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Mechanisms of INT (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl tetrazolium chloride), and CTC (5-cyano-2,3-ditolyl tetrazolium chloride) reduction in *Escherichia coli* K-12

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Abstract

The tetrazolium salts INT and CTC are frequently used as indicators of bacterial respiration. Mechanisms of tetrazolium reduction to formazan in eukaryotic cells have been proposed, however those in prokaryotes remain largely unresolved. We undertook studies to determine the sites, and degree of coupling for INT and CTC reduction in the aerobic *Escherichia coli* K-12 respiratory chain using both intact cells and inverted membrane vesicles. In addition, reduction in whole cells was assayed under anaerobic conditions which elicit different electron transport pathways. Results of experiments with inhibitors of specific electron transport components indicated that both CTC and INT were reduced prior to ubiquinone in the *E. coli* respiratory chain by the primary aerobic [succinate and NAD(P)H] dehydrogenases. INT was also reduced at ubiquinone and possibly cytochromes $b_{555,\ 556}$. Quantitative CTC reduction was more closely correlated with respiration in whole cells than INT, but the reverse was true in inverted membrane vesicles. Both tetrazolium salts were reduced to significant degrees under anaerobic conditions, particularly glucose fermentation. Mid-point reduction potential of CTC was determined to be −200 mV by redox titration. However, it appears that CTC forms a weakly fluorescent, poorly-localized intracellular formazan at redox potentials higher than ca. −200 mV. Inhibition of the aerobic terminal oxidases with KCN or azide strongly increased INT-, but significantly decreased CTC reduction in whole cells, indicating that these agents may not be useful for optimizing CTC-formazan or CTC (+) cell numbers in some bacteria. However, several other chemical agents stimulated CTC and INT reduction. These results suggest strategies for optimizing methods using these tetrazolium salts for assessment of bacterial respiratory activity.

Keywords: CTC; INT; Tetrazolium; Respiration; Electron transport; Dehydrogenase

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1. Introduction

Tetrazolium salts are often used as indicators of microbial respiratory activity, as well as viability, particularly in physiological and ecological studies. They are also utilized as differential agents in bacteriological media (e.g., TTC [2,3,5-triphenyl-2H-tetrazolium chloride] in Tergitol-7, and mE) [1]. Methods have been developed using INT, and more recently CTC, to indicate the fraction of respiring or viable bacteria in populations. This is done through reduction of the soluble tetrazolium salts to their corresponding colored (INT), or fluorescent (CTC), insoluble, intracellular formazan crystals which can

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be viewed using epifluorescence microscopy (EFM) [2–6]. INT reduction has also been used to quantify respiratory potential in microbial populations by organic extraction, and colorometric determination of formazan production [7–9]. The INT technique produces dark-red, intracellular INT-formazan (INF) crystals which can be difficult to visualize by light microscopy, particularly against an opaque background [5,10–12]. Unfortunately, this is often the type of background encountered in studies of microbial adhesion, biofilms, and sediments, as well as bacterial enumerations on membrane filters using epifluorescence microscopy. INF crystals also appear to be soluble in immersion oil [5,12]. However, the recently developed cyano-substituted, 2,3-diaryl tetrazolium salt CTC forms a fluorescent formazan (CTF) which is relatively easy to visualize against an opaque background by epifluorescence microscopy [4]. In addition, CTF is insoluble in immersion oil, and retains its fluorescence upon storage [13,14]. Direct counting of actively respiring bacteria is simplified considerably, and CTF production can be monitored by fluorescence-activated cell sorting (FACS) analysis [2,4,6,15,16].

With the increasing use of tetrazolium reduction methods for studies of prokaryotic physiology and ecology, questions arise concerning the specific cellular process(es) measured by their reduction. Although sites of reduction for INT and CTC in eukaryotic electron transport systems have been fairly well defined, those in prokaryotic cells remain largely unresolved [9,17–19]. In eukaryotic mitochondria INT has been found to be reduced by the succinate:ubiquinone oxidoreductase (SDH) complex, or cytochrome c_1 [9,18,20]. Stellmach and Severin found that CTC was reduced primarily by membrane-bound NAD(P)H-dehydrogenase (NADH-D), and possibly superoxide anion in Ascites tumor cells [21]. Kaprelyants and Kell reported CTC was reduced directly by respiratory chain dehydrogenases in *Micrococcus luteus* [3], while López-Amorós, et al. found CTC could be reduced by NAD(P)H in formalin-killed *E. coli* [15]. Although not demonstrated experimentally, SDH has been suggested as the primary site of CTC and INT reduction in prokaryotes [2,4,9]. This is primarily due to the observed stimulatory effects of succinate on CTC and INT reduction in many bacteria [5,22–24]. It is well known that various tetrazolium salts have different reduction potentials, and the redox potential of CTC is unreported [17,19,25]. Respiratory data resulting from different tetrazolium salts and assay conditions would be easier to interpret if redox potentials and site(s) of reduction were known in the prokaryotic electron transport chain. In addition, elucidation of the effects of various agents and conditions on tetrazolium reduction in prokaryotes would allow for knowledge-based optimization and interpretation of methods, particularly those used for assessments of bacterial viability [22,23].

In order to determine the mechanisms of INT and CTC reduction in a prokaryotic respiratory chain we examined the effects of various inhibitors of electron transport and uncouplers of oxidative phosphorylation with well described sites of action. In general, blockage of the electron transport chain at a point before that at which the tetrazolium salt is reduced will result in decreased reduction to formazan, while blockage after the site of reduction will increase, or produce no change in tetrazolium reduction [16–18,21]. The utility of these agents for optimization of microscopy-based methods was also investigated. In order to determine the extent of coupling between tetrazolium reduction and respiration, formazan formation was correlated with oxygen consumption measurements. These experiments were carried out in whole cells and inverted membrane vesicles of *E. coli* K-12, a prokaryote with relatively well-described aerobic and anaerobic electron transport chains [26–33] (Fig. 1). To determine whether, and to what extent CTC and INT are reduced under anaerobic conditions, whole cells were assayed under conditions designed to stimulate several different anaerobic respiratory chains, as well as glucose fermentation. In order to further characterize the chemical properties of INT and CTC, their standard mid-point redox potentials ($E'_{1/2}$), solubilities, and molar extinction coefficients (ε) in 95% ethanol were also determined.

