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Effect of Environmental pH on the Efficiency of Cellular Hybridization

(human–mouse/heterokaryocytes/multinucleate/Sendai virus fusion/lysolecithin)

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ABSTRACT **The hybridization of a human and mouse cell was strikingly pH-dependent, with a well defined optimum at (about) pH 7.6–8.0. The yield of hybrid cell colonies (1 per 500–2000 heterokaryocytes) was several hundred times greater than that obtained at pH 6.8–7.2. Although there was a significant effect on the efficiency of cell fusion, the critical time for the pH effect was in the first 4–8 days after fusion, presumably while viable hybrids were being formed from the multinucleated heterokaryocytes.**

Individual strains of mammalian cells vary widely with respect to the optimum pH for growth, measured in terms of both cell-doubling time and maximum population density (ref. 1, and H. E., manuscript in preparation). Relatively nontoxic buffers are available (2, 3) that limit the wide pH swings otherwise observed in heavy cultures; thus, it has been possible to determine the optimum pH for several specialized cell functions†.

The present paper describes the effect of environmental pH on the fusion and hybridization of human and mouse cells, both with genetic markers that permit the selective growth of the hybrid in a medium that will not support the growth of either parental cell.

MATERIALS AND METHODS

Media. Minimum essential medium (ME medium) (4) supplemented with 0.2 mM of each nonessential amino acid, 2 mM glutamine, and 10% calf serum was buffered with various organic buffers, and adjusted to the desired pH Table 1; see ref. 3). Since the petri dish cultures were kept in a CO_2 incubator at fixed CO_2 tension, the concentration of $NaHCO_3$ was varied in relation to the pH desired (Table 1). 100 IU/ml of penicillin and 50 μg/ml of streptomycin were added. HAT medium‡ (5) was buffered with the same buffers as ME medium, and was adjusted to the same pH. The degree to which the pH shifted during the1–3 days between medium changes is indicated in the tables and figures.

Parental Cells. Cl-1D mouse cells resistant to 30 μg/ml of bromodeoxyuridine (6), and 1R mouse cells resistant to 15 μg/ml of azaguanine (7) were derived from the L line. The WI 18Va2 culture of human cells had been transformed by Simian virus 40, and was resistant to 15 μg/ml of 8-azaguanine (8). The parental mutant cells were maintained in culture in the presence of the inhibitors. The frequency of back mutation was checked periodically by seeding 10^6 cells in each of 20 petri dishes, in HAT medium at pH 8.0. This procedure permitted the growth of normal cells, but not of the mutants. No colonies grew in repeated trials.

Cell culture. The methods of cell culture and the isolation of drug-resistant mutant cells have been described (5–7).

Sendai Virus-Induced Fusion (9). The parental cells were maintained at various pH values (e.g., 6.4, 6.8, 7.2, 7.6, 8.0, 8.4, 8.8) for 12–24 hr before fusion. A mixture of the pH-adapted Cl-1D and either WI 18Va2 or 1R cells (5×10^6 each) was resuspended in 1 ml of salt solution or serum-free ME medium adjusted to the desired pH and containing 2000–4000 hemagglutinating units of β-propiolactone-inactivated Sendai virus (10). The cell mixtures were kept in ice for 20–30 min, at which time there were visible clumps. The suspension was then shaken for 30 min in a water bath at 37° and centrifuged; the pellet was resuspended in either ME medium or HAT medium at the desired pH. 1×10^6 Cells in 2 or 5 ml of HAT medium at various pH values were inoculated into 35-mm plastic petri dishes or 60-mm dishes containing coverslips, respectively.

TABLE 1. *Buffers used for pH stabilization during cell fusion and hybrid colony formation (after ref. 3)*

pH	6.4	6.8	7.2	7.6	8.0	8.4	8.8
NaH_2PO_4, mM	10*	10	2	—	—	—	—
$NaHCO_3$, mM	1	2	5	15	30	40	60
"Bis–Tris"	10	—	—	—	—	—	—
"Pipes"	10	10	—	—	—	—	—
"Bes"	—	15	15	10	—	—	—
"Tes"	—	—	—	10	10	—	—
"Hepes"	—	—	10	15	15	—	—
"Hepps" (EPPS)	—	—	—	—	10	10	—
"Tricine"	—	—	—	—	—	15	15
"Bicine"	—	—	—	—	—	10	15

* Added at the indicated concentration (mM) to minimal essential medium (4), prepared without bicarbonate, supplemented with 10% serum (5% calf and 5% fetal-calf), 2 mM glutamine, 0.2 mM (each) of 7 "nonessential" amino acids, and adjusted to the indicated pH.

