Chem. Pharm. Bull. **41**(6) 1118—1122 (1993) Vol. 41, No. 6

A New Sulfonated Tetrazolium Salt That Produces a Highly Water-Soluble Formazan Dye

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A new tetrazolium compound, sodium salt of 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2*H*-5-tetrazolio]-1,3-benzene disulfonate (1a), which produces a highly water-soluble formazan dye, due to the presence of two sulfonate groups, was synthesized and its potential utility evaluated in assays of NADH and cell proliferation. The compound proved to have a similar sensitivity to 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2*H*-tetrazolium hydroxide (XTT) in the assay of NADH and was also useful as an indicator of cell viability, with less cytotoxicity than XTT, in the proliferation assay using P388 cell lines.

Keywords tetrazolium salt; formazan; water solubility; colorimetric assay; cell proliferation assay; bioreducibility

The unique property of tetrazolium salts to produce highly colored formazan dyes under reductive conditions has found wide application in clinical chemistry. The method, based on the tetrazolium salt/formazan system, is also extremely useful in the colorimetric determination of bioreducibility of cells which originates from various dehydrogenase activities. Particularly, measuring the cell viability or proliferation rate in order to evaluate the cytotoxic activity of drugs (*e.g.*, in the tumor sensitivity test) is one of the main areas of application of this assay method and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) has been used most widely because the absorption maximum (λ_{max}) of its formazan product is at a longer wavelength (565 nm) and because it can be reduced without an electron carrier.[2)] Unlike tetrazolium salts that are normally water-soluble because of the cationic character of the heterocyclic tetrazolium ring, the fomazan products are insoluble in water due to their neutrality, which allows histochemical localization of sites of enzyme activity.[3)] The water insolubility of formazan products, however, is a disadvantage when spectrophotometric measurement of formazan products in the colorimetric proliferation assay of cells is required, since an extra step needs to be added to the procedure to solubilize the formazan products.[4)] A pyridine analog, containing a pyridyl group that is linked to the tetrazolium ring, provided a limited solution to this problem of water solubility. This compound yields a water-soluble formazan at neutral to acidic pH with a λ_{max} of 539 nm, but the sensitivity was reported to be only 70% of that obtained with nitrotetrazolium blue, a common reagent used in clinical assays, for the measurement of lactate dehydrogenase activity in human serum.[5)] Paull and co-workers[6)] have developed a new sulfonated tetrazolium salt, 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2*H*-tetrazolium hydroxide (XTT), which gives a highly water-soluble formazan product; the compound has been successfully used in the assay of cell growth and drug sensitivity when cultured with tumor cell lines,[7)] as well as in cell viability and proliferation assays with normal activated T cells.[8)] As shown typically by these experiments, the water solubility of formazan products is important particularly when large-scale drug screening is attempted.

In our continuing efforts to exploit a water-soluble formazan dye, we recently synthesized a new tetrazolium compound, sodium salt of 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2*H*-5-tetrazolio]-1,3-benzene disulfonate (**1a**, Chart 1), which is a water-soluble version of the well-known 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2*H*-tetrazolium chloride (INT, **1b**). We wish to describe here the unique characteristics and merits of this compound compared with XTT as a highly sensitive tetrazolium reagent that produces a water-soluble formazan in NADH and cell proliferation assays.

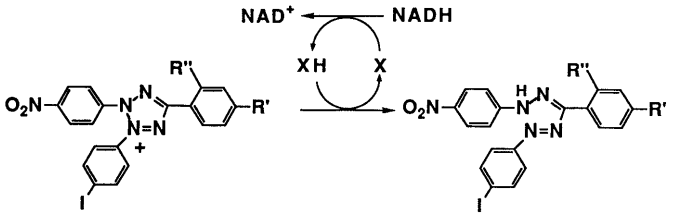

Chart 1. Reaction Scheme of Tetrazolium Salt **1a** with NADH in the Presence of 1-Methoxy PMS

