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Selective growth of some rodent epithelial cells in a medium containing citrulline

(fibroblast outgrowth/urea cycle/cancer cell biology)

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ABSTRACT **We have defined a medium (called Sun's modified Waymouth medium) that selectively cultures some rodent epithelial cells that are capable of using citrulline in place of arginine. A growth-response study of the ability of 47 different mammalian cell cultures (of mouse, rat, Syrian hamster, Chinese hamster, guinea pig, rabbit, monkey, and human origin) to use arginine or its biosynthetic precursors, ornithine, citrulline, or argininosuccinate, showed that all epithelial cells and some fibroblasts are capable of growing in citrulline medium; however, primary embryo fibroblasts and 12 established fibroblast cell lines derived from Syrian hamsters failed to grow. The citrulline medium also allowed selective outgrowth of epithelial cells, without contaminating fibroblasts, from Syrian hamster tracheal explants. This absolute nutritional difference between Syrian hamster epithelial and fibroblast cells allows citrulline medium to be used for selective cultivation of epithelial cells, which should be valuable for study of growth, differentiation, and malignant transformation of mammalian epithelial cells.**

Most human cancers are of epithelial origin. Attempts to establish epithelial cells that retain differentiated functions in culture are complicated by the overgrowth of fibroblasts (1). Heaton (2) claimed that the addition of yeast extract to culture medium could selectively inhibit fibroblast growth, and Medawar *et al.* (3) showed that an unsaturated lactone, possibly δ-hexenolactone, could inhibit the action of a growth factor required by fibroblasts. However, other investigators were unable to reproduce these observations. Thus, Buonassisi *et al.* (4) have used a procedure of alternate cell culture and animal passage to obtain epithelial endocrine cell lines. The unique functions possessed by liver cells have enabled various workers to cultivate liver epithelial cells free of fibroblasts through the addition of steroid hormones to the culture medium (5); the use of glutamine-free medium (6, 7) and selection for cobblestone-like cell morphology (8, 9) have also yielded epithelial cell cultures. Neuroblastoma cell lines have been cloned by use of a tyrosine-free medium (10), since phenylalanine could be converted to tyrosine by nerve cells. Some mouse and human skin epithelial cells have been established by inhibiting fibroblast growth with 3T3 feeder cells (11, 12). A selective medium containing D-valine has also been used to inhibit fibroblast growth, since D-amino acid oxidase was found to be present only in the specialized epithelia of many mammals (13, 14); this procedure has not been generally applicable, however.

The basis of our search for selective growth conditions was the assumption that epithelial cells can best be distinguished from fibroblasts by their differentiated functions. Enzymes in the urea cycle (Fig. 1), which involve arginine biosynthesis from ornithine through the intermediates citrulline and argininosuccinate, are unique to liver cells. Only hepatocytes can use ornithine in place of arginine in culture and in the animal; depending on the species, other types of cells are able to use citrulline in place of arginine (15). Here we report that all epithelial cells so far tested can use citrulline in place of arginine, while the ability of fibroblasts to use citrulline varies from species to species. Fibroblasts from Syrian hamsters cannot survive in citrulline medium, and fibroblasts derived from rats and Chinese hamsters degenerate after several serial passages in the medium.

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MATERIALS AND METHODS

Cell Lines and Culture Conditions. Cell lines were maintained in Waymouth medium 752/1 (KC Biological, Lenexa, KS) supplemented with L-alanine (17.8 mg/liter), L-arginine·HCl (189 mg/liter), L-asparagine (30 mg/liter), L-serine (21 mg/liter), putrescine·HCl (0.322 mg/liter), sodium pyruvate (220 mg/liter), insulin (5 mg/liter), hydrocortisone (0.1 mg/liter), $NaHCO_3$ (1.5 g/liter), and 10% fetal bovine serum (Reheis, Kankakee, IL). This modification is referred to as Sun's modified Waymouth medium. When supplemented with dialyzed fetal bovine serum, this modified medium is called arginine medium. Ornithine, citrulline, or argininosuccinate medium designates the medium in which arginine is replaced by an equimolar concentration of the precursor specified leading to arginine biosynthesis. Fetal bovine serum was dialyzed against 3 vol of Dulbecco's phosphate-buffered saline by use of an Amicon hollow-fiber cartridge with a molecular weight cutoff point of 5000. Cells were cultured under standard conditions of 37°C in a nearly 100% humidified atmosphere of 5% CO_2. Cells were passaged at a 1:10 ratio. The cell number was determined by use of a Cytofluorograf.

