with ability for maximum multiplication in the tumor, and oncolytic activity. Hemagglutinin-producing ability can be lost without the virus acquiring these two properties.

The L cell studies provide further evidence that the presence of hemagglutinin inhibitor, and not an increased ability to multiply in a host system, is the most important factor in determining hemagglutinin loss, and that as a result of prolonged passage in L cells both the HS-33 and TF-33 viruses showed a marked increase in ability to multiply in the L cell. The HS-33 virus, which was not exposed to hemagglutinin inhibitor, however, did not show a loss of hemagglutinin.

Summary. Following the observation that vaccinia virus became hemagglutinin-negative when fully adapted to the Ehrlich ascites tumor, the question arose as to whether or not the hemagglutinin loss was an intrinsic step in the adaptation process. In the present study it was possible to show that the loss of hemagglutinin by vaccinia virus and an increased ability to multiply in a host system are independent transitions, but may occur simultaneously.

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Uptake of Glucose, Amino Acids and Vitamins from a Medium by Human Amnion Cell Cultures Infected with Measles or Reovirus.* (27217)

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The only nutrients of the medium essential for synthesis of poliomyelitis virus in cell cultures have been shown to be glucose and glutamine in full-grown monolayer cultures of HeLa cells(1,2), and cystine and glucose in monkey kidney tissue cultures(3), while none are needed in concentrated suspensions of ERK cells(4). On the other hand, Eagle and coworkers(2,5) have shown that the nutritional requirements for poliomyelitis virus synthesis in HeLa cells depend on cell population density. If diluted cell suspensions are used in a medium containing glucose and glutamine as sole nutrients, rapid depletion of the free amino acid pool of the cells occurs and the capacity of the cells to synthesize the virus is greatly reduced(5). It is possible that a similar loss of amino acids may occur even with a complete medium if the nutrients are consumed during lengthy incubation of cell cultures such as is necessary for propagation of some viruses.

The present investigation is one of a series of studies dealing with the nutrients required in human amnion cell cultures for propagation of various viruses with relatively long incubation times. This report describes the changes in concentrations of glucose, amino acids and vitamins of the B-group in Eagle's minimum essential medium(5) in human amnion cell cultures infected with measles and reovirus.

Materials and methods. Cells. Human amnion cells of a continuous line, strain Utrecht, obtained from Dr. R. Doorschodt, Hygienisch Laboratorum der Rijks Universiteit, Utrecht, Holland, were grown in Roux bottles or Carrel flasks in a medium containing 20% calf serum and 0.5% lactalbumin hydrolysate in Hanks' solution. The growth medium was changed twice during the 6-day growth period. At the time of virus inoculation the number of cells was 25 to 30 million per Roux bottle containing 60 ml of medium, and 5 to 7 million per Carrel flask containing 10 ml of medium.

Cell culture medium. In all experiments where glucose, amino acids and vitamins of the B-group were determined, a single lot of Eagle's minimum essential medium contain-

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ing 0.1% bovine albumin (Bovine Plasma Albumin Fraction V, Armour Pharmaceutical Co., Ltd., England) and twice the amounts of the vitamins of the B-group was used. It was stored in 400-ml quantities at –25°C. In the preliminary experiments it was shown that after 7 days' storage of 0.2% bovine albumin at 37°C in medium complete except for the free amino acids, no amino acids could be traced. This result is in contrast to that reported by Piez *et al.*(6) for dialyzed serum.

Virus strains. A stock of the "Lang" strain of type 1 reovirus(7) containing approximately 10^8 $TCID_{50}$/ml when titrated in human embryonic kidney cell culture tubes(7) was used throughout the study. It was grown in human amnion cells in a medium containing 0.1% bovine albumin in Eagle's minimum essential medium, harvested 8 days after inoculation and stored at –30°C.

The human amnion cell-adapted Edmonston strain of measles virus was obtained from Dr. L. Philipsson, Dept. of Virology, University of Uppsala, Sweden. Several lots of measles virus containing about 10^6 $TCID_{50}$/ml were used. They were grown in human amnion cells in the same medium and under the same conditions as the reovirus, except that they were harvested on the 5th day and aliquots were stored at –60°C.

