. Recently, digital imaging microscopy methods have also been applied to cell viability testing using dyes like trypan blue that are excluded from viable cells, but enter and bind to proteins when the integrity of the plasma membrane is compromised. Dyes that enter cells and generate a fluorescent signal following binding to DNA (*e.g*., propidium iodide) and proteins are also used to measure cell viability. Tetrazolium dyes, however, are not ideal reagents for measuring the percentage of viable cells because their formazans are either crystalline which can itself damage cell membranes, or soluble and diffusible, and with the exception of CTC, non-fluorescent. Furthermore, quiescent or dormant cells that are viable are not always clearly distinguished from non-viable or dead cells.

The use of tetrazolium salts to measure superoxide production

The ability of superoxide to reduce tetrazolium salts such as NBT [71] is the basis of their application in cellular assays for measuring superoxide production and granulocytic cell function in diseases like chronic granulomatous disease [9,12,72]. Professional phagocytes generate large amounts of superoxide

following exposure to microorganisms and chemical mediators of inflammation, and this is associated with a substantial increase in cyanide-resistant oxygen consumption. This "respiratory burst" involves activation of the multi-component NADPH:oxidase enzyme complex in the plasma membrane, which transfers electrons from intracellular NADPH to molecular oxygen at the cell surface [73]. Although superoxide production at the surface of neutrophils has often been measured using ferricytochrome *c* reduction, this assay lacks sensitivity due to the high background absorbance of ferricytochrome *c* and its low extinction coefficient. In addition to cytochrome *c*, NBT has also been widely used to measure the respiratory burst of phagocytes with most dye reduction being intracellular [13]. This NBT-reducing activity has been directly linked to components of the plasma membrane NADPH oxidase using non-denaturing polyacrylamide gel electrophoresis [74]. In addition to NBT, MTT is also reduced by activated neutrophils [75], but in contrast to NBT which is primarily reduced intracellularly, 75% of MTT reduction was shown to be sensitive to SOD indicating extracellular reduction. More recently, the cell-impermeable tetrazolium dye, WST-1, has been applied as a sensitive microplate assay for measuring the respiratory burst of human neutrophils [29]. Like ferricytochrome *c*, WST-1 reduction was extensively inhibited by SOD and therefore extracellular [27,29]. Increased sensitivity of the WST-1 assay can be attributed to low background absorbance and the high extinction coefficient of WST-1. In our laboratory, we have applied the WST-1 microplate assay in both anti-inflammatory and pro-inflammatory screening.

Certain plant cells also produce a respiratory burst when confronted with incompatible pathogens, as part of a hypersensitivity response. Because the plant cell wall forms a diffusion barrier to ferricytochrome *c*, NBT which forms an insoluble formazan that is trapped inside the cell, or XTT which forms a soluble formazan, have been used to measure superoxide production kinetics by tobacco (*Nicotiana tabacum* L.) suspension cultures when challenged by compatible and incompatible pathogens [76].

Another novel observation concerns the NBT-formazan "footprints" left on the nematode parasites *T. spiralis* and *N. brasiliensis* following surface membrane contact with neutrophils, but not eosinophils, mast cells or macrophages [77]. These footprints would have resulted from localised respiratory burst activity, superoxide production and consequent NBT reduction.

An environmental application of using tetrazolium salts to measure superoxide production was highlighted by Fatima *et al.* [78] who investigated the effect of pollutants on the respiratory burst while the effect of an environmental pollutant on phagocyte activity of the freshwater catfish was determined with NBT [79].

In addition to the respiratory burst of phagocytic cells, superoxide is also produced intracellularly as an unavoidable by-product of aerobic respiration [80, 81]. This "leakage" of electrons from the mitochondrial electron transport chain results in DNA damage, lipid peroxidation and protein oxidation and will

contribute to the tetrazolium–formazan signal, depending on growth conditions and the metabolic state of the cell.

Superoxide is also produced by members of the NOX family of plasma membrane NAD(P)H oxidases other than NOX2, which is responsible for the respiratory burst [81]. For example, low levels of superoxide are produced intracellularly by NOX1 on vascular smooth muscle cells [11] and NOX4 on endothelial cells [82] and this will also contribute to tetrazolium dye reduction by these cell types. Superoxide dismutates to form H_2O_2 (K_{cat} $5 \times 10^5 M^{-1}s^{-1}$) [33] which is now a well-recognised signalling molecule involved in cell proliferation and many functional responses [81].

The ability of human spermatazoa to reduce WST-1 was investigated by Aitken *et al*. [83] who showed detectable reduction in the absence of mPMS, possibly due to low levels of superoxide production. Reduction was greatly enhanced in the presence of mPMS and the characteristics of this reduction were shown to be similar to but not identical with trans-plasma membrane reduction of WST-1 by human cell lines. With rat epididymal sperm, cytochrome P450-reductase was shown to be capable of reducing WST-1 biochemically in the presence of NADPH [61], but the contribution of this enzyme to dye reduction by intact cells is questionable because WST-1/mPMS reduction is SOD-sensitive and therefore extracellular.

The ability of tetrazolium salts like NBT and WST-1 to be reduced by superoxide generated by xanthine oxidase from hypoxanthine is the basis of their use in assays for superoxide dismutase [84,85].