Explant Cultures. Explants from tracheas or other organs obtained aseptically from 5- to 6-week-old animals were established in 24-well plates (Costar Co., Cambridge, MA) in citrulline medium. The animals used (and their sources) were: Fischer rats (Biology Division, Oak Ridge National Laboratory, Oak Ridge, TN), Syrian hamsters (Engel Laboratory, Famersburg, IN), Chinese hamsters (Bioassay System, Cambridge, MA), and guinea pigs (University of Tennessee-Comparative Animal Research Laboratory, Oak Ridge, TN).

Isolation of Lung Epithelial Cells. Each animal was perfused through the heart with Hepes/phosphate-buffered saline (150 mM NaCl/5 mM KCl/4 mM KH_2PO_4/5.5 mM glucose/10 mM Hepes) to remove blood from the lungs. The lungs were further perfused through the trachea with 0.1% Pronase (type VI, Sigma) in the same medium. Lungs were minced and pipetted in Sun's modified Waymouth medium to separate epithelial cell clusters from a matrix of fibroblasts. The cell suspension was filtered through 50- and 100-mesh stainless steel screen to remove the matrix. Epithelial cell clusters were obtained relatively free of erythrocytes in the pellet fraction after

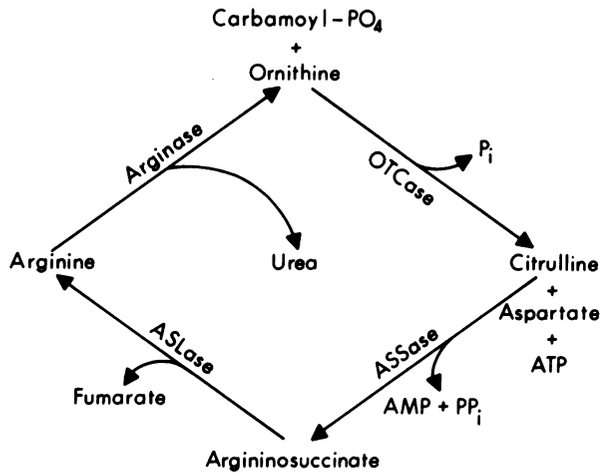

FIG. 1. The urea cycle and its enzyme system: ornithine transcarbamylase (OTCase; EC 2.1.3.3), argininosuccinate synthetase (ASSase; EC 6.3.4.5), argininosuccinate lyase (ASLase; EC 4.3.2.1), and arginase (EC 3.5.3.1).

the cell suspension was centrifuged (5 min, 160 × *g*) through the same Hepes/phosphate-buffered saline containing 0.6 M sucrose.

Growth Response of Different Cell Lines. Cells (10^5) of each line were inoculated into 60-mm plates (Corning) in ornithine, citrulline, argininosuccinate, or arginine medium. The growth of the cells in each treatment group was determined at 14 days, by which time the cells reach confluency in arginine medium. Only when cells grew to confluency within two consecutive 14-day scoring periods were they scored as positive for growth in a particular medium.

Test of Primary Epithelial Cell Growth. Clusters of isolated primary epithelial cells were plated in chamber slides in arginine-free medium for 7 days to exhaust the intracellular pool of arginine and to allow the cell clusters to attach to the substratum. The medium was then replaced with test medium (i.e., ornithine, citrulline, argininosuccinate, or arginine medium) containing [^{3}H]thymidine (0.5 μCi/ml, 1 Ci = 3.7 × 10^{10} Bq) for 48 hr. The cells were fixed in formalin (10%), coated with Kodak NTB-2, and exposed for 2 days. A ring of labeled cells surrounding a center of unlabeled cells was scored as positive for growth in that medium.

