THE AMINO ACID REQUIREMENTS OF RABBIT FIBROBLASTS, STRAIN RM3-56

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Plates 1 to 3

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ABSTRACT

Strain RM3-56 of rabbit fibroblasts was found to require arginine, cystine, glutamine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, serine, threonine, tryptophan, tyrosine, and valine for growth in a medium containing 2 per cent dialyzed serum as the only undefined component. The requirement for serine is less specific than that of the other 13 amino acids and it is partially replaced by glycine, or alanine, or by several combinations of so called accessory amino acids. The concentrations of essential amino acids which permit maximal proliferation range from 0.005 to 0.3 mM. Cystine, glutamine, lysine, tryptophan, tyrosine, valine are toxic at concentrations of 5 mM. The rate of proliferation of RM3-56 in a medium containing all 14 essential amino acids is increased significantly by the addition of alanine and to a lesser extent by the addition of aspartic and glutamic acids and glycine. A deficiency of cystine or glutamine results in cellular degeneration within 3 to 5 days, whereas the cells remain in good condition for 2 to 3 weeks in the absence of each of the remaining 12 essential amino acids. The results obtained with RM3-56 are compared with strains HeLa, L, and U12, whose amino acid requirements have been investigated under similar conditions.

The pedigree and cultural characteristics of strain RM3 fibroblasts derived from adult rabbit muscle were outlined in an earlier report (1). As a prerequisite to employing strain RM3 in studies of factors affecting virus multiplication, it was considered expedient to establish some of the nutritional requirements of this strain of cells. The general approach to this problem was fundamentally similar to that used by Fischer and Astrup (2) and Fischer (3) in studies with chick embryo fibroblasts, where an attempt was made to establish the dialyzable factors in media containing serum and embryo extract. This report deals with the amino acids required by RM3 cells for proliferation in a medium containing 2 per cent dialyzed horse serum, and the relative concentrations of each which permits optimal proliferation under these conditions.

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Methods

Media and Reagents.—Medium 56 contains 5 per cent chick embryo extract (CEE), 10 per cent normal horse serum (NHS), and 85 per cent solution S18 (Table I). *Medium 73* is composed of S16 (Table I) supplemented with 2 per cent cent dialyzed horse serum (DHS). *CEE* is prepared according to the procedure of

TABLE I

Composition of Media

Compound	Concentration		Compound	Concentration	
	S16*	S18*		S16	S18
	mM	mM		mM	mM
Alanine	1.00	0.66	Calcium chloride	1.8	1.8
α-Aminobutyric‡	0.12	0.08	Magnesium sulfate	0.8	0.8
Arginine	0.20	0.10	Potassium chloride	5.4	5.4
Asparagine	0.07	0.05	Sodium bicarbonate	26.2	26.2
Aspartic	0.11	0.07	Sodium chloride	116.2	116.2
Cystine	0.05	0.02	Sodium monobasicphosphate	1.2	1.2
Glutamic	0.11	0.07	Glucose	5.6	5.6
Glutamine	2.00	1.00	Phenol red	0.01	0.01
Glycine	0.30	0.20			
Histidine	0.05	0.02	Biotin	0.005	0.005
Hydroxyproline	0.05	0.03	Choline	0.01	0.01
Isoleucine	0.25	0.20	Folic acid	0.005	0.005
Leucine	0.25	0.10	Nicotinamide	0.005	0.005
Lysine	0.25	0.10	Pantothenic acid	0.005	0.005
Methionine	0.10	0.05	Pyridoxal	0.005	0.005
Ornithine	0.16	0.11	Riboflavin	0.0005	0.0005
Phenylalanine	0.15	0.05	Thiamin	0.005	0.005
Proline	0.13	0.09	Vitamin B12	0.0001	—
Serine	0.25	0.09			
Threonine	0.25	0.10	Glucuronic acid	0.05	—
Tryptophan	0.03	0.01	Orotic acid	0.32	—
Tyrosine	0.10	0.10			
Valine	0.25	0.10			

* Medium contains 100 units penicillin G and 50 μg. streptomycin sulfate per ml.