† Papers are in preparation describing the optimum pH for globulin secretion by mouse myeloma cells, collagen synthesis by fibroblasts, S-100 protein synthesis by rat astrocytes, interferon production, and reovirus synthesis.

‡ ME medium containing hypoxanthine, aminopterin, and thymidine, at 100 μM, 0.4 μM, and 16 μM, respectively.

TABLE 2. *Effect of environmental pH on fusion between WI 18Va2 (human) and Cl-1D (mouse) cells*

pH of medium*	Fusion induced by	Multi-nucleated cells, %†	Heterokaryocytes† % of fused cells	Heterokaryocytes† % of total population
6.4	—	2.8	3.4	0.1
	Sendai	7.2	37.0	2.66
7.2	—	3.1	3.2	0.1
	Sendai	7.6	31.1	2.36
7.6	—	3.7	5.3	0.2
	Sendai	11.5	35.0	4.0
8.0	—	3.5	2.8	0.1
	Sendai	17.2	43.6	7.5
8.4	—	3.7	2.6	0.1
	Sendai	12.3	42.9	5.3

* Medium at the indicated pH was added to cells 24 hr before fusion, during the fusion process, and for 18 hr after fusion.

† A total of 1001–1046 cells were counted at each pH value.

Lysolecithin-Induced Fusion (*11*). 5×10^6 each of Cl-1D and WI 18Va2 cells were centrifuged at $180 \times g$ at 25° for 5 min. The mixed, redispersed cells were treated at the various pH values for 1 min with 0.1 ml of lysolecithin at 400 μg/ml in serum-free ME medium containing 5 mg/ml of serum albumin, treated with 0.5 ml of 30% calf serum in ME medium to neutralize the lysolecithin, centrifuged, resuspended, and inoculated in medium at various pH values as described above.

Autoradiography. 0.2 μCi/ml of [*methyl*-^{3}H]thymidine (12.5 Ci/mmol, New England Nuclear Corp., Boston, Mass.) was added to the medium 0, 24, or 48 hr after seeding of WI 18Va2 cells, which were then grown continuously in the presence of the isotope. On autoradiography after 4 days, more than 90% of the cells showed silver grains over the nuclei. The tritium-labeled WI 18Va2 cells were then fused with unlabeled Cl-1D cells. 18 Hr after fusion, the cells on the coverslips were fixed in ice-cold ethanol–acetic acid 3:1 for 15 min, and washed for 10 min in 70% ethanol. The coverslips were mounted on gelatinized glass slides and covered with Kodak AR10 stripping film (12). After routine autoradiographic development and fixing, the slides were stained with buffered Giemsa stain at pH 5.75.

TABLE 3. *Effect of environmental pH on formation of hybrid cell colonies*

Cells fused	pH of medium	pH of culture fluid after fusion: 1 day	3 days	Average number of hybrid cell colonies per petri dish (10^6 cells) after 14 days: —	Lysolecithin	Sendai
Human x mouse	6.4	6.5	6.4	0	0	0
WI 18Va2 x	7.2	7.0	6.9	0	0	0
Cl-1D	7.6	7.4	7.3	2.3	6.4	12.1
	8.0	7.8	7.7	14.6	43.5	50.5
Mouse x mouse	6.4	6.4	6.5	0		0
Cl-1D x 1R	7.2	7.0	7.0	0.1		0.9
	7.6	7.4	7.4	0.3		3.9
	8.0	7.8	7.8	0.7		7.0
	8.4	8.0	8.0	0.4		5.7
	8.8	8.4	8.3	0		0

Cells were exposed to medium at the indicated pH for 12–24 hr before fusion, and were fused and inoculated at that pH. The medium was changed 1, 3, 6, 9, and 12 days after fusion.

TABLE 4. *Effect of change of pH after fusion on hybrid-cell colony formation of WI 18Va2 (human) and Cl-1D (mouse) cells.*

pH during fusion	pH of culture fluid after fusion: At time of inoculation	After 1 day†	After 3 days†	Average number of hybrid-cell colonies per petri dish (10^6 cells) after 14 days: —	Lysolecithin	Sendai
	6.4	6.4–6.5	6.4–6.5	0	0	0
	7.2	7.0	6.9	0	0	0
8.0*	7.6	7.4	7.3	3.0	7.5	19.7
	8.0	7.8	7.7	16.7	29.7	60.0
	8.4	8.0	7.9–8.0	13.1	24.9	46.5
	6.4	6.5	6.4	0	0	0
	7.2	7.0	6.9	0	0	0
7.2*	7.6	7.3	7.2–6.3	5.4	9.0	10.0
	8.0	7.6–7.7	7.5–7.6	9.5	17.9	21.0
	8.4	7.9–8.0	7.9	4.4	10.4	9.5

* Cells were kept at indicated pH for 24 hr before fusion.

