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Relative Growth-Promoting Activity in Tissue Culture of Co-Factors and the Parent Vitamins. (22262)

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It has been shown(1) that 2 mammalian cells in tissue culture, a human carcinoma cell (strain HeLa) and a mouse fibroblast (strain L), require nicotinamide, pyridoxal, thiamine, riboflavin, pantothenic acid, choline, and folic acid for survival and growth. The degree to which various precursors and conjugates could substitute for the corresponding vitamin in permitting the growth of the mouse fibroblast is here described.

Methods. The technics used in maintaining stock cultures, preparing replicate flasks for feeding with the experimental media, and evaluating the growth response in terms of the number of cells have been described(1-4). As in the previous vitamin experiments(1), specific deficiencies were produced by allowing the cells to grow for varying periods of time (4-14 days) in 1 liter Blake bottles, in the appropriate vitamin-free medium. During this preliminary period of vitamin depletion, the number of cells usually increased 2- to 6-fold in individual experiments; but in most of the experiments, multiplication had then stopped, and there were evidences of cell damage resulting from the vitamin deficiency (cf.(1)). The vitamin-starved cells were resuspended in the appropriate vitamin-free medium, counted, and aliquots of the suspension inoculated into a number of small replicate flasks. Twenty-four hours later, after the cells had adhered to the glass, they were fed with the experimental mixtures containing graded amounts of the vitamin or vitamin substitutes. The flasks were re-fed at 2-day intervals, and the cell count determined after 6-15 days' incubation. Since the cells at the time of the first feeding usually showed cytopathogenic evidence of vitamin depletion, it is apparent that at least 2 factors entered into the results obtained in the present experiments: the capacity of the individual compounds to revive the vitamin-depleted cells, as well as their capacity to support subsequent growth and multiplication. Accordingly, in a second type of experiment, described in the text in relation to folic acid, instead of using vitamin-depleted cells, normal cells were serially propagated in varying concentrations of the vitamin congeners. Diphosphopyridine (DPN) and triphosphopyridine (TPN) nucleotide, flavin adenine mononucleotide (FMN) and dinucleotide (FAD), and cocarboxylase were obtained from the Sigma Laboratories. The first 4 compounds were stated to be 95-98, 60-90, 100, and 65% pure, respectively; but in making stock solutions, all were handled as if they were pure material. Coenzyme A of 75% purity was obtained from the Pabst Laboratory. "Thiamin phosphate" was obtained by autoclaving a solution of cocarboxylase in 0.1 N HCl for 10 min. in a sealed tube. Crystalline pyridoxal phosphate (Palpo) was generously supplied by Dr. E. A. Peterson of the National Cancer Institute, National Institutes of Health.

Results. The results in a number of ex-

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TABLE I. Growth Response of a Mouse Fibroblast (Strain L) to Vitamin Precursors and Conjugates.

