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Differences in Inositol Requirements of Several Strains of HeLa, Conjunctival and Amnion Cells.* (24260)

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A minimal growth medium consisting of 27 factors capable of supporting the propagation of several strains of human cells for at least several months had been described by Eagle (1). Eagle and his associates(2) also reported that meso-inositol was an additional essential growth factor and that the requirement of the HeLa cell for inositol was irregular and was dependent on method of dialysis. Geyer and Chang(3) found that the HeLa and the conjunctival cells would show advanced degenerative changes after 4-7 days in Eagle's minimal growth medium, that addition of inositol prevented the appearance of cell degeneration, and that the HeLa cell regularly required inositol. Since the minimal growth medium described by Eagle is an important advance in the methodology of human cell culture, these conflicting reports prompted us to explore the basis of this irregularity. Among the factors studied are differences between strains of a cell, difference between stable laboratory cell lines and primary cell explants, contamination of cell culture by the pleuropneumonia-like organisms, and spontaneous appearance of nutritional variants and the extent of dialysis of serum. The results of these studies form the basis of this report.

Materials and methods. The methods were those described earlier(4). The minimal growth medium for determination of inositol requirement of the various strains of human cells consisted of 10% dialyzed human or horse serum in Eagles basal medium(1) plus inositol at concentration of 10 to 20 μM. The withdrawal of inositol resulted in advanced degeneration of the inositol-requiring strains within 5 to 10 days. Total inositol content of serum was analyzed, using *Saccharomyces cerevisiae* as assay organism(5).

Results. Differences in HeLa strains from several laboratories. The inositol requirement of several strains of HeLa cells, kindly furnished by other investigators, was determined. The strain maintained in the author's laboratory since 1954 invariably degenerated in the minimal growth medium without inositol. The strain obtained from Dr. B. Roizman, Johns Hopkins University, was found to propagate at approximately the same rate in the minimal growth medium with or without inositol for 14 days (Table I). Several other strains were found contaminated with a pleuropneumonia-like organism and were observed only for 7 days in the presence of the contaminant; 4 degenerated completely in medium without inositol while one gave inconclusive results.

Difference in conjunctival strains maintained in different growth media. The stock culture has been found on numerous occasions to degenerate in the assay medium without inositol(3). A subculture maintained in a 0.1 mM glucose medium (10% dialyzed serum in Eagle basal medium with the concentration of glucose reduced to 0.1 mM and with addition of inositol to a cencentration of 10-20 μM) for about 6 months failed to show similar changes. It showed a definite though small net increase in cell number during the first 2 weeks in the assay medium without inositol, and has since been propagating for at least 2 months (Table I).

Difference between a stable strain and fresh explants of amnion cells. The FL strain of

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TABLE I. Multiplication or Degeneration of Several Strains of HeLa, Conjunctival and Amnion Cells in Minimal Growth Media with and without Inositol.

Cell strain	Inositol in media	No. of cells on days No.* 0	7	14	28
HeLa (Chang)	0	18	0	0	
	10 μM	18	60	213	
HeLa (Roizman)	0	20	70	246	
	10 μM	20	105	296	
Conjunctival (stock)	0	23	0		
	10 μM	23	430		
Conjunctival (.1 mM glucose)	0	16	15	33	
	10 μM	16	41	113	
Amnion (FL)	0	17	0		
	10 μM	17	258		
Amnion (fresh explants)	0	136	49	17	15
	10 μM	136	66	22	9
	Whole serum†	136	134		334

* Avg No. of cells in thousands/culture tube.
† Same as assay medium except undialyzed serum was used.

amnion cell, isolated by Fogh(6) degenerated completely in the assay media without inositol. With cell suspensions freshly prepared from 3 human placentae by the trypsin method, no apparent difference could be observed in the assay media with or without inositol. It should be pointed out, however, the fresh amnion cell explants failed to show any net increase in number of cells over the observation period of 4 weeks in the assay media. The minimal growth medium is apparently less adequate in supporting growth of fresh amnion cell explant as compared to a medium containing undialyzed serum (Table I).