† Fresh medium at various pH values was added 1, 3, 6, 9, and 12 days after fusion and inoculation.

Cell characterization

Fusion. The percentages of multinucleated cells in the autoradiographic preparations, and the proportion of homo- and hetero-karyocytes, were determined by counts of at least 1000 cells from at least two coverslips in each experiment.

Hybridization. 10^6 of the mixed cells fused at various pH values with either Sendai virus or lysolecithin were seeded on 35-mm petri dishes in appropriately buffered HAT medium. The medium was changed at 1, 3, 6, 9, and 12 days, and the pH of the culture fluid was recorded at each change. The hybrid cell colonies were fixed, stained, and counted on the 14th day after fusion; 7–10 petri dishes were used for each determination. To confirm their hybrid nature, colonies derived from single cells were maintained in HAT medium, and recloned by seeding 100 cells per petri dish. The total number of proportion of biarmed or telocentric chromosomes was determined, and the human and mouse chromosomes in the hybrid cells were identified by the chromosomal banding technique with Giemsa stain, as described by Drets and Shaw (13).

RESULTS

The effect of pH on the fusion process

Two parental cell cultures (Cl-1D and WI 18Va2) were kept at pH 6.4, 7.2, 7.6, 8.0, and 8.4 for 24 hr; the WI 18Va2 cells were incubated in the presence of [^{3}H]thymidine. After trypsinization, portions of the two cultures were mixed in the presence of Sendai virus and were maintained at the five different pH

values during the fusion process and for the subsequent 18 hr of incubation in petri dishes. The cells were then fixed and examined by autoradiography (12).

The percentage of multinucleated cells was significantly higher when fusion was performed at pH 7.6–8.4, with a maximum of 17% at pH 8.0 (Table 2). At that pH, the average number of nuclei per multinucleated cell was also significantly increased. The yield of heterokaryocytes was similarly maximal at pH 8.0 (last column of Table 2). Qualitatively similar results, but lower yields, were obtained when the cells were kept at pH 7.2 before and after fusion, and the pH was varied only during the relatively short period of fusion with either Sendai virus or lysolecithin. In both experiments, the proportion of multinucleated cells that formed spontaneously, in the absence of either lysoleicthin or Sendai virus, was not affected by pH variation in the range 6.8–8.4.

The effect of pH on the formation of cell hybrids

The environmental pH had a striking effect on the yield of hybrid colonies, with an optimum again in the range 7.6–8.0 (Table 3). In contrast to the case of fusion, environmental pH affected not only Sendai- or lysolecithin-mediated hybridization, but spontaneous hybridization as well.

Two experiments were undertaken to determine whether the effects of environmental pH on the number of hybrid colonies ultimately formed were expressed only at the time of fusion, or in the subsequent period of cellular growth as well. Parental cells were kept at either pH 8.0 or 7.2 for 24 hr before fusion, and were fused at that same pH. The fused cells were then seeded in HAT medium at five different pH values. In both experiments, the pH at which the cells were kept *after* fusion had a marked effect on the number of hybrid cell colonies that ultimately developed (Table 4). Although the optimum in both cases was at pH 7.6–8.0, fewer hybrid colonies were formed when the cells had been fused at pH 7.2 than when fusion took place at pH 8.0. This result agrees with the effects of pH on the fusion process itself (Table 2).

To determine the critical period for the effect of post-fusion pH on cellular hybridization, cultures fused at pH 8.0 were kept at that pH for various times, then shifted back to pH 7.2. The yield of hybrid colonies increased with the length of time they were kept at pH 8.0 after fusion, with a maximum yield after 3–5 days in one experiment, and 8 days in another (curves O——O in Fig. 1). In the converse experiment, if the fused cells were immediately placed and maintained at pH 7.2, essentially no hybrid colonies developed. The hybrid cells could, however, be "rescued" by a shift of the pH back to 8.0, with progressively decreased yields in this experiment after the third day at pH 7.2 (curve ●– – –●).