‡ DL-α-aminobutyric acid; all other amino acids are of L-configuration.

Swim and Parker (4). *DHS* is prepared as follows: sterile NHS is dialyzed (seamless, cellulose tubing, Visking Corp., 0.85 inch diameter, average pore radius of 24A) at 4° C. with continuous agitation against 15 volumes of distilled water for 24 hours (water changed at 16 hours). The water is then replaced with an equal volume of Earle's saline (5) lacking bicarbonate at pH 7.2, and dialysis is continued for an additional 16 hours. *Trypsin* (Nutritional Biochemicals Corp., 2 × crystallized containing 50 per cent $MgSO_4$) is employed as a 0.01 per cent solution in a modified S18 (pH 8.0) containing 0.01 M tris (hydroxymethyl) aminoethane instead of sodium

bicarbonate. *Citric acid–crystal violet* solution for nuclear counting contains 6 per cent citric acid and 0.01 per cent crystal violet.

Preparation of Duplicate Cultures.—Stock cultures are propagated in medium 56 according to methods described previously (1). Duplicate flasks are prepared from 6 to 8 day old stock cultures as follows. The cells are released from the glass and largely dispersed by replacing the medium in each T-60 flask (6) with 5 ml. of trypsin solution, and incubating the flask at 37 C. for 5 to 8 minutes. The resulting suspension of cells is diluted with 3 volumes of medium 56 and centrifuged at 500 *g* for 3 minutes. The supernate is discarded, the cells are washed once with 10 ml. of medium, and finally resuspended by vigorous and repeated pipetting in medium 56 at a concentration of approximately 4×10^5 per ml. Clumps of cells are largely removed by passing the suspension through a sintered-glass filter (Ace Glass, Inc., Vineland, New Jersey, porosity B). The filtrate is diluted with medium 56 so as to contain 1×10^5 cells per ml. as determined by counting directly in a hemocytometer, and 2 ml. aliquots are dispensed into T-15 flasks (6) which are incubated at 37 C.

Determination of Growth Response to Experimental Media.—Medium 56 is removed from groups of 2 to 4 duplicate flasks which have been incubated for 24 hours, and the cells which adhere to the glass are washed with 2 ml. of experimental medium. Two ml. of experimental medium are then added and the flasks are incubated at 37 C. The medium is replaced on the 3rd day and the experiments are terminated on the 6th day. The cells in each flash are enumerated by a modification of the nuclear counting procedure of Sanford *et al.* (7). The experimental medium is replaced by 1 ml. of trypsin, and the flasks are incubated for 5 minutes at 37 C. so as to remove the cells from the glass. After incubation, 2 to 4 ml. (depending on the number of cells) of citric acid–crystal violet solution are added to each flask, and the nuclei are either counted immediately or the flasks are stored at 4 C. A uniform suspension of nuclei, free from cytoplasm, is obtained by shaking the flasks for 5 minutes at a rate of 120 cycles per minute on a reciprocating shaker (Eberbach and Son Co., Ann Arbor, Michigan, variable speed blood pipette shaker, equipped with a holder for T-15 flasks). Aliquots of the suspension are placed in a hemocytometer chamber immediately and the nuclei are counted.

EXPERIMENTAL AND RESULTS

It was observed that RM3-56 cells proliferate when subcultured into medium 73. To ascertain which of the amino acids in medium 73 are essential under these conditions, the growth response of RM3 to experimental media lacking in a single amino acid was determined quantitatively. The results are summarized in Table II. When either cystine or glutamine (considered as an amino acid for purposes of this discussion) is omitted from the medium, the cells not only fail to proliferate but undergo marked degenerative changes within 3 to 5 days (compare Figs. 1 and 2 with 3). On the other hand, when arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, tyrosine, and valine are omitted separately, the cells do not proliferate significantly but remain in good condition for periods of 2 to 3 weeks (Figs. 4 and 5) if the medium is replaced at regular intervals. It will also be noted in

