PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 157, 500–505 (1978)

Squamous Metaplasia of the Tracheal Epithelium in Organ Culture. II. Nutritional Influences[1] (40085)

B. T. MOSSMAN, N. HEINTZ, B. V. MACPHERSON, AND J. E. CRAIGHEAD

Department of Pathology, University of Vermont College of Medicine, Burlington, Vermont 05401

Squamous metaplasia is defined as the replacement of differentiated epithelial cells by squamous-like cells not normally present at that tissue site (1). In the respiratory mucosa, metaplasia is a common response of the epithelium to a variety of mechanical (2, 3), chemical (4, 5) and metabolic stimuli (6).

Although usually considered a benign and reversible lesion, squamous metaplasia occasionally is associated with squamous cell carcinoma *in vivo* and may, under some circumstances, constitute a distinct pre-cancerous stage in the development of this lesion. Consequently, factors affecting epithelial differentiation, and in particular, squamous metaplasia, may contribute to neoplastic transformation in some as yet unknown fashion.

Vitamin A is essential for the maintenance of a well differentiated respiratory mucosa (6, 7). In its absence, the mucociliary epithelium becomes metaplastic and keratinized. Experiments in our laboratory indicate that other nutritional influences may also play a role in the development of squamous metaplasia (8, 9). When cultured in a vitamin A-deficient, nutritionally complex medium such as Waymouth's MAB 87/3, the differentiated respiratory mucosa of the hamster trachea rapidly undergoes squamous metaplasia and keratinization. In contrast, these explants exhibit a differentiated columnar epithelium for extended periods of time when maintained in a basal medium such as Eagle's minimum essential medium (MEM) in the absence of vitamin A.

Studies reported here were undertaken to determine the nutritional constituents in Waymouth's medium, but absent from Eagle's MEM, that promote squamous metaplasia.

Materials and methods. Organ Culture Preparation. Hamster tracheal explants were prepared from 6-week-old inbred female hamsters (87.20 strain, TELACO, Bar Harbor, ME) by previously described methods (8, 9). The trachea was excised from the larynx to the bronchial bifurcation and immediately immersed in Hanks' balanced salt solution containing 100 μg Gentamicin and 50 u nystatin per ml. The trachea was then opened along the anatomic discontinuity in the cartilage rings, and individual rings were cleaved from the tissue. Each resulting C-shaped segment was cut in half to yield explants with an epithelial surface approximately 1 × 2 mm. A segment of tissue from each animal was fixed in Bouin's solution before culturing. The remaining pieces were then placed into a 35 mm plastic Petri dish and 0.4 ml of a test medium containing 50 μg Gentamicin and 25 u nystatin per ml. Explants were maintained in a 95% air–5% CO_2 water-saturated environment at 35° and the medium changed twice weekly.

Formulation of media. Table I lists the nutritional constituents present in Waymouth's MAB 87/3 medium (10) but absent from Eagle's MEM (11). These components were divided into 5 groups on the basis of their chemical or nutritional similarities. Group I consisted of insulin and glutathione. Group II contained the three water soluble vitamins, ascorbic acid, B_{12}, and biotin. Group III was made up of the nucleic acid intermediates thymidine and hypoxanthine. Group IV was composed of eight inorganic salts (KH_2PO_4, $MgCl_2 \cdot 6H_2O$, $FeSO_4$, $CuSO_4 \cdot 5H_2O$, $MnSO_4 \cdot H_2O$, $ZnSO_4 \cdot 7\ H_2O$, $(NH_4)_6Mo_7O_{24} \cdot 4\ H_2O$ and $CoCl_2 \cdot 6H_2O$). Group V consisted of eight nonessential amino acids (L-alanine, L-asparagine, L-aspartic acid, L-cysteine HCl, L-glutamic acid, glycine, L-proline, L-serine). Test media were formulated by adding each nutritional constituent, or group of constituents, to Eagle's MEM at the same concentrations as found in Waymouth's MAB 87/3. Since there are substantial differences between the two media in

[1] This research was supported by Contract NO1 CP 33360 from the Lung Cancer Division of the National Cancer Institute.

