TRICINE-Buffered Tissue Culture Media for Control of Mycoplasma Contaminants[1] (35556)

REX S. SPENDLOVE, ROBERT B. CROSBIE, STANLEY F. HAYES, AND RICHARD F. KEELER

Department of Bacteriology and Public Health, Utah State University; and USDA, ARS, VSR Division, Poisonous Plant Research Laboratory, Logan, Utah 84321

Good *et al.* (1) prepared a number of zwitterionic amino acids for use as buffers in biological systems. Investigators have used several of these buffers successfully in tissue (2–5) and organ culture (6).

This report describes our experience over the past several years with *N*-tris(hydroxymethyl) methylglycine (TRICINE) buffered tissue culture media. During these studies a fortuitous observation was made with a TRICINE-buffered monkey kidney cell culture. Vacuolation and granularity were observed to decrease in the culture when the cells were transferred to a medium containing TRICINE. An investigation showed that the cells were contaminated with mycoplasma and that growth of the cells in the presence of TRICINE eliminated the contaminants.

Materials and Methods. Cell cultures. African green monkey kidney cells (BS-C-1) reported to be free of mycoplasma were obtained from a commercial source. Orcein staining (7) of these cells in the first passage indicated a very low level of mycoplasma contamination. The mycoplasma-like particles that were observed were thought to be artifacts, however, the particles increased in number with cell passage and the cell cultures subsequently proved to be contaminated. The experiments reported below were begun with the BS-C-1 cells in their first passage after being received in this laboratory. The cells were cultured in medium 199 with Earle's salts (Grand Island Biological Co. powdered medium) containing 20% fetal calf serum. One percent of a stock solution of antibiotics was added to the medium to give 250 units each of penicillin, streptomycin, and neomycin, and 2.5 units of bacitracin/ml.

Canine kidney cells (MDCK) obtained from the American Type Culture Collection were propagated in Flow Laboratories' Auto-Pow MEM medium with Earle's salts plus 0.06% glutamine, 10% fetal calf serum, and antibiotics as described above.

To test tissue cultures for mycoplasma contamination, cell cultures grown on cover glasses were stained by the orcein method (7). When examined by phase optics, the mycoplasma appeared as small purple particles at the periphery of the cytoplasm and in the intercellular spaces.

Buffers. *N*, *N*-Bis(2-hydroxyethyl) glycine (BICINE), *N*-2-hydroxyethyl piperazine-*N'*-2-ethane sulfonic acid (HEPES); *N*-tris(hydroxymethyl)methyl-2-amino ethane sulfonic acid (TES); and TRICINE were obtained from Calbiochem.

The chemical analysis (Calbiochem) of the TRICINE (lot 840315) was: nitrogen, 7.98%, heavy metals <1 ppm; optical density of a 0.1 *M* solution at 260 mμ, 0.044; and halides expressed as chloride, 0.11%. The analysis of the HEPES buffer (lot 70081) was nitrogen 11.92%, and heavy metals <1 ppm.

Because of the unusual mycoplasma inhibitory effects of the TRICINE buffer, it was desirable to investigate its purity. Infrared (IR), melting point, and proton nuclear magnetic resonance (NMR) were used to verify that the material was indeed TRICINE and

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not some other mislabeled compound. Thin-layer chromatography (TLC) and NMR at very high gain were used to assess contamination with other compounds. IR analysis was performed on TRICINE in KBr pellets and NMR was measured with TRICINE in deuterated dimethyl sulfoxide with tetramethylsilane as an internal standard. TLC of TRICINE was done using silica gel plates of 60 μ thickness with development in the following solvents: benzene:methanol, 1:6; benzene:methanol, 1:4; chloroform:methanol, 1:4; chloroform:methanol, 3:1; pyridine:acetone:NH_4OH:H_2O, 9:6:1:4; and isopropyl alcohol:formic acid:H_2O, 15:2.5:2.5. Spots were visualized using ninhydrin in ethanol (0.1%), bromphenol blue (0.1%) in aqueous 10% phosphate buffer (pH 4.2), and a 1% potassium permanganate:2% sodium metaperiodate mixture in 2% aqueous sodium carbonate. Plates were first examined under UV light and, after spraying, under visible light.