TABLE II

Effect of Omitting Amino Acids Separately from Medium 73 on Proliferation of Strain RM3-56

Amino acid omitted	Growth index*	Amino acid omitted	Growth index*
None	5.3	Tyrosine	1.0
Arginine	1.2	Valine	0.9
Cystine	0.5	Serine	3.0
Glutamine	1.0	Alanine	4.6
Histidine	1.0	Asparagine	5.0
Isoleucine	1.6	α-Aminobutyric	5.4
Leucine	1.5	Aspartic	4.7
Lysine	1.5	Glutamic	5.1
Methionine	1.2	Glycine	4.8
Phenylalanine	1.1	Hydroxyproline	5.5
Threonine	1.1	Ornithine	5.0
Tryptophan	1.4	Proline	5.6

* Average values for 2 or more experiments.

TABLE III

Effect of Adding Amino Acids to a Modified Basal Medium on Growth of RM3-56

Additions to modified basal medium*	Growth index‡
None	1.1
Serine	3.5
I + II + III§	3.0
Serine + I + II + III	5.2
I	2.5
II	3.0
III	2.2
Alanine or glycine	2.3
Serine + I	4.6
Serine + II	4.0
Serine + III	3.3
Serine + I + II	5.3
Serine + alanine	4.7
Serine + alanine + II	5.1

* Alanine, α-aminobutyric, asparagine, aspartic, glutamic, glycine, hydroxyproline, ornithine, proline, and serine omitted from medium 73.

‡ Average of 5 or more experiments.

§ I = alanine, α-aminobutyric, asparagine; II = aspartic, glutamic, glycine; III = hydroxyproline, ornithine, proline. I, II, and III employed throughout in concentrations indicated for S16 (Table I).

Table II that the degree of proliferation is reduced significantly in the absence of serine, whereas single omissions of the remaining 9 amino acids are without effect.

The fact that proliferation is reduced in the absence of serine suggested the

possibility that the requirement for this amino acid was less specific than for the other 13 essential amino acids. This was substantiated by experiments employing a modified basal medium (Table III) which contains no serine and lacks the 9 amino acids which appeared to be non-essential when omitted separately (hereafter referred to as accessory amino acids). Under these conditions no proliferation is obtained in the absence of serine. To establish the amino acids which partially substitute for serine, the group of 9 accessory amino acids was divided into groups of 3 (groups I, II, and III in Table III),

TABLE IV

Proliferation of RM3-56 Fibroblasts as a Function of the Concentration of Essential Amino Acids

L-Amino acid	Inoculum $\times 10^4$	Amino acid concentration, mM											Concentration permitting maximal growth
		0	0.005	0.01	0.02	0.03	0.05	0.1	0.2	0.5	1	5	
		Index of proliferation after 6 days											
													mM
Arginine	15	1.2	—	3.1	—	5.2	6.3	5.3	—	6.3	5.5	4.5	0.04
Cystine	16	0.3	2.4	3.5	5.6	5.3	5.8	5.4	—	2.6	2.3	—	0.02
Glutamine	15	1.1	1.2	1.9	—	—	2.4	3.3	4.6	5.3	5.2	4.9	0.3
Histidine	18	1.1	3.9	6.1	6.1	—	—	4.6	—	5.7	—	4.3	0.01
Isoleucine	16	1.7	2.3	3.1	4.4	5.7	5.9	6.2	—	6.1	6.1	5.0	0.05
Leucine	17	1.5	—	3.3	4.1	4.5	5.4	5.7	—	—	—	4.7	0.05
Lysine	13	1.4	1.3	2.4	—	4.4	6.3	5.8	—	4.7	4.4	3.9	0.05
Methionine	19	1.1	3.6	4.7	5.8	—	5.3	—	—	5.3	5.2	4.5	0.02
Phenylalanine	17	1.1	2.7	4.4	5.3	6.3	6.3	—	—	5.3	6.3	6.3	0.03
Serine	13	3.2	—	3.4	—	4.1	5.6	5.8	5.2	5.1	5.8	5.3	0.05
Serine*	17	1.1	1.6	1.4	—	2.8	3.4	—	—	—	3.8	—	—
Threonine	17	1.1	1.6	2.4	—	4.0	5.8	5.7	—	—	5.7	4.8	0.05
Tryptophan	18	1.4	5.1	5.1	—	—	—	—	—	5.1	4.9	1.6	0.005
Tyrosine	18	1.1	2.6	3.2	5.3	4.6	5.1	—	—	4.6	4.9	1.9	0.02
Valine	18	0.9	—	1.4	—	2.2	6.3	4.6	5.8	—	5.6	3.6	0.05

