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Reduction of a tetrazolium salt, CTC, by intact HepG2 human hepatoma cells: subcellular localisation of reducing systems

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Abstract

Cell-mediated reduction of tetrazolium salts, including MTT, XTT, MTS, NBT, NTV, INT, in the presence or absence of intermediate electron carriers is used as a convenient test for animal or bacterial cell viability. Bioreduction of tetrazolium is considered an alternative to a clonogenic assay and a thymidine incorporation assay. However, correlation between clonogenic potential and capacity to reduce tetrazolium has not been demonstrated convincingly. Moreover, despite a wide use of tetrazolium viability assays, the mechanism and subcellular localisation of reducing systems or species in viable intact cells have not been fully elucidated. We report evidence indicating that a tetrazolium salt CTC can be reduced in the presence as well as in the absence of an electron carrier by viable HepG2 human hepatoma cells. CTC-formazan is formed within or at the outer surface of plasma membranes. We hypothesise that in the presence of an electron carrier the electron donors active in the reduction of CTC are located in the intracellular compartment, as well as in plasma membranes. However, in the absence of an electron carrier, the reduction occurs primarily via a plasma membrane-associated enzymatic system or species.

Keywords: Tetrazolium salt; CTC; Reductase; Plasma membrane

1. Introduction

Cell-mediated reduction of tetrazolium salts leads to corresponding formazans. These reactions are widely used as indicators of cell viability [1–4], metabolic activity [5–7], oxidative reactions (generation of oxidative species) [8,9]; they are also used for detection of reductases in cell and tissue preparations [10–15] and in cytokine assays [16]. Automated approaches (microculture tetrazolium assays, MTAs), aimed at determining proliferation and survival of cells and screening large numbers of drugs have been designed [17].

Reduction of tetrazolium salts including MTT, MTS, XTT, NBT, INT and NTV is regarded as a

Abbreviations: CTC, 5-cyano-2,3-ditolyl tetrazolium chloride; DMEM, Dulbecco's modified minimal essential medium; INT, 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2*H*-tetrazolium chloride; MB, 8-dimethylamino 2,3-benzophenoxazine (Meldola's Blue); MPMS, 1-methoxy phenazine methosulphate; MTS, 5-(3-carboxymethoxyphenyl)-2-(4,5-dimethylthiazolyl)-3-(4-sulphophenyl) tetrazolium, inner salt; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide; NBT, 3,3′-(3,3′-dimethoxy-4,4′-biphenylene)-bis [2-(4-nitrophenyl-5-phenyl)-2*H*-tetrazolium chloride; NTV, nitro-tetrazolium violet; PI, propidium iodide; PMS, phenazine methosulphate; XTT, sodium 3′-[1-[(phenylamino) carbonyl]-3,4-tetrazolium]bis(4-methoxy-6-nitro) benzensulphonic acid hydrate

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convenient test for cell viability and an alternative to a clonogenic assay and a thymidine incorporation assay [4]. The advantage is rapidity, low cost and simplicity. However, correlation between clonogenic potential and capacity to reduce tetrazolium has not been demonstrated convincingly [4,7]. Moreover, despite a wide use of tetrazolium viability assays, the mechanism of cell-mediated reduction has not been fully elucidated [4,6,18]. Understanding this mechanism is required for interpretation of MTAs.

Two different mechanisms may operate in reduction of membrane permeable and impermeable tetrazolium derivatives. MTT, the derivative which has been in use since the early work in this area probably penetrates through plasma membranes and is reduced intracellularly, in mitochondria [3,19], endoplasmic reticulum [5] and cytosol [5]. Other currently used derivatives, such as XTT, MTS are more hydrophilic than MTT due to the sulphonate groups on the former. They may not be capable of crossing plasma membranes. Thus one may expect that such hydrophilic derivatives are reduced in bulk medium or at the cell surface [3,4,13]. In the case of extracellular tetrazolium salts, several interesting questions arise: what is the reducing species and system responsible for extracellular reduction? Does this reaction correlate with cell viability and incorporation of thymidine? Does reduction of extracellular tetrazolium salts affect plasma membrane integrity? The goal of the research reported here was to establish the subcellular localisation of a system responsible for reduction of a tetrazolium salt, CTC, by intact HepG2 human hepatoma cells in vitro.

