Evaluation of 4-(2-Hydroxyethyl)-1-piperazineëthanesulfonic Acid (HEPES) as a Tissue Culture Buffer* (33543)

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In spite of its common usage in tissue culture, a $NaHCO_3$–CO_2 buffer has two important disadvantages, namely: (a) a carbon dioxide enriched atmosphere is essential if adequate pH stability is to be achieved, and (b) the 6.1 pK_a of $NaHCO_3$ results in suboptimal buffering throughout the physiological pH range.

Several attempts have been made in recent years to find a more suitable buffer. Swim and Parker (1) replaced the sodium bicarbonate of their medium with tris(hydroxymethyl)aminoethane (Tris) (2) or with glycylglycine. The growth of some established cell lines was inhibited by higher concentrations of Tris, and the cells were more granular than otherwise. Glycylglycine was not as toxic as Tris for the established cell lines, but foreskin cultures did not proliferate as well in medium that contained glycylglycine as in Tris buffered medium.

Recently Good and his associates (3) developed a series of hydrogen ion buffers covering the pK_a range 6.15–8.60. Most of these buffers are amino acids. Although the buffers have been tested primarily in studies on phosphorylation-coupled oxidation of succinate by mitochondrial preparations, it seemed reasonable to evaluate these compounds in tissue culture systems. The buffer selected for evaluation was 4-(2-hydroxy ethyl)-1-piperazineëthanesulfonic acid (HE PES). HEPES has a molecular weight of 238.3, a pK_a of 7.31 at 37°, a $\Delta pK_a/°$ of −0.014, exhibits no metal binding and is soluble to the extent of 2.25 *M* at 0°.

Another buffer developed by Good and his associates, *N*-tris(hydroxymethyl)methyl-2-amino-ethanesulfonic acid (TES) has recently been employed in an Eastern equine encephalitis chick fibroblast plaque assay (4).

Materials and Methods (1) 4-(2-hydroxy ethyl)-1-piperazineëthanesulfonic acid (HE PES) was purchased from General Biochemicals, Inc. (Chagrin Falls, Ohio) for early experiments and from Calbiochem (Los Angeles, California) for the majority of the later experiments.

(2) *Cell cultures.* Diploid cell cultures of human embryonic lung (HEL) were initiated and passaged according to the method of Hayflick and Moorhead (5). Growth medium for HEL cells consisted of Eagle basal medium (Earle salts) [BME(E)] (Gibco, Grand Island, New York) with 15% unfiltered fetal bovine serum.

Primary *Cercopithecus* and *Cynomolgus* monkey kidney (MK) cultures were prepared by a modification of the method of Youngner (6). Growth medium for MK cells consisted of medium 199 (Hanks salts) [199(H)] (Gibco, Grand Island, New York) with 15% unfiltered fetal bovine serum. BME(E) with 1% calf serum was used for the maintenance of MK cells.

The BHK-21 cells were cloned according to the method of Baron *et al.* (7). Ten clones were evaluated for their ability to grow as monolayer cultures and in suspension. Clone 4 (BHK-21/4) can be cultivated with equal facility by either method. Growth medium for BHK-21/4 cells consisted of BME(E) with 10% calf serum or Ham nutrient mixture F-12 (Gibco, Grand Island, New York) supplemented with 5% fetal bovine serum. BHK-21/4 cells were maintained in serum-free nutrient mixture F-12.

"Guinea pig spleen" (GPS) cells were grown in Earle salts, Eagle MEM vitamins, 2

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m*M* L-glutamine, 0.5% lactalbumin hydrolysate, 10% calf serum, and 100 units and 100 μg of penicillin and streptomycin, respectively. The GPS cells recently were shown to be of murine origin and probably were derived from mouse L cells (Herrick, P. R., Bauman, G., Shearer, M., Shipman, C. Jr., and Merchant, D. J., in preparation). The GPS cells were grown in suspension in either 500-ml swirled Erlenmeyer flasks or in 500-ml spinner flasks.

Detroit 6-YT cells were grown in BME(E) supplemented with 15% fetal bovine serum.

The RK-13 cells were propagated in 199(H) with 15% unfiltered fetal bovine serum.

The MA-134 cells were received from Mrs. Hope Hopps, NIH, Bethesda, Maryland. Growth medium consisted of nutrient mixture F-12 supplemented with 5% fetal bovine serum.