* Alanine, α-aminobutyric, asparagine, aspartic, glutamic, glycine, hydroxyproline, ornithine, and proline in addition to serine omitted from the medium.

which were tested singly and in various combinations. Some proliferation was obtained with each group, although group II, containing aspartic, glutamic and glycine, appeared to be the most effective. With the exception of glycine and alanine, no attempt has been made to assess the contribution made by the individual amino acids of each group, since the differences in growth response fall within the limits of experimental error. The problem is further complicated by the fact that maximal proliferation is not obtained in the presence of serine unless accessory amino acids are added to the medium (Table III). Alanine (or group I amino acids) provides the greatest stimulation to growth whereas group III (hydroxyproline, ornithine, and proline) is without

effect. Since the apparent stimulation by group II is within the limits of accuracy of the nuclear counting procedure, no attempt has been made to test the activity of the constituent amino acids.

The growth response of RM3 as a function of the concentration of each of the essential amino acids is illustrated in Table IV. With increasing concentrations of a single amino acid there is a corresponding increase in the number of cells until a maximal response is obtained at concentrations which range from 0.005 mM to 0.3 mM, depending on the amino acid. Moderate increases in the concentration of amino acids above that permitting maximal proliferation are without effect, but certain amino acids are toxic at relatively high

TABLE V

Lack of Growth Response of Strain RM3-56 to D-Amino Acids

Amino acid	L-Amino acid for maximal growth	Concentration of D- or L-amino acid*				
		0	1 × D	2 × D	5 × D + L	1 × L
		Growth index after 6 days				
	mM					
Cystine	0.02	0.9	1.6	1.6	4.7	5.1
Histidine	0.01	1.1	1.6	1.4	5.3	5.6
Isoleucine	0.03	1.9	1.3	1.8	5.5	5.3
Leucine	0.05	1.5	1.6	1.4	5.1	5.3
Methionine	0.02	1.2	1.3	1.6	4.5	6.0
Phenylalanine	0.03	1.5	1.8	1.4	4.7	4.8
Serine	0.05	3.2	3.5	2.9	5.1	5.1
Threonine	0.05	1.2	1.4	1.0	4.9	5.5
Tryptophan	0.005	1.6	1.6	1.4	4.9	4.8
Tyrosine	0.02	1.1	1.4	1.2	5.2	4.7
Valine	0.05	1.0	0.9	0.8	5.9	5.0

* Expressed as multiples of concentration of L-amino acid which permits maximal proliferation (column 2).

concentrations. Cystine inhibits proliferation at a concentration as low as 0.5 mM, and at 1 mM, marked degenerative changes occur (Fig. 6). Inhibition of growth and cellular degeneration is also produced by 5 mM tryptophan and tyrosine, whereas the same concentration of lysine or valine only inhibits proliferation. No toxic manifestations are observed with the remaining essential amino acids when added in concentrations up to 5 mM.

The results presented in Table V indicate that the D-enantiomorphs of the essential amino acids will not replace the L-isomers, and do not inhibit growth in the presence of L-amino acids when added in concentrations exceeding those of the latter by a factor of 5. The D-isomers of arginine and lysine are also inactive in this regard, as indicated by the response to graded concentrations of racemic mixtures.

