HEPES-Buffered Media in Lymphocyte Cultures[1] (35271)

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HEPES [4-(2-hydroxyethyl)-1-piperazinyl-ethane-2-sulfonic acid] was originally introduced as a hydrogen ion buffer for studies of mitochondrial metabolism (1) and became widely accepted in biochemical studies. Recently, however, it has received attention as a possibly useful tissue culture buffer (2–4). Unlike Na_2CO_3 buffer, it has a pK_a of 7.31 at 37° (2) optimal for buffering at physiological pH and does not require adjustments of atmospheric composition to maintain pH. The usefulness of HEPES as a cell culture buffer was investigated by Shipman (2). Even at concentrations higher than those required for adequate buffering capacity, HEPES was found not to be toxic to several cell lines investigated. Furthermore, although having no adverse effect on cell doubling time, HEPES-buffered media, due to its excellent buffering capacity, sustained growth of the cells at higher cell densities. Williamson and Cox (3) also reported a lack of cytotoxicity of HEPES-buffered media, which they used for cell propagation and virus titration studies. Fisk and Pathak (4) observed improved survival of cells in HEPES-buffered organ culture of adenohypophyses. Before gaining wide application in routine tissue culture, further detailed studies are needed to determine whether or not HEPES produces undesirable effects after many generations in long-term cultures. Lymphocyte cultures are, in general, of short-term duration (up to 7 days) and are commonly used in research laboratories, as well as in many hospitals, where they have become a routine procedure in immunology and transplantology. Therefore, the use of a well-standardized buffering system, not requiring costly and troublesome CO_2 gassing, would be of special value. The present studies were therefore aimed at revealing whether or not HEPES buffer could be applied to this type of culture. The stimulation of human peripheral blood lymphocytes induced by the nonspecific stimulant phytohemagglutinin (PHA), by tuberculin antigen (PPD), and in mixed allogeneic system was studied and compared in HEPES-buffered media, and in the conventional Na_2CO_3-buffered cultures.

Materials and Methods. I. Preparation of cell suspensions. A. Expts. I and II. 20–40 ml of blood were collected from healthy volunteers. The blood was defibrinized, mixed with 6% dextran (10:1; v/v), and sedimented by gravity for 2–3 hr. The leukocyte-rich serum was withdrawn, the cells were then sedimented by centrifugation and suspended in heat-inactivated fetal calf serum (FCS). In this suspension, the lymphocytes were always contaminated with granulocytes (30–50%) and with erythrocytes (up to five times more than lymphocytes). The concentration of lymphocytes was adjusted to obtain a concentration five times higher than desired in cultures. 0.2 ml of this suspension was then transferred to 12 × 75-mm plastic culture tubes (Falcon) containing 0.8 ml of medium.

B. Expt. III. Another procedure was used for experiments wherein cells were stimulated by PPD and in allogeneic mixed cultures. Confirming Johnson and Russell's observation (5), we have noted that FCS itself stimulates lymphocytes in cultures lasting longer than 5 days. The FCS was therefore omitted and all steps of culture preparation (as described under A.) were carried out on cell suspensions in autologous serum. In mixed

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lymphocyte cultures, as well as in controls, where lymphocytes from unrelated individual donors were cultured separately, the medium was supplemented with 20% of the serum pooled from both donors. Similarly, as in Expts. I and II, cells were cultured in 1-ml aliquots in plastic tubes.

C. Expt. IV. In experiments where lymphocyte stimulation was assayed by means of morphologic evaluation and autoradiography cell preparation was as follows. 20 ml of blood were collected in heparinized syringes. Blood sedimentation was carried out in upright syringes without rouleau-forming agents for 2–4 hr at 37°. Leukocyte-rich plasma was then withdrawn, centrifuged, and the cells were washed once with FCS. The cells were then incubated in culture medium plus 20% FCS in large plastic petri dishes for 1 hr and cells not adhering to the surface were collected. This procedure of isolation of lymphocytes from granulocytes was repeated, and after centrifugation the final cell suspension prepared in FCS yielded lymphocytes contaminated less than 10% with granulocytes and about 5–10% with erythrocytes. The cell suspension was adjusted with FCS to contain a concentration of lymphocytes five times higher than that desired in culture, and 0.4 ml of this suspension was added to 1.6 ml of medium in Leighton tubes.

