Cystine Requirement for Normal Poliovirus Action on Monkey Kidney Tissue Cultures.* (22685)

GEORGE R. DUBES. (Introduced by Herbert A. Wenner.)
Department of Pediatrics, University of Kansas School of Medicine, Kansas City, Kans.

With the development of medium 199 Morgan, Morton, and Parker(1) provided a chemically defined medium which has been found to maintain monkey kidney cells in tissue culture during viral action. More recently, Eagle(2) studied the growth requirements of HeLa cells and mouse L cells and devised for these 2 cell types growth media which are chemically defined except for nondialysable serum components. At this laboratory Eagle's medium for HeLa cells without serum has been found to be adequate not only for the maintenance of monkey kidney cells but also for poliovirus multiplication in these cells. Thus a relatively simple chemically defined medium is available for the study of the relationship between cell nutrition and poliovirus action. The effect of the withdrawal of specific metabolites from this maintenance medium on poliovirus action is the subject of this paper.

Materials and methods. Monkey kidney cell cultures. Trypsinized cells were grown in medium D(3) for 6 days either in test tubes or in 60-mm diameter Petri plates. After removal of the growth medium, the established cultures were washed with phosphate-buffered saline (PBS)(4): the tubes 3 times, each time with 2 ml, and the plates 3 times, each time with 4.25 ml. These cells were then maintained either on Eagle's medium for HeLa cells without serum, here referred to as Eagle's maintenance (E_m), or on E_m minus one or more of its component compounds. *Poliovirus strains.* Plaque-purified pools of 3 strains have been used. These are Akron purified pool #1 (PPl), Brooks PPl, and Mabie PPla, which are examples of antigenic types I, II, and III, respectively. These strains have been described previously (5,6,7). When not in use, the pools are stored in a dry-ice box. *Nutritional tests in tube cultures.* To each tube culture of monkey kidney cells bathed by .90 ml E_m or deficient E_m at pH 7.2 to 7.3 is added .10 ml of a 1000-fold dilution in PBS of one of the poliovirus pools. This provides inocula of 740, 8500, and 300 plaque-producing particles (PPP) per tube for Akron PPl, Brooks PPl, and Mabie PPla, respectively. Control tubes receive .10 ml PBS. The tubes are rolled at 36°C and examined for cytopathogenic action on the second day after inoculation and daily thereafter usually through the eighth day after inoculation. *Nutritional tests using the plaque method.* The method of Dulbecco and Vogt(4) as adapted for use in this laboratory(7) has been used with the modification that the plaques develop under E_m or deficient E_m agar in an incubator continuously flushed with 97% air and 3% carbon dioxide(4). *Sources of compounds tested.* All compounds were purchased from commercial firms except thiomalic acid and 2-mercaptoethylamine hydrochloride of which free samples were kindly supplied by Evans Chemetics, Inc., New York City. None of the compounds was further purified.

Results. Delay in appearance of cytopathogenic effect on monkey kidney cells in tube cultures in absence of cystine. The cytopathogenic action of Akron, Brooks, and Mabie is delayed when cystine is not supplied for the monkey kidney cell-poliovirus system (Table I). Similar experiments have shown that the omission of all 8 vitamins or of any one of the amino acids tyrosine, leucine, methionine, arginine, histidine, lysine, isoleucine, phenylalanine, threonine, tryptophane, and valine normally present in E_m does not result in any delay in the cytopathogenic action of Akron PPl. The omission of glutamine has given variable results, sometimes resulting in a short delay and at other times in no delay.

Retardation of plaque development in absence of cystine. The result, obtained in tube

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TABLE I. Time of Appearance of Cytopathogenic Effect of 3 Strains of Poliovirus on Monkey Kidney Cells in Roller Tubes in Presence and in Absence of Exogenously Supplied L-cystine.

Poliovirus strain and pool*	Time required for completion of cytopathogenic action (mean day tubes positive)					
	1		2		3	
	Cystine†	No cystine‡	Cystine	No cystine	Cystine	No cystine
Akron PPl	3.0	≧6.8	4.2	≧7.8	3.0	≧8.0
Brooks PPl	2.9	≧7.6	3.2	≧7.5	3.0	≧7.2
Mabie PPla	2.9	≧7.2	4.0	≧7.8	3.0	≧7.8

* Each virus pool was diluted 1000-fold in PBS before inoculation.
† Medium E_m with L-cystine at .20 mM.
‡ " " without L-cystine.

cultures, that exogenously supplied cystine is necessary for the normal action of the Akron, Brooks, and Mabie viruses on monkey kidney cells has been confirmed using the plaque technic (Table II).