DISCUSSION

The results of these studies indicate that rabbit fibroblasts, strain RM3-56, require arginine, cystine, glutamine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, serine, threonine, tryptophan, tyrosine, and valine for proliferation in a medium containing 2 per cent dialyzed horse serum as the only undefined constituent. It is of interest that several permanent strains of cells of human and murine origin, whose amino acid requirements have been determined under similar conditions, require the same amino acids as indicated for RM3-56 with the exception of serine (8–10). On the other hand, the Walker rat carcinoma which does not require serine has an additional requirement for asparagine (11). The fact that serine is essential for RM3-56 is believed to be a reflection of the nutritional history of the strain rather than an indication of its origin since the results of recent experiments indicate that the nutrition of mammalian fibroblasts is, within limits, a function of the composition of the medium in which they are propagated (12, 13). Similarly, the demonstration that cystine, glutamine, serine, and tyrosine are essential for fibroblasts whereas they are not required for growth or for maintenance of nitrogen balance in several mammalian species (14) is not interpreted as indicative of the degree to which nutritional synergism exists between cells possessing different physiological functions in the intact animal. In this connection, evidence was presented earlier (1) that strain RM3 is an example of fibroblasts derived from normal tissue which have undergone nutritional alterations *in vitro*. RM3-56 appears to be less fastidious than newly established strains of rabbit fibroblasts, but it remains to be determined whether this is related to its amino acid requirements.

The requirement for serine is less specific than that of the remaining 13 essential amino acids since some growth is obtained in the absence of serine when various so called accessory amino acids are present. It appears that RM3-56 can synthesize some serine from glycine or alanine (Table III), but this requires confirmation by biochemical procedures. It is difficult to assess the individual contributions of the remaining accessory amino acids to proliferation in the absence of serine because the differences in growth response are within the limits of experimental error. The fact that alanine stimulates proliferation in the presence of all 14 essential amino acids may indicate that it is also required but that a deficiency does not become apparent within 6 days. It has not been possible to investigate this possibility by propagating cells serially in experimental media since medium 73 does not permit growth of RM3-56 for more than one subculture. On the other hand, RM3-56 may be unable to synthesize alanine at a rate consistent with maximal proliferation or this culture may be composed of more than one nutritional type of cell, a proportion of which have a specific requirement for alanine. It is anticipated that the interrelationships of serine with other amino acids and the role of alanine

in cell nutrition can be evaluated by employing a variant isolated recently from a culture of RM3-56 which can be propagated serially in medium 73 (12).

The response of various strains of cells to amino acid deficiencies in relation to their cultural and morphological characteristics deserves special consideration. In the case of RM3-56 cellular degeneration is observed during the 1st week only when cystine or glutamine is omitted from the medium. Similarly, freshly explanted chick embryo fibroblasts degenerate in the absence of cystine (2, 3) or glutamine (15) under conditions which permit limited proliferation when other amino acids are omitted. Newly established strains of human fibroblasts derived from foreskin respond to amino acid deficiencies in a manner similar to that described for RM3-56. These data are in direct contrast to the results obtained with malignant cells strains HeLa (8) and L (9) and with morphologically altered fibroblasts, strain U12 (10). It is of particular interest that the adverse effects of unbalanced metabolism resulting from amino acid deficiencies appear to be more pronounced with morphologicaly altered or malignant cells than with newly established strains or permanent lines with similar morphological characteristics. The extent to which this apparent generalization is applicable and the degree to which differences in response to amino acid deficiencies reflect variations in the over-all physiology of the various strains remain to be determined.