Reduction of MTT, NBT, INT, NTV and CTC lead to formazans precipitating out of water solutions. Investigations of the sites of accumulation of formazan may assist in establishing the subcellular localisation of reducing systems. CTC seems particularly useful in this respect since CTC-formazan is fluorescent. Thus, the precise detection of small amounts of CTC-formazan is easier than detection of non-fluorescent, colour precipitates. The best sensitivity and resolution of detection of CTC-formazan in cultures of viable cells is offered by fluorescence confocal microscopy [20]. We used this method to follow the process of formation of CTC-formazan by HepG2 cells in vitro in order to establish the subcellular localisation of the site(s) of reduction of CTC.

Cell-mediated reduction of XTT, MTS and CTC (but not MTT) was reported to occur in the presence of intermediate electron carriers PMS, MPMS or MB [3,5,7,10,13,14,21]. In most cases studied, an electron carrier was necessary for reduction to occur. There were exceptions, however, where formation of formazan was detected in the absence of an intermediate electron carrier [3,5,6,18,19]. Thus, more questions arise as to the role these carriers play in reduction of extracellular tetrazolium salts by viable cells. Does the carrier move electrons between the donors located in cell interior and the extracellular tetrazolium, or between cell surface and extracellular tetrazolium as well? Do the carrier and tetrazolium affect cell metabolism, plasma membrane integrity and thus influence the parameter which is measured? We report evidence indicating that CTC can be reduced in the absence of an intermediate electron carrier while the integrity of plasma membranes is not compromised. Under such conditions, it can be demonstrated that the systems reducing tetrazolium are associated with plasma membranes as well as with an intracellular compartment.

2. Materials and methods

2.1. Reagents

CTC (5-cyano-2,3-ditolyl tetrazolium chloride) was purchased from Polysciences; MB (8-dimethylamino 2,3-benzophenoxazine, Meldola's Blue) and digitonin were obtained from Sigma. Solutions of CTC, MB and digitonin were made immediately prior to experiments.

2.2. Cell cultures

HepG2 human hepatoma cells were cultured on 20-mm-diameter, 0.17-mm-thick coverslips placed in Petri dishes. DMEM (Sigma) supplemented with 10% foetal bovine serum (Gibco) and antibiotics was used. Monolayers (1 day after seeding) were used in all experiments. Cell density was approximately 1.5×10^5/ml of medium (190 cells/mm^2).

2.3. Plasma membrane integrity assay

Integrity of plasma membranes was monitored using a propidium iodide (PI) exclusion test [22]. PI was added to cell cultures to a final concentration of 50 μg/ml. Red fluorescence of nuclei of damaged cells was detected using confocal microscopy. A culture of cells treated with digitonin (0.1 mM) served as a positive control.

2.4. Confocal microscopy

Fluorescence of PI and CTC-formazan was detected using laser scanning confocal microscopy (CLSM). We used a Bio-Rad MRC1024 system equipped with a Nikon Diaphot 300 microscope, 60× PlanApo oil immersion objective lens, 15 mW argon–krypton laser (ALC), three fluorescence detection channels and a three-colour transmitted non-confocal light detector. The 488 line and a 30% neutral density filter was used for simultaneous excitation of PI and CTC-formazan. A 565LP dichroic mirror was used for specimen epi-illumination. Fluorescence of PI in cell nuclei was collected in the region of 585–640 nm, while CTC-formazan in the region 580 up to approximately 750 nm. Coverslips with cell cultures were withdrawn from Petri dishes and mounted in a round steel holder using silicone grease. The holder was fitted in the pre-warmed microscope stage microincubator (Life Sciences Research, Cambridge, UK). The standard culture medium was replaced with DMEM without phenol red and bicarbonate, with pH adjusted to 7.4. The sample contained 250 ml of culture medium in equilibrium with air. Incubations with CTC (5 mM) were conducted at 37°C in air. During incubation transmission and fluorescence images of cells were collected in 1.5- or 2.5-min intervals. 512×512 pixels, 256 grey level images were collected using LaserSharp 2.1T software (Bio-Rad).

