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Stimulatory Effects of Glycine, L-Serine, Folic Acid and Related Compounds on Growth of Cell Cultures.* (25663)

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Twelve amino acids, L-glutamine, and infrequently L-asparagine are required for growth of cell cultures(1). Additionally, primary rhesus monkey testicular† and primary monkey kidney cultures(2) require glycine. Isolated HeLa and KB cells(3) and a strain of rabbit fibroblasts(4) require L-serine. Either glycine or L-serine is essential to the Novikoff hepatoma *in vitro*(5) and these amino acids stimulate growth of Walker carcinosarcoma 256(6) and Jensen sarcoma cultures(7).

In this communication the stimulatory effects of glycine and L-serine for a wide variety of both primary and stable line cultures are described and relationships of this requirement to folic and folinic acid are examined.

Methods and materials. Inocula for primary cultures were prepared by stirring the minced fresh tissue 2-6 hours with 0.2% trypsin (Difco 1:250) in Earle's balanced salt solution at 37°C. The cell suspension was filtered through gauze, collected by centrifugation and resuspended in whole medium. Stable line cells for inocula were prepared by trypsinization of stock cultures except Walker carcinosarcoma 256 cells which were readily shaken loose from the glass after chilling at 5°C. One ml of whole medium containing 100,000 fresh cells or 20,000 stable line cells was pipetted into 15 x 125 mm tubes which were placed in a slanted position in racks. The cultures were established (plated) by incubation for 24 hr at 37°C in an atmosphere of 8% carbon dioxide—92% air. The medium was removed and the tubes were rinsed with Earle's solution. The cells then were overlaid with 1 ml of test medium containing graduated amounts of the test compounds. Incubation was continued for 3-5 days until the cells formed a sheet or attained maximal growth in presence of optimal amounts of the test compounds. The medium was not replenished. Growth was measured by the method of Oyama and Eagle(8). The whole medium in which the cells were established was similar to that previously described(9). It contained L-isomers of essential and nonessential amino acids as well as 0.2 mM pyruvate, 10 μg adenosine, and 5% bovine serum. No p-aminobenzoic acid was included in media used. The test medium differed in that it contained dialyzed serum, 0.2 mM each of pyruvate, alpha-ketoglutarate, and oxalacetate.

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† In abstract: Tytell, A. A., Rader, Y., Krum, D. L., *Fed. Proc.,* 1958, v17, 326.

TABLE I. Stimulation of Growth of Primary and Stable Line Cultures by Glycine and L-serine.

Cultures	Added to basal medium: Nothing	Glycine	L-serine
	Relative growth (inoculum = 1.0)		
Stable lines:			
HeLa (human tumor)	8.2	9.1	9.1
KB (" ")	18.0	22.5	26.0
FL (" amnion)	8.0	8.6	9.4
Kidney (Rhesus monkey)	8.4	10.2	9.2
Heart (cynomologous monkey)	13.3	14.8	14.6
Kidney (rabbit)	10.6	11.9	11.4
Walker carcinosarcoma 256 (rat)	8.7	12.3	14.2
Strain L (mouse)	5.0	5.3	5.0
Sarcoma 180 (mouse)	5.5	5.7	5.9
Heart (chick embryo)	15.4	19.1	18.5
Primary cultures:			
Green monkey kidney	4.3	12.9	10.2
Calf kidney	3.4	4.7	4.4
" testis	5.7	8.1	8.1
Rabbit kidney	6.6	14.6	10.1
" lung	3.6	4.9	5.2
" testis	5.2	8.7	8.4
Guinea pig kidney	4.7	6.6	6.1
" " lung	5.0	11.0	11.0
" " testis	6.3	13.5	11.8
Hamster lung	4.2	6.9	6.3
Rat lung	5.9	7.6	7.6
" testis	9.3	11.9	10.8
Chick embryo kidney	2.6	6.1	6.8
" " lung	5.3	7.2	9.6
Cockerel kidney	5.7	7.5	7.7
" lung	8.8	13.6	13.1
" testis	4.4	5.7	7.8

Conditions of growth are described in text. Basal medium contained no glycine or L-serine. These compounds were added in graduated quantities up to 2 mM levels.

Adenosine and glutathione were omitted. Media for growth of avian cultures also contained 6% dialyzed chick embryo extract prepared from equal volumes of 12 day chick embryo and Earle's solution. Dialysis of serum and embryo extract was conducted as described previously(9). Earle's solution was modified in these studies to contain 3.0 g glucose and 6.46 g sodium chloride per liter.

