Amino Acid Requirements for Growth of Avian Lung Cultures.* (25416)

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Amino acid requirements for growth of a large number of stable line mammalian cells (1) primary rhesus monkey testicular† and primary monkey kidney(2) cell cultures have been determined. Morgan and Morton reported the effects of amino acids upon prolonging survival of chick embryo heart cultures in synthetic media(3). However, the amino acids essential for growth of primary avian cultures have not been reported. The present paper describes amino acid requirements for growth of primary chick embryo lung and cockerel lung cultures.

Methods and materials. Cell suspensions of 14-16 day White Leghorn chick embryo lungs and 2-6 week old cockerel lungs were prepared by stirring 2-4 hours with 0.2% trypsin (Bacto-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 a complete medium. One ml amounts of a suspension containing 50,000-100,000 cells per ml were pipetted into 15 x 125 mm tubes which were placed in a slanted position in racks. The cultures were established by incubation for 24 hours 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 amino acid. 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 compound. The medium was not replenished. Growth was measured by the protein determination method of Oyama and Eagle (4). The complete medium in which the cells were established was similar to that previously described(5). It contained l-isomers of the amino acids as well as 0.2 mM pyruvate, 10 μg adenosine per ml, 5% bovine serum, and 6% chick embryo extract. The test medium differed in that it contained dialyzed serum and dialyzed embryo extract. Adenosine and glutathione were omitted. Embryo extract was prepared from 12 day chick embryos ejected through the orifice of a 20 ml B-D Luer-Lok syringe. The brei was mixed with an equal volume of Earle's solution and after 1 hour the supernate was collected by centrifugation. Expressions of quantity of embryo extract were based on this preparation. However, this material was diluted with 2-3 volumes of Earle's solution prior to sterilization by filtration through Selas filter of 02 porosity. Dialysis of serum and embryo extract was conducted as described previously(5). Earle's solution was modified in these studies to contain 3.0 g glucose and 6.46 sodium chloride per liter.

Results. Initial investigation demonstrated the requirements of both chick embryo lung and maturing cockerel cultures for the non-dialyzable moieties of embryo extract and serum. In Fig. 1 are shown typical growth responses to dialyzed chick embryo extract. In Fig. 2 are shown the typical growth responses to dialyzed pooled bovine and chick serum. The bovine serum was superior to the chick serum. The presence of both dialyzed embryo extract and serum was essential for growth of the cultures in the test medium. High concentration of either embryo extract or of serum did not eliminate the requirement for the other component. For the amino acid studies, dialyzed embryo extract and dialyzed serum were included in the medium at 6 and 5%, respectively, in order to minimize possible contributions of amino acids by these materials.

By varying the amount of each amino acid in turn over a concentration range of 0-1.0 mM, the 13 amino acids usually required by

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† Described in abstract: Tytell, A. A., Rader, Y., Krumm, D. L., *Fed. Proc.*, 1958, v17, 326.

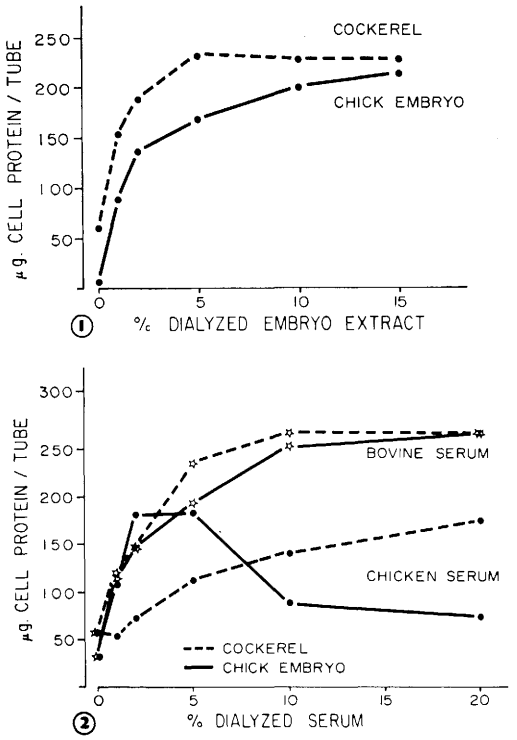

FIG. 1. Growth response of primary chick embryo and cockerel lung cultures to dialyzed embryo extract. Medium contained 5% dialyzed bovine serum.

FIG. 2. Growth response of primary chick embryo and cockerel lung cultures to dialyzed chick and bovine serum. Medium contained 6% dialyzed embryo extract.

mammalian cells(1) were found to be essential for chick embryo lung cultures. The results are presented in Table I. The optimal levels of these amino acids were similar to those for mammalian cells. Microscopic examination of cultures in the absence of any one of the essential amino acids revealed cellular deterioration. Glutamic acid up to levels of 50 mM only partially substituted for glutamine. The cockerel lung cultures were found to have amino acid requirements identical with those of the chick embryo cultures.