Experimental

Apparatus Thin-layer chromatography (TLC) was performed with Kieselgel 60 F_{254} plates (Merck, Darmstadt, Germany); A solution of *n*-butanol–acetic acid–pyridine–water (4 : 1 : 1 : 2, v/v) was used as the developing solvent. Absorbance spectra were recorded on a Shimadzu UV-210A spectrophotometer. ^{1}H-Nuclear magnetic resonance (NMR) spectra were measured on a Bruker AC-200 spectrometer, operating at 200 MHz, with 3-(trimethylsilyl)-1-propanesulfonic acid sodium salt (DSS) as an internal standard. The splitting patterns were designated as: s, singlet; m, multiplet. IR spectra were recorded in KBr disks on a Hitachi 270-30 spectrometer. Fast atom bombardment mass spectra (FAB-MS) were measured on a JEOL JMS-AX 505W mass spectrometer. Uncorrected melting points were obtained on a Yamato MP-21 melting-point apparatus.

Materials XTT was obtained from Sigma (St. Louis, MO, U.S.A.) and used as purchased. INT and 1-methoxy-5-methylphenazinium methylsulfate (1-methoxy PMS), obtained from Dojindo Laboratories (Kumamoto, Japan), were used without further purification. Stock solutions of **1a**, XTT and INT for the NADH assays were all prepared as 1 mM aqueous solutions. An aqueous stock solution of NADH was prepared at a concentration of 5 mM. The following buffers were used: 50 mM citrate (pH 5—6), 50 mM Tris (pH 7—9), or 50 mM glycine–HCl (pH 10—12).

Cell Line and Culture P388 D_1 (murine lymphoid neoplasm; American Type Culture Collection, Rockville, MD, U.S.A.) was maintained in RPMI 1640 (Gibco Laboratories, Grand Island, NY, U.S.A.) supplemented with 10% FBS (Gibco Laboratories), 200 units/ml penicillin G (Meiji Seika Ltd., Tokyo, Japan) and 100 μg/ml streptomycin sulfate (Meiji Seika). The cells were added to the medium (40 ml) in a tissue culture flask to give a concentration of 1.59×10^6 cells/ml, and then the flask incubated at 37 °C in 5% CO_2 for 12 h.

2,4-Disulfobenzaldehyde-4'-nitrophenylhydrazone, Disodium Salt A suspension containing 4-formyl-1,3-benzene disulfonic acid, disodium salt (20.3 g, 65.3 mmol) and *p*-nitrophenyl hydrazine (10.0 g, 65.3 mmol) in methanol (300 ml) was heated at 50 °C for 1.5 h. The resulting precipitate was collected, washed with a small volume of methanol and dried under vacuum to afford 20.9 g (72%) of the hydrazone as a reddish-orange powder. mp > 300 °C. TLC: *Rf* = 0.48. ^{1}H-NMR (D_2O) δ: 7.02—8.14 (m, 7H, ArH), 8.49 (1H, s, –N=CH–). IR (cm^{-1}): 1620 (C=N), 1510 (NO_2), 1330 (SO_2). FAB-MS *m/z*: 446 $[M+H]^+$.

Disodium Salt of 4-[1-(4-Iodophenyl)-5-(4-nitrophenyl)-formaz-3-yl]-1,3-benzene Disulfonate (2a) Hydrochloric acid (5.6 ml, 67.5 mmol) was added to a stirred suspension of *p*-iodoaniline (4.93 g, 22.5 mmol) in water (20 ml) which had been previously warmed to 70 °C. The suspension was cooled to 0 °C and to this was added sodium nitrite (1.58 g, 22.5 mmol) in water (20 ml) at 0—5 °C. After being stirred at 0 °C for 30 min, the solution was added to a pre-cooled solution of the hydrazone (10.0 g, 22.5 mmol) in water (200 ml); finally KOH (5.9 g, 90 mmol) in water (20 ml) was added dropwise while the temperature was kept at 0—5 °C. The solution was stirred at this temperature for 1 h and then allowed to warm up to room temperature. The pH of the reaction mixture was adjusted to *ca.* 1 with 3 M HCl. Concentrating the reaction mixture to dryness gave a residue which was taken up in 400 ml of a methanol–ethanol mixture (2 : 1, v/v). The solution was filtered and the filtrate was kept overnight at −10 °C to yield a crude product. Recrystallization from the methanol–ethanol mixture produced 7.3 g (48%) of formazan **2a** as a reddish-brown powder. mp 280—282 °C (dec.). TLC: *Rf* = 0.63. ^{1}H-NMR (D_2O) δ: 7.20—8.41 (m, 11H, ArH). IR (cm^{-1}): 1605 (C=N), 1510 (NO_2), 1340 (SO_2). FAB-MS *m/z*: 652 $[M-Na]^-$.