2. Materials and methods

2.1. Determination of INT and CTC chemical properties

All chemicals and substrates used were reagent grade or better. CTC was obtained from Polysci-

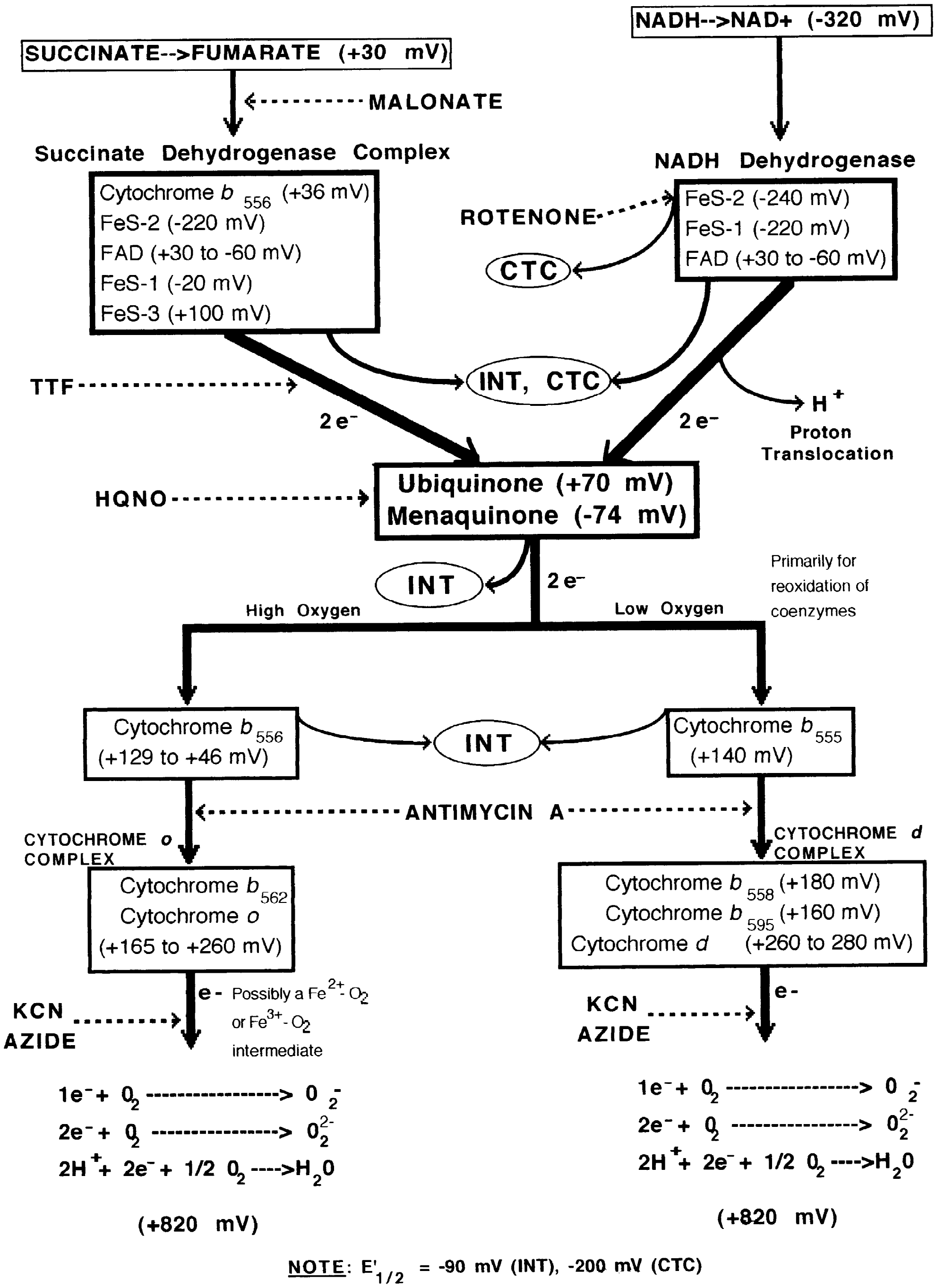

Fig. 1. Schematic of the *E. coli* aerobic respiratory chain showing the sites of action of the various inhibitors used in this study. Mid-point redox potentials for the various cytochromes, iron-sulfur centers (Fe-S), and dehydrogenases are given. Sites of reduction of CTC and INT from this study are encircled. Dashed lines indicate sites of inibition of electron transport processes. See materials and methods for chemical abbreviations. Compiled from [17–19,26,28–33,39,46,47].

ences, Inc. (Warrington, Pa.). INT and INF, as well as all substrates, fluorescent stains, and inhibitors were purchased from Sigma Chemical Co. (St Louis, Mo.).

Duplicate redox titrations were carried out in an anaerobic tent under an $N_2/CO_2/H_2$ atmosphere using 10 mM sodium dithionite as titrant and an Orion 9678BN combination redox electrode. All directly measured redox potentials were corrected by reference to the normal hydrogen electrode (NHE). INT was titrated as a control, as its redox potential has been reported previously ($E'_{1/2} = -90$ mV) [17,19,25]. Tetrazolium salts were dissolved at 0.1 mM in 50 ml, 0.1 M phosphate buffer, pH 7.0 as described by Karmarker et al. [25]. The ascorbic acid (0.1 M)-pH (pH range 2.5–11.5) method of Seidler was also used to determine the relative reducibilities of INT and CTC [35]. Molar extinction coefficients were determined by dissolving INT-(INF), and CTC-formazan (CTF) in 95% ethanol. These values were subsequently used to calculate formazan production from absorbance data. CTF was obtained by reduction of 50 ml, 0.1 mM CTC in double-distilled water (dd-H_2O) using 10 mM sodium dithionite. The precipitate was washed 3 times with dd-H_2O and lyophilized.

2.2. Growth conditions

Escherichia coli K-12 was grown aerobically in 350 ml Erlenmeyer flasks with vigorous aeration (300 rpm) at 25°C to late-log phase in 100 ml glycerol (8 g/l)-mineral medium supplemented with 1 μM selenic acid and sodium molybdate [35]. *E. coli* was also grown anaerobically in the same medium, except glycerol was replaced by glucose. All subsequent manipulations of anaerobic cultures and experiments were performed in a $N_2/CO_2/H_2$ atmosphere. All media for anaerobic experiments were equilibrated for at least 24 h under anaerobic atmosphere prior to use. Cells were washed 3 times by centrifugation at 4,000 X *g*, and resuspended in 0.85% NaCl (pH 6.5) at ca. 10^{10} cells/ml. Subsamples for aerobic tetrazolium salt experiments were removed after vigorous aeration (300 rpm on a gyratory shaker) for 10 min at 25°C. Plate counts were performed by spot-plating subsamples (exposed without inhibitors for the same period, and in the same buffer as respective assays) onto R2A medium (Difco Laboratories, Detroit, MI) and incubating for 48 h at 25°C.