	Period of initial starvation in appropriately vit.-deficient medium, days	Degree of cellular multiplication during period of depletion*	Inoculum of vit.-depleted cells, × 10^4	Period of secondary incubation, days	Degree of multiplication* in media containing indicated conc. (M) of specific vitamin, precursor or conjugate								Approximate max effective conc., M
					10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}	10^{-11}	0	
Nicotinic acid	10	2.7	25	6	5.2	5.6	3.0	1.0				.7	10^{-6}
Nicotinamide	10	2.7	25	6	2.6	6.2	4.2	.8				.7	"
DPN	10	2.7	25	6	6.0	6.0	2.2	1.1				.7	"
TPN	10	2.7	25	6	3.1	4.3	2.0	.9				.7	"
Pyridoxine†	8	3.1	12	9		8.8	10	10.8	5.7	.3		.97	10^{-8}
Pyridoxal†	8	3.1	12	9		12	10.4	10.2	8.25			.97	"
Pyridoxamine†	8	3.1	12	9		6.7	10.6	5.0	1.0			.97	10^{-7}
Pyridoxal phosphate†	8	3.1	12	9		9.1	6.4	2.4	1.3			.97	10^{-6}
Thiamin	12	4.1	18	8	7.2	9.3	8.6	8.5	5.8	2.0	.6	.55	10^{-8}
Thiamin phosphate	12	4.1	18	8		2.9	3.7	3.7	3.0	1.7	.2		"
Cocarboxylase	12	4.1	18	8	6.0	7.6	7.5	8.6	3.4	1.2	.6		"
Riboflavin	7	.8	14	7				7.6	9.0	2.8		1.1	10^{-9}
FMN	7	.8	14	7		8.0	13.4	7.6	7.9	3.6	1.3	1.1	"
FAD	7	.8	14	7			15.7	9.5	4.0	2.2	1.5	1.1	10^{-8}
Pantothenic acid	7	1.4	18	15	8.3	9.5	10.9	11	.33			0	"
CoA	7	1.4	18	15	7.4	7.9	7.1	.14				0	10^{-7}
Folic acid	14	3.9	16	6		10.6	9.6	4.6	2.0	2.1		.6	10^{-7} to 10^{-8}
Citrovorum factor (natural) †	14	3.9	16	6		11	10.8	10.8	9.2	2.2		.6	10^{-9}†
PABA	14	3.9	16	6		.9		.8		.8		.6	

* Referred to inoculum as 1. † Gravimetric concentration (g/ml) rather than molar.

periments with the mouse fibroblast are given in Table I. Each experiment there shown is illustrative of several others, with qualitatively similar results.

1. *Nicotinamide,* nicotinic acid, DPN and TPN all permitted the growth of mouse fibroblasts previously depleted of the vitamin by 10 days' starvation. Although the 4 compounds were equally active in terms of their effective concentrations, more growth was usually obtained with nicotinamide and DPN than with nicotinic acid or TPN.

2. *Pyridoxal* and pyridoxine were essentially equivalent in their growth-promoting activity. Although pyridoxamine and pyridoxal phosphate could substitute for pyridoxal, with the preparations here used somewhat higher concentrations were necessary.

3. *Thiamine* and cocarboxylase proved equivalent, both with respect to the concentrations required for maximal growth, and the amount of growth obtained at that optimal concentration. Smaller amounts of growth were, however, observed with "thiamine phosphate."

4. *Riboflavin* and FMN proved equivalent in growth-promoting activity. With FAD, however, significantly higher concentrations were required for a comparable effect.

5. *Pantothenic acid* proved approximately 10 times more active than the 2 samples of coenzyme A used in these experiments (Fig. 1). It is of interest that a similar quantitative difference in the activity of pantothenic acid and coenzyme A was noted by Dewey and Kidder(5) for *Tetrahymena pyriformis* and *Colpidium campylum.*

6. *Folic acid* was regularly less active than natural citrovorum factor in terms of the amounts required for the revival and growth of the vitamin-depleted cells. p-Aminobenzoic acid (PABA) proved wholly inactive (Fig. 1).

To determine whether PABA would permit the survival and growth of normal cells, not previously depleted of folic acid or its derivatives, normal cells, not previously damaged by folic acid depletion, were serially propagated in varying concentrations of folic acid and of PABA. Even under these circumstances, however, PABA failed to permit the continuing growth of the cells in the absence of folic acid.

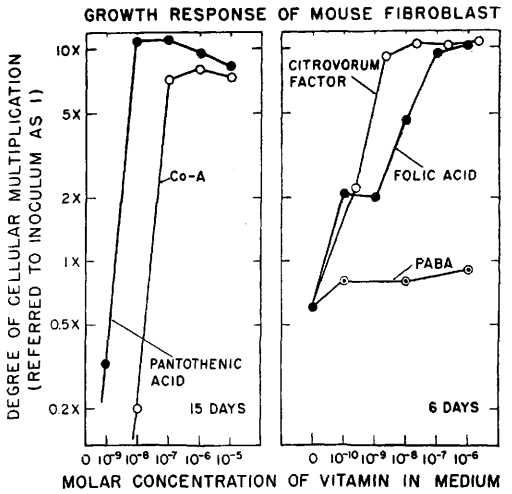

FIG. 1. Varying growth response of mouse fibroblast. (a) Pantothenic acid and coenzyme A; (b) Citrovorum factor, folic acid and PABA.