Results. In Table I are shown stimulatory effects of optimal amounts of glycine (0.1-0.2 mM) and L-serine (0.5-1.0 mM) upon growth of a number of primary and stable line cultures. The results are expressed in terms of maximal growth attained relative to 24 hour plated cells. The majority of cultures responded appreciably to additions of glycine or L-serine to the medium. Effects of either compound were detectable at 0.01 mM concentrations. Growth was often depressed by 0.5-1.0 mM glycine and 1.0-2.0 mM L-serine. Although glycine and L-serine were interchangeable in their role as stimulatory nutrients, the influence of one compound was found to be superior to the other in many instances. These differences possibly express interconversion rates of glycine and L-serine. In general, stable line cells were stimulated 10-50%. Only the mouse strain "L" and sarcoma 180 cells failed to respond significantly. The majority of primary cultures were stimulated 50-200%.

Dimethyl glycine and sarcosine at 0.01-10 mM concentrations substituted for glycine only to a slight extent in growth of Walker carcinosarcoma 256 and primary green monkey kidney. Glyoxylic acid was somewhat more active but again response was much inferior to that with glycine or L-serine.

Eagle reported replacement of the glycine requirement of monkey kidney cultures by folinic acid(1). This prompted examination of the effects of folic and folinic acids on glycine requirement for maximal growth of representative cultures. In Fig. 1 are shown relative effects of folic acid, glycine, and L-serine upon growth response of typical cultures. Stimulation by glycine and L-serine is evident. Increased concentration of folic acid (0.5-1 μg/ml) also resulted in slightly improved growth for the majority of cultures although the test medium routinely contained 0.2 μg folic acid per ml. Maximal effect of folic acid usually was attained at 5-20 μg/ml. Folinic acid‡ in experiments conducted simultaneously stimulated primary green monkey kidney and chick embryo lung to a similar degree at 0.01-0.1 μg/ml. With the remaining cultures, 1-5 μg was necessary to produce effects comparable to those of higher concentrations of folic acid. Nevertheless, neither folic acid nor folinic acid potentiated growth of the majority of cultures equal to that of glycine or L-serine. Walker carcinosarcoma 256 was particularly unresponsive to folic and folinic acid. Primary green monkey kidney cells

‡ Leucovorin, formyl tetrahydropteroylglutamic acid. Lederle Labs, Am. Cyanamid Co., N. Y.