2.3. Substrates, inhibitors, and uncouplers

Inhibitors were added as 100 or 10 μl aliquots of 10 or 100X solutions, respectively, to give the following concentrations: KCN (2 mM), sodium azide (50 mM), rotenone (ROT, 1 mM), malonate (MAL, 100 mM), thenolyltrifluroacetone (TTF, 1 mM), carbonyl cyanide m-chlorophenylhydrazone (CCCP, 10 μM), antimycin A (20 μM), and 2-heptyl-4-hydroxyquinoline-N-oxide (HQNO, 0.2 mM). Rotenone, TTF, CCCP, HQNO, and antimycin A were freshly prepared as 100X solutions in ethanol. Controls with ethanol alone showed no significant ($p<0.05$) effect on formazan producton (final ethanol concentration did not exceed 1%). Superoxide dismutase (SOD) was used at a final concentration of 175 units ml^{-1} (Sigma Chemical Co., St Louis, Mo.). Concentrations of substrates for anaerobic incubations were 10 mM each of glucose, formate, glycerol, and fumarate. Combinations were as shown in Fig. 4. R2A broth without phosphate (R2A-P) was used at 0.1-strength, final concentration. Nitrate was present as 40 mM KNO_3. Assays were performed in 10 mM potassium phosphate buffer containing 0.85% NaCl, 5 mM $MgCl_2$, at pH 6.5. The pH of all whole cell assay solutions was adjusted to 6.5 prior to cell and tetrazolium salt addition. Substrate and inhibitor controls (no cells or vesicles present) showed no detectable formazan production after 4 h incubation as measured spectrophotometrically (see below). Cells were used at a final concentration of ca. 10^9 cells ml^{-1}. Cells for nutrient assays were allowed to react (vigorous agitation/aeration in the presence of substrate) for 1 h prior to tetrazolium salt addition in order to allow cells time to begin metabolizing the particular substrate(s) available. Respiration rate of whole cells was 104 nmol O/min/mg protein. Freshly prepared CTC and INT solutions were added at a final concentration of 1 mM. Incubations were performed in duplicate at 25°C with vigorous aeration for 2 h in the dark (4 h for anaerobic incubations). Reactions were stopped by addition of 3.7% formaldehyde (final concentration) and immersion in an ice bath. Formaldehyde alone showed no detectable formazan production. Samples for Respiration/Electron Transport System (R/ETS) ratio measurements (see below) were stopped by addition of 5% trichloroacetic acid (TCA) final concentration. Formaldehyde killed (15 min exposure) controls were performed for all assays. Subsamples (91 μl) for CTC-reduction assays were diluted in filter-sterilized distilled water, filtered through 0.22 μm pre-blackened Nuclepore polycarbonate membranes (no. 110656), and counterstained with 10 $\mu g\ ml^{-1}$ DAPI (36). Subsamples for INT assays were diluted as above and air-dried as heat-fixed smears using 20 μl aliquots. These were counterstained with 100 $mg\ l^{-1}$ acridine orange (AO) in 2 mM Tris buffer (pH 7.0) for 3 min and viewed under both epifluorescence and bright field microscopy. Samples for epifluorescence direct counts were viewed using a Leitz Ortholux II microscope equipped with Leitz filter blocks B2, N2.1, and H3 to visualize DAPI, CTC, and INT/AO, respectively. INT (+) cells contained red INT-formazan deposits when viewed under bright-field microscopy, while CTC (+) cells contained fluorescent-orange CTC-formazan deposits. At least 600 cells in ≥10 random fields were counted at 1,250 X for each filter. Error was calculated as described previously [36].

Samples for spectrophotometric determination of formazan (0.9 ml) were centrifuged in a high-speed microcentrifuge (5000 X g, 10 min) and supernatants discarded. Pellets were then resuspended in 95% ethanol and disrupted by sonication for 15 s using a sonicator microtip (Fisher Scientific Sonic Dismembrator 50) at 30% power, and stored at 4°C overnight. One to 3 ml of ethanol was required to completely extract formazan from cell pellets, depending upon the amount of formazan present. Cell pellets retained no detectable formazan after the above extraction procedure. Spectrophotometric measurement of formazan standards in ethanol showed no degradation for up to 3 days at 4°C. Suspensions were then centrifuged (9000 X *g*, 10 min) and absorbance of supernatants determined at 450 nm for CTC, and 485 nm for INT using an HP 8452A spectrophotometer. Spectrophotometric measurement of formazan standards in ethanol showed no degradation for up to 3 days at 4°C. Cell pellets retained no detectable formazan after the above extraction procedure.

It should be noted that tetrazolium reduction and oxygen consumption were determined using separate aliquots from a common cell or vesicle suspension, not concurrently on the same suspension. Several experiments were performed measuring these parameters concurrently, but no discernable effects on endogenous respiration rates by CTC or INT were observed. However, we have observed significant tetrazolium salt-induced bactericidal effects and sublethal injury (as measured by recoveries on selective and nonselective medium) during these studies, dependent upon the exogenous substrate (data not shown).

2.4. Inverted membrane vesicles

Three liter cultures of *E. coli* K-12 grown aerobically as described above were harvested in late log phase and inverted membrane vesicles, as well as vesicles depleted in Mg^{2+}-dependent ATPase, were prepared by the method of Burstein et al. [35]. Depleted, inverted-vesicles lack Mg^{2+}-dependent ATPase activity, but retain membrane-bound dehydrogenase and electron transport activities [35,37]. Study of inhibition of membrane processes is facilitated through the use of *inverted* membrane vesicles, which avoids problems related to inhibitor permeability through intact cell membranes [38,39]. Duplicate assays were performed in a final volume of 1.0 ml, 10 mM phosphate buffer (pH 6.5), containing 5 mM magnesium acetate. Inhibitor concentrations used were the same as those used for intact cells. Sites of action are shown in Fig. 1. Vesicle protein (0.25 mg, final concentration 0.25 mg $protein\ ml^{-1}$) was added, and allowed to react with agents for 30 min before tetrazolium salt addition. Protein was determined by the micro-BCA method [40]. Respiration rates were measured at 181 nmol O/min/mg protein with NADH as substrate, and 357 nmol O/min/mg protein using succinate. Freshly prepared INT and CTC were added to a final concentration of 1 mM. Exogenous substrates were then added to final concentrations of 10 mM succinate, or 0.6 mM NADH which was generated using 50 μg alcohol dehydrogenase, 1% absolute ethanol, and 0.6 mM NAD^{+} [39]. There was no detectable formazan production by these substrates in the absence of vesicles. Oxygen consumption rates remained constant throughout the 1 h incubations with both succinate and NADH. Reactions were performed at 25°C for 1 h in the dark, and were stopped by the addition of 5% TCA (final concentration). TCA-treated controls were performed using succinate and NADH as reductants. There was no detectable formazan produced by TCA-treated vesicles, or vesicles in the absence of succinate or NADH. Membranes were pelleted at 175,000 X *g* for 1 h, and resuspended in ethanol to dissolve formazans as described above for intact cells. Debris was pelleted by repeating centrifugation, and the supernatants used for absorbance measurements.