Inoculation procedures. Bottles or flasks were washed free of growth medium 3 times with Hanks' solution and incubated for 2 to 4 hours at 36°C in this solution to starve the cells in an amino acid- and vitamin-free medium. The cells were infected with 2.0 ml (Roux bottles) or 0.5 ml (Carrel flasks) of undiluted measles or reovirus. After incubation for 1 hour at 36°C, the unadsorbed virus was washed twice with Hanks' solution and 60 ml of the final medium was added to Roux bottles and 10 ml to Carrel flasks. The pH of the medium was kept at 7.0 to 7.2 with sodium bicarbonate. Periodically specimens were taken from the medium of 2 to 4 virus-infected bottles or flasks, pooled, centrifuged and stored at –25°C. Identical specimens were taken from the medium of the control bottles or flasks.

Viral assays. Reovirus was assayed by the hemagglutination method in tubes described in detail elsewhere(7). To 0.4 ml of serial 2-fold dilutions of virus, 0.2 ml of a 0.75% suspension of type O human erythrocytes was added. After a thorough shaking, the tubes were kept at room temperature for 2 hours. The sedimentation pattern was recorded and endpoint taken according to Chanock and Sabin(8).

The infectivity titer of measles virus was determined in human amnion cell culture, using 4 tubes per 10-fold dilution of virus. The medium in the tubes was 5% horse serum, and 5% tryptose phosphate broth in Eagle's minimum essential medium. The medium was changed once, after 3 to 5 days' incubation at 36°C. The final microscopic reading was made in 10 to 14 days.

Determination of glucose. Glucose was determined by an enzymatic method(9).

Vitamin assays. Assays of the vitamins of the B-group were carried out microbiologically according to Barton-Wright(10), except in the case of inositol, which was determined by the method of Norris and Darbre(11). Pantothenate, nicotinamide, riboflavin and folic acid were determined acidimetrically and thiamine turbidimetrically, with lactic acid bacteria as test organisms. Pyridoxal was determined gravimetrically with a mould as test organism. Concentrations of the vitamins are expressed as percentages of the original biological activity, because in some cases, when international reference vitamins were used as standards, these microbiological determinations showed less than the expected activity in the fresh final medium, although all reagents in the medium were measured on an analytical balance. To insure at least the levels of activity advocated by Eagle(5), and also to decrease the volume of the samples needed for assays, double the recommended amounts of vitamins were added to the batch of medium used in this study.

Determinations of amino acids. Amino acids were determined by paper chromatographic methods. For desalting, the specimens were run through a column of ion exchange resin (Amberlite Resin, CG-120, 100-200 mesh, BDH, England) treated with 2.6 N HCl. Salts were washed out with distilled water and amino acids were eluted with 5 N ammonia. Albumin remained on the column. Ammonia was evaporated from the amino

acid eluate in a water bath at 100°C. The 2-dimensional chromatograms were run on Whatman No. 1 paper by the descending technic. The first solvent was n-butanol–methyl ethyl ketone—17 N ammonia—water (5:3:1:1 v/v)(12). The second solvent was n-butanol—glacial acetic acid—water (63:10:2). Arginine, however, was run separately by the one-dimensional ascending technic, using 77% ethanol with 1% diethylamine as solvent(13). The amino acid spots were stained with 0.5% ninhydrin solution and eluted with 70% ethanol, and the absorptions were measured with a Unicam spectrophotometer at 570 mμ.

Results. In both infected cell cultures and controls glucose was completely utilized in 60 to 70 hours. A typical experiment with reovirus is shown in Fig. 1. Between 6 and 48 hours after addition of the final medium, glucose concentrations were lower in the reovirus-infected samples than in controls, but after 48 hours they were approximately the same in both. The respective virus titres in the same experiment are shown in Table II. Consumption of glucose in measles and reovirus-infected cultures was not compared in the same experiment but the difference between infected and uninfected cultures appeared to be the same with these 2 viruses.