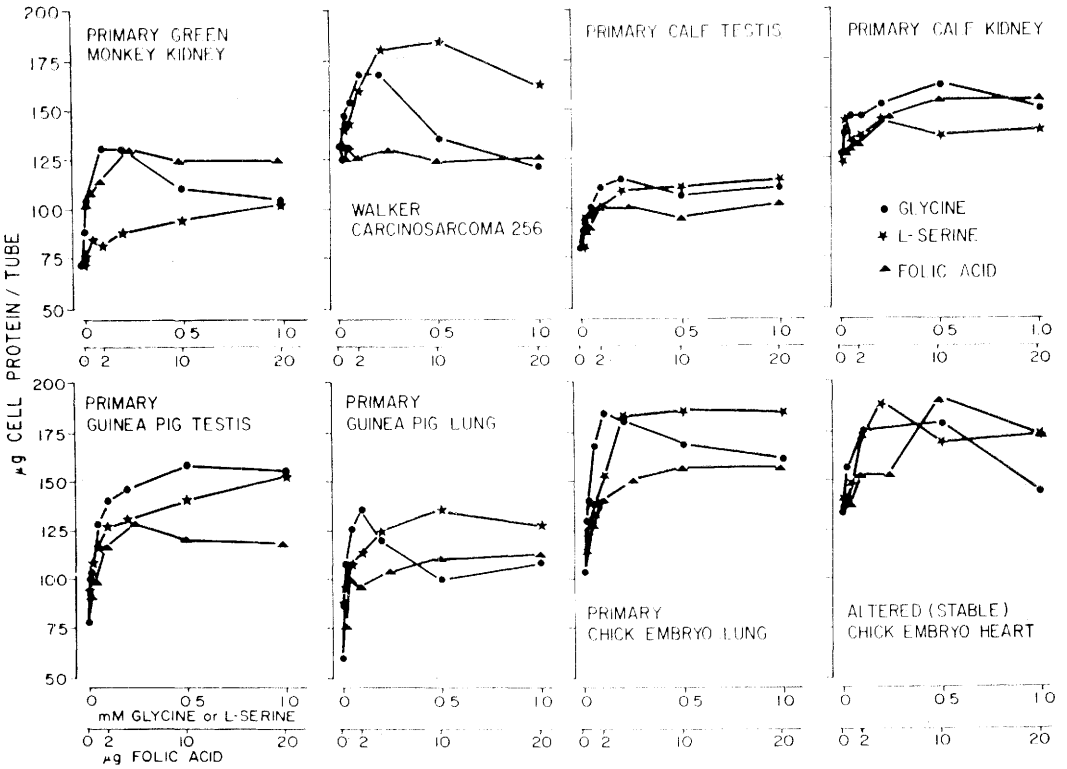

FIG. 1. Growth response of cell cultures to glycine, L-serine, and folic acid.

were particularly responsive to folic and folinic acid, and 2 μg and 0.01 μg respectively of the factors sufficed to promote growth equal to that obtained with glycine.

A more detailed inquiry into the contributions of glycine and L-serine to the cell economy and relation of these amino acids to folic acid in the present tissue culture system appeared desirable. The Walker carcinosarcoma 256 and primary green monkey kidney cells were grown in media which contained 10^{-3} mM amethopterin (4-amino-N^{10}-methylpteroylglutamic acid) and was devoid of folic acid. Initial experiments established that purines, pyrimidines, and glycine were required for growth under these conditions, as shown previously for other cells by Hakala (10,11).

The ability of various purines and derivatives to support growth of cells supplied with glycine and thymidine is shown in Table II. For tumor cells and the primary kidney cells, adenine, adenosine, ATP, hypoxanthine and inosine were effective in approximately equimolar quantities. Responses were determinable at 0.01 mM and maximal at 0.05-0.1 mM. Guanine was only slightly active for primary green monkey kidney. Guanosine and guanylic acid in further experiments were ineffective. Studies with media from which folic acid was omitted but which contained no amethopterin yielded similar results.

Table III shows capacity of several pyrimidines to support growth of the Walker carcinosarcoma and monkey kidney cells in medium containing glycine and adenosine. Both cultures utilized thymidine and thymidylic acid. Thymine was slightly beneficial to the kidney cultures but not to the tumor cells. Cytosine and uracil did not effectively fulfill the pyrimidine requirements. In later experiments 5-methyl-deoxycytidine was shown completely interchangeable with thymidine. These compounds exhibited activity at levels as low as 0.001 mM. Because of this high sensitivity, thymidine requirements could not be demonstrated consistently in media which did not contain amethopterin. Apparently trace quantities of folic acid residual in cells or test medium permitted synthesis of the

TABLE II. Growth Response to Purines by Primary Green Monkey Kidney and Walker Carcinosarcoma 256 Cells in Media Containing Amethopterin, Glycine and Thymidine.

Conc., mM	Adenine	Adenosine	ATP	Hypoxanthine	Inosine	Guanine
	Primary green monkey kidney cells (μg cell protein/tube)					
.0	26	26	26	26	26	26
.005	60	30	30	36	28	
.01	96	64	54	74	60	24
.02	140	110	98	110	120	26
.05	180	172	152	184	166	20
.1	168	192	144	178	156	40
.2	156	176	128	172	162	58
	Walker carcinosarcoma 256 (μg cell protein/tube)					
.0	16	16	16	16	16	16
.01	64	36	44	42	42	18
.02	118	86	66	100	82	16
.05	154	152	136	150	154	16
.1	166	136	140	168	156	16
.2	152	116	80	168	156	12

Basal medium contained .2 mM glycine, .05 mM thymidine, 10^{-3} mM amethopterin. L-serine and folic acid were omitted.

small quantities of thymidine essential to the cells.

Similar experiments to determine the requirement for glycine were conducted in media containing 0.05 mM each of thymidine and adenosine. Growth was negligible at zero levels of glycine, detectable at 0.01 mM, and optimal at 0.1-0.5 mM as observed previously in the glycine stimulation studies in media containing folic acid. However, L-serine was totally ineffective in replacing the glycine. p-aminobenzoic acid did not spare or replace the folic acid requirement of the cultures.

TABLE III. Growth Response to Pyrimidines by Primary Green Monkey Kidney and Walker Carcinosarcoma 256 Cells in Media Containing Amethopterin, Glycine and Adenosine.