II. Preparation of media. The media (Eagle's basal medium, BME, with Earle's salt, and 199 medium with Hanks' salt, both concentrated 10 times), antibiotics, Na_2CO_3, serum and L-glutamine were purchased from Gibco (Grand Island, N.Y.). HEPES was obtained from Calbiochem (Los Angeles, Calif.; lot no. 920336).

In bicarbonate-buffered media, Na_2CO_3 was included to a final concentration of 2.2 g/liter (18.9 m*M*) in BME and 0.35 g/liter (3.3 m*M*) in 199 medium. HEPES-buffered media were prepared as follows: 100 ml of concentrated media were diluted with distilled water to an approximate volume of 700 ml. HEPES was dissolved (9.53 g in BME; 2.38 g in 199 medium) and the volume was adjusted to 1 liter with distilled water. The final concentration of HEPES was 40 m*M* in BME and 10 m*M* in 199 medium. These concentrations ensured optimal isotonicity of media based on Earle's or Hanks' salt solutions, respectively, when Na_2CO_3 was substituted by HEPES (2). After adding L-glutamine (final concentration 2 m*M*) and antibiotics (penicillin, 100 units/1 ml; streptomycin, 100 μg/ml), the pH was adjusted with 6 *N* NaOH, with an accuracy of $\pm$0.02 pH units. Considering $-0.014\ \Delta pK/°$ value for this buffer, the pH readings performed at room temperature were corrected to 37° (-0.2 pH units difference). All media were sterilized by filtration through Millipore filters (pore size 0.45 μ).

III. Cell incubations. All cultures were prepared in duplicate. PHA (P) (Difco, Detroit, Mich.) was included at a final concentration of 2 μl of standard solution per 1 ml of culture PPD (Parke-Davis, Detroit, Mich.) was at a concentration of 10 μg/ml. Na_2CO_3-buffered cultures were incubated with loosened caps in an incubator with a continuous flow of 5% CO_2 in air. Parallel HEPES-buffered cultures were incubated with loosened caps in a moistened chamber. Thymidine-methyl-^{3}H (New England Nuclear Corp., Boston, Mass.) (sp act, 6.7 Ci/mmole) was added at a final activity of 0.25 μCi/ml for 6 hr before terminating the cultures, unless stated otherwise in the figure legends. The pH of all terminated cultures was measured.

The cultures from plastic tubes were harvested by twice washing with ice-cold saline and then frozen. After thawing, they were suspended by treatment with a vortex mixer, transferred to 10-ml glass tubes, and washed twice with 5% cold TCA and with cold absolute methanol. Cell pellets were then dissolved in 1 ml of Soluene (Packard Instrument Co.) and the radioactivity was measured in an LS-200 Beckman liquid scintillation counter using Omnifluor-(New England Nuclear Corp.) toluene (4 g/liter). The mean value of duplicate cultures is presented in the figures.

Cell cultures from Leighton tubes were terminated by washing twice with cold saline and then by smearing onto gelatinized slides. Before washing with saline, however, part of the cell suspension from each culture was assayed for viability using the trypan blue exclusion test. The smears were fixed in

methanol and then stained with Giemsa. Other smears were fixed in ethanol–acetic acid (9:1), washed with 5% cold TCA for 10 min, with unlabeled thymidine (1 m*M*), and finally with distilled water (4 hr). The autoradiographs were prepared using AR10 Kodak film, and were exposed in dry CO_2 atmosphere at 4° for 10 days. After developing they were counterstained with hematoxylin.

The percentages of blast and labeled cells were calculated by evaluation of 1000 mononuclear cells from smears or from autoradiographs of each culture; the mean values of duplicate cultures are presented (Fig. 4).

Results. Expt. I (Fig. 1). This experiment was designed to establish the optimal pH for lymphocyte transformation in HEPES-buffered cultures. Cultures of control and PHA-treated lymphocytes in BME adjusted to pH values ranging from 7.1 to 7.7 were treated with ^3H-thymidine for 16 hr before harvesting, and the incorporated radioactivity was assayed by liquid scintillation as described under Methods. Surprisingly, the