The toxicity of each buffer was tested against several established mammalian cell lines. TRICINE showed little toxicity at concentrations as high as 0.025 *M* in a single passage. Because of its low toxicity and relatively low cost, TRICINE was selected for these studies.

In preliminary experiments, the optimal buffer system for open air cultivation of tissue cultures was found to be TRICINE at concentrations between 0.005 and 0.010 *M* in combination with 0.005 *M* $NaHCO_3$. The TRICINE concentration in the experiments described was increased to more effectively inhibit mycoplasma. The buffer concentrations in the inhibition experiments were as follows: TRICINE used alone, 0.023 *M*; TRICINE in combination with bicarbonate, 0.015 *M* and 0.005 *M*, respectively; and $NaHCO_3$ used alone as a control, 0.017 *M*.

Cultures containing TRICINE were incubated in an air atmosphere, while cultures buffered with bicarbonate without TRICINE were incubated in air containing 5% CO_2.

Mycoplasma. Three species of human mycoplasma, *Mycoplasma hominis, M. orale,* and *M. salivarium* were obtained from Dr. Charles Smith of the University of Utah Medical Center. Classification of these organisms was confirmed by a growth inhibition test (8) using paper discs saturated with anti-mycoplasma sera (BBL).

The broth used in culturing the mycoplasma was a modification of that described by Chalquest (9). It contained 2.3 g of PPLO broth base, 1.0 g of yeast autolysate and 0.5 g of Bacto starch (all obtained from Difco); 0.05 g of thallium acetate, 0.05 g of Trypticase (BBL); 10.0 ml of horse serum, 1.0 $\times$ 10^5 units of penicillin and 0.1 g of coenzyme I (Calbiochem)/100 ml. To prepare the agar medium, 3.4 g of PPLO agar base was substituted for the PPLO broth base.

To confirm the enhancing effect of HEPES on mycoplasma growth (1) and to further test the inhibiting effect of TRICINE, these buffers were added at various concentrations to PPLO broth and agar media as shown in the Results section. Tubes containing 30-ml volumes of broth were inoculated with mycoplasma and were incubated at 37° for 8 days. Inocula containing 0.05 ml of each broth culture were then transferred to the surface of PPLO agar containing one of the specified buffer systems. *Mycoplasma orale* and *M. salivarium* cultures were incubated at 37° in a Torbal anaerobic jar in an atmosphere of 5% CO_2 and 95% N_2; *M. hominis* cultures were held at 37° in an air atmosphere.

Results. BS-C-1 and MDCK cells were grown in an air atmosphere in media containing a TRICINE–$NaHCO_3$ buffer. The cell yield was comparable to that of cultures grown in a bicarbonate-buffered medium incubated in a closed system. The growth in a medium buffered with only TRICINE, however, was somewhat less, and continuous growth of the cells in this medium had a deleterious effect. The BS-C-1 cells degenerated during the fifth or sixth passage while MDCK cells usually degenerated in the sixth. Table I shows results obtained with BS-C-1 cells. Degenerating cells recover when transferred to a medium buffered with bicarbonate, and can subsequently be cultured again for 3 to 5 passages in a medium containing only TRICINE as a buffer.

When BS-C-1 cells that were heavily contaminated with *M. hominis, M. Orale,* or *M. salivarium* were cultured in media containing TRICINE, the number of mycoplasma decreased. The results obtained with *M. orale-*

TABLE I. Yield of BS-C-1 Cells When Grown in Media with Different Buffer Systems.

Cell passage no.	Cell yield/culture ($\times 10^6$)		
	$NaHCO_3$–CO_2	TRICINE	TRICINE–$NaHCO_3$
1	3.8	3.7	3.9
2	4.0	3.5	3.8
3	4.1	3.2	4.2
4	3.9	2.5	3.9
5	4.0	3.0[a]	3.9
6	3.9	3.3[a]	3.8
7	3.9	3.3	3.9
8	3.8	3.3	3.8
9	4.0	3.2	3.8

[a] The cells began to degenerate during passage 4, so they were cultivated in a medium buffered with bicarbonate during passages 5 and 6. The cultures were then returned to the TRICINE-containing medium.

infected cultures are shown in Figs. 1–3.