The concentration of the essential amino acids which permits maximal proliferation of RM3-56 varies over a range of 0.005 to 0.3 mM. These results are quantitatively similar to those obtained with strains HeLa (8), L (9), and U12 (10) under analogous experimental conditions. The toxic manifestation elicited by high concentrations of certain amino acids confirms the results of early studies by Burrows and Neymann (16). RM3-56 resembles strains HeLa, L, and U12 in that the degree of toxicity varies with the structure of the amino acid and is a function of its concentration. It should be noted, however, that as in the case of the concentration of the individual amino acids which permits maximal proliferation, each strain of cells possesses a certain degree of individuality.

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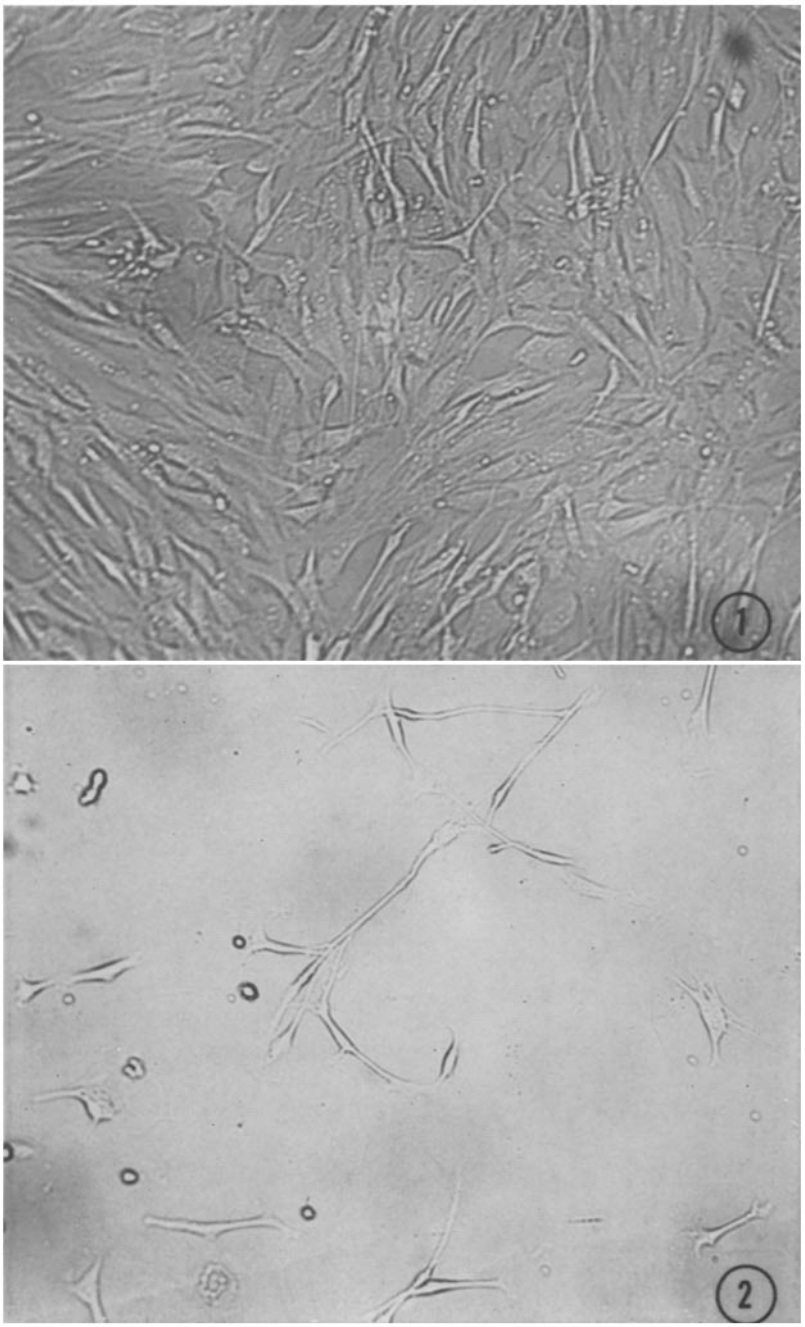
EXPLANATION OF PLATES

The magnification of each figure is approximately 150.

