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A DEFINED MAINTENANCE MEDIUM FOR SUPPORTING CHICK FIBROBLAST MONOLAYERS AND FOR PLAQUE FORMATION BY VENEZUELAN AND EASTERN EQUINE ENCEPHALITIS VIRUSES

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The nutritive requirements for growth of tissue cells have been well defined by numerous investigators including Eagle (1955, 1959) and Evans et al (1956). A complex combination of serum, amino acids, salts, glucose, and vitamins is necessary for cell proliferation. However, once the tissue cells have proliferated sufficiently to establish confluent cell monolayers, the nutritional requirements for the maintenance of cell viability appear to be far simpler than those required for cellular growth.

This report describes an agar overlay medium consisting of a balanced salts solution and 2 amino acids, L-cystine and L-histidine·HCl, that is able to maintain chick fibroblast monolayers in a viable condition and support the formation of plaques by Venezuelan and Eastern equine encephalitis viruses.

METHODS

Virus strains.—Venezuelan (VEE) and Eastern (EEE) equine encephalitis virus strains were used. The origin and properties of these viruses were described by Brown (1963). Virus seeds were prepared in 10-day chick embryos and used as a 10% chick embryo suspension in Difco beef heart infusion broth (BHIB).

Preparation of chick fibroblast monolayers.—Chick fibroblast (CF) monolayers were prepared from minced, trypsinized 10-day-old chick embryos. Twenty to 30 million cells contained in 5 ml growth medium were added to 60-mm glass Petri dishes. The growth medium consisted of 0.5% lactalbumin hydrolyzate, 0.1% yeast extract, Hanks' balanced salts solution, 10% calf serum, and 0.14% sodium bicarbonate. Confluent CF monolayers were obtained after 24 hours incubation at 37 C in an incubator supplied with a 5% carbon dioxide-95% air mixture. All experiments were performed on 24-hour CF monolayers.

The monolayers were washed twice with phosphate-buffered saline (PBS) and then infected with appropriate dilutions of VEE or EEE virus prepared either in BHIB, PBS, or gelatin-saline solution (0.1% Difco gelatin in 0.8% sodium chloride). Virus was absorbed for 30 minutes, and then the cultures were overlaid with the appropriate media. Plates were incubated at 37 C in an atmosphere of 5% carbon dioxide and 95% air for 2 days. The monolayers were then stained with 1:10,000 aqueous neutral red solution in 1% agar to determine cell viability and to detect plaques.

Preparation of media.—All balanced salts solutions were prepared in 10× strength. The amino acid stock solutions were prepared at 100 times the concentrations recommended by Nagle et al (1963). Cystine was dissolved in 0.2 M HCl. All stock solutions were sterilized by filtration through sintered glass filters.

The various media were prepared

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from the sterile stock solutions as double strength concentrations of the desired medium. Sterile, double strength, melted Noble's agar solution (2.2%) was added to an equal volume of the medium just before use.

RESULTS

The defined medium described by Nagle et al (1963) for the growth of cell cultures in suspension was modified by increasing the sodium bicarbonate concentration to 0.14%, including 7 additional amino acids (alanine, aspartic acid, cystine, glutamic acid, glycine, proline, and serine), and omitting methocel. This medium, when used in an agar overlay for CF monolayers infected with either VEE or EEE virus, maintained the cells in an excellent state and permitted the formation of a maximum number of plaques. The requirement for the individual constituents of this medium for cell viability and plaque formation was then systematically examined by omitting one component at a time from an otherwise complete growth medium. The individual amino acid requirements for monolayer maintenance and plaque formation are shown in table 1. Deletion of all the amino acids from the complete medium caused the death of uninfected cell monolayers within 48 hours, and no plaques were detectable in cultures that had been infected with VEE or EEE virus. These results show that the CF monolayers died only in the absence of cysteine or cystine. None of the other amino acids were necessary for plaque formation or maintenance of cell viability. Cystine, being more stable in stock solution than cysteine, was routinely used in all subsequent media described in this report.