The BS-C-1 cells were also obtained from Mrs. Hope Hopps. Several growth media were evaluated and nutrient mixture F-12 supplemented with 5% fetal bovine serum was found to be superior to the other media tested. BS-C-1 cells could be maintained for 30 days using F-12 medium without serum.

Puppy salivary gland (PSG) cells were derived from 1- to 3-day-old puppies. Growth medium for PSG cells consisted of BME(E) supplemented with 15% unfiltered fetal bovine serum and 1% Eagle MEM nonessential amino acids.

Chick embryo fibroblasts derived from 10-day-old embryos were prepared essentially according to the method of Rubin (8). Growth medium consisted of 199(H) supplemented with 10% tryptose phosphate broth and 8% newborn calf serum (Hyland Laboratories, Los Angeles, California).

The KB and HEp-2 cells were grown in suspension in calcium-free Eagle basal medium (Eagle salts) supplemented with 15% tryptose phosphate broth and 10% calf serum. Growth medium for monolayer cultures consisted of BME(E) with 20% calf serum.

The LM cells were cultivated in BME(E) with 15% fetal bovine serum.

(3) *Viruses.* The HPV-77 strain of rubella virus was employed. Sendai virus was obtained through the courtesy of Dr. Francis Payne, The University of Michigan, Ann Arbor, Michigan. Polyoma virus was kindly supplied by Dr. Sarah Stewart, NCI, Bethesda, Maryland.

(4) pH *and oxidation–reduction potential (ORP) measurements* were made utilizing Radiometer instruments PHM-26 or PHM-27. The ORP measurements were made using platinum and saturated calomel electrodes at a pH of 7.20 and a temperature of 22°. Both pH and ORP measurements were made using a water-jacketed beaker to maintain the desired temperature. The buffer solutions and the unknown solutions were stirred by means of a nonheating magnetic stirring device.

(5) *Tonicity measurements* were performed with an Advanced Instruments Osmometer model no. 65-31 using 2-ml samples.

(6) *Cell counts.* Total cell counts were made with a model B Coulter counter equipped with 100-μ orifice. Viable counts were determined by means of trypan blue dye exclusion.

Results. The buffering capacity over the pH range of 6.8–7.8 of medium 199 buffered with various concentrations of $NaHCO_3$ or HEPES is illustrated in Fig. 1. A comparison of the slopes indicates that medium 199 with 0.01 *M* HEPES represents a degree of buffering intermediate between that seen with medium 199 (Hanks salt) and medium 199 (Earle salts).

The effect of 0.001, 0.01, 0.025, and 0.1 *M* HEPES upon the microscopic appearance of selected primary, human embryonic diploid and established cell lines is recorded in Table I. Coverslip cultures were grown in the medium to be evaluated and were withdrawn and stained (May-Grünwald Giemsa) for subsequent examination. A diffuse cytoplasmic vacuolization was observed in five of eight cultures growing in 0.1 *M* HEPES buffered media. This vacuolization was not seen with the lower concentrations of HEPES nor with the $NaHCO_3$ buffered media.

Since it had been ascertained that 0.01 *M*

Medium	Buffer	ml of Titrant required to raise the pH one unit
199 (H)	None	0.8
199 (H)	0.35g $NaHCO_3$/L	1.0
199 (H)	.001M HEPES	1.3
199 (H)	.01M HEPES	6.6
199 (E)	2.2g $NaHCO_3$/L	11.0
199 (H)	.1M HEPES	52.0