0037-9727/78/1573-0500$01.00/0

TABLE I. CONSTITUENTS PRESENT IN WAYMOUTH'S MAB 87/3 BUT ABSENT FROM EAGLE'S MINIMUM ESSENTIAL MEDIUM (MEM).[a]

		Mg/L
Group I	INSULIN AND GLUTATHIONE	8.00
	Insulin	15.00
	Glutathione	
Group II	VITAMINS	
	Ascorbic acid	17.50
	Vitamin B_{12}	.20
	Biotin	.02
Group III	NUCLEIC ACID INTERMEDIATES	
	Thymidine	8.00[b]
	Hypoxanthine	25.00
Group IV	INORGANIC SALTS	
	KH_2PO_4	208.0
	$MgCl_2 \cdot 6H_2O$	240.0
	$FeSO_4$	.45
	$CuSO_4 \cdot 5H_2O$	.50
	$MnSO_4 \cdot H_2O$	.016
	$ZnSO_4 \cdot 7H_2O$	.030
	$(NH_4)_6MO_7O_{24} \cdot 4H_2O$	.025
	$CoCl_2 \cdot 6H_2O$	.022
Group V	NONESSENTIAL AMINO ACIDS	
	L-Glutamic acid	150.0
	L-Aspartic acid	60.0
	L-Serine	12.80[b]
	Glycine	50.00
	L-Proline	50.00
	L-Cysteine-HCl	90.00
	L-Alanine	11.20[b]
	L-Asparagine	24.00

[a] Formulations in GIBCO catalog, (1973–1974), Grand Island, NY, pp. 110, 120.
[b] Corrections to original formulation.

the concentrations of glucose, riboflavin, thymine and choline chloride, the concentrations of these four constituents were adjusted to the higher concentration in the Waymouth's formulation. Media were sterilized by Millipore filtration under nitrogen pressure and osmolality was determined with a Fiske Osmometer. The experimental media ranged between 285 and 310 mOsm/kg water. Since these values were within the normal range of osmolalities found in commonly used tissue culture media (12), adjustment of experimental media was not considered necessary.

Histological techniques. Explants from each experimental group were harvested after 4 weeks, fixed in Bouin's solution, dehydrated in alcohol and embedded in paraffin. Sections (5 μm) were stained using hematoxylin and eosin and evaluated according to the following morphologic criteria. Specimens exhibiting a ciliated columnar epithelium that showed no metaplastic changes were classified as differentiated respiratory epithelium (Fig. 1A). If metaplastic changes affected less than 15% of the total epithelial surface, it was described as focal squamous metaplasia (Fig. 1B). Specimens with greater than 15%, but less than 50% metaplasia were designated as moderately metaplastic, whereas those lesions affecting greater than 50% of the epithelium were defined as extensive (Fig. 1C). Explants were assigned a numerical score from one (differentiated) to four (extensive metaplasia) to make quantitative characterization of groups feasible. Keratinization, identified as accumulations of flattened, desquamated, nucleated or anucleated surface cells, was also noted (Fig. 1D).

Results. Addition of Single Groups of Nutrients. In a series of parallel experiments, explants were cultured in: (a) MEM, (b) MEM with one of the individual groups of constituents added (Groups I–V), (c) MEM plus all five groups of additives (simulated Waymouth's MAB 87/3) or (d) the nutritionally complex medium, Waymouth's MAB 87/3. Nineteen to 38 specimens from each experimental group were graded; the inclusion of an equal number of explants for each test group in the analysis was not possible because of sporadic fungal contamination of cultures and imperfections in histologic preparations.

Figure 2A indicates the relative proportion of the specimens with metaplastic changes after 4 weeks. Explants cultured in MEM usually showed a differentiated mucociliary epithelium, whereas those maintained in Waymouth's MAB 87/3 or the medium fabricated to simulate Waymouth's (MEM plus Groups I–V) exhibited extensive metaplasia. The addition of insulin and glutathione (Group I) or the vitamins (Group II) to MEM failed to enhance the development of squamous metaplasia. On the other hand, Group III (the nucleic acid intermediates), Group IV (the inorganic salts), and Group V (the nonessential amino acids) produced an apparent increase in metaplasia.

