Minimal Vitamin Requirements of Rabbit Fibroblasts, Strain RM3-73. (23085)

R. F. HAFF* AND H. E. SWIM† (Introduced by R. F. Parker)
Department of Microbiology, School of Medicine, Western Reserve University, Cleveland.

Isolation of a subline of rabbit fibroblasts, designated RM3-73, from the established stock strain RM3-56(1) has been described.‡ Strain RM3-73 appears to be less fastidious than RM3-56 since it can be propagated serially in a medium containing a small quantity of dialyzed serum as the only undefined component, whereas RM3-56 requires both whole serum and chick embryo extract for continuous proliferation. As a basis for comparing nutritional requirements of the two strains, it was of interest to determine some of the factors required by RM3-73. This strain is particularly suitable for nutritional studies since the cells can be propagated for indefinite periods in a medium which contains a minimal quantity of undefined constituents. Thus, some of the restrictions which are imposed by inadequate basal media are eliminated.‡ This report describes the vitamins required by strain RM3-73 in a medium containing 2% dialyzed serum.

TABLE I. Composition of Medium 73.

Compound*	Conc., mM	Compound	Conc., mM
Alanine	1.00	Calcium chloride	1.8
α-Aminobutyric†	.12	Magnesium chloride	.8
Arginine	.20		
Asparagine	.07	Potassium chloride	5.4
Aspartic	.11		
Cystine	.05	Sodium bicarbonate	26.2
Glutamic	.11		
Glutamine	2.00	Sodium chloride	116.2
Glycine	.30	Sodium monobasiephosphate	1.2
Histidine	.05		
Hydroxyproline	.05	Glucose	5.6
Isoleucine	.25	Glucuronic acid	.05
Leucine	.25	Orotic acid	.32
Lysine	.25	Phenol red	.01
Methionine	.10		
Ornithine	.16	Biotin	.005
Phenylalanine	.15	Choline	.01
Proline	.13	Folic acid	.005
Serine	.25	Nicotinamide	.005
Threonine	.25	Pantothenic acid	.005
Tryptophan	.03	Pyridoxal	.005
Tyrosine	.10	Riboflavin	.0005
Valine	.25	Thiamin	.005
		Vit. B_{12}	.0001

* Medium also contains 100 units of penicillin G and 50 μg of streptomycin sulfate/ml and 2% (v/v) dialyzed horse serum.

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

TABLE II. Effect of Omitting Vitamins Separately from Medium 73 on the Proliferation of RM3-73.

	Proliferation index after 7 days*			
	Passage			
Vit. omitted	1st	2nd	3rd	4th
Folic acid	6.6	1.0	D†	
Nicotinamide	.8	D		
Pantothenic acid	4.4	.8	D	
Pyridoxal	6.3	6.4	6.1	5.4
" ‡	4.1	1.6	.5	D
Riboflavin	.5	D		
Thiamin	7.6	7.0	3.3	D
Biotin	9.3	7.0	7.2	7.9
Choline	7.2	7.3	6.2	8.1
Vit. B_{12}	8.2	8.4	7.6	8.3
None	7.6	7.2	8.3	7.5

* Avg value for 2 experiments.

† D = Extensive degeneration of cells and counts not performed.

‡ Alpha-aminobutyric, asparagine, aspartic, glutamic, glycine, hydroxyproline, ornithine, and proline in addition to pyridoxal omitted from medium 73.

Methods. Stock cultures of RM3-73 were propagated on glass in medium 73 (Table I) according to methods described previously (1). Each experimental medium was evaluated by propagating the cells serially in a group of flasks (1 to 4 T60 and 4 T15 flasks). The medium was replaced on the third and fifth days, and subcultures were prepared at intervals of 7 days by the use of trypsin.‡ The degree of cellular proliferation for each 7-day interval was determined by the nuclear counting procedure.‡

Results. The results obtained when vitamins of medium 73 are omitted separately from the medium are summarized in Table II. The degree to which rate of proliferation de-

* Former U. S. Public Health Service Research Fellow, National Microbiological Institute.