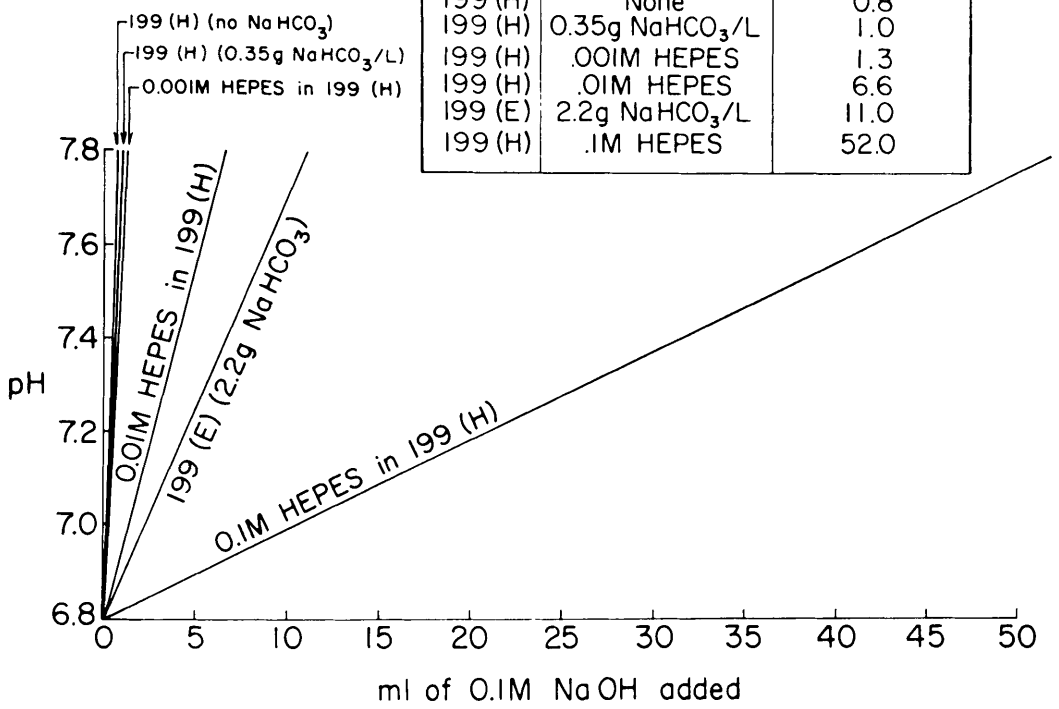

FIG. 1. Buffering capacities of medium 199 containing $NaHCO_3$ or HEPES.

HEPES provided adequate buffering capacity and was not cytotoxic to monolayer cultures, an experiment was devised to study the growth characteristics of suspension cultures of GPS cells. Three hundred ml of 0.01 *M* HEPES buffered medium (pH 7.20) and 300 ml of $NaHCO_3$ buffered medium (1 g of $NaHCO_3$/liter) were placed in 500-ml spinner flasks (Bellco, Vineland, New Jersey). The medium was allowed to equilibrate at 37° and was inoculated with sufficient washed GPS cells to produce a density of approximately 250,000 cells/ml. The $NaHCO_3$ buffered system was closed to the atmosphere by means of a rubber stopper whereas the HEPES buffered system was open to the atmosphere by means of a stainless steel closure.

The results of this experiment are shown in Fig. 2. The population doubling time of cells in the HEPES buffered medium was not significantly different from that seen in the $NaHCO_3$ buffered medium (34–35 hr). The maximum cell density and the viability, however, were higher in the HEPES buffered culture (1.6×10^6 cells/ml, 90% viable) than in the $NaHCO_3$ buffered culture (1.1×10^6 cells/ml, 85% viable). A nearly constant pH for 90 hr was maintained by using HEPES whereas the usual decreasing pH extending over approximately 1 pH unit was seen with the $NaHCO_3$ buffered medium.

The influence of HEPES upon the oxidation–reduction potential of Eagle basal medium, nutrient mixture F-12 and medium 199 is shown in Table II. HEPES had a small effect on the ORP of well poised media. A shift to a lower E_h was seen with increasing concentrations of HEPES.

Table III illustrates the influence of HEPES upon the tonicity of Eagle basal medium, nutrient mixture F-12 and medium

TABLE I. Effect of HEPES upon the Microscopic Appearance of Selected Primary, Human Embryonic Diploid and Established Cell Lines.

Name of cell line and tissue of origin	Species	Molarity of HEPES			
		0.001	0.01	0.025	0.1
Primary cells					
Chick embryo fibroblasts	Chicken	—[a]	NC[b]	—	—
Monkey kidney	*Cercopithecus* sp.	NC	NC	NC	—
Monkey kidney	*Cynomolgus* sp.	NC	NC	NC	—
Puppy salivary gland	Dog	—	NC	—	—
Human embryonic diploid cell lines					
Human embryonic lung	Human	NC	NC	—	NC
Established cell lines					
BHK-21/4 (kidney)	Hamster	NC	NC	—	DCV[c]
BS-C-1 (kidney)	*Cercopithecus* sp.	NC	NC	—	DCV
Detroit 6-YT (bone marrow)	Human	—	NC	—	—
"GPS" (connective tissue)	Mouse	—	NC	—	—
HEp-2 (carcinoma, larynx)	Human	NC	NC	NC	NC
KB (carcinoma, oral)	Human	NC	NC	NC	DCV
LM (connective tissue)	Mouse	NC	NC	—	NC
MA-134 (kidney)	*Cercopithecus* sp.	NC	NC	—	DCV
RK-13 (kidney)	Rabbit	NC	NC	—	DCV