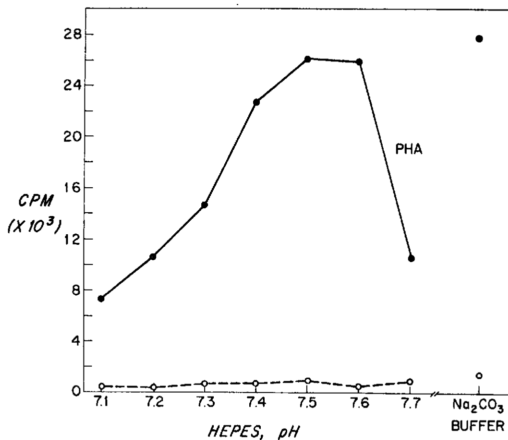

FIG. 1. Stimulation of lymphocytes (as measured by ^3H-thymidine incorporation) in Na_2CO_3- and HEPES-buffered media. 10^6 lymphocytes [control (O- -); or with PHA (●—)] were grown in 1-ml cultures prepared on Eagle's medium buffered with HEPES at different pH. A similar set of cultures was prepared on the same medium buffered with Na_2CO_3. Cultures were terminated on the third day and ^3H-thymidine was included for the last 16 hr of incubation. Mean values of duplicate cultures are presented.

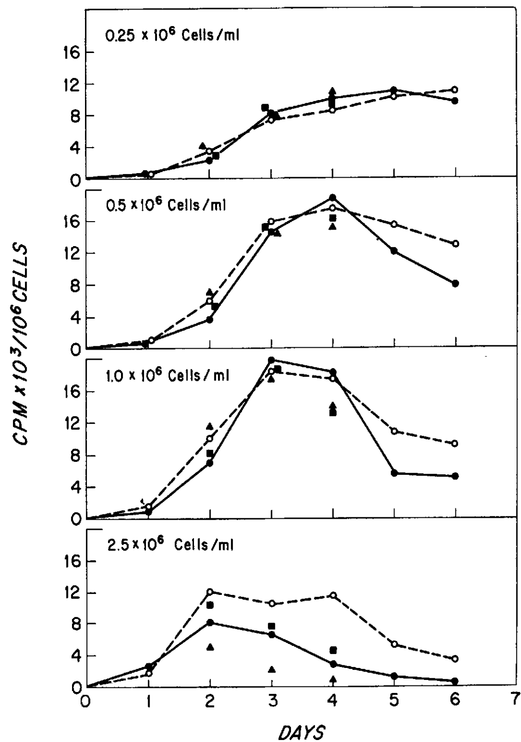

FIG. 2. The rate of lymphocyte stimulation (as measured by ^3H-thymidine incorporation) in cultures of various cell concentration, buffered with Na_2CO_3 and with HEPES. ^3H-thymidine was present in cultures during the last 6 hr. (O—) Eagle's medium, Na_2CO_3 buffer; (O- -) Eagle's medium, HEPES buffer; (▲) 199 medium, Na_2CO_3 buffer; (■) 199 medium, HEPES buffer.

maximal ^3H-thymidine incorporation by cells in HEPES-buffered cultures appeared to be at a relatively high pH (7.4–7.6). At pH 7.5 it was almost as high as in the cultures buffered with Na_2CO_3. The experiment was repeated using cultures in 199 medium with substantially similar results. In all further experiments on HEPES-buffered cultures the media were adjusted to pH 7.5.

HEPES itself does not stimulate lymphocytes, as in all control cultures in HEPES-buffered media, incorporation of ^3H-thymidine was even lower than in Na_2CO_3-buffered control cultures.

Expt. II (Fig. 2). The rate of lymphocyte stimulation in HEPES and in Na_2CO_3-buffered cultures was compared. The lymphocytes were cultured at different con-

centrations ($0.25–2.5 \times 10^6$/ml) in BME and 199 media. They were harvested every day (up to day 6) and ^{3}H-thymidine was included during the last 6 hr. The number of counts per culture was later recalculated per 10^6 cells initially in culture, to allow a better evaluation of the differences in the stimulation of lymphocytes at various densities. The highest stimulation was obtained at a concentration of 0.5 and 1.0×10^6 cells/ml. The peaks of stimulation appeared to occur at different times, depending on the cell density in culture. At a concentration of 0.25×10^6 cells/ml the peak was between days 5–7; at 0.5×10^6 cells/ml, on day 4; at 1.0×10^6, on day 3; and finally, at 2.5×10^6, on day 2. Incorporation of ^{3}H-thymidine was essentially similar in cultures buffered either with Na_2CO_3 or with HEPES, except in a few cases. Specifically, at the highest cell concentration (2.5×10^6/ml) after the second day of culturing, stimulation of lymphocytes in HEPES-buffered cultures (both in BME and 199 media) was markedly higher than in similar cultures buffered with Na_2CO_3. At concentrations of 10^6 and 0.5×10^6 cells/ml, cultures buffered with HEPES showed higher incorporation of ^{3}H-thymidine on days 5 and 6 than parallel Na_2CO_3-buffered cultures.