Preparation of Cell Extracts. Cells were washed twice with 0.9% saline and detached from the plates through treatment with 0.01% trypsin for 5 min at 37°C. Cells (2 × 10^7) were suspended in distilled water, frozen and thawed four times, and centrifuged at 20,000 × *g* for 20 min. Enzyme assays were performed in the supernatant fraction. Protein was measured by the biuret method (16).

Assay of Argininosuccinate Synthetase Activity. The activity of argininosuccinate synthetase [L-citrulline:L-aspartate ligase (AMP-forming), EC 6.3.4.5] was determined by the method of Lockridge *et al.* (17). The reaction mixture contained, in a total volume of 0.5 ml, 10 mM Tris·HCl, 10 mM $MgCl_2$, 100 mM L-citrulline, 0.2 mM L-aspartate, 5 mM ATP, 1 × 10^6 cpm of [^{14}C]aspartate (250 Ci/mol, Schwarz/Mann), and 10 μl of cell extract that contains ≈100 μg of protein. [^{14}C]Aspartate was purified in a Dowex 1-X8 acetate column as described (17). The enzyme assay was initiated by the addition of cell extract. After 1 hr of incubation at 37°C, the reaction mixture was boiled for 5 min to terminate the reaction. Acetic acid (1 M, 0.05 ml) was added to the boiled reaction mixture, which was incubated for 2 days at 37°C to convert argininosuccinate to its anhydride form. The anhydride was separated from [^{14}C]aspartate by elution from a Dowex 1-X8 acetate resin column, then collected for measurement of radioactivity. Enzyme activity was expressed as nmol of argininosuccinate produced per hr per mg of protein.

RESULTS

Growth of Different Cell Cultures in Medium Containing Arginine or Its Precursors. A total of 47 different mammalian cell cultures consisting of primary and secondary cultures, as well as established fibroblast and epithelial cell lines from humans, primates, and rodents, have been tested for their ability to grow in ornithine, citrulline, argininosuccinate, and arginine media (Table 1). None of the cells tested could use ornithine, and the addition of carbamoyl phosphate and ornithine to allow the formation of citrulline did not affect cell survival. All cell cultures showed optimal growth in argininosuccinate and arginine media. Both fibroblasts and epithelial cells of human and mouse origin were able to grow in citrulline medium.

Primary epithelial cells from rat trachea, lung, and kidney, as well as the transformed tracheal cell lines 1000 W and 909, grew in citrulline medium. The epithelial origin of these cells and their characteristics have been documented (18). Primary lung fibroblasts derived from Fischer rats also were able to use citrulline; however, this ability was lost after one passage (at a 1:10 ratio) in culture. The failure of two continuous rat cell lines, NRK and XC, to grow in citrulline might be due to their extremely low argininosuccinate synthetase activity (Table 2) during the long period of establishment as continuous cell lines. We were unable to obtain other cell lines derived from Fischer rats to test their ability to grow in citrulline medium.

Both primary epithelial cells and fibroblasts from lung and trachea of Chinese hamsters were able to grow in citrulline medium, while the secondary fibroblasts from lung and trachea lost the ability to do so. Such loss of ability to use citrulline might explain the failure of Chinese hamster cell lines V79 and CHO to grow in citrulline medium, even when 10^7 cells were tested for up to 3 weeks.

Unlike cultures from other species, Syrian hamster cultures displayed a distinct difference between the abilities of fibroblasts and epithelial cells to grow in citrulline medium. Embryo fibroblast cultures and 12 fibroblast cell lines did not grow in citrulline medium, even when tested at a density of 5 × 10^5 cells per 100-mm plate for up to 21 days. Primary cultured fibroblasts derived from different organs also failed to use citrulline. In contrast, two skin epithelial cell lines and primary epithelial cells derived from lung and trachea grew in the citrulline medium. Electron microscopic studies of these two continuous epithelial skin cell lines revealed the presence of intermediate junctions, a characteristic of epithelial cells but not of fibroblasts (19). Furthermore, when 5 × 10^6 cells of two epithelial cell lines, HAE-1 and HAE-2, were injected into *nude* mice, anaplastic tumors were induced within a month (unpublished observation).