2.5. Oxygen consumption

Oxygen consumption was measured using a Gilson 5\6 oxygraph with a Clark-type electrode [39]. All experiments were performed in a 2 ml-water-jacketed cell at 25°C. Oxygen saturation was measured using double-distilled water and corrected for temperature and barometric pressure [1]. Zero-oxygen calibration was performed using a saturated sodium sulfite solution. All assays were carried out in the same solutions used for tetrazolium reduction assays. TCA-(membranes), and formaldehyde-(whole cells) treated controls showed no detectable oxygen consumption or formazan formation.

2.6. Calculations

In order to correlate oxygen consumption with formazan production it was necessary to convert values obtained to the number of electron equivalents (eq) required for reduction. The values 2 $\mathrm{eq\,mol^{-1}}$ formazan, and 4 $\mathrm{eq\,mol^{-1}}$ O_2 were used [8,9,19]. The degree of coupling between tetrazolium reduction and oxygen consumption (nmol formazan-equivalents $\mathrm{nmol^{-1}}$ O_2-equivalents) was expressed as the respiration/electron transport system (R/ETS) ratio [9]. To determine the relative effects of various agents on tetrazolium reduction, all whole cell-, or vesicle-formazan values were compared to no-inhibitor or no-substrate controls. Percent deviations from controls were calculated using the equation: [mol formazan$_{\text{(inhibitor or substrate)}}$ – mol formazan$_{\text{(control)}}$/mol formazan$_{\text{(control)}}$] X 100. Statistical significance of differences between treatments and controls was determined using a one-tailed student's *t*-test assuming unequal variance ($\alpha = 0.05$) [41].

3. Results and discussion

3.1. Chemical properties of CTC/INT and their formazans

The physical and chemical properties of CTC and INT, and their corresponding formazans determined in this study are listed in Table 1. During reduction CTC underwent a distinct three-phase transition, from soluble and dark-orange, to a brilliant-orange colloidal suspension (ca. −50 mV), and finally to a dark-orange-red formazan precipitate (ca. −200 mV). During the colloidal phase, redox potential readings took ca. 10 min to increase and stabilize after dithionite addition. No such effects were noted with INT which underwent a two-phase transition, from dark-red and soluble, to the insoluble purple-red formazan (ca. −90 mV). In the presence of ascorbic acid as a reducing agent, CTC was reduced at pH≥4, while INT ($E'_{1/2} = -90$ mV) was reduced at pH ≥5, indicating CTC was more easily reduced than INT [19]. Formazan formation was greatest between pH 5.5–8.5 for INT, and pH 5–10 for CTC. While different tetrazolium salts can be compared on the basis of standard mid-point reduction potentials ($E'_{1/2}$) and reducibilities obtained under identical assay conditions, actual reduction potentials will vary dependent on test conditions (pH, concentration) [3,19]. In general, the less negative a tetrazolium salt redox potential the more easily it is reduced [19,34]. However, results indicated that CTC was slightly more reducible than INT. This may be due to the formation of a colored, colloidal intermediate during CTC reduction at ca. −50 mV. Tetrazolium salts undergo tetrazolinyl-radical cation intermediate stages during reduction, and it appears that this form is more stable and produces a diffuse, poorly-localized formazan at redox potentials higher than ca. −200 mV during CTC reduction (Fig. 1) [18,19]. This would explain the evenly-dispersed, weakly-fluorescent intracellular deposits observed in whole cell CTC reduction assays in the presence of

Table 1
Physical and chemical properties of CTC and INT reduction and their formazans. Molar extinction coefficients, solubilities, and CTC redox potential were determined in this study

Chemical property	Tetrazolium salt		Tetrazolium formazan	
	CTC	INT	CTF	INF
$E'_{1/2}$ (mV)[a]	-200 (-50?)	-90	—	—
Solubility[b]	ca. 50 mM	ca. 5 mM	ca. 200 μM	ca. 250 μM
Color	Colorless to light yellow	Colorless to light yellow	Red-orange	Purple-red
λ_{max}	ND[c]	ND	450 nm	485 nm
$\epsilon_{(90\%\ EtOH)}$	ND	ND	1.624×10^4 l/mol•cm	6.781×10^3 l/mol•cm
Fluorescence	NF[d]	NF	Ex: 380 nm Em: 602 nm	NF

[a] Mid-point reduction potential (pH 7.0, 25°C) with reference to the normal hydrogen electrode. Possible CTF production at ca. -50 mV (see text).

[b] Tetrazolium salt solubilities at 25°C were determined in distilled water, formazan solubilities were in 95% ethanol (formazans are water insoluble). CTC solubility varied with solvent (i.e. seawater, distilled water, etc.).

[c] ND, Not determined.

[d] NF, not fluorescent.

certain inhibitors (antimycin A, KCN, CCCP) (see below). Similar results have been observed using mendola blue ($E_{1/2} = -110$ mV) as an artificial electron donor in *Listeria monocytogenes* and Erlich ascites tumour cells [42–44]. We have also observed this phenomenon in the presence of phosphate over ca. 10 mM in *E. coli* [22,45]. Ethanol-extractable CTF in the absence of CTC (+) cells may also be due to this phenomenon. This was observed when azide inhibited formazan production to 10% of the control value of CTF, but no CTC (+) cells could be detected (Fig. 3).