The results presented in Table I are those of representative quantitative experiments. Variations in the results of different experiments did exist; they were the date of onset of degeneration, date of apparent completion of degeneration and actual net increase in cell number. However, the pattern of advanced degeneration of inositol-requiring strains and varying degree of multiplication of inositol-independent strains when tested in the inositol-free medium has been consistently observed in every experiment. Clone lines from the Roizman and Chang strains of HeLa as well as the stock and .1 mM glucose conjunctival cells also gave similar results.

Isolation of inositol-independent variant through deliberate selection. The relative frequency of inositol-independent cells present in the inositol-requiring culture was explored using a method similar to that used successfully for isolation of carbohydrate variants(7). At each attempt, 3 to 5 million cells from the inositol-requiring culture were exposed to the minimal growth medium without inositol; the inositol-free medium was replenished on the 3rd, 5th and 7th days, then at weekly intervals until the appearance of inositol-independent variant or the end of 2 months if no variant developed. Advanced degenerative changes regularly appeared on about the 7th day which proceeded to completion except in one instance. Seven attempts have been made with the inositol-requiring HeLa strain; no inositol-independent variant has yet been isolated. With the conjunctival cell, only 1 of 12 trials was successful; several colonies of inositol-independent cells appeared about 2 weeks after onset of advanced degeneration of the parent culture. This variant continued to propagate in the inositol-free medium increasing by about 10-fold per week for about 6 months, after which it degenerated completely.

Influence of extent of dialysis of serum. Four sera were dialyzed against 40 times their volume of 0.85% NaCl with stirring at about 20°C for 2 hours and aliquots were further dialyzed against 8 more changes of 0.85% NaCl for 2 days at 4-10°C. These sera were tested for their ability to prevent degenerative changes of the inositol-requiring HeLa and conjunctival cells; both cells showed advanced degenerative changes whether serum dialyzed for 2 hours or 2 days was used. This is in agreement with the reported high concentration (1 to 4 μM) of inositol required by these cells(2,3). Three of these sera were analyzed for inositol. A sizable portion of serum inositol is not dialyzable and most of the dialyzable inositol is removed after 2 hours (Table II). Thus, the inositol-"free" medium contained about 0.5 μg of non-dialyzable inositol per ml which is presumably not utilized by the inositol-requiring cells; and, the inositol-"independent" cell may have be-

TABLE II. Total Inositol Content of Dialyzed Sera.

Serum	Total inositol, μg/ml Pre-dialyzed	Dialyzed 2 hr	Dialyzed 2 days
Horse #1	8.4, 8.0*	5.2, 5.0	4.9, 5.3
Human (pool)	9.0, 9.0	not assayed	5.3, 5.0
" #1	8.8, 9.1	5.0, 4.1	3.7, 3.1

* Results of duplicate samples.

come independent of added inositol through its acquired property to use the non-dialyzable inositol.

Presence of vitamins in extensively dialyzed serum. The finding that about one-half of the serum inositol is not dialyzable prompted us to determine the presence of other vitamins in a dialyzed serum. A horse serum dialyzed against 10 changes of 10 times its volume of 0.85% NaCl at 0-10°C for 96 hours with constant stirring was assayed (Wisconsin Alumni Research Foundation) with the following results (expressed as amount per 100 ml dialyzed serum): 37 units Vit. A, 60 U.S.P. units Vit. D, 16 mg choline, 0.3 μg Vit. B_{12}, about 30 mμg biotin, 1.4 μg folic acid, 2 μg of pyridoxine, 58 μg pantothenic acid and 1.2 μg thiamine.

Lack of correlation between PPLO contamination and inositol requirement. Some cell strains are known to have been contaminated by pleuropneumonia-like organisms(8) without any associated signs of contamination such as cell degeneration, turbidity of medium and rapidly decreasing pH. It is perceivable that some of the nutritional studies reported previously may have been made with PPLO contaminated cells. It seems, therefore, desirable to determine if inositol requirement is related to PPLO contamination. Table III tabulates the status of PPLO contamination and inositol requirements of various cell strains; no relationship could be established. However, the effect of PPLO contamination on minimal concentration of inositol required cannot be determined from these data.