Explant Cultures. The apparent selectivity of citrulline medium for growth of Syrian hamster epithelial cells but not fibroblasts was examined further by use of explant cultures of various organs or tissues that contain both cell types. Explant cultures established from organs other than trachea did not show selective growth of epithelial cells free from fibroblasts in citrulline medium. Fibroblasts migrated out from organ explants much faster than did epithelial cells, leading to outgrowth of fibroblasts even in ornithine medium. However, in 185 tracheal explants tested, only epithelial cells grew in citrulline medium after continuous observation for a month. Fig. 2 shows the outgrowth in citrulline medium of cells from tracheal explants derived from Syrian hamsters, Chinese hamsters,

Table 1. Growth of mammalian cells in ornithine, citrulline, argininosuccinate, and arginine media

Animal	Cell type (history*)	Culture (origin)	Growth in medium specified				Source of cells
			Ornithine	Citrulline	Argininosuccinate	Arginine	
Mouse	Fibroblast (finite, 2°)	Sme (embryo)	−	+	+	+	This laboratory
		Blc (embryo)	−	+	+	+	This laboratory
	Fibroblast (continuous)	3T3 (embryo)	−	+	+	+	S. A. Aaronson
		RFM (embryo)	−	+	+	+	This laboratory
		RAG (kidney)	−	+	+	+	P. A. Lalley
	Epithelial (continuous)	ME (skin)	−	+	+	+	T. J. Slaga
Rat	Fibroblast (finite, 1°)	RLF-1° (lung)	−	+	+	+	This laboratory
	Fibroblast (finite, 2°)	RLF-2° (lung)	−	−	+	+	This laboratory
	Fibroblast (continuous)	NRK (kidney)	−	−	+	+	This laboratory
		XC (muscle)	−	−	+	+	This laboratory
	Epithelial (finite, 1°)	RLE-1° (lung)	−	+	+	+	This laboratory
		RTE-1° (trachea)	−	+	+	+	This laboratory
		RKE-1° (kidney)	−	+	+	+	This laboratory
	Epithelial (continuous)	K16 (liver)	−	+	+	+	I. B. Weinstein
		1000W (trachea)	−	+	+	+	A. C. Marchok
		9-0-9 (trachea)	−	+	+	+	A. C. Marchok
Syrian hamster	Fibroblast (finite, 1°)	SHE-1° (embryo)	−	−	+	+	This laboratory
	Fibroblast (continuous)	X-2 (embryo)	−	−	+	+	This laboratory
		X-5 (embryo)	−	−	+	+	This laboratory
		X-6 (embryo)	−	−	+	+	This laboratory
		β-PL-4 (embryo)	−	−	+	+	This laboratory
		β-PL-6 (embryo)	−	−	+	+	This laboratory
		BP-2 (embryo)	−	−	+	+	This laboratory
		4N-2 (embryo)	−	−	+	+	This laboratory
		4N-3 (embryo)	−	−	+	+	This laboratory
		MCA-3 (embryo)	−	−	+	+	This laboratory
		MCA-5 (embryo)	−	−	+	+	This laboratory
		MCA-24 (embryo)	−	−	+	+	This laboratory
		MG-5 (embryo)	−	−	+	+	This laboratory
	Epithelial (finite, 1°)	SHT-1° (trachea)	−	+	+	+	This laboratory
		SHL-1° (lung)	−	+	+	+	This laboratory
		SHK-1° (kidney)	−	+	+	+	This laboratory
	Epithelial (continuous)	HAE-1 (skin)	−	+	+	+	D. L. Berry
		HAE-2 (skin)	−	+	+	+	D. L. Berry
Chinese hamster	Fibroblast (finite, 1°)	CHLF-1° (lung)	−	+	+	+	This laboratory
	Fibroblast (finite, 2°)	CHLF-2° (lung)	−	−	+	+	This laboratory
	Fibroblast (continuous)	V79 (lung)	−	−	+	+	This laboratory
		CHO (ovary)	−	−	+	+	This laboratory
	Epithelial (finite, 1°)	CHLE-1° (lung)	−	+	+	+	This laboratory
		CHTE-1° (trachea)	−	+	+	+	This laboratory
Guinea pig	Fibroblast (finite, 2°)	GP-2° (lung)	−	+	+	+	This laboratory
Rabbit	Fibroblast (continuous)	CCL-60 (lens)	−	+	+	+	This laboratory
Monkey	Fibroblast (finite, 2°)	Pongo (skin)	−	+	+	+	This laboratory
		Gorilla (skin)	−	+	+	+	This laboratory
Human	Fibroblast (finite, 2°)	WI-38 (lung)	−	+	+	+	L. Hayflick
	Fibroblast (continuous)	H5578T (breast tumor)	−	+	+	+	W. K. Yang
	Epithelial (continuous)	ALAB (breast tumor)	−	+	+	+	W. K. Yang