† Aided by a grant from The National Foundation for Infantile Paralysis.

‡ Unpublished experiments.

TABLE III. Proliferation of Strain RM3-73 as a Function of Concentration of Individual Vitamins.

Vitamin	Passage No.	Vitamin conc., mM × 10^{-3} 10	1	.1	.01	.001	mM × 10^{-3} permitting optimal growth
		Proliferation index*					
Folic acid	3-4	7.1	9.3	D†			1
Nicotinamide	2-3	7.0	6.3	3.8	D		1
Pantothenate	2-3	7.1	7.5	7.4	3.3	D	.1
Pyridoxal‡	3-4	4.1	5.0	5.2	2.1	D	.1
Riboflavin	2-3		7.1	8.9	3.3	D	.1
Thiamin	4-5	6.9	7.2	7.6	4.4	D	.1

* Avg values for passages indicated in Column 2.
† D = Cells degenerated.
‡ α-aminobutyric, asparagine, aspartic, glutamic, glycine, hydroxyproline, ornithine, and proline in addition to pyridoxal omitted from medium 73.

clines in each successive culture passage is a function of the particular vitamin omitted, and this appears to be unrelated to concentration of that vitamin which permits optimal proliferation (see Table III). On continued serial propagation of RM3-73 in the absence of one of the essential vitamins, the cells gradually degenerate (compare Fig. 1, 2, and 3 with Fig. 4) until lysis occurs. It will be noted in Table II that pyridoxal presents a special case since, except for a modest decrease in the rate of proliferation, there is no apparent requirement for this vitamin when omitted separately from medium 73. On the other hand, when α-aminobutyric, asparagine, aspartic, glutamic, glycine, hydroxyproline, ornithine and proline are omitted in addition to pyridoxal, the cells fail to proliferate for more than 2 passages. It is also of interest that pyridoxal is the only vitamin which inhibits proliferation when employed in 100 times the concentration permitting optimal growth (Table III). This group of so-called accessory amino acids which exerts a sparing action on the requirement for pyridoxal is not required for the continuous proliferation of RM3-73 in the presence of the vitamin. The group of accessory amino acids are effective however in increasing rate of proliferation of RM3-73 in the presence of pyridoxal as indicated by the results presented in Table III. The data in Table II which indicate that biotin, choline, and vit. B_{12} are not essential were substantiated by propagating RM3-73 cells for 15 passages in a modified medium 73 lacking these components. In a similar experiment, it was demonstrated that neither glucuronic nor orotic acid is required by RM3-73.

Growth response of RM3-73 as a function of the concentration of each of the essential vitamins is illustrated in Table III. Determinations of this type are complicated by the fact that one or more passages are usually required to deplete the cells of the vitamin and its conjugates when suboptimal levels are supplied in the medium. The data presented in Table III (after 2 to 5 passages in experimental media) are thus representative of the responses anticipated on continued proliferation. This was substantiated in part by experiments employing suboptimal concentrations (1 x 10^{-5} mM) of pantothenic acid and riboflavin. Under these conditions, the level of proliferation indicated in Table III was maintained for at least 8 passages.

Discussion. The foregoing data indicate that folic acid, nicotinamide, pantothenic acid, pyridoxal, riboflavin, and thiamin are essential for the continuous proliferation of rabbit fibroblasts strain RM3-73. It is apparent that these represent only the minimal requirements of this strain of cells, since additional vitamins may be contributed by unidentified factors supplied by the dialyzed serum. The degree to which the growth rate of RM3-73 declines with each successive culture passage in the absence of an essential vitamin (Table II) is indicative of the rate at which the cells are depleted of the vitamin and its derivatives. This type of response to a nutritional deficiency illustrates the importance of supplementing information obtained in short-term experiments (5-10 days without preparing subcultures) on cell culture nutrition with data procured in studies which are conducted for a sufficient period to permit serial cultivation in the experimental media. In view of the extensive degenerative changes observed when cells are propagated in the absence of an essential vitamin, it is considered that the procedure employed in these studies is somewhat more reliable than that of de-