Delay in poliovirus propagation in absence of cystine. Curves which show the propagation of Akron PPl on monkey kidney tissue cultures in the presence and in the absence of cystine were obtained by plaque assays of daily harvests of supernatant fluids from appropriate cultures. These curves show that the propagation of Akron is delayed in the absence of cystine to about the same extent as is the cytopathogenic effect of Akron. This slower propagation in the absence of cystine probably is not due to any interference with virus adsorption (see below). It may be due either to a decreased rate of virus multiplication or virus release or both.

TABLE II. Plaques Formed by Akron PPl, Brooks PPl, and Mabie PPla on Monkey Kidney Cells in Presence and in Absence of Exogenously Supplied L-cystine.

Poliovirus strain and pool	Virus conc.	Plaques/plate	
		E_m*	E_m without L-cystine
Akron PPl	10^{-3}	—	1
	10^{-4}	†	0, 0
	10^{-5}	20, 25	0
	$10^{-5.4}$	11	—
Brooks PPl	10^{-4}	—	0
	10^{-5}	63, 28	0, 0
	10^{-6}	3, 4	0, 0
Mabie PPla	10^{-4}	46, 46	5, 1
	10^{-5}	8, 4	1

* Contains L-cystine at .05 mM.
† Too many to count.
— = Not done.
The plaques developed for 4 days at about 35°C. In addition to the differences in assay found above, the plaques that were formed on E_m without cystine were smaller than those formed on E_m.

TABLE III. Amount of L-cystine Necessary for Normal Cytopathogenic Action by Akron PPl on Monkey Kidney Cells.

Conc. (mM) of L-cystine in maintenance medium	Time required for completion of cytopathogenic action (mean day tubes positive)
0	≧9.1
.001	≧9.4
.004	≧9.9
.016	6.6
.064	4.3
.256	3.7

Each tube received .10 ml Akron PPl at 10^{-3} conc. in PBS.

Concentration of cystine necessary for optimal cytopathogenic action by Akron PPl. In Table III are presented data which show that the lowest concentration of L-cystine which will give optimal or near-optimal cytopathogenic action is about 10^{-4} M. The amount of cystine or "cystine-activity" in the inoculum itself has been estimated by the assay of autoclaved (10 lbs/in^2 for 10 minutes) samples of Akron PPl, Brooks PPl, and Mabie PPla, using the poliovirus-monkey kidney tissue culture system. In this way it was found that the amount of "cystine-activity" introduced into the system by the usual inoculum of 10^{-3} virus concentration is far too small to affect significantly the determination of the concentration of cystine necessary for optimal cytopathogenic effect.

Tests of compounds as possible substitutes for cystine. Seventeen compounds were selected on the basis of their metabolic relationship with cystine or simply because they possessed a chemical structure somewhat similar to that of cystine. These compounds were

TABLE IV. Activity of 17 Compounds in Replacing Cystine for Hastening Cytopathogenic Action of Akron PPl on Monkey Kidney Cells.

Activity relative to L-cystine		
Approximately equally active	Less active on a molar basis	Inactive‡
L-cysteine hydrochloride	DL-cystathionine*	DL-α-alanine
Glutathione	DL-homocysteine thiolactone hydrochloride†	Cysteic acid
		Glycine
		DL-homocystine (toxic; .1 mM)
		2-mercaptoacetic acid
		2-mercaptoethanol (toxic; .5 mM)
		2-mercaptoethylamine hydrochloride (toxic; .05 mM)
		DL-methionine§
		DL-serine
		Sodium sulfate
		" sulfite
		" thiosulfate
		Thiomalic acid

* About 8 times less active.
† " 20 " " " .
‡ Inactive at conc. of .10 and 1.0 mM except for the 3 compounds which were noticeably toxic for monkey kidney tissue cultures as designated above. Following the notation of toxicity is the highest relatively nontoxic conc. tested and found inactive.
§ Normally present in E_m at .05 mM.

tested for the ability to substitute for cystine in the latter's capacity to hasten the cytopathogenic action of Akron PPl. The results obtained permit the grouping of the compounds into 3 categories (Table IV). The possibility that the relatively low activities of DL-cystathionine and of DL-homocysteine thiolactone hydrochloride may be due to small amounts of contaminating substances, *e.g.*, cystine, has not been excluded.