* 1° refers to primary cultures; 2° refers to secondary cultures.

Table 2. Argininosuccinate synthetase activity in cultured rodent epithelial and fibroblast cells

Cell line	Specific activity,* nmol/hr per mg protein
Syrian hamster fibroblasts	
BP-2	0.50
MG-4	0.27
4N-2	0.34
Syrian hamster epithelial cells	
HAE-1	31.90
HAE-2	23.82
Rat fibroblasts	
XC	0.36
NRK	0.20
Rat epithelial cells	
9-0-9	18.79

* Average of two independent assays.

and rats. Only cells from Syrian hamster explants were pure epithelial cells without contaminating fibroblasts. Although 5 of 15 rat explant cultures showed pure epithelial cell growth in the first 1–2 weeks, this was followed by the outgrowth of fibroblasts, as observed earlier by Marchok *et al.* (18) under different experimental conditions. Thus, it appears that citrulline medium provides a simple and defined system to establish pure epithelial cells from Syrian hamster tracheal explants as well as from monolayer cell cultures.

Clonal Growth of Epithelial Cell Lines from an Excess of Syrian Hamster Primary Fibroblasts. As a test of whether transformed epithelial cells could form distinct colonies from a background of fibroblasts, reconstruction experiments were carried out in which 100 transformed skin epithelial cells, designated HAE-1 or HAE-2, were mixed with 10^5 Syrian hamster embryo fibroblasts in citrulline medium. After 4 weeks, the cultures were fixed and stained. Embryo fibroblasts did not grow in citrulline medium; however, epithelial cell lines HAE-1 and HAE-2 exhibited 15–25% cloning efficiency, which was enhanced to ≈45% when these cells were cocultured with 1×10^5 fibroblasts (Table 3). Ten colonies exhibiting epithelial cell morphology from each mixing experiment were cloned and grown for karyotypic analysis to identify their origin. HAE-1 and HAE-2 cells recovered from these reconstruction experiments were highly polyploidic, with modal chromosome numbers of 72 and 70, respectively, which are characteristic of the original HAE-1 and HAE-2 model cells. The embryo fibroblasts maintained a diploid constitution ($2N = 44$) for about 20 passages in arginine medium. These reconstruction experiments, therefore, demonstrated that transformed epithelial cells could be selectively grown in citrulline medium from a culture containing a large excess of fibroblasts.

Argininosuccinate Synthetase Activity in Representative Epithelial and Fibroblast Cell Lines. Although it is convenient to test the cells' growth response to citrulline as a means to distinguish epithelial cells from fibroblasts, it is important to provide some quantitative difference between the argininosuccinate synthetase activity of these two cell types. Argininosuccinate synthetase activity was measured in eight cell lines derived from Syrian hamster and rat (Table 2). Fibroblasts have, on the average, 1–2% of the enzyme activity of the corresponding epithelial cells. Thus, the wide difference in argininosuccinate synthetase activity between fibroblasts and epithelial cells appears to account for their different growth response in citrulline medium.