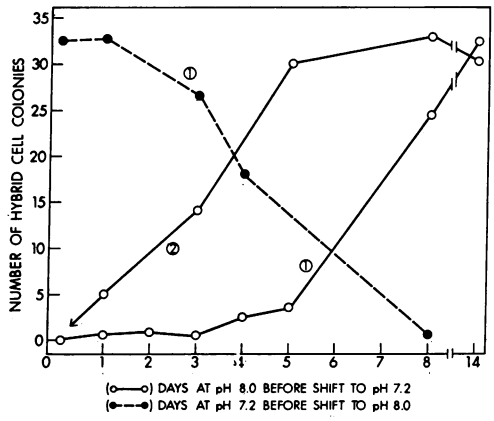

FIG. 1. The effect of environmental pH after cell fusion in the development of hybrid cell colonies. *Exp. 1.* Human (WI 18Va2) and mouse (Cl-1D) cells were fused with Sendai virus at pH 8.0, and plated at both pH 7.2 and 8.0. Medium was changed daily, and the pH was shifted at the indicated times (*data shown as lines labeled* ①). The pH of the culture fluid at the time of medium change varied between 7.5 and 7.9 for the pH 8.0 medium, and between 6.9 and 7.2 for the pH 7.2 medium. *Ordinate:* Number of hybrid-cell colonies per 5×10^5 cells fused. *Exp. 2.* As in Exp. 1, except that the medium was changed 1, 3, 5, 8, and 11 days after inoculation. The pH of the culture fluid at the time of medium change varied between 7.5–7.8 for the pH 8.0 medium, and between 6.8–6.95 for the pH 7.2 medium. The yield of hybrid colonies in this experiment was four times greater than is indicated by the ordinate scale (*line labeled* ②).

DISCUSSION

In the extensive literature on the methodology of somatic cell fusion and hybridization (10) (14), little attention has been paid to the effects of environmental pH. Bratt and Gallaher (15) have described an increased yield of homokaryocytes in chicken cells fused in the presence of Newcastle disease virus at pH 8.0–8.4; but, in general, when bicarbonate-buffered salt solution or growth medium is used for fusion, the pH at the time of fusion could be anywhere from 6.8–8.0, depending on population density and the balance between acid production and CO_2 loss (see ref. 3). There are similarly no data on the frequency of interspecific hybrid colony formation in relation to the pH of the medium during the period of incubation.

The data presented here indicate that the fusion process itself is pH-dependent, with a significantly larger yield of heterokaryocytes at pH 7.6 and 8.0 than at pH 7.2. Maintenance of pH 7.6–8.0 in the culture medium after fusion had an even greater effect on the formation of hybrid cell colonies, whether the cells had fused spontaneously or in the presence of lysolecithin or Sendai virus. When cells were fused at pH 8.0, and maintained at an average of about pH 7.85 through the entire subsequent 14 days (see Table 4), the yield of interspecific hybrid cell colonies in one experiment was 60 colonies per 10^6 of the mixed cells. This yield was reduced to 20 if the fused cells were placed at pH 7.6, and no colonies formed at pH 7.2 or 6.8. The critical time for this striking pH effect was the first 4–8 days after fusion. Cells that had been at pH 8.0 could then be shifted back to pH 7.2 without an effect upon the yield of hybrid cell colonies, while cells that had been at pH 7.2 could no longer be "rescued" by a shift to pH 8.0. In the system here studied, the formation of viable hybrid cells from heterokaryocytes was, at best, both inefficient and slow. Over a period of 4–8 days, one in 500–2000 heterokaryocytes developed into a hybrid cell capable of sustained replication, and at least one element in that process was markedly pH-dependent.

It is to be noted that the conditions generally used for cell fusion and hybridization, with cells suspended and inoculated at high population density in bicarbonate-buffered medium, predispose to rapid acidification (pH 7.2 and less). In that pH range, the yield of viable hyrbid cells was extremely small, fractional in relation to that obtained at pH 8.0.

The optimum pH of 7.6–8.0 for both fusion and hybridization does not coincide with the optimum for the growth of either the parental cells used in the present experiments or