[a] — = not tested.
[b] NC = no change as compared with the $NaHCO_3$–CO_2 buffered control.
[c] DCV = diffuse cytoplasmic vacuolization.

199. Although 0.01 *M* HEPES produces a physiological osmotic pressure with a medium utilizing Hanks salts, approximately 0.02 and 0.04 *M* HEPES, respectively, would be required to restore correct tonicity to media containing a F-12 salt base and Earle salts.

Dual titrations with rubella virus in RK-13 cells were performed using HEPES and $NaHCO_3$ buffered growth and maintenance media. Virtually identical end points were achieved.

HEPES (0.01 *M*) buffered saline (HBS) prepared according to the formula given in Table IV has been used for a wide variety of purposes including hemagglutination tests. No differences in hemagglutinating end points have been observed with either polyoma virus or Sendai virus when compared with a phosphate buffered saline system.

Discussion. In order to evaluate any buffer in a meaningful manner two essential aspects must be considered, namely (a) what is the buffering capacity in the desired range,

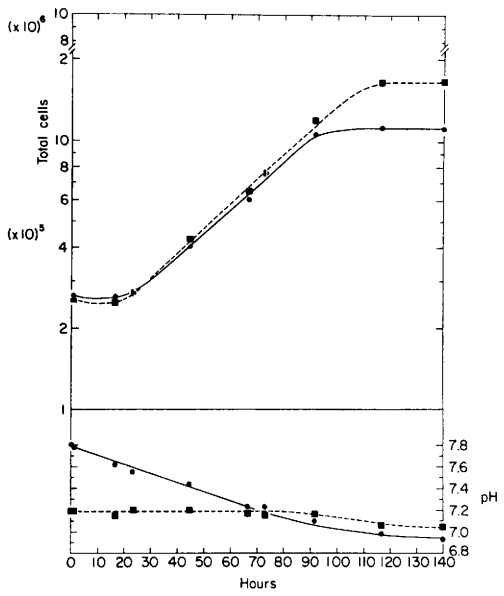

FIG. 2. Growth in suspension of GPS cells in $NaHCO_3$ buffered (●) and HEPES buffered (■) media; (lower) the corresponding pH is shown for each medium.

TABLE II. Influence of HEPES upon the Oxidation–Reduction Potential (ORP) of Eagle Basal Medium, Nutrient Mixture F-12 and Medium 199.[a]

Medium	$NaHCO_3$ (g/liter)	HEPES (M)	E(+mV)	E_h(+mV)[b]
Eagle basal medium, Earle salts	2.2	—	230	477
	—	0.001	219	466
	—	0.01	211	458
	—	0.1	194	441
Ham nutrient mixture F-12	1.176	—	(198)[c]	445
	—	0.001	138	385
	—	0.01	126	373
	—	0.1	115	362
Medium 199, Hanks salts	0.35	—	153	400
	—	0.001	146	393
	—	0.01	145	392
	—	0.1	146	393

[a] All measurements were made with platinum and saturated calomel electrodes at a pH of 7.20 and a temperature of 22°.

[b] $E_h = E + 247$ mV where 247 mV is the potential of the saturated calomel electrode against the standard hydrogen electrode.

[c] Initial reading—the value decreased as a function of time.

and (b) what are the biological side effects of this buffer?

HEPES, with a pK_{a2} of 7.3 at 37° and a solubility in excess of 2 M at 0°, has a greater buffering capacity than either $NaHCO_3$ (pK_a 6.1 at 37°) or Tris (pK_a 7.9 at 37°) when used at a physiological pH.