2.5. Spectroscopic measurements

CTC (0.5 mM) was dissolved in PBS. A suspension of crystals of CTC-formazan in PBS was obtained by reduction of CTC (0.5 mM) by ascorbate (2.5 mM). Absorption spectra (200–800 nm) of CTC and CTC-formazan were collected using an HP 8542 UV/VIS spectrophotometer. Fluorescence emission and excitation spectra of CTC and CTC-formazan were measured using Perkin–Elmer LS50 spectrofluorometer.

2.6. Data analysis

CTC-formazan was detectable by confocal microscopy due to its red fluorescence. The growth of CTC-formazan crystals was observed by registering time sequences of images of the same areas of the cell culture. Sequences were collected and analysed using automated routines of the TimeCourse software (Bio-Rad). For analysis, 10–15 regions embracing cell membranes, cell interiors or surrounding medium were selected in each experiment. The mean intensities of red fluorescence in each region were plotted against time.

Detection of cell edges and thus the position of plasma membranes was based on transmitted light images. The position of crystals of formazan relative to plasma membranes was determined by overlaying the confocal images of red fluorescence over non-confocal transmitted light images taken through the cell centre. It follows that, depending on the shape of individual cells and position of a confocal slice, the edges of cells on confocal slices overlapped or fell just inside the edges on transmitted light images.

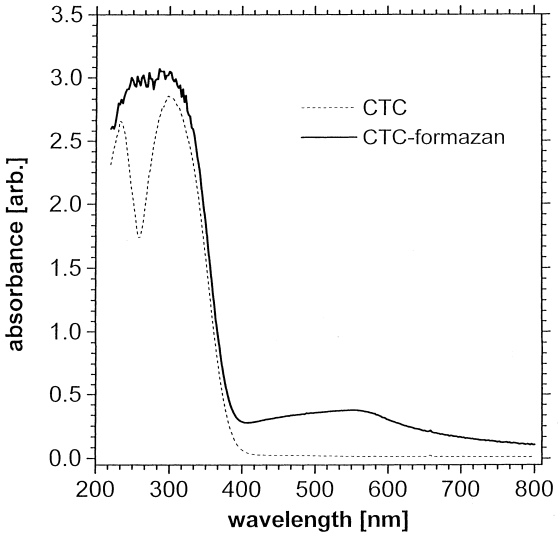


Fig. 1. Absorbance of CTC (0.4 mM) and CTC-formazan crystals formed in air-equilibrated PBS, at room temperature, following addition of ascorbate (1 mM).

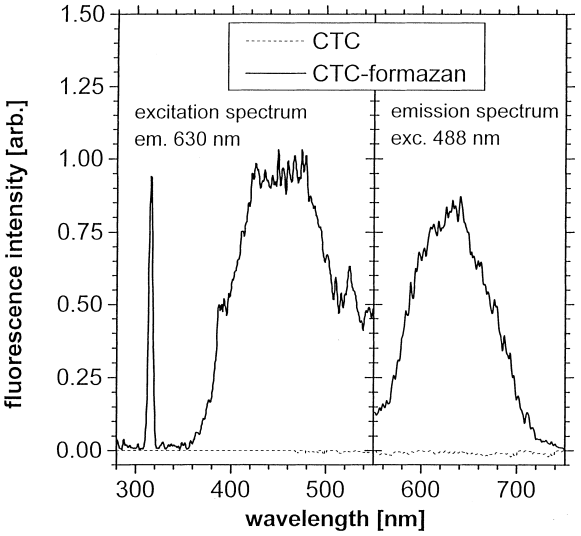


Fig. 2. Excitation (em. 630 nm) and emission (exc. 488 nm) spectra of CTC (0.5 mM, dissolved in PBS) and CTC-formazan (a suspension of crystals).

3. Results

3.1. Spectroscopic properties of CTC

CTC readily dissolves in water. The solution absorbs in the UV region, up to 400 nm, with two maxima at approximately 216 and 300 nm (Fig. 1).