Due to the ordinal nature of the histologic assessment, a nonparametric multiple comparison procedure was used to search for differences in squamous metaplasia between pairs of experimental groups at the 95% simultaneous level of confidence (13). A significant increase in metaplastic activity was found with use of Waymouth's medium, the

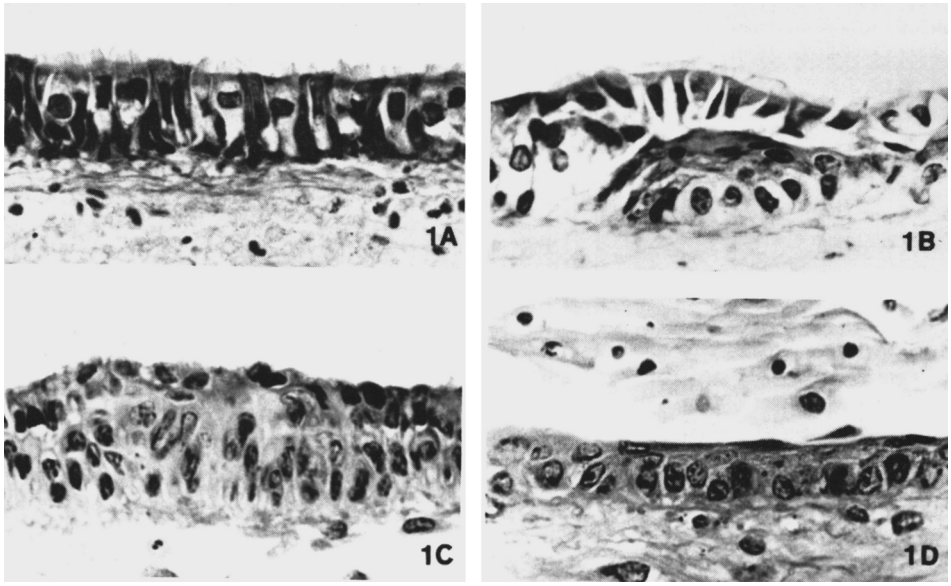

FIG. 1. Examples of morphologic criteria for evaluation of explants. A) Differentiated columnar epithelium, ×1160. B) Focal basal cell metaplasia, MEM with addition of proline, ×1112. C) Diffuse squamous metaplasia, MEM with addition of Groups I–V, ×1080. D) Squamous metaplasia with keratinization, MEM with addition of Groups I–V, ×1120. Explants were maintained in culture for 4 weeks.

simulated Waymouth's medium and MEM plus Group V (as compared to MEM).

Similar effects were observed when keratinization was evaluated. Thus, when Group V was added to MEM, the results did not differ significantly from those obtained with Waymouth's and the simulated Waymouth's medium. Keratinization in explants maintained in MEM with either Group III or Group V was more prominent than with MEM alone. These differences were statistically significant (14, 15).

Addition of groups of nutrients in combination. Because Groups III, IV, and V ranked nearest to Waymouth's in the induction of metaplasia and keratinization, combinations of the three were investigated to determine whether or not their effects were additive (Fig. 2B). Differences between combinations were examined using metaplasia and keratinization as a basis for assessment (13, 15). Although the nucleic acid intermediates of Group III and the inorganic salts of Group IV appeared to produce an increase (not statistically significant) in metaplasia when added individually to MEM (Fig. 2A), metaplasia was not more prominent when the two groups were combined (Fig. 2B). Similar results were obtained when both Group IV and Group V were added to MEM. Increased degrees of metaplasia and keratinization developed in explants maintained in MEM with Groups III and V and in those cultures in the simulated Waymouth's medium. We conclude from these observations that Group IV (the inorganic salts) does not contain essential nutritional constituents required for the development of squamous metaplasia and keratinization.

Addition of individual amino acids. Because the nonessential amino acids comprising Group V were most effective in promoting squamous metaplasia and keratinization, an attempt was made to determine whether an individual amino acid or several different amino acids induced these changes. Each of the amino acids (L-alanine, L-asparagine, L-aspartic acid, L-cysteine HCl, L-glutamic acid, glycine, L-proline and L-serine) was added individually to MEM at a concentration of 3.72 mM (i.e, the total concentration of nonessential amino acids in complete Waymouth's medium).