The type of diluent used in the preparation of virus dilutions had a profound effect upon the ability of an overlay medium containing cystine as the sole amino acid to support viable monolayers. If virus dilutions were prepared in either gelatin-saline or PBS, this medium was unable to support viable monolayers. However, if BHIB was used as the virus diluent, the monolayers remained viable and supported virus plaque formation. These data indicate that BHIB supplies some nutritional factors necessary for cell maintenance but is deficient in either cystine or cysteine.

The next experiment was designed to determine what amino acids besides cystine were necessary to maintain cell viability when either gel-saline or PBS was used as a diluent. Individual amino acids were added to the cystine-containing overlay medium at concentrations described by Nagle et al, and cell viability was determined by staining with neutral red after 48 hours incubation. Of the amino acids tested, only L-histidine ·HCl in combination with cystine pro-

TABLE 1.—The effect of omissions of individual amino acids from defined medium upon plaque formation by VEE and EEE viruses*

Amino acid omitted	Concentration tested, mg per liter	VEE plaques	EEE plaques
All		−	−
L-cystine	75‡	−	−
L-cysteine·HCl	75	−	−
L-arginine·HCl	100	+	+
L-histidine·HCl	60	+	+
L-isoleucine	150	+	+
L-leucine	300	+	+
L-lysine	300	+	+
L-methionine	60	+	+
L-phenylalanine	120	+	+
L-threonine	135	+	+
L-tryptophan	60	+	+
L-tyrosine	120	+	+
L-valine	150	+	+
L-glutamine	450	+	+
Glycine†	60	+	+
L-alanine†	120	+	+
L-serine†	150	+	+
L-aspartic†	270	+	+
L-glutamic†	315	+	+
L-proline†	115	+	+
Complete		+	+

+, monolayers viable or plaques present; −, monolayers dead or no plaques counted. BHIB used as virus diluent.
* Venezuelan and Eastern equine encephalitis viruses.
† Levels of these amino acids were recommended by S. C. Nagle (personal communication).
‡ Levels of amino acids used were recommended by Nagle et al, 1963.

vided the factors necessary for cell survival as well as for plaque formation by either EEE or VEE virus. Cystine alone or in combination with other amino acids failed to support viable CF monolayers.

The optimal concentration of cystine and histidine·HCl was established by determining the concentration required for each amino acid to produce maximum plaque counts with VEE or EEE viruses (table 2). As previously noted, all monolayers died in the absence of cystine, despite the presence of high levels of histidine. On the other hand, if histidine was omitted from the medium, high levels of cystine supported plaque formation by both viruses, although in reduced numbers. The addition of low concentrations of both amino acids together resulted in the highest plaque counts. Increasing concentrations of either cystine or histidine failed to increase the number of plaques further. On the basis of these data the concentrations of cystine and histidine used routinely for all media described hereafter were 75 and 60 mg per liter, respectively.

The requirement of CF monolayers for the other individual constituents of Nagle's medium was also determined.

TABLE 2.—The effect of increasing concentrations of L-cystine and L-histidine·HCl added to Nagle's salts solution on plaque formation by VEE and EEE viruses*

Histidine ·HCl, mg per liter	Cystine, mg per liter				
	0	37.5	75	150	300
			VEE		
0	−†	—	—	21‡	45
30	—	65	50	46	66
60	—	45	64	52	67
120	—	58	60	72	62
240	—	69	70	72	65
			EEE		
0	—	—	—	36	63
30	—	102	87	90	78
60	—	108	121	102	88
120	—	87	102	83	81
240	—	99	110	85	87

* Venezuelan and Eastern equine encephalitis viruses.
† Monolayers dead.
‡ Number of plaques per plate.