Sodium Salt of 4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2*H*-(5-tetrazolio]-1,3-benzene Disulfonate (1a) Hydrochloric acid and *n*-butylnitrite were sequentially added to a pre-cooled (0 °C) solution of formazan **2a** (3.5 g, 5.5 mmol) in methanol (120 ml) with stirring. The solution was allowed to warm to room temperature and stirred overnight. After being treated with activated charcoal, the reaction mixture was concentrated to dryness to leave an oily residue, to which tetrahydrofuran was added to prompt crystallization. The crude product thus obtained was redissolved in methanol and treated again with activated charcoal. The methanolic solution was concentrated to give a colorless oil. Addition of ether yielded crystals, which were collected and dried to give 2.35 g (66%) of **1a** as a yellowish powder. mp > 300 °C. ^{1}H-NMR (D_2O) δ: 7.42—8.45 (m, 11H, ArH). IR (cm^{-1}): 1630 (C=N), 1540 (NO_2), 1360 (SO_2). FAB-MS *m/z*: 651 $[M]^+$. *Anal.* Calcd for $C_{19}H_{11}IN_5NaO_8S_2$: C, 35.0; H, 1.7; N, 10.8. Found: C, 34.5; H, 1.9; N, 9.8.

NADH Assay To a mixture containing **1a** (0.1 mM) and 1-methoxy PMS (5 μM) in 5 ml of Tris–HCl buffer (50 mM, pH 8.0), 0—50 μl of NADH (final concentrations, 0—50 μM) was added and the solution incubated at 22 °C for 5 min. The absorbance was measured at 438 nm (pH 8.0). The pH was then raised to 12.5 by adding 100 μl of 4 M NaOH and the absorbance at 593 nm at this pH was recorded against a reagent blank prepared in the same manner but without adding NADH.

Cell Proliferation Assay A solution of **1a** was prepared at a concentration of 1 mM in phosphate buffered saline containing 1-methoxy PMS (20 μM), then sterilized (with a 0.22-μm filter) and stored at 4 °C. Four ml of this solution of **1a** was immediately added to a culture medium (final volume, 40 ml) which contained 8.9×10^4 cells/ml after 12-h pre-incubation, and the cells were then incubated for an additional 100 h. An aliquot (4 ml) was taken to count the number of the cells grown in the medium ($> 1.0 \times 10^7$ cells/ml) using a Bürker–Türk hemocytometer (Nitirin, Tokyo, Japan) and the absorbance of the aliquot supernatant, obtained by centrifuging, was measured at 428 nm.

Results and Discussion

The presence of the two sulfonate groups confers water-solubility on formazan **2a** as well as on the tetrazolium salt **1a** that has a negative net charge. Although there was the possibility that the two sulfonate functionalities might substantially affect the reduction potential of the tetrazolium salt, through further decreasing electron density of the cationic tetrazolium nucleus, we reasoned that, although one sulfonate group might be enough for the formazan product to be water-soluble, the tetrazolium salt with only one sulfonate group would not have a sufficient water-solubility presumably due to intramolecular salt formation. In addition, a compound with the structure of **1a** is readily prepared by synthesis because of the commercial availability of the starting disulfonated aldehyde. Formazan **2a** was synthesized from 4-formyl-1,3-benzene disulfonic acid following a general method[3] which involves hydrazone formation and subsequent diazonium coupling. As expected, formazan **2a** has a water-solubility greater than 0.1 M. Oxidation of **2a** was carried out with *n*-butyl nitrite to give tetrazolium salt **1a** in a moderate overall yield.