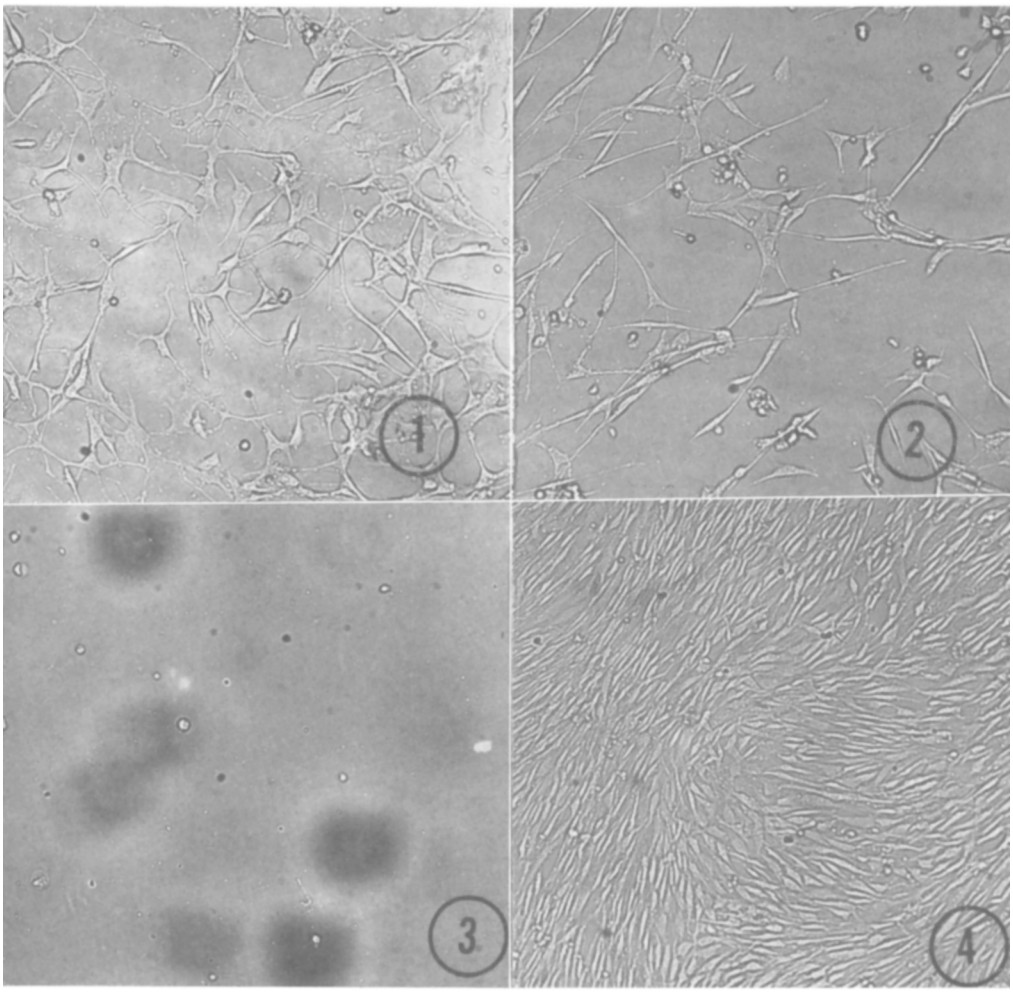

FIG. 1. RM3-73 cells after 7 days in absence of nicotinamide.
FIG. 2. " " " " " " " " riboflavin.
FIG. 3. " " " 14 " " " " pantothenic acid.
FIG. 4. A typical 7-day culture of RM3-73 in medium 73.

termining the concentration of vitamin required to restore the active proliferation of severely depleted cells(2).

RM3-73 resembles certain microorganisms(3,4) in that its requirement for pyridoxal is replaced for at least 5 passages (representing approximately 3 x 10^4 fold increase in number) by a group of eight amino acids which are otherwise not required for the continuous propagation of this strain of fibroblasts under the conditions employed. The data are consistent only with the interpretation that amino acids reduce the requirement for pyridoxal to a minimal level since the dialyzed serum added to the medium may not be entirely free from the vitamin.