TABLE 3.—The effect of decreasing concentrations of sodium chloride and glucose on chick fibroblast monolayer viability and plaque formation

Concentration, %	VEE*	EEE*
Sodium chloride		
0	−†	−
0.07	−	−
0.15	−	−
0.30	±	±
0.60	+	+
0.74	+	+
0.89	+	+
1.04	+	+
1.18	±	±
1.34	±	±
1.48	−	−
Glucose		
0	−	−
0.01	+	+
0.10	+	+
0.50	+	+

Medium consisted of L-cystine, L-histidine·HCl, and Nagle's salts solution from which sodium chloride and glucose were omitted.
* Venezuelan and Eastern equine encephalitis viruses.
† −, monolayers dead; ±, monolayers viable but reduced plaque count; +, monolayers viable and maximal plaque count.

With cystine and histidine as the only amino acids present, the other components were deleted one at a time to determine the effect on cell viability and plaque formation. In the absence of sodium chloride or glucose the monolayers died. The requirement for sodium chloride for the maintenance of cell viability may not be a reflection of nutritional requirement, but rather may be due to the change in osmolarity resulting from its omission. However, this aspect was not investigated. Omission of the vitamin mixture, salts, and sodium pyruvate had no adverse effect upon the monolayer viability or the plaque counts. However, in the absence of potassium chloride, magnesium chloride, and calcium chloride the monolayers stained poorly with neutral red, and visualization of plaques was difficult.

The quantitative requirements for sodium chloride and glucose for the maintenance of CF monolayers are shown in table 3. A minimal level of at least 0.6% of sodium chloride was required for cell maintenance, and a maximum concentration of 1.04% could

TABLE 4.—Composition of defined maintenance medium

Component	Mg per liter
Nagle's salts solution	
Sodium chloride	7400
Glucose	1000
$NaH_2PO_4 \cdot H_2O$	100
$CaCl_2 \cdot 2H_2O$	265
$MgCl_2 \cdot 6H_2O$	275
KCl	400
Sodium bicarbonate	1400
L-cystine	75
L-histidine·HCl	60
Penicillin	100,000 units
Streptomycin	100
Phenol red	10
Agar	11 g

be tolerated without killing the cells or adversely affecting the virus plaque count. A low level of glucose (0.01%) was sufficient to hold CF monolayers viable and permit maximum plaque formation by VEE and EEE viruses. The highest concentration of glucose tested (0.5%) had no deleterious effect upon the monolayers nor upon the plaque count.

The concentration of sodium chloride and glucose in any of the well known balanced salts solutions falls within the limits established in table 3, which suggests that adding the 2 amino acids to a standard balanced salts solution would result in a satisfactory maintenance medium for CF cells. Cystine and histidine were added to Hanks', Earle's, and Gey's balanced salts solutions containing 1.1% agar, and then each solution was used to overlay infected monolayers. The data obtained demonstrated that any of the balanced salts solutions combined with the 2 essential amino acids supported living CF monolayers and permitted equally good plaque counts.

The composition of the defined maintenance medium devised in this study is shown in table 4. The glucose-salts mixture is the same described by Nagle et al (1963). However, this mixture can be replaced with any of the balanced salts solutions commonly used in tissue culture laboratories. L-cystine and L-histidine·HCl were the only amino acids found to be essential for CF cell viability under the conditions tested here.

Plaque counts of VEE and EEE viruses were approximately equal in the defined and in the complex overlay media (table 5). The values shown are the average plaque counts of 3 to 4 replicate plates. The data show some variation, but the average ratio of counts on complex medium to those on defined maintenance medium was approximately 1.0, indicating that plaque counts on both media were equivalent. Plaque size of VEE and EEE viruses on the maintenance medium equalled that obtained on the lactalbumin hydrolysate medium.

Infected CF monolayers overlaid with the defined maintenance medium and incubated at 37 C in a 5% carbon dioxide-95% air mixture have remained viable and plaques have been easily discernible up to 10 to 12 days. Earle's L cell monolayers and a hamster kidney cell line available in our laboratories have remained viable up to 7 days when overlaid with this medium.