Discussion. The finding that strains of HeLa cell maintained independently in different laboratories differed in their inositol requirement seemed to explain satisfactorily the conflicting reports on the inositol requirement and the adequacy of Eagle's minimal growth medium. That such a difference should exist is not surprising since human cells in continuous cultivation resemble unicellular organisms(9) and since variability is an attribute of many unicellular organisms undergoing prolonged cultivation. Considering that the conditions for growth of these cells are not clearly defined, it is surprising that differences in the specific requirement for a growth factor between stock cultures of a human cell line have not been reported earlier. The ability of the inositol-requiring conjunctival cell, after being maintained in the 0.1 mM glucose medium which contains about 2 μg of inositol per ml, to propagate in the inositol-free medium deserves some emphasis. This suggests that the inositol-requiring culture acquired its inositol-independent property through a mechanism other than the deliber-

TABLE III. Lack of Correlation between PPLO Contamination and Inositol Requirement.

Cell strain*	Date tested	PPLO†	Inositol requirement
HeLa§ (Chang)	1956	+	Required
	1957	—	"
	1958	—	"
" (Roizman)	1957	—	Not required
	1958	—	" "
" (Eagle)	1957	+	Required
" #1 (Eagle)	1958	+	"
" #2 (Eagle)	1958	+	Inconclusive‡
" (Kunz)	1958	—	Required
" S3 (Barton)	1958	+	"
Conj.§ (wild)	1956	+	Required
	1957	—	"
	1958	—	"
" (.1 mM glucose)	1958	—	Not required

* Type 3 adenovirus produced characteristic cytopathogenic changes in all strains listed.

† — indicates failure to demonstrate PPLO by methods currently used (to be published by Dr. E. S. Murray). It is not certain when and where these cells were contaminated by PPLO prior to actual testing.

‡ No degeneration could be observed after 7 days in inositol-free medium; experiment was not continued because it is undesirable to work with PPLO contaminated cultures.

§ These cultures had been treated with 50 units of tetracycline/ml medium for about 2 wk around Jan. 1957 and have since been tested at regular intervals and found negative for PPLO contamination in presence of 50 units of penicillin and 50 μg of streptomycin/ml medium. Such treatment has not been regularly successful in eliminating PPLO from all cultures.

ate use of a defined selecting environment. The difficulty in isolating an inositol-independent culture through deliberate selection using an inositol-free medium tends to support this hypothesis. It is conceivable that unsuspected changes in the nutritional requirement of a cell culture could occur and thereby contribute toward further conflicting results. The role played by the serum protein component of the minimal growth medium is not known. A large number of substances, biologically active in low concentration are known to be present in dialyzed serum. Some of these substances are the enzymes, vitamins, hormones and trace metals. Thus, medium containing serum proteins should be considered non-chemically defined. Sera from various sources are known to influence cell propagation in a number of ways(4,10). The failure to demonstrate inositol as an essential for fresh explants of human amnion cell under the described experimental condition is also of interest. It suggests that caution is desirable in the generalization of data obtained through study with stable laboratory strains of human cells.

Summary. Under the described experimental conditions, qualitative differences in the inositol requirement of the following human cells have been found: 1) between several wild strains of HeLa cell obtained from several laboratories; 2) between a wild strain of conjunctival cell and a subculture propagating in a low glucose medium, and 3) between a stable laboratory strain and fresh explants of human amnion cells. The significance of these findings was discussed.

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Deposition of Fluoride in Soft Tissues Following Skeletal Saturation.* (24261)

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The significant reductions in dental caries as a result of communal fluoridation can no longer be questioned. However, the biological effect of fluoride ingested at the level used to fortify fluoride-deficient water on tissues other than the dental structures has received little attention because: (1) determination of these micro-quantities of fluoride requires specialized analytical technics; and, (2) the almost immeasurably small quantities of fluoride found in tissues other than the teeth and skeleton had until recently been considered negligible. In addition, fluoride ingested at the level of 1 μg/ml is thought to be detoxified rapidly by excretion in the urine and incorporation into the skeleton. Recently, however, Muhler(1) and Buttner and Muhler(2) have shown that certain dietary factors (ascorbic acid and fats) increase fluoride retention both in the skeleton and in certain specialized soft tissues. It is important, then, to

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