The number of vitamins required by RM3-73 is less than that required by other permanent strains of cells which have been studied under similar conditions. Strains L and HeLa require choline(2) in addition to those indicated for RM3-73 (Table III) and several strains require i-inositol(5). Human fibroblasts, strain U12-79, require both choline and i-inositol.§ It is of considerable interest in this connection, that RM3-73 was isolated

§ Unpublished experiments.

by a process of selection from strain RM3-56 which requires whole chick embryo extract and horse serum in addition to the defined components of medium 73.‡ This suggests the possibility that additional vitamins are required for the RM3-56 strain of rabbit fibroblasts.

Summary. A strain of rabbit fibroblasts designated strain RM3-73 requires folic acid, nicotinamide, pantothenic acid, pyridoxal, riboflavin, and thiamine for continuous proliferation in a medium containing 2% (v/v) dialyzed horse serum. The concentration of each vitamin which permits optimal proliferation was determined by propagating the cells serially in media containing graded concentrations of the compound. A group of eight amino acids which are not required for serial propagation in the presence of pyridoxal replaced the requirement for this vitamin under experimental conditions employed.

1. Haff, R. F., and Swim, H. E., PROC. SOC. EXP. BIOL. AND MED., 1956, v93, 200.

2. Eagle, H., *J. Exp. Med.*, 1955, v102, 595.

3. Snell, E. E., and Guirard, B. M., *Proc. Nat. Acad. Sci.*, 1943, v29, 66.

4. Stokes, J. L., and Gunness, M., *Science*, 1945, v101, 43.

5. Eagle, H., Oyama, V. I., Levy, M., and Freeman, A., *ibid.*, 1955, v123, 845.

Received February 15, 1957. P.S.E.B.M., 1957, v94.

Viremia in Coxsackie B Meningitis. (23086)

ALEXIS SHELOKOV AND KARL HABEL

U. S. Department of Health, Education and Welfare, Public Health Service, N.I.H., Nat. Inst. of Allergy and Infectious Diseases, Bethesda, Md.*

Viruses of the Coxsackie B group have been frequently isolated from the stools and less frequently from the throat and the spinal fluid of aseptic meningitis patients. However, demonstration of viremia has received only a passing mention in the literature(1,2). The present report describes isolation of a Coxsackie B2 virus from the serum of a patient 3 days after onset of a febrile illness, but 5 days prior to the onset of frank meningitis.

The patient, a 36-year-old male physician engaged in virus research, became ill in November 1955 while investigating an outbreak of a disease characterized by fever, dizziness, chest pain, myalgia and sometimes nuchal discomfort. His studies included examination of patients, collection and testing of blood, pharyngeal and fecal specimens, some of which were found to contain Coxsackie B2 virus.

Methods. Monkey kidney cultures prepared by a modified Youngner technic(3) were used both for virus isolation and neutralization tests. The cells were grown in the lactalbumin hydrolysate-calf serum medium recommended by Melnick(4) and after 4 days of incubation were changed to a maintenance medium in which the concentration of the calf serum was decreased by one half. The human amnion cell cultures were prepared according to a procedure described by Takemoto and Lerner(5). In virus isolation attempts, 0.3 ml of undiluted sera and spinal fluid were added to several tubes each; the throat swabs in Hanks' solution were first treated with 500 u penicillin, 500 μg streptomycin, and 100 u mycostatin per ml; the stools were emulsified to make 10% suspension in Hanks' solution and treated with 1,250 u penicillin and streptomycin both before and after centrifugation at 8,000 rpm for 30 minutes. Serum-virus neutralization tests employed mixtures of equal volumes of appropriate serum and virus dilutions representing 100 tissue culture infectious doses which after 1 hr. at room temperature were inoculated into culture tubes. All sera used in neutralizations, including serial specimens from the patient, were heated at 56°C for 30 minutes.

* Lab. of Infectious Diseases.