3.2. *Tetrazolium reduction/respiration ratios*

R/ETS ratios indicate the moles of respiratory electron flow (determined by oxygen consumption, R) per mole of tetrazolium salt reduced (ETS). Thus, R/ETS ratios reflect the degree of coupling between these two processes [9]. Although R/ETS ratios varied with time of incubation, the generally narrow range of this variation indicated that tetrazolium reduction reflected a fairly constant fraction of respiratory activity over 1 h incubation (particularly after 20 min). Using inverted membrane vesicles, R/ETS values with NADH as the substrate were ca. 45–60 for CTC, and 8–14 for INT over 1 h incubation (Table 2). Using succinate as the substrate R/ETS ratios for CTC and INT were ca. 35–60, and 4–10, respectively. Thus, the degree of coupling was roughly similar for individual tetrazolium salts with either succinate, or NADH as the primary electron donor. These results also indicated INT reduction was more tightly coupled than CTC to respiratory electron flow in inverted membrane vesicles by a factor of ca. 5–10 (Table 2). In contrast, R/ETS ratios for whole cells indicated CTC reduction was more tightly coupled to respiratory electron transport than INT by a factor of ca. 10 (Table 2). R/ETS ratios obtained using these systems were also higher than those reported previously using bacterial cell homogenates [8,9]. These results suggest INT and, to a lesser extent, CTC reduction may be limited by penetration through the intact cell envelope (substantivity) to the site(s) of reduction in whole cells [23]. Alternatively, CTC reduction may be more closely linked than INT to dehydrogenases active during endogenous respiration, or may be inhibited by relatively low transmembrane electrochemical gradient ($\Delta\mu_{H}+$) in inverted vesicles compared to whole cells (see below). One additional possibility is differential NADH-D and SDH activities (or the general reduction environment) due to changes in membrane lipids during vesicle formation [10]. Vesicle respiration rates were greater than those for whole cells (104 nmol/min/mg) on a per mg protein basis, with succinate (357 nmol/min/mg) stimulating oxygen consumption more than NADH (181 nmol/O/min/mg). Considering that membrane vesicles lack cytoplasmic protein, this observation is not surprising. Slightly lower R/ETS ratios after 20 min incubation using succinate as compared with

Table 2
Ratios of 0_2 respiration to formazan production for whole cell and inverted membrane vesicle tetrazolium reduction assays

	Respiration/ETS[a]					
	Whole cells[b]		Inverted membrane vesicles			
	Endogenous (Oxidant)		NADH[c] (Oxidant)		SUCCINATE[d] (Oxidant)	
Time (min)	CTC	INT	CTC	INT	CTC	INT
5	2.4±0.24	47.7±3.2	42.2±3.5	11.8±3.4	59.2±4.1	10.5±1.0
10	5.2±0.79	74.3±4.5	46.2±2.5	13.7±2.1	57.0±3.7	8.7±0.77
20	5.7±0.54	76.5±5.1	47.8±2.2	13.1±1.3	38.2±2.4	4.9±1.2
40	7.8±0.97	115±7.1	58.0±3.2	7.8±1.1	34.7±3.3	2.9±0.97
60	8.9±0.76	91.3±4.3	57.6±2.8	9.1±1.9	39.2±3.1	3.6±1.4

[a] Respiration/ETS = mol 0_2 eq./mol formazan eq.
[b] Respiration rate = 104 nmol O/min/mg protein.
[c] Respiration rate = 181 nmol O/min/mg protein.
[d] Respiration rate = 357 nmol 0/min/mg protein.

NADH as the respiratory substrate indicated reduction of CTC and INT was more closely correlated with SDH than NADH-D activity in vesicles.

3.3. *Effects of inhibitors on tetrazolium reduction*

Mid- and terminal-chain blockage were used to determine sites of CTC and INT reduction. In general, blockage of electron transport below the site of tetrazolium reduction (i.e., closer to the terminal oxidase) should either increase, or not effect the amount of formazan produced compared to controls [17,18]. Blockage prior to the site of reduction (i.e., closer to the primary dehydrogenase) should decrease formazan production (Fig. 1). Effectors of ATP synthesis (ADP, ATPase depleted membranes), and a proton conductor (CCCP) were also utilized to determine their effects on tetrazolium reduction. Inhibition of respiratory electron transport processes using inverted membrane vesicles indicated site(s) of CTC and INT reduction under defined dehydrogenase (SDH or NADH-D) activities, while experiments using whole cells indicated site(s) of reduction during endogenous respiration (Figs. 1, 2, 3a) [39].

The production of both INF and CTF in inverted membrane vesicles using succinate or NADH as the reductant indicated CTC and INT reduction was mediated through activities of both membrane-bound SDH-, and NADH-D. Rotenone treatment strongly increased CTF, but decreased INF production in vesicles, suggesting both CTC and INT were reduced by NADH-D, but that CTC was reduced prior to the FeS-2 center of NADH-D in this system (Figs. 1 and 2) [33,39]. Decreased production of both formazans in rotenone-treated whole cells also indicated reduction by NADH-D (Fig. 3a).

Malonate inhibition (nearly 100% for CTC), and TTF stimulation of CTF and INF production in vesicles indicated both CTC and INT were also reduced by SDH, prior to the ubiquinone-binding subunit (Figs. 1 and 2) [28,46,47]. The nearly 7-fold

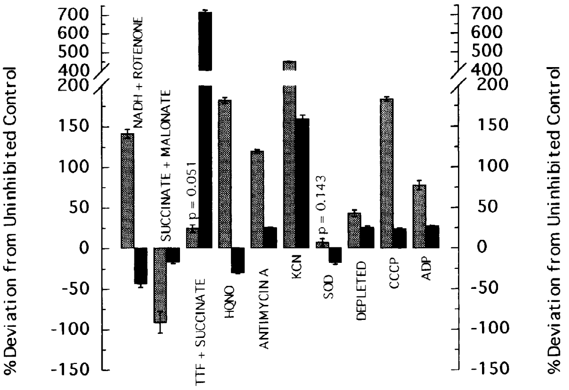

Fig. 2. Effects of various inhibitors on CTC (shaded bars), and INT (solid bars) reduction in *E. coli* inverted membrane vesicles. Data are expressed as percent deviations in formazan production from substrates (NADH or succinate) alone. Sites of inhibition are listed in Table 2. P-values are ≤ 0.05 except where noted. Error bars represent deviation from the mean ($n = 2$). Calculations and abbreviations are as described in materials and methods.