No significant differences in ^{3}H-thymidine incorporation were observed in parallel cultures made on BME or 199 media at cell concentrations up to 1.0×10^6/ml, irrespective of the buffer used. At 2.5×10^6/ml, however, stimulation of lymphocytes was considerably higher in BME in both buffer systems. The pH of all cultures was measured at the time of termination. In HEPES-buffered cultures, when lymphocytes were at a concentration of 1.0×10^6 or below, no change of pH greater than 0.05 units was observed. The only exception was at 10^6 cells/ml in 199 medium, when the pH dropped to 7.4 on the fourth day from 7.5. At 2.5×10^6 cells/ml, in BME–HEPES cultures, the pH was constant (7.5) throughout the first 4 days of culturing, and dropped 0.1 and 0.2 units on days 5 and 6, respectively. In 199–HEPES cultures the pH decreased to 7.4 on day 3 and to 7.2 on day 4.

Due to the rapid escape of CO_2 from Na_2CO_3-buffered cultures (when exposed to air) their pH could not be measured accurately. The pH of these cultures was evaluated by comparison with standard samples of phenol red in freshly prepared HEPES media of known pH. In general, Na_2CO_3-buffered cultures showed markedly larger changes in pH than the parallel HEPES cultures. This was especially pronounced in cultures of high cell density. Thus, in cultures made in BME–Na_2CO_3 medium, at cell concentrations of 2.5×10^6/ml, the pH dropped below 7.0 after 2 days of incubation. In 199–Na_2CO_3 medium, the decrease of pH at the same time was even greater (below 6.8). In Na_2CO_3-buffered cultures, at a lymphocyte concentration of 10^6/ml, the drop in pH was also observed (below 7.0), but only after 5 days of incubation.

In summarizing this experiment the following conclusions can be drawn. (i) The decrease of pH in older cultures and in those cultures of higher cell density is much more pronounced in Na_2CO_3-buffered media than in parallel cultures buffered with HEPES. This is also reflected by ^{3}H-thymidine incorporation. In all these cases higher incorporation was observed in HEPES-buffered cultures. (ii) The rate of lymphocyte stimulation in cultures of lower cell density is essentially similar in Na_2CO_3- and HEPES-buffered media. (iii) At lower cell densities the peaks of stimulation appear later, irrespective of whether cultures are buffered with HEPES or with Na_2CO_3.

Expt. III (Fig. 3). The lymphocyte stimulation by PPD and in mixed allogeneic cultures was studied. In both cases slightly higher ^{3}H-thymidine incorporation was observed in Na_2CO_3-buffered cultures. Incorporation of ^{3}H-thymidine in control cultures, in the absence of stimulants, was also higher in Na_2CO_3-buffered media.

Expt. IV (Fig. 4). Finally, the stimulation of lymphocytes was evaluated by morphologic criteria and by autoradiography. Cells were grown in Leighton tubes at various densities (calculated per 1 mm^2 of the tube bottom surface area) and assayed on days 2, 3, and 4 of culturing. The morphology of the cells from HEPES- and Na_2CO_3-buffered cultures was identical. Any morphologic changes that would be suggestive of a cytotoxicity of