TABLE III. Influence of HEPES upon the Tonicity of Eagle Basal Medium, Nutrient Mixture F-12 and Medium 199.[a]

Medium	$NaHCO_3$ (g/liter)	HEPES (M)	Tonicity (milliosmoles/kg)
Eagle basal medium,	2.2	—	284
Earle salts	—	0.001	241
	—	0.01	253
	—	0.1	372
Ham nutrient	1.176	—	285
mixture F-12	—	0.001	264
	—	0.01	272
	—	0.1	385
Medium 199,	0.35	—	276
Hanks salt	—	0.001	270
	—	0.01	280
	—	0.1	364

[a] All measurements were made at the pH of the corresponding $NaHCO_3$-buffered medium.

When preparing HEPES buffered solutions, the $-0.014\ \Delta pK/°$ must be considered in determining both the buffering capacity and the operating pH. Thus a buffer adjusted to pH 7.3 at 22° would have a pH of approximately 7.5 at 0° and 7.1 at 37°. This phenomenon is, of course, not unique with HEPES and is exhibited by most buffer systems. Although the $\Delta pK_a/°$ of HEPES is not as low as $NaHCO_3$ (—0.009) it is considerably lower than Tris (—0.031).

The evaluation of the biological side effects of a buffer is much more difficult since these side effects will depend upon the particular cell employed and the methodology utilized. Although it does not appear that HEPES

TABLE IV. Formulation of 0.01 M HEPES Buffered Saline (HBS).[a]

NaCl (g)	8.00
KCl (g)	.40
Na_2HPO_4 (g)	.10
Dextrose (g)	1.00
HEPES (g)	2.38
Distilled water (ml)	1000

Adjust pH to 7.20 at 22° with NaOH

[a] The tonicity of this salt solution is approximately 290 milliosmoles/kg.

produces toxic manifestations as measured by its effect upon ORP, population doubling time, cell viability, or influence on efficiency of virus titrations, the diffuse cytoplasmic vacuolization induced in some cultures by high concentrations of the buffer could possibly represent an undesirable side effect. Since the use of 0.1 *M* HEPES significantly raises the osmotic pressure of the medium, it was felt that this vacuolization could be a generalized effect due to the hypertonicity of the medium. This hypothesis was tested by adding sucrose to $NaHCO_3$ buffered nutrient mixture F-12 until the osmotic pressure was 385 milliosmoles/kg. Cells grewn in this medium exhibited a diffuse cytoplasmic vacuolization indistinguishable from that produced by 0.1 *M* HEPES buffered medium. Transmitted-light interference microscopy of living cultures was utilized to eliminate the possibility that the vacuoles might represent an artifact introduced in fixation or staining.

HEPES should have particularly wide spread applicability in at least two systems. Firstly, the use of a buffer which does not require an enriched atmosphere is of advantage in steady-state operation where pH, ORP and other physiological parameters are controlled. Since pH would be dependent upon the composition of the atmosphere, the ORP could be controlled with nitrogen or oxygen purging without a concomitant rise in pH. Secondly, a wide variety of virus assays which normally require incubation in a CO_2 enriched atmosphere could now be performed in ordinary incubators.

Summary. The amino acid 4-(2-hydroxy ethyl)-1-piperazineëthanesulfonic acid (HEPES) has been evaluated as a tissue culture buffer. HEPES has a molecular weight of 238.3, a pK_{a2} of 7.31 at 37°, a $\Delta pK/°$ of −0.014, exhibits no metal binding and is soluble to the extent of 2.25 *M* at 0°. HEPES (0.01 *M*) produced adequate buffering in all cell systems tested. HEPES was not toxic to primary monkey kidney, chick embryo fibroblasts or puppy salivary gland cells, human embryonic lung, BHK-21/4, BS-C-1, Detroit 6-YT, GPS, HEp-2 KB, LM, MA-134, or RK-13 cells. The GPS cells were grown in suspension in HEPES and $NaHCO_3$ buffered media. Although the population doubling times were not significantly different, a higher maximum cell density and viability were seen in the HEPES buffered system. HEPES had a minimal effect on the oxidation–reduction potential of well-poised media. HEPES had no effect on rubella virus titrations or on hemagglutination assays of polyoma or Sendai virus. The formulation of a HEPES buffered salt solution is presented.

Addendum. After this manuscript was prepared a short communication appeared in the literature (9) describing the use of HEPES and TES as tissue culture buffers. When used at a final concentration of 28 m*M*, HEPES produced no apparent cytotoxicity and did not alter the efficiency of virus infectivity titrations.

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