The combined results of duplicate experiments are presented in Table II. Extensive squamous metaplasia and keratinization de-

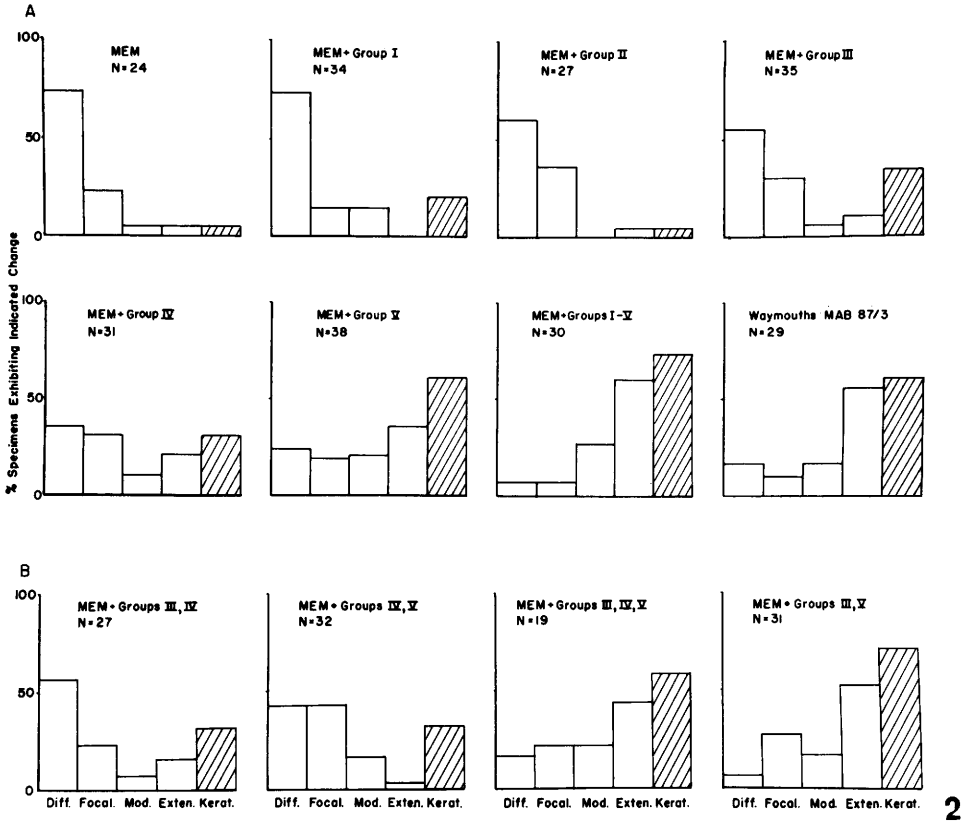

FIG. 2. Features of the epithelium in hamster tracheal organ cultures 4 weeks after maintenance in Eagle's MEM with supplementation. A) Single group additive experiments. B) Combination group experiments. The percentage of the epithelium showing metaplasia was graded on observance of five or more serial sections from each tissue. N = numbers of explants graded in each group.

TABLE II. ADDITION OF NON-ESSENTIAL AMINO ACIDS TO MEM. PERCENTAGE OF SPECIMENS EXHIBITING INDICATED CHANGES.[a]

	Experiment I					
Amino acid (3.72 mM)	No. Exam.	Diff.	Focal	Mod.	Exten.	Kerat.
L-Alanine	26	61	39			7
L-Asparagine	25	80	20			0
L-Aspartic acid	35	74	26			2
L-Cysteine HCl	25	100				0
L-Glutamic acid	40	55	23	13	10	18
Glycine	29	44	51	3		3
L-Proline	31	74	26			3
L-Serine	23	61	17	18	4	22
L-Serine (1.86 m*M*)	22	73	9	5	14	18
L-Serine (7.44 m*M*)	14	86	14			7
L-Serine (18.5 m*M*)	18	28	67	6		6
Group V	56	27	19	18	32	48
Mem	24	71	21	4	4	4

[a] After 4 weeks in culture, the percentage of the epithelium exhibiting squamous metaplasia and keratinization was graded independently by two observers as described in the text.

veloped only in explants maintained in medium supplemented with either L-glutamic acid or L-serine. Epithelia cultured in MEM with the addition of L-alanine, L-asparagine, L-aspartic acid, L-cysteine or L-proline failed to develop metaplasia affecting greater than 15% of the epithelium. The majority of these cultures exhibited a differentiated mucosa.

Obvious necrosis of epithelial cells and collapse of the cartilage ring were seen in explants cultured in L-cysteine. Cell death has been noted by others (16) in monolayer cultures exposed to cysteine at concentrations of > 1.0 m*M*.

L-Serine was added to MEM at different concentrations (1.86, 3.72, 7.44 and 18.5 m*M*) to determine whether or not its effects were dosage-dependent. At all concentrations but the lowest, cytotoxic changes were evident in explants.