Conc., mM	Thymidine	Thymidylic acid	Thymine	Uracil	Cytosine
	Primary green monkey kidney cells (μg cell protein/tube)				
.0	30	30	30	30	30
.001	80	84	36		
.005	156	156	50		
.01	196	198	54	44	46
.02	200	202	68		
.05	202	192	84	40	50
.1	196	198	84	44	
.2	168	176	88	46	44
	Walker carcinosarcoma 256 (μg cell protein/tube)				
.0	10	10	10	10	10
.001	24	26	8		
.005	192	140	8		
.01	174	186	10	12	6
.02	176	172	14		
.05	162	162	14	6	8
.1	152	158	8	6	8
.2	138	138	14		

Basal medium contained .2 mM glycine, .05 mM adenosine and 10^{-3} mM amethopterin. L-serine and folic acid were omitted.

Discussion. Some question may be raised concerning the adequacy of cofactors involved in bio-synthesis of glycine and L-serine. Eagle(12,13) reported the optimal folic acid requirement for stable line cells grown in media devoid of glycine and L-serine to be approximately 0.004-0.04 μg/ml. Haff and Swim reported 0.4 μg/ml to be optimal for repeated subculture of a strain of rabbit fibroblasts(14). Eagle customarily included in his medium 1 μg/ml of each required vitamin (15). Most frequently other media employed in tissue culture have contained 0.1 μg folic acid/ml(16). In the initial studies on glycine and L-serine stimulation described here, folic acid was present at 0.2 μg/ml. Increasing this concentration to 0.5 and 1 μg/ml improved growth to some extent in absence of glycine or L-serine. Nevertheless, maximal stimulation by folic acid generally was not attained until the level reached 5-20 μg/ml. This maximal growth was similarly attained by inclusion of 0.01-5 μg of folinic acid. This active form of folic acid is synthesized from it and is often effective at lower concentrations (17). Fortification of the coenzyme system involved in glycine and L-serine synthesis, therefore, augmented growth of the cultures. However, the enhancement usually was not equivalent to that produced by the amino acids, glycine and L-serine. Evidently syn-

thesis of these compounds remained a limiting factor. The possibility existed that supplementation of the medium with purines and thymidine, other products of folic acid systems, might increase efficiency of glycine synthesis. Addition of these compounds at various levels to the medium in absence of glycine or L-serine did not stimulate as did the amino acids. It may be concluded that glycine and L-serine are desirable constituents of media designed for maximal growth of a wide variety of cell cultures.

Hakala demonstrated replacement of the folic acid requirement of HeLa, sarcoma 180, and J-111 cells by glycine, purines and thymidine(10,11). The work reported here both on the rat Walker carcinosarcoma 256 and primary green monkey kidney cells agreed with her findings in major respects. Adenine and hypoxanthine and their derivatives were effective for the various cell cultures at 0.02-0.1 mM. Guanine was very slightly active. Guanosine and guanylic acid were slightly active for sarcoma 180 while the Walker carcinosarcoma 256 and primary green monkey kidney cell did not respond. Thymidine, thymidylic acid and 5-methyl-deoxycytidine were equally active. As suggested by Hakala, deamination mechanisms for 5-deoxycytidine probably exist in the cultures. In contrast to Walker carcinosarcoma 256, sarcoma 180, and J-111 the green monkey kidney cells were somewhat benefited by thymine. HeLa cells were reported to grow slowly on thymine. In the present work L-serine, unlike glycine did not support cells in media containing amethopterin, thymidine, and adenosine. Omission of L-alanine, pyruvate, alpha-ketoglutarate, and oxalacetate did not depress growth.

As suggested by Lockhart and Eagle(3) leakage of glycine or L-serine may take place from isolated cells in media devoid of glycine or L-serine. Accordingly, growth of isolated cells could fail through loss of compounds which easily escape from the cell and are synthesized at limited rates. Glycine or L-serine has been found in this laboratory to be essential rather than stimulatory for the isolated Walker carcinosarcoma 256 cell. Very small inocula approximating isolated cells may respond to glycine or L-serine as essential amino acids. By contrast, the greater quantities of glycine and L-serine which leak from massive cultures in deficient medium are conserved in utilizable concentrations in the medium. The inocula employed in our studies were at practicable levels which produced sheets of cells within 3-5 days.

Summary. Growth of the majority of a wide variety of primary and stable line cultures was stimulated significantly by inclusion of glycine or L-serine in the medium. Folinic acid or high levels (5-20 μg/ml) of folic acid also accomplished this effect for most cultures, although generally not to the same extent as did amino acids. The requirement of Walker carcinosarcoma 256 and of primary green monkey kidney cells for folic acid was fulfilled by glycine, thymidine, and adenine or several related compounds. L-serine could not replace glycine under similar conditions. The implications of these findings are discussed.

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