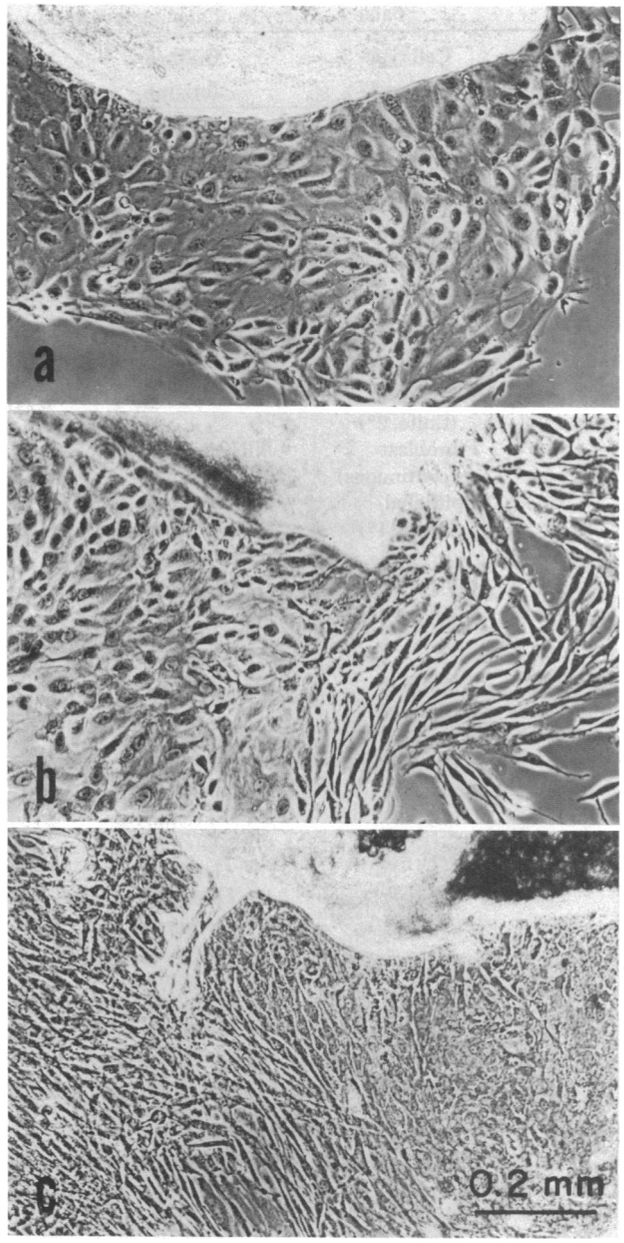

FIG. 2. Growth of tracheal explant cultures from (*a*) Syrian hamsters, (*b*) Chinese hamsters, and (*c*) rats in citrulline medium.

DISCUSSION

In the absence of a selective culture method for epithelial cells free from contaminating fibroblasts, epithelial cell lines were established based on morphological criteria such as the so-called cobblestone arrangement of cuboidal cells. However, established Buffalo rat liver cells with a cuboidal shape were shown later to be fibroblast cells (7). Several morphologically epithelial cell lines listed by the American Type Culture Collection were subsequently identified as sublines of HeLa cells (20). Selective cultivation of epithelial cells must be rigorously performed to avoid contaminating fibroblast growth. The D-valine medium reported by Gilbert and Migeon (13) to allow selective culturing of epithelial cells but not fibroblasts was found in our laboratory to support the growth of fibroblasts such as CHO and 3T3 cells after a lag period of 1–2 days (unpublished results).