Discussion. In the absence of either serum or vitamin A, the differentiated respiratory mucosa undergoes squamous metaplasia when cultured in an enriched synthetic medium such as Waymouth's MAB 87/3. Since these changes do not develop in a basal medium such as Eagle's MEM or Dulbecco's medium (8), one is obliged to conclude that the nutritional additives in the more complex medium stimulate cellular proliferation and squamous metaplasia. This investigation was undertaken to define the specific nutritional supplements responsible for these effects.

Although studies using serum-free chemically defined media have contributed to our understanding of the nutritional requirements of continuous lines of mammalian cells, a paucity of information exists concerning the growth requirements of primary cell or organ cultures (17). Since the epithelium of tracheal organ cultures is comprised of a diverse population of cells, the specific nutritional needs of each cell type are difficult to assess. Moreover, little is known concerning the relationship of cellular nutrition to differentiation.

It would appear that nutrients possessing the capacity to stimulate cell growth are important in the generation of squamous metaplasia in respiratory mucosa. Since the vitamin A stores in the epithelium probably are limited, the manifestation of vitamin A deficiency develops readily. Our observations indicate that nucleic acid intermediates, in combination with nonessential amino acids, contribute significantly to the development of squamous metaplasia in vitamin A-deprived tracheal cultures. This might be related to a nonspecific growth-stimulatory effect of these constituents as is observed when they are added to monolayer cultures (18, 19). Possibly the *de novo* synthesis of nucleic acid intermediates requires expenditures of energy by the cell that can be alleviated in part by the addition of preformed intermediates.

The most pronounced enhancement of squamous metaplasia and keratinization was produced by the addition of nonessential amino acids to the culture medium. Although termed "nonessential" *in vitro* as they are not required for the growth of strain L or HeLa cells in monolayer culture (11), these amino acids influence protein synthesis and energy metabolism (17). They can be incorporated directly into proteins, utilized as biosynthetic precursors for important regulatory metabolic intermediates, or serve as allosteric effectors of various anabolic reactions stimulating cell proliferation (20). Although several laboratories report that specific essential amino acids stimulate protein synthesis and mitotic activity in cell cultures (21–23), little work has been done to evaluate the effects of individual nonessential amino acids on growth and differentiation. In monolayers of fetal muscle cells, Matheson and Pahira (24) documented an increase in myotube formation and an approximate doubling of DNA and protein synthesis with addition of all nonessential amino acids to MEM. Similarly, Griffiths and Pirt (25) have observed that a mixture of serine, alanine and glycine increases the cell yield in suspension cultures of mouse LS cells when added to medium containing essential amino acids.

In our experiments, L-serine and L-glutamic acid had more striking effects on metaplasia and keratinization than other nonessential amino acids. These observations can be related to nutritional studies using cells in monolayer. Phytohemagglutinin (PHA) or antigen-stimulated human lymphocytes (26), rabbit fibroblasts (27) and cells from the limb-buds of rabbit embryos (11) have a requirement for serine in addition to essential amino acids. In clonal populations of HeLa cells, nonessential amino acids also become essential for optimum growth and survival (28). In these experiments, the addition of serine alone is able to replace the group mixture. Presumably, under normal circumstances, serine is lost from the intracellular pool into the medium at a rate exceeding the capacity of the cells to synthesize the amino acid (11).

Glutamine is the growth-limiting essential amino acid in most cell cultures because of its rapid uptake by the cells and its instability in culture medium (29). However, cells can be adapted to growth at high levels of glutamic acid when substituted for glutamine (30). Demars has observed increased amounts of the enzyme glutamine synthetase in these cultures (31).

Insulin and glutathione did not promote squamous metaplasia in explanted tracheal tissue. Although insulin stimulates growth in some mammalian cell lines (32), insulin and glutathione failed to affect epithelial differentiation in our experiments. The vitamins also seemed unimportant. Since the amounts of these components required for maximal growth of many cell lines are small (32), supplementation probably does not affect growth in an additive fashion. Ascorbic acid, vitamin B_{12} and biotin are not absolute requirements for the sustained growth of mammalian cells *in vitro* (30).

