Amino Acid Requirements of the Novikoff Hepatoma *in vitro*. (24542)

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Amino acid requirements of a number of mammalian cell strains have been determined under tissue culture conditions. These not only included some serially-propagated cells of human origin(1,2) but also neoplasms of rat(3) and mouse(4), monkey testicular and kidney cells(5,6), and rabbit fibroblast(7). In general, the essential amino acids seemed to follow a similar pattern, *i.e.*, the 8 essential amino acids (lysine, leucine, isoleucine, threonine, valine, phenylalanine, tryptophan, and methionine) as well as arginine, histidine, tyrosine, cysteine, and glutamine. The present report describes experiments designed to determine the amino acids necessary for growth and survival of the Novikoff hepatoma *in vitro*.

Methods. The stock tumor* was carried intraperitoneally in Holtzman rats. Six days after tumor transplantation, the animals were sacrificed and initial inoculum for tissue culture was prepared from freshly excised tumor. Details of experimental technics have been described(3,10), and basal medium 5a was used. This medium was the same as previously reported(11) except that dialyzed pooled human serum was used in lieu of bovine serum, all amino acids were the L-isomer, L-serine included in substrate at a concentration of 0.25 mM, and i-inositol was added at 36.0 μg/ml final medium. Since the pH of substrate was a vital factor in growth of Novikoff hepatoma *in vitro*(12), the flasks were plugged with gauze stoppers and incubated in a flowing atmosphere of 7% CO_2 in air. The initial inoculum was 10,000 cells/T-15 flask (total volume 2 ml), and growth response was determined by a whole cell count.

Results. The effect of omitting individual amino acids from the medium can be seen in Table I. It was apparent that the 13 amino acids (including glutamine) previously reported to be required by a number of cell lines (1,2,4) were essential for growth of Novikoff hepatoma *in vitro*. The essentiality of glycine was not clear-cut, however, since growth was observed, but it was obviously inferior to that obtained in a complete medium. When the principal source of amino acid nitrogen was restricted to contain only 13 essential amino acids, only a cell maintenance condition was observed during 8-day incubation period. Some growth was obtained by adding 0.25 mM serine to the restricted medium, but supplementing with 0.1 mM glycine resulted in a 56-fold increase in cells during this time. When both glycine and serine were added, growth was comparable to a complete medium containing the 21 amino acids and amides in basal medium 5a (Fig. 1). From this, it was apparent that glycine and serine definitely stimulated growth of Novikoff hepatoma, and glycine was more effective than serine.

While amino acid requirements of several cell lines cultured *in vitro* seemed to follow a general trend, further investigations into this area revealed many interesting differences. For example, Walker tumor required asparagine(8), rabbit fibroblast, serine(7), and monkey testicular cell, glycine(5) in addition to the 13 amino acids or amides reported essential for HeLa tumor(1) and Strain L(4). Further, chick heart fibroblast did not require glutamine or isoleucine; and alanine, aspartic

TABLE I. Effect of Omitting Individual Amino Acids from Substrate on Growth of Freshly Excised Novikoff Hepatoma.

Amino acid	Growth* in 8 days	Amino acid	Growth* in 8 days
Control	24.0	Phenylalanine	1.8
Arginine	4.2	Tyrosine	1.8
Histidine	1.6	Tryptophan	.2
Lysine	.9	Cysteine	0
Glycine	8.4	Methionine	.9
Alanine	24.9	Glutamine	.4
Serine	28.9	Asparagine	34.6
Threonine	.4	Aspartic acid	30.0
Valine	.9	Glutamic acid	33.5
Leucine	1.1	Proline	31.5
Isoleucine	.2	Hydroxyproline	29.5

* Initial inoculum referred to as 1.

* Kindly supplied by Dr. Alan C. Sartorelli, McArdle Memorial Laboratory, Madison, Wis.

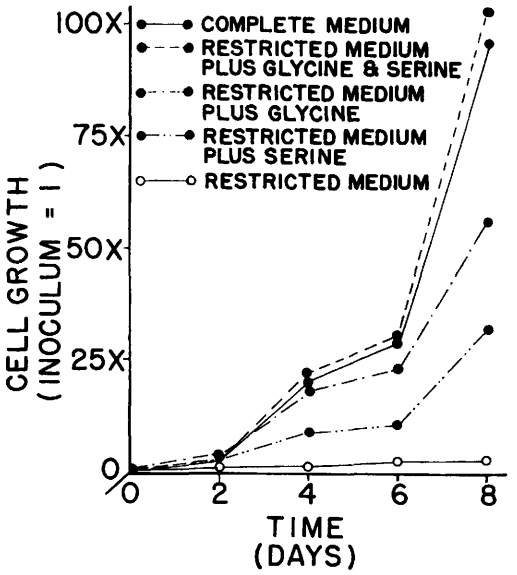

FIG. 1. Growth response of Novikoff hepatoma to glycine and serine. Amino acids in the restricted medium were only those essential for growth.

acid, glutamic acid, proline, and hydroxyproline inhibited growth of this cell line(9). In the present studies, no growth was obtained when both glycine and serine were deleted from the substrate, suggesting that one (possibly glycine) was essential for growth, but the presence of serine in the substrate could partially spare this requirement.