The BS-C-1 cells before inoculation with *M. orale* are shown in Fig. 1. A low level of the contaminating mycoplasma discussed above is apparent. These cells were infected with *M. orale* and incubated until large numbers of mycoplasma were evident (Fig. 2). After the cells were cultured 5 times in a medium containing TRICINE without bicarbonate there was no visible evidence of mycoplasma contamination (Fig. 3). The cells were then transferred twice in a bicarbonate-buffered medium to prevent degeneration of the cultures. After 4 additional passages in a TRICINE-buffered medium, mycoplasma could not be isolated nor detected by orcein staining.

Manchee and Taylor-Robinson (10) reported that growth of T-strain mycoplasma was enhanced when they were cultivated in media containing HEPES buffer. Consequently, the effect of HEPES and TRICINE buffers was tested on the growth of *M. hominis*, *M. orale*, and *M. salivarium* as shown in Table II. Agar plates inoculated from the broth tubes were analyzed for growth.

Growth of all three mycoplasma was enhanced by HEPES and inhibited by TRICINE. The enhancing effects of HEPES was confirmed by the following results. *Mycoplasma hominis* colonies appeared in cultures containing HEPES buffer 1 or 2 days earlier than in control cultures without HEPES. Neither *M. orale* or *M. salivarium* formed colonies when HEPES was omitted from both the broth and the agar media. In other experiments it was possible to obtain colony growth without HEPES by increasing the inoculum size.

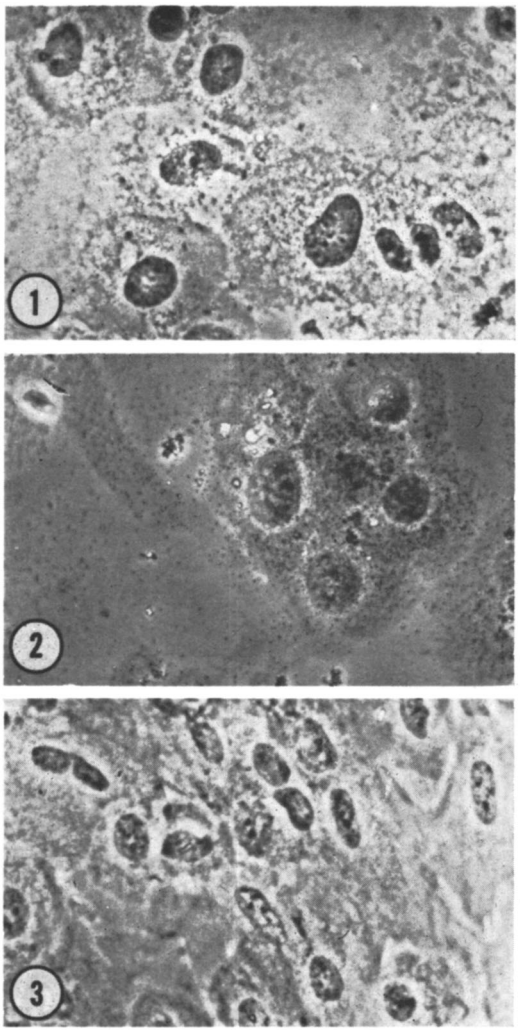

FIG. 1. Orcein-stained BS-C-1 cells in first passage. Cells cultured in a bicarbonate-buffered medium.

FIG. 2. Orcein-stained BS-C-1 cells after infection wih *M. orale*. The culture was buffered with bicarbonate.

FIG. 3. Orcein-stained BS-C-1 cells after infection with *M. orale* and growth for 5 passages in a medium containing TRICINE.

TABLE II. Enhancement of Mycoplasma Growth by HEPES and Inhibition of Growth by TRICINE.[a]

Buffer[b] conc (*M*)		Growth[c]		
Broth medium	Agar medium	*M. hominis*	*M. orale*	*M. salivarium*
0	0	+	—	—
0	H (0.050)	+	+	+
0	H (0.025)	+	+	±
0	T (0.05)	—	—	—
0	T (0.023)	±	—	—
0	T (0.015)	±	—	—
H (0.05)	0	+	+	+
H (0.025)	0	+	+	±
H (0.05)	H (0.05)	+	+	+
H (0.025)	H (0.025)	+	+	±
H (0.1)	T (0.1)	—	—	—
H (0.075)	T (0.05)	—	—	—
H (0.05)	T (0.023)	—	—	—
H (0.025)	T (0.015)	—	—	—
T (0.015)	0	—	—	—
T (0.1)	H (0.1)	—	—	—
T (0.05)	H (0.075)	—	—	—
T (0.023)	H (0.05)	—	—	—
T (0.015)	H (0.025)	—	—	—

[a] Final examination of agar plates made 2 weeks post inoculation.