The potential utility of **1a** in colorimetric assays of cell bioreducibility was evaluated first by quantifying NADH with this compound. The reaction scheme is illustrated in Chart 1, in which a two-electron reduction of **1a** with NADH is mediated by an electron carrier, 1-methoxy PMS. The assay of NADH with **1a** is based on the fact that the amount of formazan **2a** produced is proportional to the analyte, NADH.

Figure 1 shows the absorption spectra of the reactions of **1a** with various amounts of NADH. After being incubated with NADH in the presence of 1-methoxy PMS at pH 8.0 (spectra a—c), the pH of the reaction mixture was raised to 12.5 (spectra d—f). The formazan product **2a**, having a λ_{max} at 438 nm with a molar absorptivity (ε) of approximately 3.7×10^4 at pH 8.0, exhibits an intense color at the longer wevelength of 593 nm ($\varepsilon = 5.7 \times 10^4$) upon addition of aqueous alkali. The bathochromic shift of absorption at alkaline pH (438→593 nm) is ascribed to a possible deprotonation at the secondary amine site of the formazan nucleus whose pK_a value is lowered due to the presence of the two electron-withdrawing sulfonate groups. While the λ_{max} of 438 nm of **2a** is shorter than that of either XTT (470 nm) or INT (515 nm), it is longer at alkaline pH than that of MTT (565 nm) which has the longest λ_{max} of

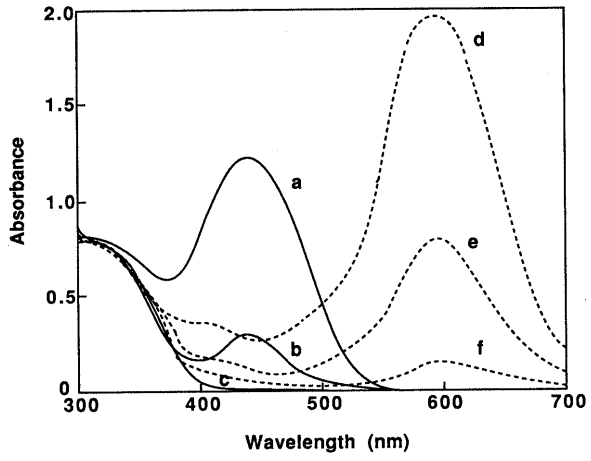

Fig. 1. Absorption Spectra of **1a** in the NADH Reduction, Mediated by 1-Methoxy PMS

See the experimental section for the procedure. The reactions were carried out at 22 °C and pH 8.0 (a—c); the solutions were then brought to pH 12.5 (d—f) with aqueous NaOH. The concentrations of NADH are: a, d: 50 μM; b, e; 20 μM; c, f: blank.

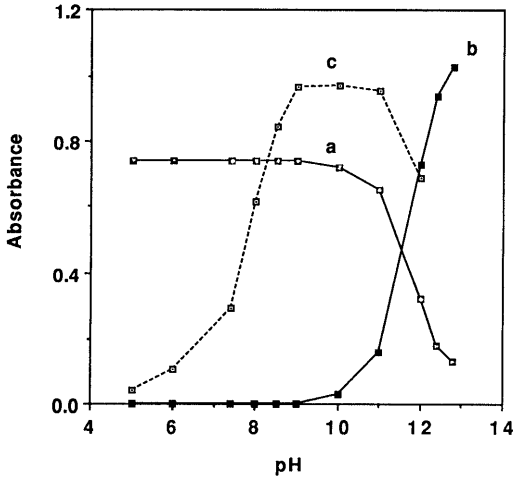

Fig. 2. pH–Absorbance Profiles of Formazan **2a** and NADH Reduction of **1a**, Mediated by 1-Methoxy PMS

(a) formazan **2a** (20 μM) at 438 nm; (b) formazan **2a** (20 μM) at 593 nm; (c) reductions of **1a** (0.1 mM) with NADH (30 μM), mediated by 1-methoxy PMS (5 μM), were carried out for 5 min at 22 °C and monitored at 438 nm.