PLATE 1

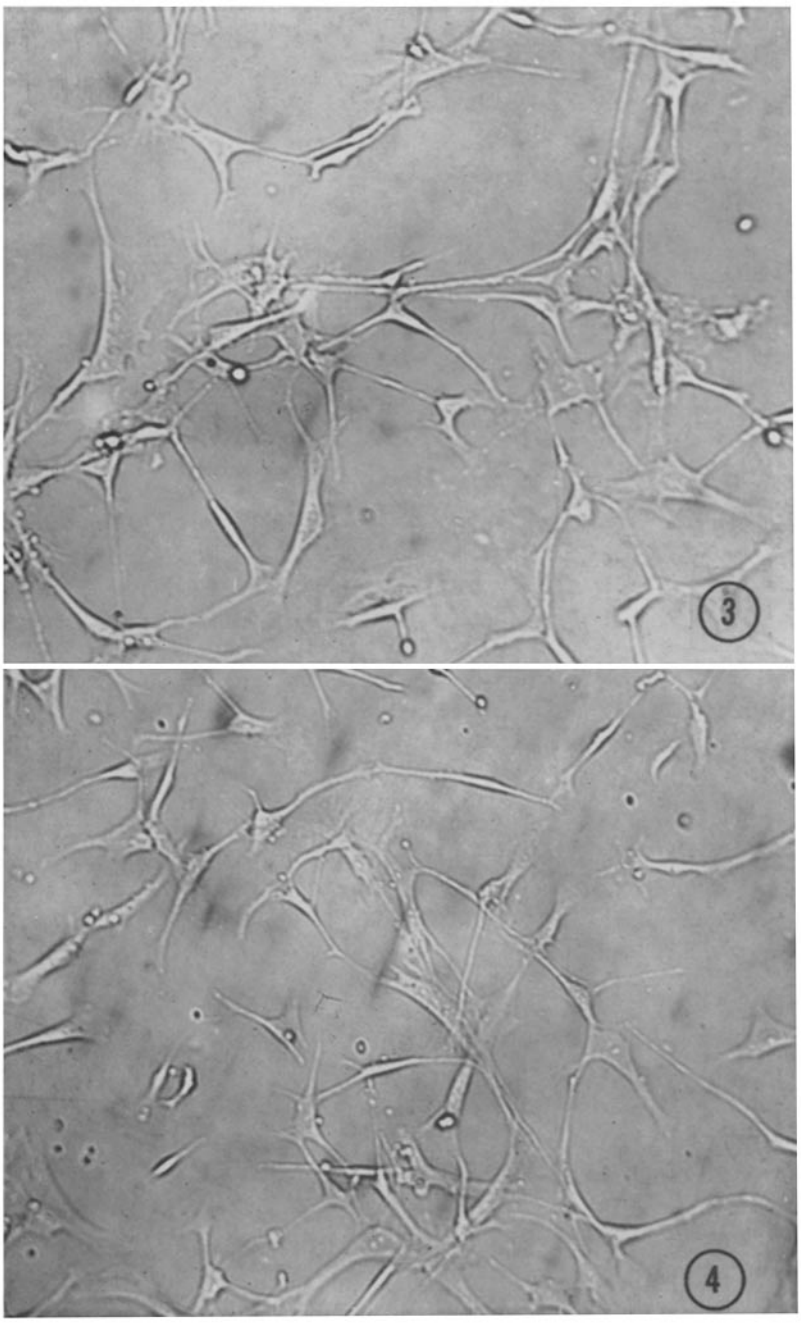
FIG. 1. A typical culture of RM3-56 after 7 days in medium 73.
FIG. 2. RM3-56 after 7 days in absence of glutamine.