[b] H = HEPES, T = TRICINE, 0 = control without buffer.

[c] A ± designation = 1–4 colony-like formations observed; a + designation = too many colonies to count.

The only evidence of any growth when TRICINE was present in either the broth or the agar was 1 or 2 questionable colony-like formations with *M. hominis*.

IR and NMR analysis (Figs. 4 and 5) established that TRICINE used was indeed *N*-tris(hydroxymethyl) methylglycine and not some other mislabeled product. The compound had the appropriate IR hydroxyl band at 3320 cm^{-1}, N–H band at about 3200, C–H band at 2800, carboxyl absorption between 1600–1650, and primary alcohol absorption at about 1060. NMR integration showed 13 protons. Three were in a broad low band centered at 5.2 δ which was lost on treatment with D_2O and was, thus, attributable to the 3 OH protons; two were in a singlet at 3.48 δ attributable to the methylene protons adjacent to the carboxyl group; one was at 3.27 δ (the proton on the N); and six were in a multiplet centered at 2.48 δ and were thus attributable to the six methylene protons adjacent to the hydroxyls. An additional broad envelope at about 2.3 δ (1 proton) may have been the carboxyl proton. The analysis verified that the compound was TRICINE.

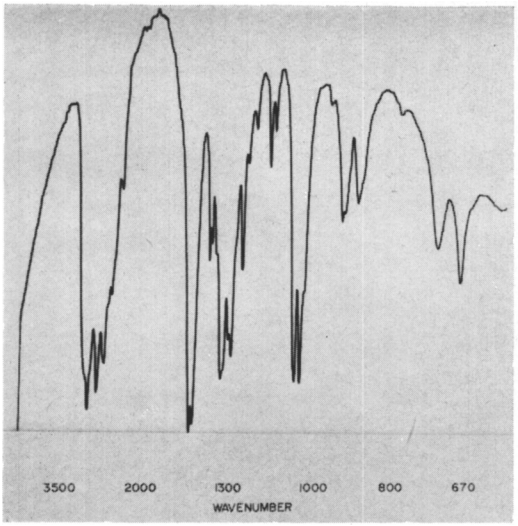

FIG. 4. Infrared spectrum of TRICINE.

Analysis for purity of TRICINE suggested that if contamination by other organic compounds were present, the level was below approximately 0.5%. Thus, no unassignable peaks were seen on NMR analysis even with the gain and sample concentration very high. Discernible peaks would have been evident for contaminant protons if the contaminant were 0.5% or greater. Further, TLC analysis showed no extraneous spots when TRICINE was chromatographed in 6 solvents and sprayed with 3 separate spray reagents specific for amino acids, basic compounds, and polyhydroxy compounds.

The melting point was sharp at 188–189°. This compared favorably with the 187° melting point reported by Good (11), and further verified the excellent purity of the TRICINE.

Discussion. HEPES, TES, and TRICINE, hydrogen ion buffers developed by Good *et al.* (1), have been used successfully in cultivating a variety of cells (2–6) and in the