Summary. A number of the co-factors here tested (FMN, DPN, cocarboxylase) had essentially the same activity as the corresponding vitamin (riboflavin, nicotinamide and thiamine, respectively) in promoting the growth of the mouse fibroblast, in terms of either the effective concentration or the amount of growth obtained. Several vitamin conjugates (FAD, TPN, coenzyme A and pyridoxal phosphate) proved significantly less active than the parent vitamin (riboflavin, nicotinamide, pantothenate, and pyridoxal, respectively). The present experiments provide no information as to the degree to which these differences may reflect only variations in cell permeability, rather than partial blocks in the utilization of these specific co-factors, or in their conversion to a more active form. Natural citrovorum factor, however, was somewhat more active than folic acid. Of the vitamin congeners or precursors here tested, pyridoxine and pyridoxal were essentially equivalent in activity; nicotinic acid was significantly less active than either nicotinamide or DPN in terms of the amount of growth obtained, although the effective concentrations were of the same order of magnitude; while PABA was wholly inactive as a substi-

tute for folic acid. As with the co-factors, the present experiments do not exclude differential cell permeability as the basis of these differences.

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Viral Susceptibility of a Human Carcinoma Cell (Strain KB). (22263)

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The cultivation of a human epidermoid carcinoma of the floor of the mouth (strain KB) by direct implantation in a fluid medium has been described(1). As will be here shown, this cell line has proved susceptible to a number of human and animal viruses.

Methods. The basal medium used in these experiments was the same as that used in the original isolation, and consisted of the amino acids, vitamins and salts essential for the growth of the HeLa cell, at the concentrations optimal for that cell(2-5). In the initial cultivation this was supplemented with 10% whole human serum. In the present experiments, the same basal medium was supplemented with 4% whole horse serum, in order to avoid the complication introduced by the presence in pooled human serum of antibodies to some of the viruses. Four-day cultures of the cell line in T-15 flasks(2), then containing approximately 4.5 x 10^6 cells, were washed twice with the horse serum medium, and refed with 2.5 ml of fresh medium. One-tenth ml of the viral suspensions of Table I, diluted as there indicated, were then added. The medium was then replaced at 2-day intervals. This first passage test was terminated 7 or 8 days after the original inoculation. The times required for the earliest cytopathogenic effect, and for the destruction of more than 75% of the cells, are indicated in Table I. Fluids harvested at the time of maximal cytopathogenic effect (or after 7 to 8 days in the case of those viruses with no apparent effect) were then similarly inoculated into somewhat larger (T-30) culture flasks containing approximately 4.2 x 10^6 cells at time of inoculation. The virus inoculum in this second passage was 0.5 ml in a total of 5 ml. As in the first passage, the fluids were changed every other day, beginning the day after inoculation. The time required for the earliest visible cytopathogenic effect, and for the almost total destruction of the cells is shown for each virus in the last 2 columns of Table I. Material harvested from this second passage at the time of maximal cytopathogenic effect (or after 6 days in the case of those viruses with no apparent effect) was assayed for viral content by either complement-fixation, pathogenicity in tissue culture, or animal inoculation. The results of those tests are summarized in Table II.

Results. Cytopathogenic effects were produced in the KB cell in both first and second passages by type 1 poliomyelitis (Mahoney), herpes simplex, vaccinia, types 1, 3, and 4 adenoidal-pharyngeal-conjunctival (APC) viruses, lymphocytic choriomeningitis (LCM), and encephalomyocarditis (EMC) viruses (*cf.* Tables I and II). In all these, the titers of the second passage fluids indicated that the viruses had multiplied. The titer of complement-fixing antigen produced by the type 1, 3 and 4 APC viruses was significantly higher than had previously been obtained in

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