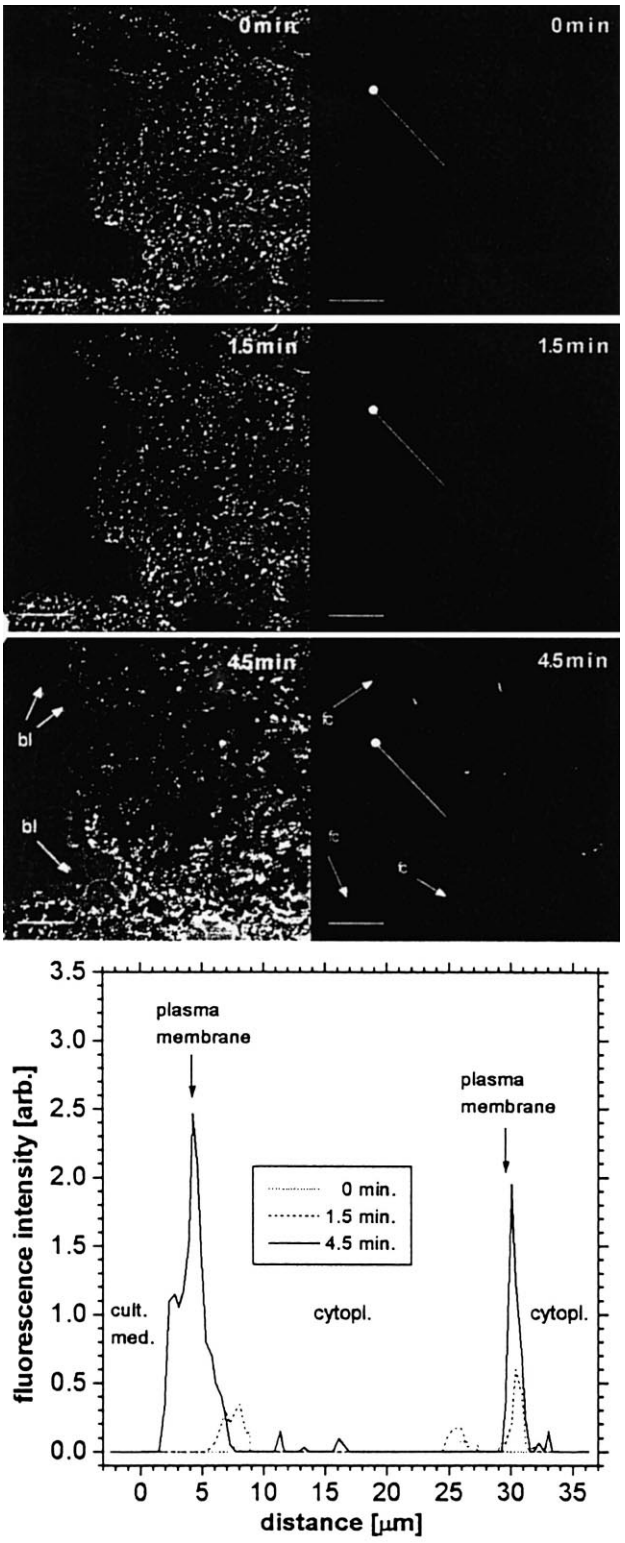


Fig. 3. Formation of crystals of CTC-formazan in a monolayer culture of intact HepG2 cells, in the presence of an electron carrier. Scale bar: 10 μm. Left column: transmitted light (488 nm) images of HepG2 cells. Images were collected at times indicated following the addition of CTC. As time progresses, the cytoplasm absorbs more blue light due to penetration of MB into cells. Blebs forming from plasma membranes at the end of the incubation period are indicated (bl). Right column: confocal images of red fluorescence in HepG2 cells shown in the left column. Thickness of the optical sections 1.2 μm. Small crystals of CTC-formazan (fc) are forming near cell surface. No red fluorescence of PI can be detected in cell nuclei indicating that the integrity of plasma membranes was not compromised. Small red dots in cytoplasm of the image at time 0 are due to autofluorescence. Bottom: intensities of red fluorescence in culture medium surrounding the cells, in plasma membranes and in cytoplasm. Intensities of red fluorescence were measured along the white lines, starting with a dot, which were marked in confocal images (right column). The position of cell plasma membranes is marked with arrows. Red fluorescence of CTC-formazan is concentrated at the plasma membranes, no formazan is detected in medium or cell interior.