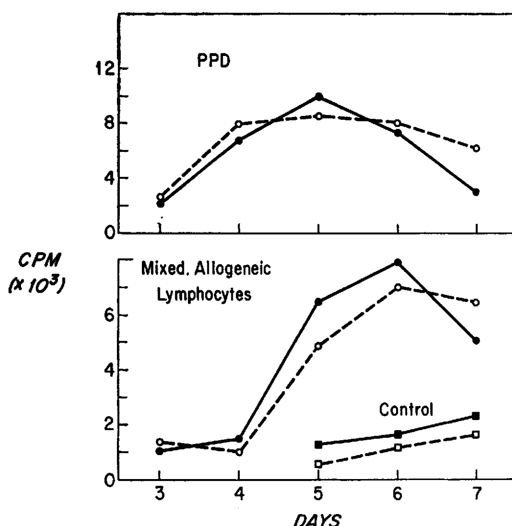

FIG. 3. Stimulation of lymphocytes (as measured by ^{3}H-thymidine incorporation) by PPD and in mixed cultures. 1-ml cultures of lymphocytes (10^6 cells) were prepared in Eagle's medium buffered with Na_2CO_3 (—); or with HEPES (- - - -). ^{3}H-thymidine was added 4 hr before termination of cultures. In PPD and mixed cultures, mean values of duplicates are presented; in controls to mixed cultures, the mean values were obtained from duplicate cultures of each donor.

HEPES were not detected. The mitotic figures were normal. Chromosomal preparations made from HEPES cultures did not show any obvious chromosomal abnormalities. Within the range of 10^2–10^4 cells/mm^2 the number of cells synthesizing DNA, or evaluated morphologically as lymphoblasts, was essentially similar in media buffered with HEPES or with Na_2CO_3. Again, however, a much higher transformation was observed in HEPES-buffered cultures when the cells were incubated at very high density (10^5/mm^2).

This experiment also confirmed the previous observation that at low cell densities transformation is delayed in time (6); this being evident considering either the number of cells engaged in DNA synthesis or the transformation index.

In all cultures at concentrations up to 10^4 cells/mm^2, whether buffered with HEPES or Na_2CO_3, less than 10% of the cells stained with trypan blue. At a density of 10^5/mm^2, however, in HEPES-buffered cultures, 80–90% were viable, while in cultures buffered with Na_2CO_3, the number of cells that stained with trypan blue increased to 20, 25, and 40% on days 2, 3, and 4 of culturing, respectively.

No change of pH was observed in cultures buffered with HEPES during 4 days of incubation except in one culture at a density of 10^5 cells/mm^2 where the pH decreased by 0.15 units (to 7.35) on day 4. At the same time, in Na_2CO_3-buffered cultures, the pH dropped to 6.9 and 6.6 in cultures of cell density 10^4 and 10^5/mm^2, respectively.

Discussion. The advantages of HEPES as a buffer for tissue culture have been fully discussed by Shipman (2). The present results indicate that HEPES-buffered media

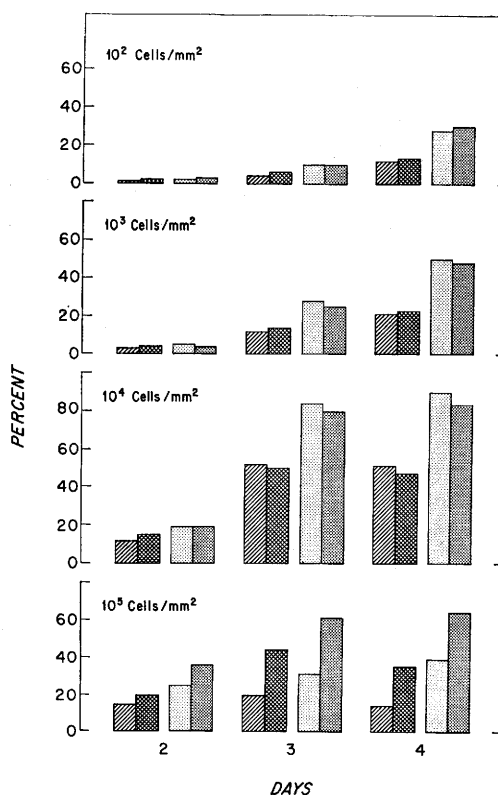

FIG. 4. The percentage of blast cells (dotted bars) and DNA-synthesizing cells (striped and cross-hatched bars) in cultures of lymphocytes stimulated by PHA, buffered with Na_2CO_3 (all left bars in the pairs) and with HEPES (right bars). Purified lymphocytes were incubated at various densities in Eagle's medium and the cultures were terminated on days 2, 3, and 4. ^{3}H-thymidine was included during the last 6 hr of culturing.

can be used for culturing lymphocytes and for studies of their stimulation. HEPES itself does not stimulate lymphocytes. The stimulation induced by a nonspecific agent such as PHA, or by antigens, proceeds with almost the same rate in HEPES as in Na_2CO_3-buffered cultures. In crowded cultures buffered with HEPES, the viability of lymphocytes and their rate of transformation is greatly facilitated compared with cultures of the same density buffered conventionally with Na_2CO_3.