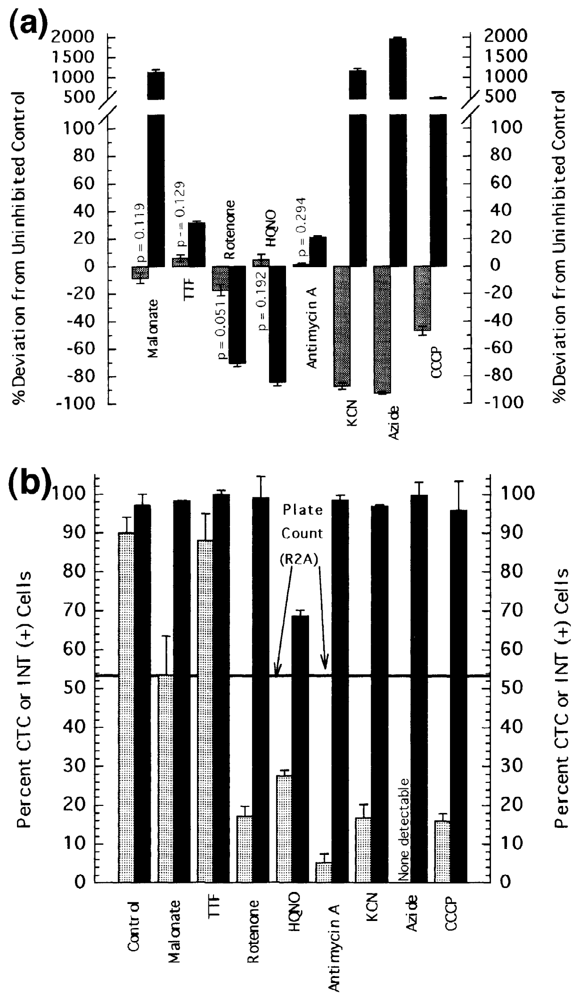

Fig. 3. Effects of inhibitors on aerobic CTC (shaded bars), and INT (solid bars) reduction in whole cells of *E. coli*. Formazan production values are expressed as a percentage deviations from control without inhibitors (a). P-values are ≤ 0.05 except where noted. Percent CTC (+) cells by epifluorescence microscopy (b). Horizontal line indicates the percent of total AODC cells enumerated by R2A plate counts. Error bars represent (a) deviation from the mean ($n = 2$), and (b) standard deviation. See Materials and methods (Section 2) for calculations and abbreviations.

increase in INF formation with TTF indicated INT reduction was tightly coupled to SDH activity, consistent with R/ETS ratio results (see below) (Fig. 2). Using whole cells, SDH inhibition by malonate or TTF had no significant effect on CTC-, while increasing INT-reduction 10-fold (Fig. 3a). Thus, while CTC and INT were reduced by both SDH and NADH-D in vesicles, CTC reduction did not appear to be mediated primarily by SDH in whole cells. Increased INF formation in malonate-treated whole cells may be due to stimulation of other dehydrogenases active during endogenous respiration with $E'_{1/2}$ values higher than that of INT, such as formate dehydrogenase ($E'_{1/2} = -105$ mV) [31].

Inhibition at ubiquinone (HQNO) and cytochrome *b* (antimycin A) increased CTC reduction in vesicles, while no significant effect was observed using whole cells (Fig. 2, Fig. 3a). This indicated that CTC was reduced prior to ubiquinone in the respiratory chain, as has been reported for *Micrococcus luteus* (Fig. 1) [2,39]. In both vesicles and whole cells INF formation was decreased by HQNO, but strongly increased by inhibition at points further along the transport chain (antimycin A, cyanide, azide), indicating INT reduction by ubiquinone and possibly cytochrome $b_{555,\ 556}$ (Figs. 1–3a) [31,39]. In contrast, KCN and azide inhibition of cytochromes *o* and *d* strongly decreased CTF formation in whole cells, suggesting CTC reduction by the terminal oxidases (Fig. 3a) [26,31]. Similar results have been reported with azide-treated *Pseudomonas diminuta* [4]. Considering the $E'_{1/2}$ of CTC (-200 mV), it appeared unlikely that the terminal oxidases could directly reduce CTC, as their mid-point potentials are ca. $+70$ to $+280$ mV (Fig. 1). In addition, inhibition of electron carriers previous to the terminal oxidases increased CTC reduction (HQNO, antimycin A in vesicles), an observation which contradicts reduction after these points by the terminal oxidases. SDH and NAD-H are indirectly inhibited by blockage of terminal oxidases, and cyanide is known to block FeS-centers in several enzymes directly [18, 31,47,48]. However, SDH and NADH-D inhibition by KCN are not consistent with increased reduction of both CTC and INT in KCN-treated vesicles, and inhibition by azide (which does not effect FeS-centers) in whole cells (Figs. 2 and 3a).

These observations may be due to a reduced (more positive) transmembrane potential due to the action of KCN and azide. Inhibition of the terminal oxidases also inhibits the major site of proton consumption (in the formation of H_2O by terminal oxidases) and $\Delta\mu_H+$ generation in *E. coli* cells (Fig. 1). This gradient, formed across the membrane, can be significant (-10 to -180 mV, negative inside) and contributes to the overall redox environment in which tetrazolium reduction takes place [15,19,26,30,31]. CTC reduction is expected to be more sensitive to an increase in redox potential than INT due to CTC's lower $E'_{1/2}$, and thus may be selectively inhibited to some degree by agents such as KCN and azide which increase transmembrane potential. The observed decreased CTF-, and increased INF-formation by collapsing $\Delta\mu_{\mathrm{H}}+$ in whole cells with CCCP support this hypothesis (Fig. 3a). Although CTC reduction increased in CCCP-treated inverted vesicles, proton consumption is on the outside surface of the membrane in buffered solution, inhibiting normal (i.e., whole cell, or non-inverted vesicle) $\Delta\mu_{\mathrm{H}}+$ formation, but still allowing some degree of uncoupling (Fig. 2) [35]. Dismutation of superoxide anions in vesicles using SOD had no effect on CTC reduction, while slightly decreasing INF formation (Fig. 2). INT reduction may thus be mediated to some extent by production of superoxide anions as hypothesized by Seidler [19]. However, it should be noted that intracellular CTC reduction by superoxide anion in *whole cells* cannot be ruled out. Uncoupling (CCCP), depletion of ATPase, and ADP all increased CTF and INF formation in vesicles (Fig. 2). Since these conditions are all known to increase respiratory electron transport activity, it appears CTC and INT reduction was linked to electron transport processes which were, in turn, linked to ATPase activity [35,37,39].