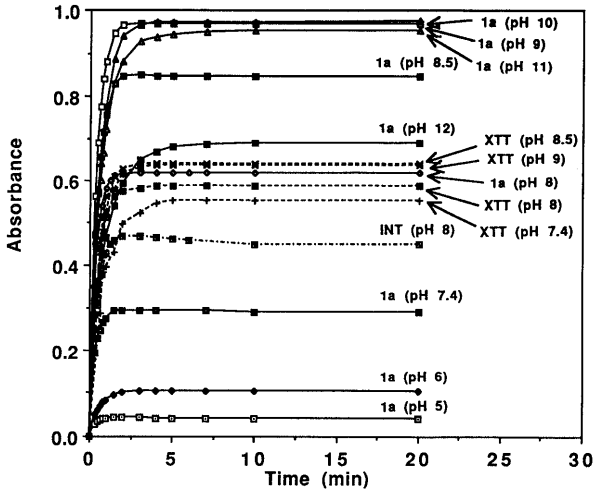

Fig. 3. Time Courses of the NADH Reductions of **1a** Mediated by 1-Methoxy PMS at Various pH Values in Comparison with XTT and INT under the Same Reaction Conditions

The reactions of **1a** (0.1 mM) with NADH (30 μM), in the presence of 1-methoxy PMS (5 μM), were carried out at 22 °C and monitored at 438 nm. The reactions of XTT and INT were monitored at 470 nm (for XTT) or 515 nm (for INT).

all the tetrazolium salts currently in use.

Figure 2 depicts the pH profiles of formazan **2a** absorbance observed both at 438 and 593 nm and of the reduction of **1a** with NADH, in the presence of 1-methoxy PMS, measured at 438 nm. The increase in the absorbance of **2a** at 593 nm (curve b, deprotonated form) corresponds to the decrease observed at 438 nm (curve a, protonated form) and from this the pK_a value of the secondary amine functionality of **2a** is estimated to be approximately 11.5. Formazan **2a** thus exists mostly in its deprotonated from above pH 12. A formazan product of XTT, which also carries two sulfonate groups at different aromatic positions, gives a similar pH profile due to deprotonation at the heterocyclic nucleus. With the ε value at 593 nm being 1.54-fold greater than that observed at 438 nm, it is clear that measuring the absorbance (ΔA) of **2a** at higher pH allows NADH to be assayed with a higher sensitivity ($\Delta A/\Delta$[NADH]), although cellular reductions of tetrazolium salts are usually carried out at neutral pH. Because of this pH differential, a rate assay both at 593 nm and neutral pH is infeasible and, in addition, an end-point procedure offers a more sensitive assay than does rate assay. The ε value of **2a** at 438 nm, as indicated in the figure, is constant over a broad pH range below 10. From the pH–absorbance profile of the NADH reduction of **1a** (curve c), the optimum pH for the reaction is in the range, 8.5 to 10.5. At higher pH values, the profile is similar to that of **2a** observed at the same wavelength of 438 nm, whereas it is in sharp contrast to **2a** at a pH below 8, with a large decrease in absorbance. These observations are rationalized by assuming a diminished production of **2a** at lower pH. Unlike **1a**, XTT gives a relatively constant sensitivity toward NADH at neutral pH under the same conditions (data not shown).