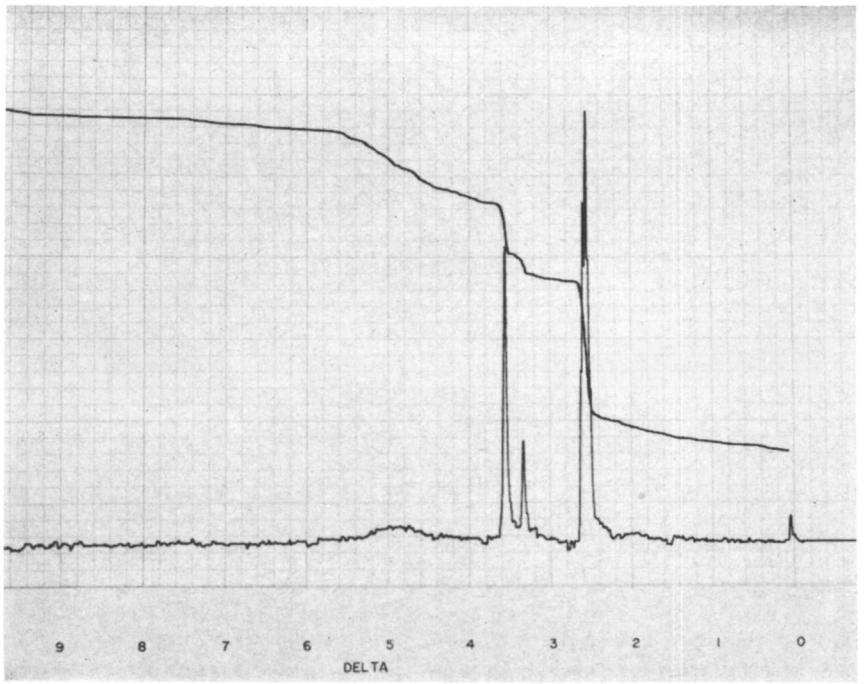

FIG. 5. Nuclear magnetic resonance spectrum of TRICINE.

assay of 7 different viruses (2–4). In all of the above investigations the buffers were reported to be as good as, or better than, a bicarbonate–CO_2 buffer system.

An air–CO_2 atmosphere is not required when these new hydrogen ion buffers are used in culturing cells. Consequently, the problems encountered in incubating and manipulating cultures in the presence of CO_2 are circumvented.

This communication confirms the usefulness of TRICINE in tissue culture (5), and describes a method for using TRICINE to eliminate certain mycoplasma contaminants from tissue cultures.

TRICINE and TRICINE–bicarbonate buffer systems were used in culturing BS-C-1 and MDCK cells. When TRICINE was used without bicarbonate, and the cells were incubated in an air atmosphere, cells from both lines started to degenerate after 5–6 passages. This probably reflects the requirement of the cells for more CO_2 than was provided in the medium. The fact that the cells recovered rapidly when the TRICINE was replaced with a bicarbonate-containing medium supports this assumption. Cells cultivated in media containing TRICINE and bicarbonate do not show this degeneration. These observations are consistent with studies by several groups of investigators (12–18) who demonstrated that bicarbonate is essential for *in vitro* mammalian cell growth.

In addition to BS-C-1 and MDCK cells, human amnion (FL), human diploid lung, mouse (L) and rainbow trout gonad (RTG-2) cells have been propagated by us in TRICINE–bicarbonate-containing media. All of the cells grew well; however, the L cells dislodged prematurely from the culture container. This may have resulted from a calcium deficiency, since TRICINE has chelating activity (1).

Inhibition of mycoplasma by TRICINE buffer is surprising since Manchee and Taylor-Robinson (10) observed an increase in the number of viable T-strain mycoplasma in broth, and an increase in colony size on agar when HEPES, another substituted amino acid buffer, was added to the media used for propagation. They postulated that the HEPES buffer was augmenting growth by stabilizing the pH at 6.8. The enhancing effect of HEPES was confirmed by us under

conditions where TRICINE inhibited growth. These results suggest that the stimulation of growth by HEPES involves more than pH control.

A study is underway to determine the mechanism by which TRICINE inhibits mycoplasma growth. Elucidation of this mechanism may help define additional differences between mycoplasma and other organisms and could facilitate development of new chemotherapeutic methods for mycoplasma diseases.

TRICINE is synthesized by Calbiochem according to the procedure of Good (11) using tris(hydroxymethyl) aminomethane, monochloroacetic acid, methanol, and water with about 80% yield. It is recrystallized from methanol and water. Thus, the likely contaminants of significance from a concentration point of view would include these starting materials plus any unknown products of the reaction which would probably contain nitrogen and/or polyhydroxyl functions. The preparative recrystallization procedure would eliminate all but traces of these. NMR analysis confirmed that no organic contaminant was present at over 0.5% of the original TRICINE. TLC analysis verified that no significant level of amino acid, basic (nitrogen-containing amines, *etc.*) or polyhydroxylated type contaminants were present. Thus, for the antimycoplasma activity to be due to an organic contaminant, rather than the TRI CINE itself, seemed remote.

Summary. Monkey kidney (BS-C-1) and canine kidney (MDCK) cell cultures were inoculated with *Mycoplasma hominis, M. orale*, and *M. salivarium*. Growth of these mycoplasma was inhibited when the cultures were propagated in media buffered with TRICINE or a TRICINE–bicarbonate combination.

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