Further metabolic differences can be distinguished between cell lines if one would consider the results of sparing experiments. Attempts to spare the tyrosine requirement of Novikoff cells by increasing amounts of phenylalanine yielded negative results. However, glutamine, cysteine, and arginine requirements could be spared (Table II). While glutamic acid at extremely high concentrations (20 mM) was toxic, it could partially spare the glutamine requirement at intermediate levels (5-10 mM). Glutamic acid also could partially spare the glutamine requirement for monkey testicular cell(5), while glutamic acid and glutamine were readily interconvertible in monkey kidney cell(6). Further, aspartic acid and asparagine spared the glutamine requirement for monkey kidney cells, but these compounds were ineffective for Novikoff hepatoma (Table II) or Walker tumor(8).

The requirement of cysteine for Novikoff cells was alleviated with moderate amounts of sodium thioglycolate, as well as sodium sulfite, sodium bisulfite, sodium thiosulfate, and sodium sulfide, but methionine, cysteic acid, or sodium sulfate was not effective. Combinations of the inorganic substances with further supplementation of serine did not improve cellular growth markedly. Similar evidence for sparing cysteine requirement for a number of cell lines was reported by Eagle(6) but chick heart fibroblast has shown an absolute requirement for cysteine or cystine(13,14).

Of particular interest were the arginine-sparing experiments. While ornithine was ineffective, supplementing an arginine-deficient

TABLE II. Some Typical Sparing Experiments with Freshly Excised Novikoff Hepatoma *In Vitro*.

		Concentration (mM)					
		0	.5	1	5	10	20
Deficiency	Supplement	Growth in 8 days, inoculum = 1					
Glutamine	Glutamic acid	.7	1.6	1.6	5.3	6.7	.2
	Aspartic "	.4	.7	.4	.0	.4	.0
	Asparagine	.4	.2	.0	1.6	.8	.4
Cysteine	Methionine	.2	.4	.2	.4	.0	.9
	Sodium thioglycolate	.9	12.0	11.3	8.2	5.3	2.2
	Cysteic acid	.2	0	.0	.0	.0	.0
	Na_2SO_4	.2	.4	.2	.2	.0	.0
	Na_2SO_3	.0	12.0	8.4	1.6	.4	.0
	$NaHSO_3$	.0	8.9	10.4	3.3	.4	.4
	$Na_2S_2O_3$	.2	1.1	9.3	10.4	6.2	8.8
	Na_2S	.2	.4	.6	10.9	9.6	7.8
Arginine	Citrulline	1.6	5.3	13.5	47.7	42.6	49.1
	Ornithine	1.6	2.9	1.8	.2	.7	.2

Control growth range = 86.5-90.4

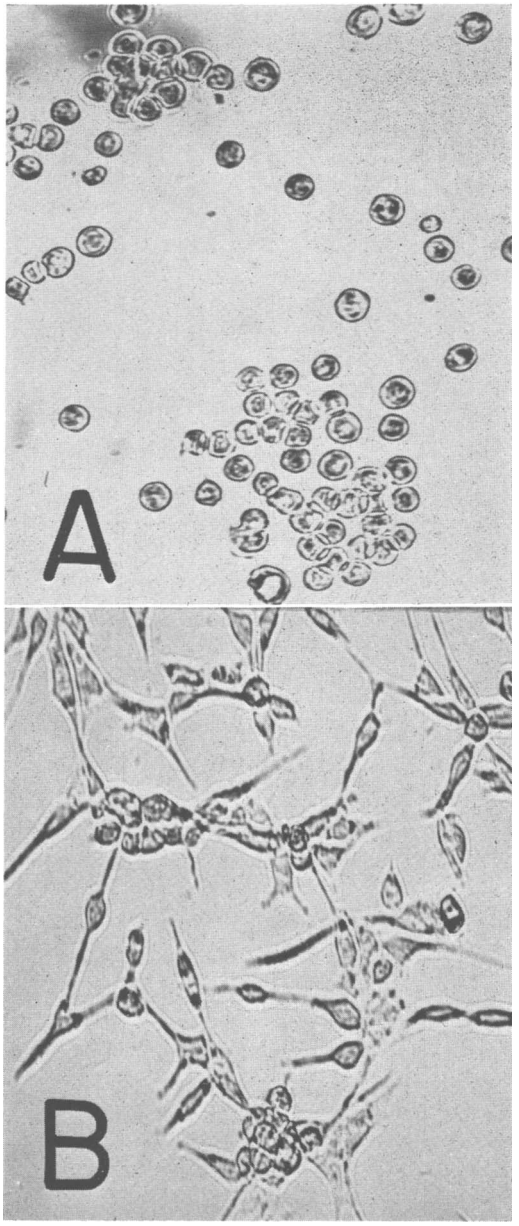

FIG. 2. Novikoff hepatoma after 96 hr incubation. A—complete medium 5a; B—complete medium 5a – arginine. 240×.