The time courses of the reduction of **1a** with NADH in the presence of 1-methoxy PMS at various pH values were compared with those of XTT and INT under identical conditions (Fig. 3). The formation of the formazan from **1a**, XTT and INT is very rapid, all being complete within a few minutes and there seems to be no decrease in formazan

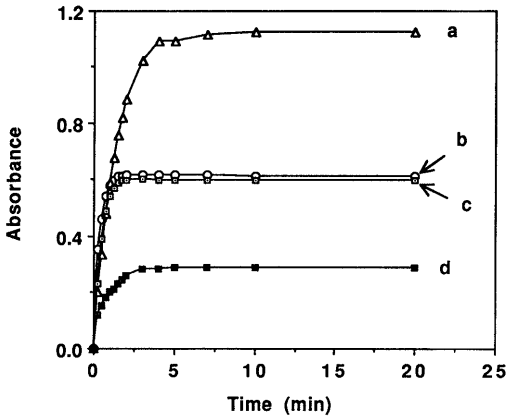

Fig. 4. Time Courses of the NADH Reductions of **1a**, Mediated by 1-Methoxy PMS in the Presence of Various Detergents

The reactions were carried out as described in Fig. 3 with a 0.1% (w/v) detergent at pH 8.0. (a) CTMAB; (b) blank; (c) Triton X-100; (d) SDS.

once a plateaus has been reached. As described above, incomplete formazan production gives rise to a poorer sensitivity for **1a** at acidic pH, since neither a decreased reaction rate nor a lower ε value for **2a** is observed in these experiments.

In order to maximize the sensitivity in terms of NADH, we next examined the effect of detergents on the reduction rate of **1a** with NADH using Triton X-100, cetyltrimethylammonium bromide (CTMAB) and sodium dodecyl sulfate (SDS) as a non-ionic, cationic, or anionic detergent, respectively (Fig. 4). While Triton X-100 does not have a significant effect on the reaction, both CTMAB and SDS are found to be effective in a positive or negative manner, respectively. With CTMAB, the sensitivity is enhanced 1.84-fold compared with the control reaction. Although to a lesser extent, enhancement of the sensitivity of **1a** could also be observed in the presence of bovine serum albumin.

Figure 5 compares the standard curves of the absorbances *versus* NADH concentrations for the reductions

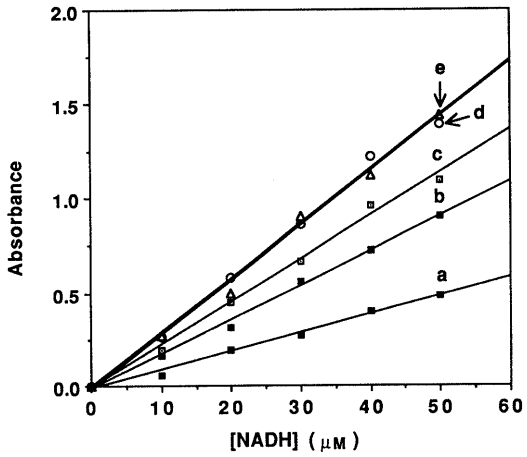

Fig. 5. Comparison of the Standard Curves between **1a**, XTT and INT for NADH Reductions, Mediated by 1-Methoxy PMS

The reactions of the tetrazolium salt (0.1 mM) in the presence of 1-methoxy PMS (5 μM) were carried out at pH 8.0 for 5 min at 22 °C. (a) INT (515 nm); (b) XTT (470 nm); (c) **1a** (438 nm); (d) **1a** (593 nm); (e) XTT (590 nm).

TABLE I. Comparison of the Sensitivity of **1a** towards NADH with XTT and INT in the Presence of 1-Methoxy PMS[a)]

pH	**1a**[b)]	**1a**[c)]	XTT[d)]	XTT[e)]	INT[f)]
7.4	10.6 (0.993)	13.9 (0.993)	19.2 (0.998)	30.6 (0.998)	—
8.0	22.8 (0.997)	28.9 (0.997)	18.4 (0.998)	29.0 (0.997)	10.0 (0.996)
9.0	31.3 (1.000)	41.6 (0.999)	19.2 (1.000)	32.2 (0.999)	—
8.5	30.6 (0.998)	41.8 (0.992)	19.4 (1.000)	32.4 (0.999)	—

a) The values in parentheses are correlation coefficients (*r*). The reactions were carried out as described in the experimental section with 0—50 μM NADH. The sensitivities are determined as Δ absorbance/Δ[NADH] (mM) at *b*) 438 nm; *c*) 593 nm; *d*) 470 nm; *e*) 590 nm and *f*) 515 nm.