3.4. Direct counts

In general, direct counts of CTC and INT (+) aerobic cells reflected formazan production trends compared to controls (Fig. 3). However, cell CTC (+) counts were decreased significantly using HQNO and antimycin A, while CTF production was unaffected (Fig. 3). This suggests that factors other than the total amount of CTF produced affect formation of fluorescent CTF crystals. Size and number of fluorescent CTF crystals per cell was highly variable depending on the particular inhibitor used. Several inhibitors, notably HQNO, antimycin A, cyanide, azide, and CCCP resulted in very faintly fluorescent CTF which was evenly distributed throughout cells. Formation of visible INF crystals appeared less sensitive to the effects of inhibitors than CTF (Fig. 3). However, visualization of INF crystals was difficult compared to fluorescent CTF, particularly when deposits were small. Using control cells, plate counts on R2A indicated 52.6±1.4% of total cells (AODC) were able to form colonies on this medium while INT and CTC (+) cell numbers were 97.0±2.2, and 89.9±0.9% of total cell numbers, respectively (Fig. 3b). INT (+) cell numbers were consistently higher than plate counts, even in the presence of inhibitors (Fig. 3b). TTF, rotenone, and azide were the most effective agents for increasing INT (+) cell counts using EFM (Fig. 3b). CTC (+) cell numbers were greatest using TTF, and in the uninhibited control (Fig. 3b). All CTC (+) cell numbers were significantly below plate counts, except for the control, malonate, and TTF samples (Fig. 3b). This may have been due to the use of 1 mM CTC, as maximal formazan production has been found at 2–6 mM in several bacteria (4, 42). The use of cyanide or azide to increase tetrazolium reduction (as has been suggested for INT) will increase INF formation significantly [5,9,16,17,49] (Fig. 3). However, this technique may not always be appropriate for CTC reduction assays in intact bacterial cells [21,44].

3.5. CTC and INT reduction under anaerobic conditions

Tetrazolium reduction in whole cells was assayed under anaerobic conditions which induce 4 different electron transport pathways: Glucose fermentation, formate dehydrogenase-fumarate reductase (> +30 mV), glycerol dehydrogenase-fumarate reductase, and nitrate reductase (+20 to +220 mV) under endogenous respiration (Fig. 4) (29). R2A-P was added as a general source of substrates. Glucose fermentation and R2A-P medium (which contains glucose and pyruvate) stimulated reduction of both tetrazolium salts over 100%, indicating that the various dehydrogenases postulated to be active during glucose fermentation reduce CTC, and particularly INT. Induction of the formate dehydrogenase-fumarate reductase pathway increased CTC reduction to the greatest degree (Fig. 4). CTC is most likely reduced by formate dehydrogenase (−145 to −360 mV) in this system [29]. Stimulation of CTC reduction by formate is similar to results found with

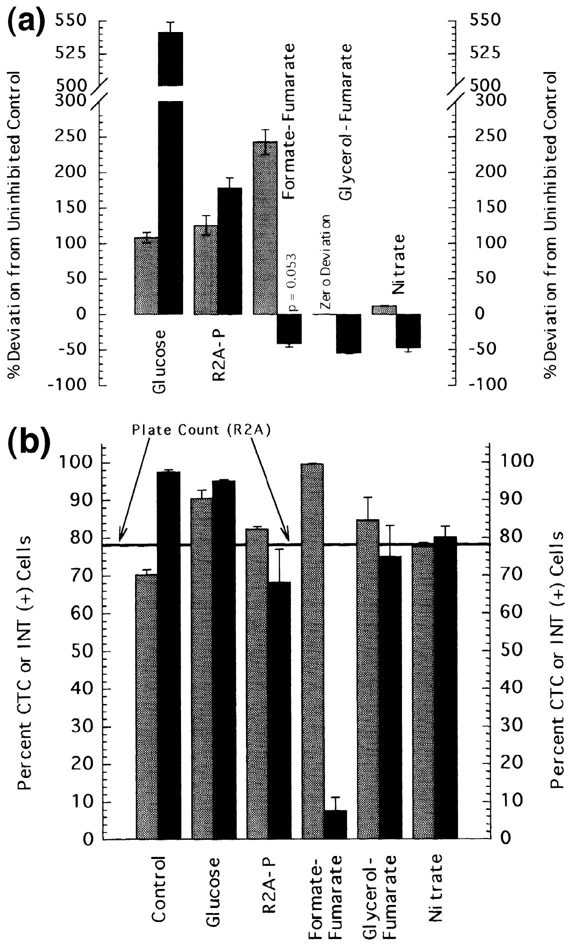

Fig. 4. Effects of various substrates and electron acceptors on CTC (shaded bars) and INT (solid bars) reduction by whole *E. coli* cells under anaerobic conditions. Formazan production values are expressed as a percentage deviations from control without substrate(s) (a). P-values are ≤ 0.05 except where noted. Percent CTC (+) cells by epifluorescence microscopy (b). Horizontal line indicates the percent of total AODC cells enumerated by R2A plate counts. Error bars represent (a) deviation from the mean ($n=2$), and (b) standard deviation. See Materials and methods (Section 2) for calculations and abbreviations.

aerobic *Campylobacter jejuni* cells [50]. Glycerol-fumarate and nitrate had little, or no effect on CTF formation, but CTC (+) cell numbers with glycerol-fumarate increased 5–10% over control values (Fig. 4). In contrast, INF formation, and INT (+) cell numbers decreased with fumarate or nitrate as the terminal electron acceptor (Fig. 4a). Numbers of CTC (+) and INT (+) cells were above ca. 70% under all anaerobic incubation conditions tested (except INT in the presence of formate-fumarate) (Fig. 4b). Glucose and formate-fumarate produced the largest numbers of CTC (+) cells, while INT (+) numbers were greatest in the control and glucose samples (Fig. 4b). With the exception of the control, CTC (+) numbers were equal to, or greater than plate counts on R2A in all cases. Thus, substrate addition appeared necessary for CTC (+) counts to exceed plate counts when using 1 mM CTC. INT (+) cells numbers were 5–20% greater than plate counts in the control, glucose, and nitrate samples (Fig. 4b). INT and CTC were reduced to significant degrees under all anaerobic conditions employed in this study, with glucose particularly stimulating formazan production.

In summary, studies using both inverted membrane vesicles and whole cells indicated CTC and INT were reduced by both SDH and NADH-D (Fig. 1). However, the specific sites involved in the reduction of CTC and INT were somewhat different since CTC appeared to be reduced by dehydrogenases other than SDH in whole cells. In addition, INT could be reduced by ubiquinone, possibly cytochrome $b_{555,556,}$ superoxide anion, and perhaps other primary dehydrogenases which donate electrons to ubiquinone (Fig. 1). These results are consistent with reduction sites inferred from INT and CTC mid-point redox potentials, as well as being similar to sites determined for eukaryotic mitochondrial electron transport chains, and *Micrococcus luteus* (Fig. 1) [2,9,18,19,31]. Both CTC and INT were reduced under most anaerobic conditions tested, particularly glucose fermentation (Fig. 4a). Coupling of tetrazolium reduction to respiration (as measured by oxygen consumption) differed between whole cells (CTC>INT) and inverted membrane vesicles (INT>CTC), but in general ranged between 10 and 40 pairs of respiratory electrons for every tetrazolium molecule reduced. CTC reduction appeared more sensitive to effectors of intracellular redox potential than INT in whole cells, and appeared to produce a weakly-, or non-fluorescent formazan at redox potentials higher than ca. −200 mV. Also, use of inhibitors of terminal oxidases (i.e., cyanide, azide), and uncouplers may not be useful to maximize whole cell prokaryotic CTC reduction assays.