Following addition of ascorbate the CTC solution gradually changed colour to orange due to formation of CTC-formazan. The CTC-formazan precipitated out of solution within 5 min of incubation. The absorption curve of a suspension of CTC-formazan crystals has a band in the UV region extending up to 400 nm (Fig. 1) (one has to note, however, that the absorption curve of CTC-formazan refers to a suspension of particles rather than a solution and, as such, may be a combined effect of light absorption and dispersion). When excited with blue light (488 nm) CTC exhibited no fluorescence while CTC-formazan emitted light in the broad region extending from 500 to 750 nm, with a maximum at approximately 630 nm (Fig. 2). Excitation spectrum of CTC-formazan has a narrow peak at 318 nm and a broad region extending from 360 to 550 nm (Fig. 2).

3.2. *Reduction of CTC by HepG2 cells in the presence of an electron carrier*

Cell-mediated reduction of CTC was investigated in monolayer cultures of HepG2 cells. CTC, an electron carrier Meldola's Blue, and a plasma membrane integrity indicator PI, were added to culture medium.

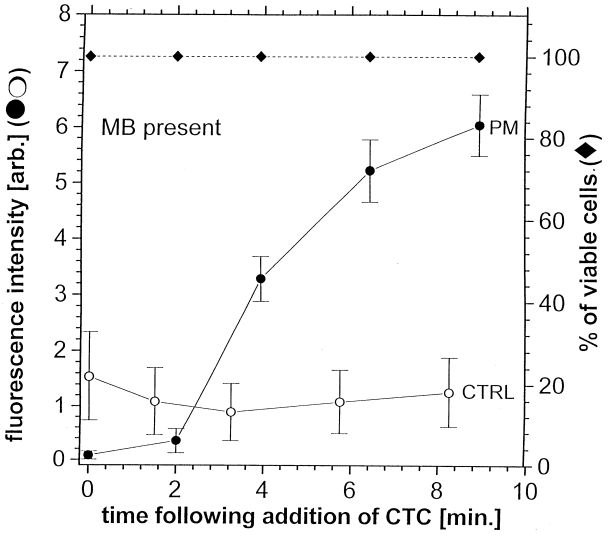


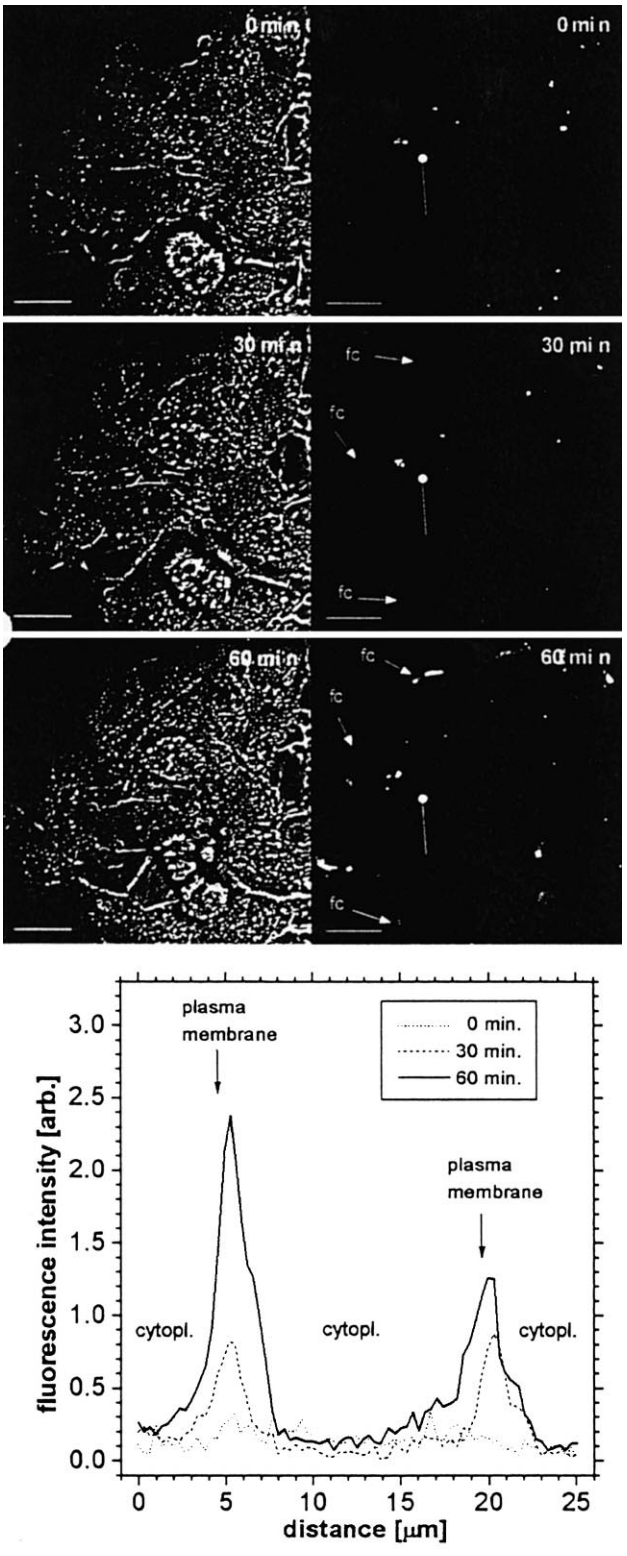
Fig. 4. Kinetics of formation of CTC-formazan in the presence of MB (left axis) and a proportion of cells with intact plasma membranes (right axis). PM, intensity of red fluorescence measured in the regions of cell plasma membranes; CTRL, red fluorescence of plasma membranes measured in a parallel control experiment, in the absence of CTC.

Within a few minutes of addition of CTC, crystals of CTC-formazan were formed in the regions of cell plasma membranes (Fig. 3). These crystals measured up to 2 μm in length and were always adjacent to plasma membranes of HepG2 cells. The whole sequence of images that were collected demonstrates that the crystals were formed