Microbiological applications of tetrazolium dye reductions

Traditional microbiological enumeration techniques such as colony counts on plate employing selective media are time consuming and do not account for viable non-culturable cells found in many microbial ecosystems [86,87].

A number of different tetrazolium dyes have been used to distinguish between dormant and metabolically active microbial cells. Most respiring micro-organisms are able to reduce tetrazolium dyes in their electron transport chain, generating results within hours. For example, MTT has been used to test the antibacterial properties of fungal extracts [88] and the effects of antimicrobial peptides on growth of *Candida albicans* [89]. INT, which was first used to measure respiratory capacity of individual bacteria in fresh-water lakes [90], has been applied to the measurement of respiratory activity of planktonic organisms in marine environments [91] and of microorganisms in groundwater [92].

Other studies have used XTT [93] and TTC [94] to test the efficacy of antimicrobials and for microbial ecotoxic finger printing [95].

CTC, which produces an insoluble fluorescent formazan, has been used in conjunction with flow cytometry to assess the effect of antibiotics on human pathogens like *Staphylococcus aureus* and *Pseudomonas aeruginosa* [96] and to

determine numbers of metabolically active food poisoning organisms like *Escherichia coli 0157:*H7 [97]. CTC has also been used successfully to visualise and quantify respiring microbial cells numbers in aquatic habitats like seawater, ground water and fresh water [98], in drinking water [99] and in soil [100] as well as in determining the risks of biodeterioration in old stone buildings [101].

In our laboratory, we have used WST-1/mPMS to identify and partially characterise an electron transport system in the plasma membrane of microbial cells and compared this with mammalian plasma membrane transport [34]. In the budding yeast *Saccharomyces cerevisiae*, dye reduction per unit surface area (milliA450 min^{-1} $[mm^2]^{-1}$) under both aerobic and anaerobic conditions was 3% of that of the human leukaemia cell line, HL60. *Escherichia coli* was found to reduce the dye at an even lower rate of 0.2% that of HL60 cells under aerobic conditions and 0.4% under anaerobic conditions. However, unlike the mammalian system, WST-1/mPMS reduction by these microbial cells was unaffected by rotenone (Herst and Hermiz, unpublished results), demonstrating a lack of the rotenone sensitive mammalian respiratory complex I in *S. cerevisiae* [102], and the presence of alternative NADH dehydrogenases in *E coli* [103]. Dye reduction by *E. coli* under hypoxic conditions was found to be more resistant to cyanide and azide than under normoxic conditions (Herst and Hermiz, unpublished results), reflecting the structural differences between the two terminal oxidases, cytochrome b_o and b_d which are expressed under normoxic and hypoxic conditions respectively [104].

In summary, the reduction of tetrazolium dyes by microorganisms will depend on the particular dye used, the organism, its growth phase and metabolism, as well as nutrient availability and growth conditions. Species-specific contribution to overall microbial productivity in an ecosystem must therefore include consideration of the dye-reducing ability of each species involved. As the fraction of actively respiring cells of each species and their contribution to ecosystem productivity varies enormously, analysis of complex microbial communities by tetrazolium dye reduction alone has limited value [92,105–108].

Summary and Conclusions

The wide use of tetrazolium dyes in cell biology belies our ignorance about their biological chemistry and the nature of their cellular reduction. With the rapidly increasing use of these dyes as convenient and inexpensive tools in cell microculture applications, and the introduction of new generation tetrazolium dyes that are reduced to soluble formazans that equilibrate rapidly in the cell culture medium, there is an urgent need to understand their bioreduction so that their use can be appropriately targeted. We propose that the net charge on the dye molecule is the primary factor responsible for cellular uptake by, or exclusion from the cell via the plasma membrane potential. Other factors

that contribute to cellular uptake and reduction are reducibility of the tetrazole ring and the overall lipophilicity of the molecule. These considerations together with the cellular dye-reducing properties lead us to the conclusion that MTT and other positively charged tetrazolium salts like NBT are reduced primarily intracellularly by oxidoreductase enzymes, the majority of which utilize the reduced pyridine nucleotide, NADH. In contrast, tetrazolium dyes that are negatively charged and have a mandatory requirement for an intermediate electron acceptor, including XTT and WST-1, are reduced at the level of the plasma membrane and most likely at the cell surface by trans-plasma membrane electron transport. Although both MTT and WST-1/mPMS reduction are driven by intracellular NADH, the source of the NADH appears to differ in that WST-1/mPMS reduction is more highly dependent on the malate/aspartate shuttle that links mitochondrial TCA cycle NADH with the extramitochondrial space. The use of tetrazolium salts in cell proliferation assays and in drug testing applications is discussed, as is their employment in measuring superoxide production by the respiratory burst of phagocytic cells and by cardiovascular cells that express other NOX family proteins. Whereas NOX2 uses intracellular NADPH, other members of this family use both NADH and NADPH. Last, tetrazolium salts have been used widely in microbiological applications relating to metabolic and respiratory activity, but these applications are often confounded by the plethora of microbial species and metabolisms involved, particularly where environmental screening is concerned.

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