of **1a**, XTT and INT under the same conditions. For **1a** and XTT, the reactions were performed at pH 8 and then terminated by the addition of aqueous alkali (final pH = 12.5); the absorbances were measured at two different wavelengths corresponding to the protonated and deprotonated formazan product. When observed either at 438 or 593 nm, the tetrazolium salt **1a** shows a good linear response with correlation coefficient (*r*) of 0.997 over the range 0—50 μM NADH. The sensitivities at various pH values toward NADH, given by the slopes of the curves in the figure, are summarized in Table I. At or above pH 8, the sensitivity of **1a** at 438 nm (or 593 nm) is higher than XTT, but only approximately half as high at pH 7.4. Unlike **1a** or XTT, INT, which is non-sulfonated, does not change its absorption spectrum in response to pH (λ_{max} = 515 nm at pH 8.0), and its sensitivity and the water-solubility are poor.

Since the usefulness of **1a** in the colorimetric assay of NADH was demonstrated, a correlation between the amount of formazan product and the number of viable cells in the growth assay was examined using **1a** and XTT under identical conditions with the P388 cell line, in which an optimum concentration of 1-methoxy PMS as determined for XTT[8)] was used (Fig. 6). The cells were incubated in the presence of the tetrazolium salt and the absorbances of the formazan products were measured at 428 nm (**1a**) or 468 nm (XTT), which were the respective λ_{max} values under these cellular reduction conditions. After 48-h incubation (1.57×10^6 cells/ml for **1a**), tetrazolium salt **1a** continues to

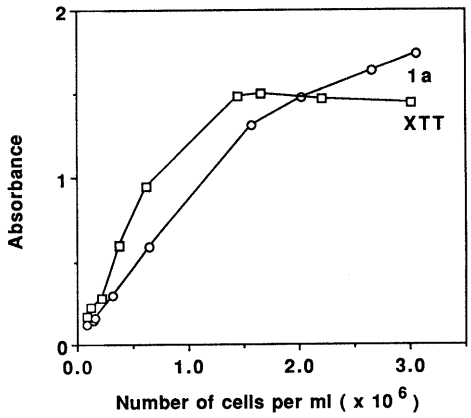

Fig. 6. Cell Proliferation Measurement of P388 Cell Line Using **1a** in Comparison with XTT

The assay procedure for **1a** is described under Experimental and the absorbance was measured at 428 nm; the measurement with XTT at 468 nm was performed in an identical fashion.

be metabolized to the formazan product, whereas no more formazan production is observed with XTT, thereby indicating that tetrazolium salt **1a** exhibits a greater dynamic range in terms of the number of cells and a better correlation between formazan production and cell number ($r = 0.981$) than does XTT. It is also noted that the cell proliferation rate with XTT becomes much smaller compared with **1a** after 48 h (1.45×10^6 cells/ml), which could indicate that the formazan product of **1a** is less cytotoxic than XTT.

In summary, these experiments prove that **1a** offers the advantage of a simplified assay procedure due to the water-solubility of its formazan product compared with MTT which at present is widely used in cell growth assays. Tetrazolium salt **1a** was found to be roughly as sensitive as XTT both in the NADH and cell proliferation assays. However, under different assay conditions, XTT was demonstrated to be more sensitive than MTT in a similar experiment with Sarcoma 180 cells.[9)] Tetrazolium salt **1a** therefore promises to be of value as a viability indicator in cell proliferation assays. To further increase the potential of **1a**, our efforts are currently being focused on developing an analogue of **1a** whose λ_{max} is shifted bathochromically.

Acknowledgement The authors are grateful to Mr. Hideyuki Tominaga for his technical assistance.

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