The search for selective growth conditions is based on the assumption that epithelial cells can be best distinguished from fibroblasts by their specific differentiated functions exhibited

Table 3. Clonal growth of transformed epithelial cells cocultured with an excess of Syrian hamster embryo fibroblasts in citrulline medium

Epithelial cell line tested	No. of cells seeded		Average no. of colonies per plate
	Fibroblasts	Epithelial cells	
HAE-1	10^5	0	0
	10^5	100	43
	0	100	13
HAE-2	10^5	0	0
	10^5	100	44
	0	100	25

in culture. Based on the growth response of epithelial cells to the urea cycle metabolic intermediates, the failure of fibroblasts to use citrulline indicates a deficiency of argininosuccinate synthetase activity. Determinations of this enzyme activity of fibroblast and epithelial cells derived from Syrian hamsters and rats (Table 3) confirmed this point.

Citrulline medium selects for Syrian hamster epithelial cells and serves as their physiological marker. With some technical modifications, it is also applicable to Chinese hamster and Fischer rat cells (unpublished observation). Thus, it appears that all mammalian epithelial cells can use citrulline through the action of argininosuccinate synthetase. The general applicability of citrulline medium for selecting fibroblast-free epithelial cells depends on the expression of argininosuccinate synthetase in fibroblasts, which is a species-specific characteristic (Table 1). Using the citrulline medium, we have established lung epithelial cell lines from Fischer rats and Syrian hamsters and skin epithelial cells of Syrian hamster origin after treatment with dimethylnitrosamine (unpublished results).

The selective growth of some rodent epithelial cells in citrulline medium can provide a tool for studying growth, differentiation, and malignant transformation of various epithelial cells; for testing the effects of drugs on such cells; and for isolating epithelial cell-tropic RNA tumor viruses. Furthermore, since citrulline medium selects against all established fibroblast cell lines derived from Syrian hamsters, Chinese hamsters, and rats, it should be useful as a selective system in somatic cell hybridization studies.

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1. Carrel, A. & Burrows, M. T. (1910) *J. Am. Med. Assoc.* **55,** 1379–1381.
2. Heaton, T. B. (1926) *J. Pathol. Bacteriol.* **29,** 293–306.
3. Medawar, P. B., Robinson, G. M. & Robinson, R. (1943) *Nature (London)* **151,** 195.
4. Buonassisi, V., Sato, G. & Cohen, A. I. (1962) *Proc. Natl. Acad. Sci. USA* **48,** 1184–1190.
5. Waymouth, C., Chen, H. W. & Wood, B. G. (1971) *In Vitro* **6,** 371.
6. Kulka, R. G., Tomkins, G. M. & Crook, R. B. (1972) *J. Cell Biol.* **54,** 175–179.
7. Leffert, H. L. & Paul, D. (1973) *J. Cell. Physiol.* **81,** 113–124.
8. Coon, H. G. (1968) *J. Cell Biol.* **39,** 29A.
9. Williams, G. M., Weisburger, E. K. & Weisburger, J. H. (1971) *Exp. Cell Res.* **69,** 106–112.
10. Breakefield, X. O. & Nirenberg, M. W. (1974) *Proc. Natl. Acad. Sci. USA* **71,** 2530–2533.
11. Rheinwald, J. G. & Green, H. (1975) *Cell* **6,** 317–330.
12. Rheinwald, J. G. & Green, H. (1975) *Cell* **6,** 331–344.
13. Gilbert, S. F. & Migeon, B. R. (1975) *Cell* **5,** 11–17.
14. White, M. T., Hu, A. S. L., Hamamoto, S. T. & Nandi, S. (1978) *In Vitro* **14,** 271–281.
15. Eagle, H. (1959) *Science* **130,** 432–437.
16. Henry, R. J., Sobel, C. & Berkman, S. (1957) *Anal. Chem.* **29,** 1491–1495.
17. Lockridge, O., Spector, E. B. & Bloom, A. D. (1977) *Biochem. Genet.* **15,** 395–407.
18. Marchok, A. C., Rhoton, J. C., Griesemer, R. A. & Nettesheim, P. (1977) *Cancer Res.* **37,** 1811–1821.
19. Farquhar, M. G. & Palade, G. (1963) *J. Cell Biol.* **17,** 375–412.
20. Jones, H. W., McKusick, V. A., Harper, P. S. & Wuu, K. D. (1971) *Obstet. Gynecol.* **38,** 945–949.