exclusively at cell surfaces. No crystals were formed in cell interior or in the bulk medium. The growth of the intensity of fluorescence of CTC-formazan at cell surfaces is shown in Fig. 4. An example of the intensities of fluorescence of CTC-formazan measured in culture medium surrounding the cells, in the regions of plasma membranes and in the cytoplasm is shown in Fig. 3, bottom. Transmitted light images indicate that MB was entering cells (Fig. 3). During the incubation with MB and CTC plasma membrane integrity was not compromised as demonstrated by the absence of PI fluorescence from cell nuclei (Fig. 3). However, formation of membrane blebs [23] was detected (Fig. 3). This phenomenon indicated that damage to plasma membranes or cytoskeleton occurred, although it was not yet manifested by the increased plasma membrane permeability.

No reduction of CTC was detected in the absence of cells indicating that the medium alone did not have a capacity to reduce CTC.

3.3. *Reduction of CTC in the absence of an electron carrier*

HepG2 cells are capable of reducing CTC to CTC-formazan in the absence of an electron carrier. In cultures without MB, CTC-formazan was still gradually formed at cell surfaces over a period of 1 h (Figs. 5 and 6). The size of crystals was below the resolution of an optical microscope (i.e. below approximately 250 nm). The intensities of red fluorescence in plasma membranes and in the intracellular compartment are shown in Fig. 5, bottom. These data demonstrate that the presence of MB was not a prerequisite for reduction of CTC by HepG2 cells. Both in the presence and in the absence of MB the crystals of CTC-formazan were formed in the regions of plasma membranes. The integrity of plasma membranes was not compromised (Fig. 6). The rate of reaction was approximately 13 times slower in the absence than in the presence of MB.