Careful analysis of the present results (Figs. 1–4) shows, however, that the highest peaks of stimulation in cultures of moderate cell density were in media buffered with Na_2CO_3. Apart from the experiments reported here, HEPES-buffered media have also been used in this laboratory for culturing of lymphocytes in other studies. In these experiments also, in cultures of optimal cell density, higher stimulation was sometimes observed in Na_2CO_3-buffered media (up to 20% in comparison with HEPES cultures). We cannot explain this phenomenon, although it seems unlikely that HEPES itself could suppress stimulation. We have used both 199 and Eagle's media, with HEPES concentrations of 0.01 and 0.04 *M* respectively. If HEPES itself would suppress stimulation (or would be subtoxic to the cells) the differences described would be more pronounced at 0.04 *M* HEPES concentration. In fact, if differences were observed, they were always within the same range in Eagle's or in 199 medium. It seems more likely, therefore, that either carbonate ions, or the slight changes in pH within the physiological range that occur in Na_2CO_3-buffered cultures, could facilitate the cell stimulation.

The variation of the results between identical duplicate cultures was always much lower in HEPES-buffered media. It is suggestive, therefore, that the variation in the stimulation rate of lymphocytes in the identical cultures could, to a large extent, be attributed to a local change of pH in cultures.

Relatively high pH (7.4–7.6) was found to facilitate maximal stimulation in HEPES-buffered cultures. The pH of optimally stimulated cultures in Na_2CO_3-buffered media was between 7.2 and 7.4, as judged by comparison of the phenol red color with standard tubes. This method of pH evaluation is, however, not very accurate, and the changes of pH during incubation of Na_2CO_3-buffered cultures were also higher than in HEPES cultures, which does not enable one to establish the precise range of optimal pH for lymphocyte stimulation in Na_2CO_3-buffered cultures.

The stimulation of lymphoyctes in noncrowded cultures proceeded at the same rate in Eagle's and in 199 media, using either buffer system. In cultures of high cell density, however, cell survival and their stimulation was much better in Eagle's medium. This medium, based on Earle's salt solution, has a much higher buffering capacity, and thus is more advantageous for denser cultures.

Eagle's medium, when buffered with HEPES, thus offers an extremely high buffering capacity, which permits the growth of lymphocytes at high density and for longer times without the necessity of changing the medium. HEPES, at a concentration of 40 m*M*, does not seem to be toxic to lymphocytes during 7 days of culturing.

The phenomenon that fewer lymphocytes undergo stimulation in cultures of low cell density was described by Moorhead *et al.* (7) and by Ling (8). However, the present experiments (when cultures were assayed every day during 6 days' incubation) would indicate that peaks of maximal lymphocyte stimulation occur at different times, depending on the original cell density. In crowded cultures, the time required for the stimulated lymphocyte to pass through G_1 to S phase (DNA synthesis assay, Fig. 2) or to undergo morphologic transformation to a blast cell (Fig. 4) seems to be much shorter. This phenomenon does not seem to be related to the differences in pH of the cultures (which could result from the various cell densities in Na_2CO_3-buffered cultures) as it also occurs in HEPES-buffered cultures, where the pH is stable and fully controlled during all incubation. Presumably the cell–cell interaction (6, 7, 9) plays a primary role in this phenomenon.

Summary. HEPES [4-(2-hydroxyethyl)-1-piperazinyl-ethane-2-sulfonic acid] was stud-

ied as a possible hydrogen ion buffer in cultures of human peripheral blood lymphocytes. It was found the HEPES fulfills the demands required, namely:

1. It is not toxic to lymphocytes within the range of concentration (10–40 m*M*), which ensures excellent buffering capacity.

2. It does not itself stimulate lymphocytes.

3. It essentially does not affect the rate of lymphocyte stimulation induced by the nonspecific stimulant (phytohemagglutinin), antigen (tuberculin), or in mixed allogeneic cultures.

In comparison with bicarbonate buffer, HEPES offers greater buffering capacity within the physiological range of pH. Due to this, HEPES-buffered media maintained constant pH even in very crowded cultures, ensuring better cell survival and stimulation. The maximal stimulation of lymphocytes induced by PHA was observed in HEPES-buffered cultures at a pH range of 7.4–7.6.

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