Tetrazolium salts are converted to formazans by all reducing systems possessing actual redox potentials more negative than the tetrazolium/formazan system [19]. Therefore, use of CTC and INT in reducing environments (sediments, media containing reducing agents [thioglycollate, ascorbate, etc.]) where in situ redox potentials are lower than the $E_{1/2}$ values of the tetrazolium salts will lead to non-biological tetrazolium reduction [4,19]. This could explain observations of INF on non-living detrital material in benthic suspensions, and CTC reduction by some growth media [21,51]. In addition, bacterial exopolymeric material (alginate from *Pseudomonas aeruginosa*) has been reported to reduce CTC and INT in the absence of viable cells [52]. This may be due to non-biological reduction by ionized carboxyl-, or pyruvyl-groups in these polysaccharides, particularly under acidic conditions.

In this study reduction of INT and CTC appeared closely linked to bacterial respiratory activity. However, significant intracellular reduction of both tetrazolium salts was observed in formaldehyde-treated cells if sufficient time (5–15 min) had not passed before CTC or INT addition (data not shown). This effect was much more pronounced for INT than CTC, requiring ca. 15 min contact with formaldehyde to completely eliminate INT-, and 5 min to eliminate CTC-reduction (CTF produced was diffuse and poorly fluorescent). Similar results were noted using INT in a recent study of tetrazolium reduction in *Campylobacter jejuni* by Boucher et al. [50]. This may be related to ‘nothing dehydrogenase’ activity noted in several histochemical studies attributed to residual reduction potential in fixed cells [19,20,53]. Since INT would continue to be reduced at higher redox potentials than CTC as the potential dissipates (i.e. >100 mV, <90 mV), this may explain more persistent INT reduction after fixation. No detectable tetrazolium reduction was observed in the presence of nutrients or formalin without cells. Therefore, to prevent residual formazan formation it is important to fix cells with formaldehyde for at least 10–15 min prior to tetrazolium salt addition. This differs from results using R2A as a nutrient source reported by Bovill et al., although the concentration of R2A in the present study was 10-fold lower [42].

Formazan production by inverted membrane vesicles suggests spontaneous tetrazolium reduction by ‘nonviable’ cell membranes. However, it should be emphasized that vesicles are produced under reducing conditions (dithiotreitol) in order to maintain the reduced state of electron-transport components [35]. Indeed, a reducing environment is important to obtain vesicles with active dehydrogenases [35,38,39]. In the environment (under non-reducing conditions) ETS components would be expected to oxidize, degrade, and become nonfunctional. However, reduction of CTC or INT by membrane-bound ETS components (in the absence of viable cells) in reducing environments such as sediments cannot be ruled out.

Information obtained using CTC and/or INT to assess respiratory activity in prokaryotic cells will vary depending on the organism and assay conditions. For instance, it appears some active bacteria do not reduce INT, or CTC under certain conditions [23,24,36,54]. This could be related to low tetrazolium salt substantivity (association with cell components, permeability), unique respiratory pathways (for instance those bacteria which can respire, but don’t contain a complete Kreb’s cycle), or low cellular $\Delta\mu_{\mathrm{H}}+$ (particularly using CTC) in some bacteria [19,23].

To date CTC reduction has been reported in *E. coli*, *Salmonella typhimurium*, *Yersinia enterocolitica*, *Enterococcus faecalis* [36], *Klebsiella pneumoniae* [16], *Paracoccus denitrificans* [13], *Micrococcus luteus* [2], *Pseudomonas fluorescens* [55], *Pseudomonas putida* [4], *Campylobacter jejuni* [49], *Listeria monocytogenes* [42], *Listeria innocua* [13], *Shigella flexneri*, *Vibrio cholerae* (non-01) [56], *Vibrio vulnificus*, and *Helicobacter pylori* [24]. However, considering the relatively variable nature of prokaryotic respiratory chains compared to eukaryotes, differences in tetrazolium reduction between organisms are to be expected. Therefore, selection of an appropriate tetrazolium salt for an application, and interpretation of data should take into account the histochemical properties of these indicators of respiration in prokaryotic systems.

From the results of this study, application of methods using tetrazolium reduction for assessments of respiratory activity and/or cell viability may benefit from the following suggestions: For each system in which tetrazolium salt reduction is to be used, it is essential to run cell-free, and/or killed-controls to check for abiotic reduction to formazan. Choice of killing agent should take into account that certain agents, such as cyanide and other respiratory inhibitors, will increase tetrazolium reduction in some situations. Killing-agents should also have sufficient time to act on cells prior to addition of tetrazolium salt so that residual reduction potential can dissipate/oxidize. In this study at least 15 min. with 3.7% formaldehyde was required. Use of non-defined substrates such as yeast extract, peptone, beef extract, etc., should be avoided when possible, or carefully controlled, as these may contain reducing substances which will lead to abiotic tetrazolium reduction. The use of chemical reducing agents (ascorbic acid, thioglycollate, etc.) should also be avoided when possible, or care taken as to reaction pH and redox potential. The use of tetrazolium reduction to formazan for assessing cellular viability, particularly on unknown mixed bacterial populations, should be viewed with some caution. This is due to individual and population cellular metabolic diversity, which may result in viable cells which either do not reduce the particular tetrazolium salt at all, or in insufficient quantities. Intracellular formazan deposits are only visible microscopically when sufficient tetrazolium salt has been locally-reduced. It also appears that CTC forms a diffuse, weakly-fluorescent formazan under some incubation conditions [22]. The use of respiratory inhibitors or uncouplers such as TTF, cyanide, or CCCP, which appear to significantly increase formazan production, may prove quite useful for optomizing tetrazolium reduction methods where microscopic examination of cells is required. Addition of carefully selected substrates (particularly in systems where availability limits respiratory activity) to stimulate respiratory activity should also aid in optomizing tetrazolium reduction assays. Carefully designed controls, and choice of appropriate inhibitors and substrates for use in assays, will result in more accurate and meaningful tetrazolium reduction data.

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