Table I illustrates experiments with vitamin assays. With one exception, no significant change was observed in concentrations of vitamins. The exception was nicotinamide, which was depleted from the medium relatively rapidly. In 4 days concentrations of nicotinamide fell to below 10% of initial biological activity, the values corresponding to about 0.2 μg/ml, which can no longer be quantitatively estimated by the microbiological method used. In other similar experiments concentrations of nicotinamide in samples taken 1 day after addition of the final medium were about 50% of initial concentrations, and at 4 days 10% to 20% while no activity could be traced at 7 days or thereafter. In many experiments the decrease was slightly more rapid in the virus-infected samples than in the controls. Concentrations of choline are not shown in Table I, since methionine interferes to a certain extent with the assay of this vitamin. However, choline concentrations appeared unchanged.

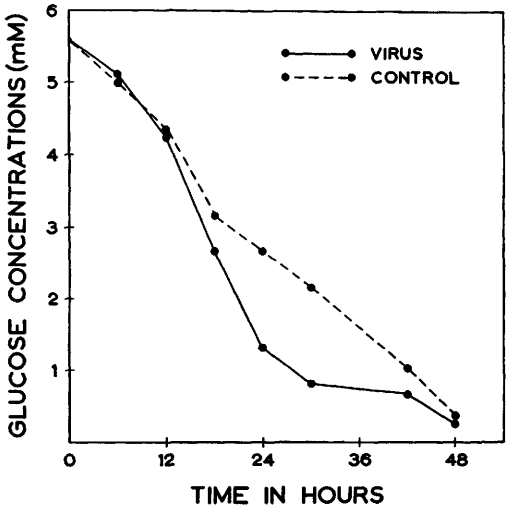

FIG. 1. Glucose concentrations (mM) in Eagle's minimum essential medium in reovirus-infected human amnion cell cultures and controls.

TABLE I. Vitamin Concentrations (% of Initial Biological Activity) and Infectivity Titers in Eagle's Minimum Essential Medium in Measles Virus-Infected Human Amnion Cell Cultures and Uninfected Controls.

	Time after addition of final medium			
	4 days		7 days	
	Virus	Control	Virus	Control
Folic acid	83	97	101	101
Inositol	81	82	81	81
Nicotinamide	<10	<10	<10	<10
Pantothenate	98	112	95	105
Pyridoxal	132	128	104	79
Riboflavin	85	85	86	86
Thiamine	69	93	70	89
Infectivity titer in medium	6.0		6.5	
Infectivity titer in cells*	7.0		7.5	

* Frozen and thawed twice.

Amino acid concentrations in a representative experiment are shown in Table II. During the observation period of 48 hours, 2 amino acids (cystine and tryptophane) disappeared temporarily from the samples of both virus-infected and control cell cultures. The disappearance of cystine was very rapid. In the first sample of the virus-infected cell culture, taken 6 hours after addition of the final medium, no cystine could be traced. In some other experiments arginine also disappeared in 48 hours and at 3 days glutamine concentrations of virus-infected and control

TABLE II. Amino Acid Concentrations (mM) and Hemagglutination Titers in Eagle's Minimum Essential Medium in Reovirus-Infected Human Amnion Cell Cultures and Uninfected Controls.

	Time in hr after addition of final medium																	
	0		6		12		18		24		30		42		48			
	v	c	v	c	v	c	v	c	v	c	v	c	v	c	v	c		
Arginine	.60	.60	.47	.54	.50	.39	.50	.41	.40	.34	.14	.24	.02	.13	.08	.02		
Cystine	.10	.10	0	.02	.02	0	.01	.01	.02	.03	.01	0	.01	.01	.05	.01		
Glutamine	2.00	2.00	1.63	1.65	2.07	2.02	3.04	2.60	2.33	2.00	2.65	3.09	3.41	2.90	2.91	2.12		
Histidine	.20	.20	.15	.17	.18	.13	.19	.16	.14	.16	.13	.19	.16	.11	.11	.12		
Isoleucine	.40	.40	.34	.32	.28	.37	.31	.26	.18	.20	.17	.24	.14	.15	.19	.15		
Leucine	.40	.40	.30	.37	.32	.24	.23	.31	.19	.27	.18	.18	.16	.12	.18	.10		
Methionine	.10	.10	.08	.08	.08	.07	.07	.08	.05	.07	.04	.06	.03	.03	.04	.05		
Phenylalanine	.20	.20	.17	.15	.16	.17	.16	.15	.12	.13	.15	.17	.11	.12	.11	.13		
Threonine	.40	.40	.31	.47	.43	.31	.33	.44	.34	.38	.25	.34	.23	.30	.27	.28		
Tryptophane	.05	.05	.03	.03	.02	.03	.02	.02	0	.01	0	.02	.02	.03	0	0		
Tyrosine	.20	.20	.14	.17	.15	.14	.14	.13	.11	.11	.11	.15	.12	.12	.11	.12		
Valine	.40	.40	.31	.40	.36	.31	.27	.33	.23	.28	.18	.23	.22	.21	.21	.14		
Hemagglutinin titer			0		0		4		32		96		256		512			

v = virus-infected. c = control.