Summary. The mucociliary epithelium of the adult hamster trachea undergoes squamous metaplasia in organ culture when maintained in a complex, chemically-defined medium (Waymouth's MAB 87/3) without serum. This change fails to develop in explants cultured in Eagle's minimum essential medium (MEM) (8, 9). To determine the factors promoting squamous metaplasia, nutritional constituents in Waymouth's MAB 87/3 medium but not present in MEM were divided into five groups: (a) insulin and glutathione, (b) vitamins C, B_{12} and biotin, (c) nucleic acid intermediates, d) inorganic salts and e) nonessential amino acids. Test media were formulated by adding each group or combinations of groups to Eagle's MEM. Organ cultures were maintained in the test media for 4 weeks and then examined histologically. The nonessential amino acids were most important in enhancing metaplasia and keratinization. When nonessential amino acids were added individually to MEM, L-glutamic acid and L-serine seemed most influential in inducing metaplastic changes.

The authors acknowledge the excellent technical assistance of Brenda W. Ley, Bonnie J. MacLeod, Bettie M. Clements and Herman C. West.

1. Robbins, S. L., "Pathologic Basis of Disease", W. B. Saunders, Philadelphia, (1974).
2. Sanderud, L., Acta Pathol. Microbiol. Scand. **47,** 345 (1958).
3. Sara, C. A., and Reye, R. D., Med. J. Austral. **2,** 328 (1969).
4. Auerbach, O. et al., New Engl. J. Med. **265,** 253 (1961).
5. Gould, V. E., Wenk, R., and Sommers, S. C., Cancer **28,** 426 (1971).
6. Wolbach, S. B., and Howe, O. R., J. Exp. Med. **42,** 753 (1925).
7. DeLuca, L., et al., Cancer **30,** 1326 (1972).
8. Mossman, B. T., and Craighead, J. E., Proc. Soc. Exp. Biol. Med. **149,** 227 (1975).
9. Mossman, B. T., Ley, B. W., and Craighead, J. E., Exp. Mol. Pathol. **24,** 405 (1976).
10. Gorham, L. W., and Waymouth, C., Proc. Soc. Exp. Biol. Med. **119,** 287 (1965).
11. Eagle, H., Science **130,** 432 (1959).
12. Waymouth, C., *In Vitro* **6,** 109 (1970).
13. Miller, R. G., "Simultaneous Statistical Inference", p. 165, McGraw-Hill Book Co., Inc., New York (1966).
14. Dixon, W. F. and Massey, F. J., Jr., "Introduction to Statistical Analysis", p. 324, McGraw-Hill Book Co., Inc., New York (1969).
15. Feller, W., "An Introduction to Probability Theory and Its Application", Vol. 1, John Wiley and Sons, New York (1957).
16. Nishiusch, Y. et al., *In Vitro* 312, 635 (1976).
17. Ham, R. G., In Vitro **10,** 119 (1974).
18. Lamar, C., and Imm, B., J. Nutr. **191,** 1589 (1971).
19. Lambert, K., and Prit, S. J., J. Cell Sci. **17:** 397 (1975).
20. Lehninger, A. L., "Biochemistry" (2nd ed.), Worth Publishers, Inc. New York (1975).
21. Hume, S. P., and Lamb, J. F., Proc. Physiol. Soc. p 46 (1974).
22. Litwin, J., Exp. Cell Res. **72,** 566 (1972).
23. Ellem, K. A. O., Fabrizio, A. M., and Jackson, L. G., Biochim. Biophys. Acta **209,** 425 (1970).
24. Matheson, D. W., and Pahira, J. J., *In Vitro* **10:** 371 (1975).
25. Griffiths, J. B., and Pirt, S. J., Proc. Roy. Soc. B. **168,** 421 (1967).
26. Waithe, W. I. et al., Cell. Immun. **17,** 323 (1975).
27. Haff, R. F., and Swim, H. E., Fed. Proc. **15,** 591 (1956).
28. Lockhart, R. J., and Eagle, H., Science **129,** 252 (1959).
29. Patterson, M. K., *in* "Growth, Nutrition and Metabolism of Cells in Culture", (G. H. Rothblatt and V. J. Cristofalo, eds.), Vol. 1, p. 171, Academic Press, New York (1972).
30. Eagle, H., *in* "General Physiology of Cell Specialization", (D. Mazia and A. Tyler, eds.), p. 151, McGraw-Hill Book Co., Inc., New York (1963).
31. Demars, R., Biochim. Biophys. Acta **27,** 435 (1958).
32. Higuchi, K., and Robinson, R. C., In Vitro **9,** 114 (1973).

Received March 28, 1977. P.S.E.B.M. 1978, Vol. 157.