A study of the other amino acids and derivatives in the test medium demonstrated the stimulatory effects of glycine, L-serine, L-alanine, and pyruvate. Growth response of chick embryo lung cultures is shown in Table II. Addition of 0.1 mM of either glycine or L-serine to the test medium from which both these amino acids had been omitted resulted in marked stimulation. Furthermore, inclusion of 0.1 μg folinic acid or 5-10 μg folic acid per ml (the test medium contained 0.2 μg folic acid per ml) stimulated the cultures in the absence of added glycine or L-serine, although maximal growth was not attained. Addition of either 0.01 mM L-alanine or 0.1 mM pyruvate to the test medium from which L-alanine and keto acids had been omitted resulted in stimulation. Oxalacetate or alpha-ketoglutarate at 0.2 mM also replaced alanine. Lactate, isocitrate, citrate, cis-aconitate, malate, succinate, fumarate, glutamate, aspartate, folinic acid, and higher levels of folic acid, pyridoxine and pyridoxal did not substitute for L-alanine. Pyridoxal phosphate (1-10 μg/ml) did stimulate the chick lung cells to a limited extent in the absence of L-alanine. Thus a medium containing the 13 essential amino acids also required glycine, L-alanine or related compounds to attain growth comparable to that on the original test medium which contained 20 amino acids. Other "non-essential" amino acids were omitted from the medium without affecting growth. Primary cockerel lung cells responded to glycine, L-alanine, and

TABLE I. Amino Acid Requirements for Growth of Primary Chick Embryo Lung Cultures.

Amino acid	Concentration of amino acid (mM) 0	.01	.02	.05	.1	.2	.5	1.0
	Growth response (inoculum = 1)*							
L-arginine	1.5	4.0	6.3	8.6	9.5	9.8	9.3	9.3
isoleucine	1.7	3.1	6.0	10.0	12.6	12.3	12.7	14.0
leucine	2.3	4.6	6.0	9.7	14.4	15.7	15.4	11.3
lysine	2.9	4.9	7.0	9.9	14.0	14.6	14.7	14.7
methionine	1.5	3.3	5.8	7.0	7.4	7.5	7.3	5.2
phenylalanine	2.5	4.0	6.4	6.6	7.0	7.3	7.8	6.1
threonine	1.7	3.2	4.2	6.3	7.6	7.2	5.2	4.6
tyrosine	3.4	6.6	11.0	14.4	14.7	15.0	14.6	13.1
valine	.8	2.9	7.3	7.0	7.9	8.9	8.3	8.3

	Concentration of amino acid (mM) 0	.1	.5	1.0	1.5	2.0	5.0
L-glutamine	1.7	4.3	11.0	13.3	14.7	14.6	15.6

	Concentration of amino acid (mM) 0	.001	.002	.01	.02	.05	.1	.2
L-cysteine	1.6	2.6	3.3	7.9	10.9	14.0	14.3	13.1
histidine	2.4	3.7	4.1	7.6	12.3	14.7	14.4	14.7
tryptophane	1.9	2.9	4.9	14.3	15.5	16.4	16.0	14.7

* Inoculum refers to 24 hr plated cells subjected to the test medium.

TABLE II. Stimulatory Effects of Glycine, L-serine, L-alanine, and Pyruvate on Primary Chick Embryo Lung Cultures.

Compound	Concentration (mM)									
	0	.001	.005	.01	.02	.05	.1	.2	.5	1.0
	Growth response (inoculum = 1)*									
Glycine†	9.4			10.4	11.4	12.6	13.0	12.4	11.4	11.2
L-serine†	9.4			11.2	11.8	12.6	15.0	15.2	17.2	16.8
L-alanine‡	8.3	9.8	12.3	15.1	15.0	15.1	15.5	14.5		
Pyruvate‡	8.3			10.9	12.0	13.0	14.1	14.1	13.4	13.4

* Inoculum refers to 24 hr plated cells subjected to test medium.

† Test medium contained 13 essential amino acids plus L-alanine, pyruvate, L-asparagine, L-aspartate, L-glutamate, L-proline, L-hydroxyproline.

‡ Test medium contained 13 essential amino acids plus glycine, L-serine, L-asparagine, L-aspartate, L-glutamate, L-proline, L-hydroxyproline.

related compounds in the same manner as did the embryonic cells.

Discussion. A major difference in the nutritional requirements of avian and mammalian cultures which has received detailed study appears to be a factor or factors associated with the macromolecular constituents of embryo extract(6,7). Additional factor(s) associated with the nondialyzable portion of serum are required by both groups of cells. The serum factor(s) were not species specific. In fact, bovine serum was more effective than chick serum and did not inhibit at higher levels as did chick serum upon occasion. The requirement for embryo extract was not confined to embryonic tissues but applied as well to the maturing cockerel cells. However, these studies were over brief growth periods on a gross population of primary cells. Adaptation or stabilization processes in continued culture could result in strains with different properties. At present primary chick embryo lung cultures have proved highly satisfactory in this laboratory in assay procedures in studies of embryo extract growth factor(s). Kutsky has prepared growth-promoting proteins from tissue extracts which were assayed using explants or subcultured cells(6,7).