4. Discussion

The experiments described above were carried out in order to establish the subcellular localisation of the site(s) of reduction of CTC. We will discuss experimental evidence supporting the view that CTC is reduced to formazan within or on surfaces of plasma membranes of intact cells, by enzyme system(s) residing in cell interior as well as associated with plasma membranes.

4.1. Penetration of CTC through plasma membranes

The extracellular or intracellular reduction of tetrazolium and subsequent deposition of formazan may depend on the ability of tetrazolium to cross plasma membranes. To our knowledge, penetration of tetrazolium salts into intact viable cells has not been measured directly. In the case of CTC, reports exist referring to both intracellular formation of formazan in bacteria detected by optical microscopy [24,25] and extracellular deposition of CTC-formazan formed on plasma membranes of Ehrlich ascites cells [13,14]. The presence of a stable positive charge in the tetrazolium salt molecule [26] may be expected to limit the ability of tetrazolium salts to penetrate freely through intact plasma membranes of viable cells. In our experiments, the integrity of plasma membranes of HepG2 cells was carefully monitored and shown not to be compromised during incubation with CTC and MB. Under these conditions, CTC-formazan was detected entirely in the regions of plasma membranes. In a series of control experiments,

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Fig. 5. Formation of crystals of CTC-formazan in a monolayer culture of HepG2 cells, in the absence of an electron carrier. Scale bar: 10 μm. Left column: transmitted light (488 nm) images of HepG2 cells Right column: confocal images of red fluorescence in HepG2 cells shown in the left column. Thickness of optical sections, 1.2 μm. CTC-formazan (fc) is forming near cell surface. No red fluorescence of PI can be detected in cell nuclei indicating that the integrity of plasma membranes was not compromised. Bottom: intensities of red fluorescence in plasma membranes and in the cytoplasm. Intensities of red fluorescence were measured along the white lines, starting with a dot, which were marked in confocal images (right column). The position of cell membranes is marked with arrows. Red fluorescence of CTC-formazan is concentrated at the plasma membranes, no formazan is detected in cell interior.

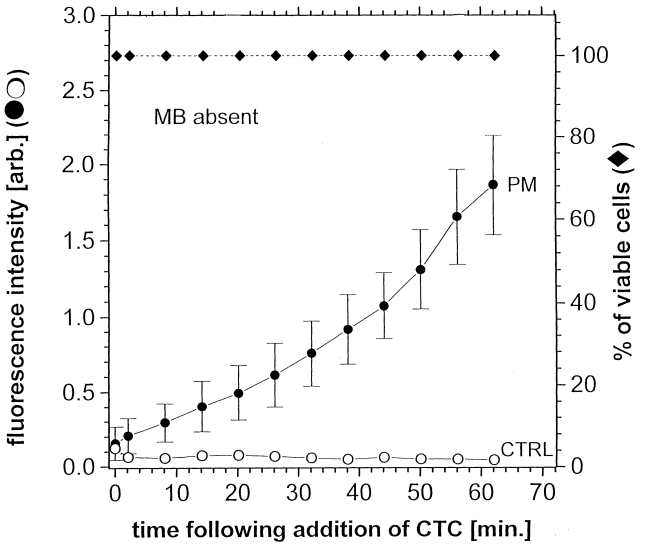

Fig. 6. Kinetics of formation of CTC-formazan in the absence of an electron carrier (left axis) and a proportion of cells with intact plasma membranes (right axis). PM, intensity of red fluorescence in the regions of plasma membranes; CTRL, red fluorescence measured in a parallel control experiment, in the absence of CTC.

plasma membranes were rendered permeable by treatment with digitonin. Under these conditions, we detected formation of crystals of CTC-formazan intracellularly (data not shown). This observation is consistent with the observation of Stellmach [13] who reported an increased rate of reduction of CTC by Ehrlich ascites cells challenged with digitonin. Thus, it seems possible that during incubations with intact HepG2 cells reported here CTC remained in the extracellular space.