cell cultures were 0.1 mM or less. Lysine, however, could not be determined in the 2 chromatograms used. Concentrations of the other amino acids decreased to a lesser extent. No consistent significant differences were noted in utilization of amino acids between virus-infected and uninfected cell cultures. After 4 days the concentrations of many amino acids, including cystine, histidine, phenylalanine and tryptophane began to increase. The increase was greater in the virus-infected cell cultures than in the controls. In addition to the amino acids initially present, considerable amounts of other amino acids, such as alanine and serine, appeared in the medium in 4 to 7 days. Table III shows the development of the total amino acid pool in the medium of virus-infected cultures and in controls in an experiment in which the cells were not starved before virus inoculation.

TABLE III. Total Amino Acid Pool in Eagle's Minimum Essential Medium in Measles-Infected Human Amnion Cell Cultures and Controls. Extinction values at 570 mμ of diluted amino acid eluates (see *Methods*).

	Time in days after addition of final medium					
	0	1	2	3	5	7
Virus-infected	.611	.398	.323	.038	.237	.291
Control	.611	.496	.393	.046	n.t.	n.t.

n.t. = not tested.

Discussion. These experiments indicated that none of the metabolites of Eagle's minimum essential medium except glucose were consumed in significantly greater amounts by human amnion cell cultures infected with measles or reovirus than by uninfected controls. Even the glucose concentrations were approximately the same 48 hours after the change of the final medium and thereafter. However, the total, although temporary, disappearance or great depletion of some metabolites of the medium in both infected and uninfected cell cultures at an early stage suggests that increased concentrations of certain metabolites may be necessary in human amnion cell cultures for maximal yield of viruses which require a lengthy incubation. Whether or not there is a temporary disappearance of the intracellular free amino acid pool(14) and whether or not it is critical for maximal

virus yields remains, however, to be determined.

It is interesting to note the temporary disappearance of cystine within a few hours of addition of the final medium. As shown by Rappaport(3), cystine is the only essential amino acid of the medium for synthesis of poliomyelitis virus in monkey kidney tissue culture. Furthermore, the effect of cystine on the thermal stability of poliomyelitis virus has been shown by Pohjanpelto(15).

The disappearance of nicotinamide cannot be explained by the possible instability of this vitamin in Eagle's minimum essential medium, because it has been stored up to 9 weeks at 37°C without loss of microbiological activity. A similar loss in concentration of nicotinamide has been found with 2 strains of HeLa cells.

Becker *et al.*(16) have reported that primary trypsinized human amnion cell cultures infected with poliomyelitis virus consume more glucose than uninfected cultures. In the present study the increased uptake of glucose by human amnion cells of a continuous line infected with measles or reovirus was also apparent during the first 24 hours. In both infected and uninfected cultures, however, the total disappearance of glucose occurred at approximately the same time, usually in 60 to 72 hours.

Summary. The uptake of glucose, amino acids and vitamins from Eagle's minimum essential medium by human amnion cells of a continuous line infected with measles or reovirus and uninfected controls was determined. In both infected and uninfected cultures glucose was completely utilized in 60 to 72 hours. Between 6 to 48 hours the infected cultures consumed more glucose than did the controls. Concentration of nicotinamide in both the infected cell cultures and controls decreased in 4 days to below 10% of initial value. No significant changes in concentrations of other vitamins were observed. Of the amino acids, arginine, cystine and tryptophane disappeared temporarily or were greatly depleted in 70 hours. Glutamine concentrations began to fall after 48 hours and this amino acid was almost completely utilized in 3 days. No consistent significant differences were observed in utilization of amino acids by infected and uninfected cell cultures.

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