DISCUSSION

The nutrition of tissue cells and the nutritive requirements for virus replica-

TABLE 5.—Comparison of plaque counts of VEE and EEE viruses* on chick embryo cell monolayers overlaid with lactalbumin hydrolysate medium and with minimal maintenance medium

VEE			EEE		
Minimal	Lactalbumin hydrolysate†	Ratio‡	Minimal	Lactalbumin hydrolysate†	Ratio‡
25	32	0.78	33	36	0.92
134	110	1.21	89	102	0.87
73	105	0.70	43	36	1.19
			111	124	0.89
210	171	1.33	175	153	1.14
63	36	1.75	137	141	0.97
69	75	0.92	87	102	0.78
108	127	0.85	203	218	0.93
Average		1.08			0.96

* Venezuelan and Eastern equine encephalitis viruses.
† 0.5% lactalbumin hydrolysate, 0.1% gelatin, 0.1% yeast extract, 0.14% sodium bicarbonate, 1.1% Noble's agar, in Hanks' balanced salts solution.
‡ Plaque count on minimal defined maintenance medium/plaque count on lactalbumin hydrolysate medium.

tion are intimately related. Virus synthesis is dependent upon the presence of certain substances either already in the cell or readily available from the growth medium. The amino acid pool within the cell appears to be in dynamic equilibrium with the medium, and the amount of amino acids within the cell is related to the composition of the growth medium (Eagle and Piez, 1962). Darnell and Levintow (1960) presented evidence that poliovirus was synthesized directly from the free amino acid pool within the cell and that cellular protein did not participate.

Once the cell monolayers are fully grown, cells can be maintained in a viable state and maximal virus synthesis can be achieved in the presence of relatively simple media. Maximal viral titers of foot and mouth disease virus were obtained on primary bovine calf kidney cultures fed a medium consisting only of a balanced salts solution and glucose (Pledger and Polatnick, 1962). Similar results have been reported for poliovirus synthesis on monkey kidney cells fed a balanced salts solution, glucose, and glutamine (Eagle and Habel, 1956), and Coxsackie B virus was grown successfully in cultured monkey heart cells fed with a medium consisting of a balanced salts solution, glutamine, and cystine (Tyndall and Ludwig, 1963). Although these reports were concerned with virus replication in a liquid medium, they support the observation made in this study that maximal plaque formation by VEE or EEE virus can also be achieved on established CF monolayers overlaid with an agar medium consisting of a salts solution, glucose, L-cystine, and L-histidine·HCl.

Glucose and sodium chloride were essential for the viability of all monolayers regardless of origin. However, the requirement for amino acids varied with the type of cell and virus used. Cell-virus systems other than those mentioned here require the presence of a number of different amino acids for virus replication (Halonen, 1962; Tankersley, 1964).

The medium described in this report was developed as part of a more comprehensive study to determine what nutritional factors influence virus replication. If host cells are fed on a medium that just fulfills their nutritive requirements for maintenance of viability, then infection with a virus may impose an additional nutritional stress upon the host cell. This deficiency may be expressed as an inhibition of virus plaque formation. Plaque formation by VEE and EEE viruses was maximal in the defined maintenance medium described here, which indicates that under these conditions CF cells possess the capacity to support virus replication without requiring an exogenous source of nutrients. However, the requirement for cystine and histidine for VEE or EEE virus replication has not been established, because in the absence of either of these amino acids the monolayers die. High levels of cystine can replace histidine for cell monolayer maintenance, but virus plaque counts are reduced (table 3), suggesting that histidine may participate directly in virus replication. Studies are now in progress to determine whether these amino acids do play a role in virus synthesis or whether they merely provide the essential nutrients for the maintenance of cell viability.

SUMMARY

A defined maintenance medium that supports chick fibroblast (CF) cell monolayers in a viable state for at least 10 days and permits the formation of plaques by Venezuelan (VEE) and Eastern (EEE) equine encephalitis viruses is described. The medium consists of a balanced salts solution, glucose, 2 amino acids (L-cystine and L-histidine ·HCl), sodium bicarbonate, and agar. Plaque counts by VEE and EEE viruses

on CF monolayers overlaid with this medium compared favorably with those obtained on a complex overlay medium.

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