4.2. The site(s) of CTC reduction

Following reduction of CTC, crystals of CTC-formazan precipitate out of solution. Thus, in cultures of HepG2 cells the crystals are probably formed at the sites of reduction. Analysis of the confocal images recorded during incubations of intact cells with CTC, in the presence and in the absence of MB, indicate that formation of CTC crystals took place at or near plasma membranes. Therefore we conclude that the process of reduction of CTC occurs at the cell surface or within cellular plasma membranes.

Deposition of CTC-formazan might result from exocytosis of crystals formed in cell interior. Exocytosis of formazan crystals by neuronal cells was reported [6]. This mechanism, however, was not involved in deposition of CTC-crystals on the surface of HepG2 cells. The time sequence images we collected clearly demonstrate that in HepG2 cell cultures the crystals of CTC-formazan were neither formed intracellularly nor were they transported across cytoplasm.

4.3. The role of an intermediate electron carrier

In the majority of published reports, reduction of tetrazolium was carried out in the presence of intermediate electron carriers PMS, MPMS and MB. The most effective electron carrier in CTC reduction was MB [14]. MB was also effective in the system we studied. However, in HepG2 hepatoma cells, unlike in Ehrlich ascites cells [13,14], MB was not required for reduction of extracellular CTC to formazan accumulating in plasma membranes. This observation indicates that one of the putative reducing systems was located in plasma membranes. This system did not require an exogenous electron carrier.

Formation of CTC-formazan was faster in the presence of Meldola's Blue than without this electron carrier. MB is known to be capable of undergoing reversible reduction and oxidation [27]. MB may also cross intact plasma membranes [21,27]. Analysis of transmitted light images suggests that MB indeed penetrated into cells. There is no direct evidence demonstrating that MB in reduced form can diffuse out of cells. However, one may put forward a hypothesis that MB carried electrons between intracellular donors and extracellular CTC. In this case, the presence of a membrane permeable electron carrier would enable electron transfer from intracellular electron donors onto the extracellular tetrazolium. This mechanism would constitute an example of formazan produced at a distance from reducing enzyme(s).

4.4. The reducing system/species

Depending on the presence or absence of an electron carrier CTC may be reduced by different reducing systems.

In the presence of MB, electrons may be trans-

ported by an electron carrier from mitochondria or cytosol to the extracellular tetrazolium. Mitochondrial [3,19,28] as well as cytosolic [7,19] and ER-associated [5,19] oxidoreductases were shown to be involved in the reduction of MTT. It is possible that these enzymes may also be involved in the reduction of CTC via MB as a membrane permeable electron carrier.

In the absence of MB, the reduction may be performed by a plasma membrane reducing enzyme system or a short-lived reducing species. The enzyme systems capable of transferring electrons directly onto CTC may involve plasma membrane oxidoreductases, including oxidoreductase NAD(P)H: ferricyanide [29–32] and NAD(P)H: O_2 [33,34]. These enzymes are present in fibroblasts, adipocytes [29] and hepatocytes [34,35]. Plasma membranes of intact HepG2 cells have a capacity to reduce another extracellular acceptor of electrons – a nitroxide free radical Cat1 [36]. Further studies are needed to establish if CTC and other tetrazolium salts can serve as artificial acceptors of electrons for plasma membrane oxidoreductases in hepatocytes.

A possibility exists that CTC was reduced by superoxide radical [37] produced by HepG2 cells. The radical which is generated in mitochondria and elsewhere in the cell cannot diffuse over distances long enough to reach extracellular CTC. However, NADH oxidase activity was detected in plasma membranes of hepatocytes [35] and hepatoma cells [38]. Therefore, one may also hypothesise that CTC was reduced by oxygen radicals generated within plasma membranes.

5. Conclusions

Based on the data presented in this report we conclude that: (1) CTC may not penetrate through intact plasma membranes of HepG2 cells; (2) it is reduced to CTC-formazan within or on the surface of plasma membranes; (3) the reducing systems or species capable of reduction of CTC to formazan appear to be located both intracellularly and in plasma membranes; (4) in the presence of a membrane permeable intermediate electron carrier reducing systems in both compartments may be involved; and (5) in the absence of an electron carrier, CTC is reduced exclusively by an as yet unidentified plasma membrane reductase.

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