Avian cells in common with mammalian cells exhibited requirements for 13 essential amino acids. Avian cells also were stimulated by glycine, L-serine and to a lesser degree by 0.1 μg folinic acid or 5-10 μg per ml of folic acid. These stimulatory effects of glycine, L-serine, folinic acid and higher concentrations of folic acid frequently have been encountered in this laboratory in a wide variety of mammalian cultures. Substitution of glycine by folinic acid for growth of primary monkey kidney cultures has been reported (8).

An outstanding difference in the micromolecular requirements for optimal growth of avian cultures was the marked stimulation by small quantities of L-alanine. Relatively high levels of pyruvate, oxalacetate, alpha-ketoglutarate could replace the alanine requirement perhaps by contribution to its biosynthesis. Pyridoxal phosphate improved growth in some measure in the absence of L-alanine although the test medium contained a total of 1 μg per ml of pyridoxine and pyridoxal. The participation of this cofactor in transamination reactions in production of L-alanine(9) may account for its activity. Isolated Walker carcinosarcoma 256 cells have been shown to require pyruvate, oxalacetate, or alpha-ketoglutarate, although alanine could not substitute for these compounds(5).

Summary. Primary chick embryo and maturing cockerel lung cultures were found to grow in a test medium containing dialyzed chick embryo extract, bovine serum, and a variety of small molecular constituents. The avian cultures required the 13 amino acids usually found to be essential for mammalian cells. They also were markedly stimulated by glycine or L-serine and by small quantities of L-alanine. Folinic acid and higher levels of folic acid partially replaced the glycine requirement for optimal growth. Pyruvate, oxalacetate, or alpha-ketoglutarate, replaced the requirement for L-alanine. Pyridoxal phosphate partially replaced the L-alanine.

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Rectal Absorption of 6-Alpha-C^{14}-H_3-Prednisolone.* (25417)

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Administration of adrenocortical steroids per rectum in treatment of ulcerative colitis has produced encouraging results(1-11). In general, the primary mode of action of this form of therapy has been attributed to a local effect, as in the topical treatment of certain dermatologic conditions(2,5,6,10,14) though Liddle(13) and Nabarro *et al.*(4) have demonstrated absorption of some forms of hydrocortisone given by rectum. Our first experience with rectal administration of steroids was in Feb., 1958 during a preliminary evaluation of 6-methyl-prednisolone. The response was surprisingly gratifying. The subsequent availability of radioactive 6-methyl-prednisolone (6-alpha-C^{14}-H_3-prednisolone) prompted studies to (1) estimate amount of steroid absorbed from the rectum, (2) determine the rapidity with which it might be absorbed, and (3) length of time it remained within the body, if absorbed.

Methods. Administration: One milligram (3.2 microcuries) of 6-alpha-C^{14}-H_3-prednisolone, dissolved in one ml of 95% ethanol, was drawn into a 20 ml syringe. Forty mg of 6-methyl-prednisolone sodium succinate, dissolved in 1 ml of water, were rapidly drawn into the same syringe; (in case 4 this step was eliminated). Fifteen milliliters of tap water were drawn into the same syringe. The contents of the syringe were mixed rapidly and emptied by gravity into a soft rubber catheter, whose tip had been placed in the rectum of the patient lying on his left side. The catheter drained into the rectum completely within one minute and then was withdrawn; no patient evacuated the rectum in the succeeding 24 hours. *Analysis of urine*: Two successive 24 hour urine collections were made as separate voided specimens. The volume and time of each voided urine were recorded. Aliquots (0.5 ml) from each specimen were counted in a Packard Tri Carb Scintillation Counter as disintegrations per minute; total disintegrations/minute per voided specimen were calculated from these data. All voided specimens for each 24 hour period were pooled and mixed. Nine hundred ml aliquots from each 24 hour pool were extracted for the neutral lipid fraction. *Extraction of urinary corticosteroids*:† (12) The urinary pH was adjusted to 5. One-tenth volume of acetate buffer (pH 5.0) was added. Hydrolysis with 300 units of spleen beta-glucuronidase/ml progressed 5 days. The hydrolyzed urine was adjusted to pH 1 with sulfuric acid and continuously extracted with ether for 48 hours. The ether extract was washed 3 times with each 1/10 volume 2N sodium hydroxide and 1/10 volume of 5% sodium chloride. Each hydroxide or salt washing was back extracted with ether. Each "back extract" was added to the original ether extract. The total ether extract was evapo-

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† Modification of the method of Dobriner made by Attallah Kappas—personal communication.