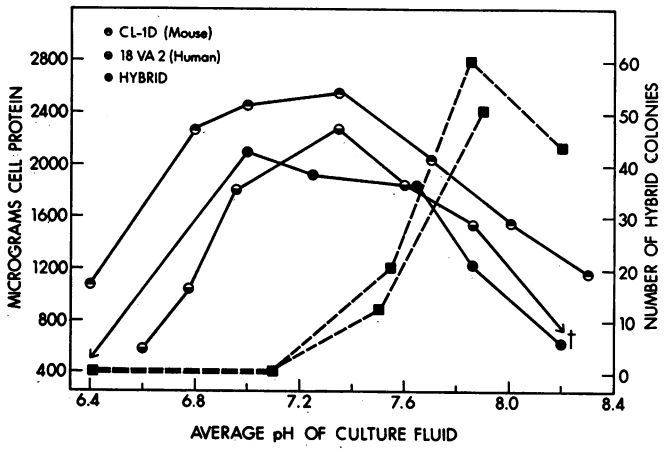

FIG. 2. The effect of environmental pH on cellular growth (⊖, ⊖, O) and on cellular hybridization by Sendai virus (■). The medium adjusted to, e.g., pH 6.4, 6.8, 7.2, 7.6. 8.0, and 8.4 was changed daily for the growth experiments, and on days 1, 3, 6, 9, and 12 for the hybridization experiments (Tables 3 and 4). The pH values plotted are the average pH during the period of exponential growth, or during the first 3 days after cell fusion, respectively. The growth of Cl-D (mouse) cells ⊖——⊖ was twice that shown on the ordinate scale. *Ordinates: (left)* μg of cell protein per 15-cm² flask after 6–9 days (——); *(right)* number of hybrid colonies per 10^6 fused cells after 14 days (■- - -■).

their hybrids. Measured by rate of growth, maximum population density, and plating efficiency, all the cells used in these experiments (including the hybrid progeny) had a pH optimum for growth of about 7.3, at which pH there was little or no effective hybridization, while the rate of growth was markedly depressed at pH 8.0, optimal for both cell fusion and hybridization (Fig. 2).

The specific step in hybrid cell formation from heterokaryocytes that is sensitive to pH remains to be determined. It is not known that the organic buffers used here actually penetrate into the cell, nor indeed has it been established that the intracellular pH necessarily mirrors that of the medium. It is possible that the striking effects of environmental pH on cellular growth, fusion or hybridization may be mediated at the plasma membrane rather than within the cell interior.

If the results reported here with three types of parental cells prove general,§ the efficiency of interspecific somatic cell hybridization can be greatly increased by appropriate control of environmental pH, and genetic analysis can be correspondingly facilitated. As one aspect of this problem, studies are in progress on the effect of environmental pH on the loss of chromosomes from human–mouse hybrids.

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1. Ceccarini, C. & Eagle, H. (1971) *Proc. Nat. Acad. Sci. USA* **68**, 229–233.
2. Good, N. E., Winget, G. D., Winter, W., Connolly, T. N., Izawa, S. & Singh, R. M. M. (1966) *Biochemistry* **3**, 467–477.
3. Eagle, H. (1971) *Science* **174**, 500–503.
4. Eagle, H. (1959) *Science* **130**, 432–437.
5. Littlefield, J. W. (1966) *Exp. Cell Res.* **41**, 190–196.
6. Dubbs, D. R. & Kit, S. (1964) *Exp. Cell Res.* **33**, 19–28.
7. Miggiano, V., Nabholz, M. & Bodmer, W. (1969) *Wistar Inst. Symp. Monogr. n9* 61–76.
8. Weiss, M. C., Ephrussi, B. & Scaletta, L. J. (1968) *Proc. Nat. Acad. Sci. USA* **59**, 1132–1135.
9. Harris, H. & Watkins, J. P. (1965) *Nature* **205**, 640–646.
10. Steplewski, Z. & Koprowski, H. (1970) *Methods Cancer Res.* **5**, 155–191.
11. Croce, C. M., Sawicki, W., Kritchevsky, D. & Koprowski, H. (1971) *Exp. Cell Res.* **67**, 427–435.
12. Rogers, A. W. (1967) *Techniques of Autoradiography* (Amer. Elsevier Pub. Co., New York), 1st ed., pp. 240–252.
13. Drets, M. E. & Shaw, M. W. (1971) *Proc. Nat. Acad. Sci. USA* **68**, 2073–2077.
14. Coon, H. G. & Weiss, M. C. (1969) *Proc. Nat. Acad. Sci. USA* **62**, 852–859.
15. Bratt, M. A. & Gallaher, W. R. (1969) *Proc. Nat. Acad. Sci. USA* **64**, 536–543.

NOTE ADDED IN PROOF

§ Results qualitatively and quantitatively similar to those here reported for human–mouse and mouse–mouse hybridization have since been obtained in hamster–rat and mouse–hamster pairings. The cells used were a hamster Maloney-virus-transformant MSVHT-1 BUCL-1 (TK^-) hybridized with rat hepatoma SU 5AH ($HGPRT^-$), and mouse IT 22 (TK^-) hybridized with a hamster polyoma transformant PYY-TG ($HGPRT^-$). In both cases, when cells were fused at pH 8.0 and then placed at various pH values, there were about ten times as many hybrid colonies at pH 8.0 as at pH 7.2. TK, thymidine kinase; *HGPRT*, hypoxanthine-guanine phosphoribosyl transferase.