Elimination of adsorption as the step at which cystine is effective. Particles of Akron PPl were adsorbed for 30 minutes at 36°C onto monolayers of monkey kidney cells, which had been washed 3 times with PBS (4.25 ml washing), in the presence of 3 different media: PBS, E_m without cystine, and E_m with L-cystine at .50 mM. The fraction of the particles adsorbed was the same in the 3 media. This indicates that the presence of exogenously supplied cystine is effective at some postadsorptive phase(s) of the viral infection.

Discussion. Davies *et al.*(8) studied the effect of dietary amino acid deficiencies in mice on the course of poliomyelitis following intracerebral inoculation of a mouse-adapted strain of Lansing poliovirus. They found that a deficiency in any one of 8 amino acids (tryptophane, isoleucine, valine, phenylalanine, histidine, threonine, methionine, and leucine) resulted in some delay in the onset of symptoms, in a decrease in the total number of mice paralyzed by the 28th day postinoculation, and in an increase in the number of mice which died without showing characteristic signs of poliomyelitis. A lysine deficiency, however, had very little or no effect on the course of the disease. Their deficient mice and one of their control nondeficient groups received no cystine, cysteine, or glutathione. Their other control group received only the small amount of cystine present as residues in the casein fed. It is difficult to relate this work to the studies performed *in vitro* because of the manifold differences in system and technic. Recently Eagle and Habel have reported that salts, glucose, and glutamine are sufficient and necessary for poliovirus propagation on HeLa cells. A comparison of their findings with the results herein reported suggests that the cystine and possibly the glutamine requirements for poliovirus action depend on the nature of the host cell and/or the nature of the poliovirus strain. Further investigations, however, are needed to clarify the situation. Rappaport(9) has developed a medium containing salts, glucose, cysteine, and six other amino acids for the maintenance of monkey kidney tissue cultures during polio-

virus propagation. Furthermore, she has found that cysteine can replace the 6 other amino acids for poliovirus propagation. The combined evidence strongly indicates that cysteine and/or its disulfide derivative cystine play an important role in the process of poliovirus propagation in monkey kidney tissue cultures. These results suggest the possibility of finding cystine antimetabolites which would delay poliovirus action *in vitro* and perhaps *in vivo*. With only the information available at this time it is somewhat premature to speculate as to the reason for this cystine requirement. One reasonable working hypothesis is that the available endogenous cystine, either free or in peptide bondage, simply does not meet the demand for the cystine residues of the protein portions of the newly-forming poliovirus particles.

Summary. The cytopathogenic action of the poliovirus strains Akron, Brooks, and Mabie on either tube or Petri plate cultures of monkey kidney cells is considerably delayed when cystine is omitted from the maintenance medium. Omission of all the vitamins or of any one of the 12 other amino acids except glutamine normally present in the maintenance medium resulted in no delay in the appearance of the cytopathogenic effect of Akron PPl. Omission of glutamine resulted in a short delay or no delay. It has further been shown that the propagation of Akron is slower in the absence of cystine. The lowest concentration of L-cystine which will give optimal or near-optimal cytopathogenic action by Akron is about 10^{-4} M. Glutathione and L-cysteine hydrochloride can replace cystine at about the same concentration; DL-homocysteine thiolactone hydrochloride and DL-cystathionine can replace also but only at higher molar concentrations. None of 13 other compounds was found to replace cystine. The adsorption of Akron onto monkey kidney cells is not affected by the absence of cystine.

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Role of the Adrenal in Response of Rats to *Orinase*.* (22686)

W. E. DULIN, E. H. MORLEY AND J. E. NEZAMIS.
(Introduced by M. H. Kuizenga.)
Department of Endocrinology, The Upjohn Co., Kalamazoo, Mich.

Adrenocortical and medullary hormones both counteract the hypoglycemic effects of insulin(1,2,3). Excessively high doses of the hypoglycemic agent *Orinase* do not produce a hypoglycemic response(4).† These observations suggested that the adrenals may respond to high doses of *Orinase* with increased secretions. Therefore, experiments were designed to study the role of the adrenal cortex and medulla in modifying the hypoglycemic reaction of rats to this drug.

Methods. Male rats obtained from the Upjohn colony (Sprague-Dawley ancestry) were used throughout these experiments. The intact animals weighed 140-160 g at the time of treatment with *Orinase*. Adrenalectomies and

* Upjohn tradename for 1-butyl-3β-tolylsulfonylurea.

† Personal communication from W. L. Miller.