(Haff and Swim: Amino acid requirements of rabbit fibroblasts)

PLATE 2

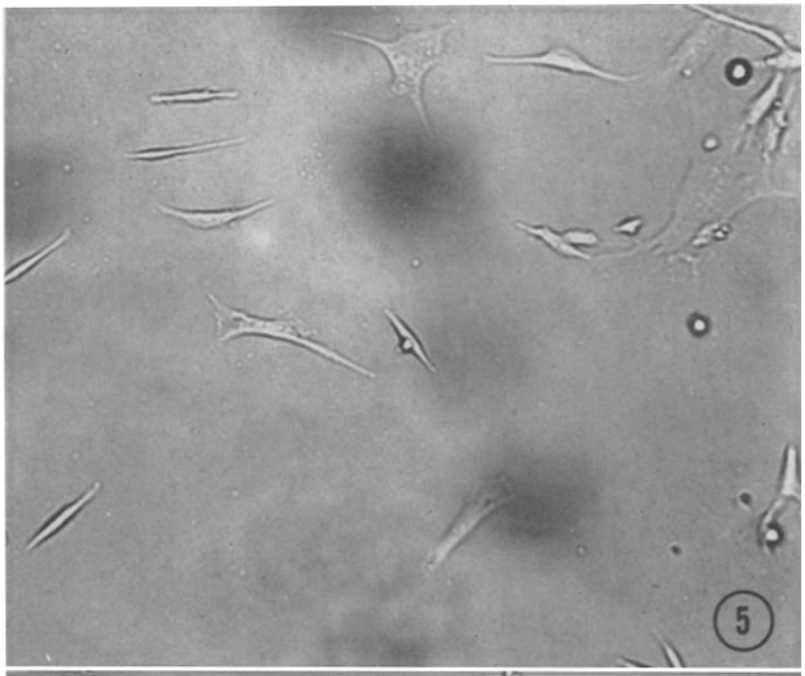
FIG. 3. RM3-56 after 14 days in the absence of arginine.
FIG. 4. RM3-56 after 12 days in absence of leucine.

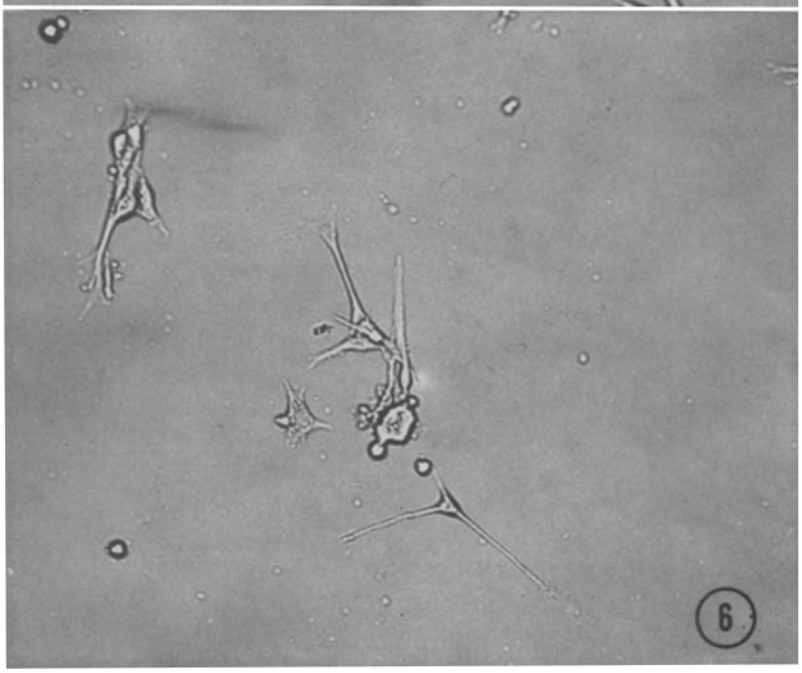


(Haff and Swim: Amino acid requirements of rabbit fibroblasts)

PLATE 3

FIG. 5. RM3-56 after 6 days in absence of cystine.
FIG. 6. RM3-56 after 7 days in presence of 1 mM cystine.





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