medium with citrulline resulted in excellent growth of Novikoff hepatoma *in vitro*. At the highest level of citrulline tested (20 mM), a 49-fold increase in cells was obtained during the 8-day incubation period. Since this neoplasm arose from hepatic tissue, it appeared that a part of metabolic sequences of the Krebs-Henseleit cycle was still operating in the resultant tumor. Citrulline was previously reported to be inactive in replacing arginine for Walker tumor *in vitro*(15), but Morgan(16) has shown it to be active for chick heart fibroblast.

Some mention should be made of the morphological appearance of Novikoff cells in arginine-deficient medium. In a complete medium the cells were rounded, non-granular in appearance, with many mitotic figures, but adhesion to the glass surface was poor. Many cells exhibited balloon-like extensions of the cell surface, previously noted by Hotchin(12). As the growth period was extended, numerous giant cells appeared with granulated cytoplasm and enlarged nuclei. When arginine was omitted from the substrate, the rounded cells were no longer present; and the culture consisted almost entirely of fibroblasts or spindle cells (Fig. 2). They were less refractile and practically all cells adhered to the surface of glass vessel. Pathological examination indicated that these cells were neoplastic in nature and were not derived from normal stroma of the tumor.† Furthermore, this phenomena appeared to be reversible. For example, when cells cultured in an arginine deficient medium for 4 days were placed in a complete medium, they became rounded, many mitotic figures were apparent, and cell population increased. Attempts, however, continuously to culture fibroblasts for further studies failed, since the cells would usually die after 6-10 days in an arginine-deficient medium. Relation of arginine to morphology of Novikoff hepatoma cells *in vitro* merits further investigation.

Summary. Novikoff hepatoma requires 12 amino acids and glutamine for growth *in vitro*. When both glycine and serine were deleted from the medium, no growth occurred. In presence of serine some growth was apparent, but glycine was more effective in stimulating tumor cell proliferation. Glutamic acid and sodium thioglycolate could partially spare glutamine and cysteine requirements, respectively, and several sulfur-containing inorganic salts could partially replace cysteine. Argi-

† We are indebted to Dr. E. S. Irvine for pathological examinations.

nine could be replaced by citrulline but ornithine was not active. When arginine was deleted, the typically rounded cell of Novikoff hepatoma was no longer apparent; but a predominance of fibroblasts remained attached to the surface of the flasks.

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Creatine Metabolism in Hyperthyroidism.* (24543)

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Present knowledge of the influence of hyperthyroidism on creatine metabolism has been summarized(1). It was pointed out that the disease leads to an elevated creatinuria and reduction in concentration of muscle creatine. Several possible explanations for this metabolic defect were suggested although no direct experimental evidence supported them. The present report presents the results of a study of creatine metabolism in hyperthyroidism utilizing radioactive creatine precursors in a manner similar to that employed in a study of creatine metabolism in Vit. E deficiency (3).

Methods. Two series of experiments were conducted. In the first, Sprague-Dawley rats of both sexes, weighing initially between 80 and 100 g were given a diet of laboratory chow. Four rats were given daily subcutaneous injections of 0.5 mg of sodium thyroxine. Four other rats were given no injections and served as controls. After 3 weeks the rats were each injected intraperitoneally with 100 microcuries of glycine-1-C^{14} (specific activity 0.81 microcuries per μmole) per kilo of body weight. The animals were killed 2 or 3 hours after the injection. Since there appeared to be no significant difference in results obtained from animals killed 2 hours after glycine injection as compared to those killed after 3 hours, the results were combined. Glycocyamine and creatine concentrations and specific activities were determined as previously described(3). In the second series of experiments, weanling Sprague-Dawley rats of both sexes were given a purified diet consisting of casein, 18 g; sucrose, 74.5 g; hydrogenated vegetable fat (Crisco), 3 g; cod liver oil, 2 g; salt mix(2), 2 g; choline chloride, 0.1 g; thiamine chloride, 1.5 mg; riboflavin, 1.5 mg; niacin, 6 mg; inositol, 30 mg; calcium pantothenate, 3 mg; pyridoxine hydrochloride, 1.5 mg; biotin, 15 μg; 2 methyl 1, 4 naphthoquinone, 75 μg; Vit. B_{12}, 10 μg. Each rat was given 10 mg alpha

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