STUDIES ON THE FACTORS ESSENTIAL TO THE INITIATION AND MAINTENANCE OF MULTIPLICATION OF PSITTACOSIS VIRUS (6BC STRAIN) IN DEFICIENT CELLS IN TISSUE CULTURE*

By JOHN D. HARE AND HERBERT R. MORGAN, M.D.

(*From the Department of Bacteriology, The University of Rochester School of Medicine and Dentistry, Rochester, New York*)

(Received for publication, January 11, 1954)

During the course of a number of experiments on the growth of the 6BC strain of psittacosis virus in cultures of chick embryo tissues (1–4), virus multiplication as measured by the amount of virus appearing in culture fluids was found to diminish markedly after an initial period of rapid proliferation until very little or no virus could be demonstrated in culture fluids. At this time if the cellophane discs, which served as an inert support on which the cells proliferated, were removed from representative cultures in which no virus was appearing and the adhering tissues were stained and examined microscopically, it was found that in a number of instances many healthy appearing fibroblasts remained. On the stained discs from many of these cultures, psittacosis elementary bodies were seen within the fibroblasts, indicating that some of these cells were still infected. Since it had been assumed that virus would grow as long as any host cells remained and that, therefore, all available cells would have been destroyed, the finding of a considerable number of intact fibroblasts, some containing visible virus, was unexpected. Because this seemed to represent a basic alteration in the behavior pattern of this particular host-cell-virus complex, it was thought of interest to study the conditions under which psittacosis virus could remain within chick embryo fibroblasts in tissue culture without active multiplication and subsequently to attempt to induce its proliferation by altering the extracellular environment for which this culture technic is so well adapted. In the work presented here, it has been possible to show that cells cultivated in nutritionally deficient media lose their ability to support the growth of psittacosis virus and that a variety of nutrient materials will restore this capacity.

Materials and Methods

Virus.—The 6BC strain of psittacosis virus, originally obtained from Dr. K. F. Meyer, which had been repeatedly passed in eggs by the yolk sac route, was used. A uniform source

* These studies were supported by a contract among the Office of Naval Research, Department of the Navy, and The University of Rochester, NR 135-091, and by the Ernest L. Woodward Fund.

of virus was obtained by infecting 7-day-old embryonated eggs and harvesting the yolk sacs after 4 to 6 days of incubation at 34° to 36°C. These yolk sacs were pooled, homogenized, and diluted to give a 20 per cent suspension by weight in beef heart infusion broth and finally stored in glass ampoules in a dry icebox until used. Dilutions of this standard preparation were made in Hanks's balanced salt solution (5) in order to obtain a final concentration of virus in the culture fluids of from 10^{-2} to 10^{-3} LD_{50} per ml.

Tissue Cultures.—The tissue culture technic used here was a modification of that described previously (3, 6). 9- to 10-day-old chick embryos were minced with scissors and washed with four 10 ml. changes of Hanks's balanced salt solution to which was added 0.0125 ml. of a 1.4 per cent sodium bicarbonate solution per ml. The washed tissue was planted under the cellophane discs in 10 ml. Erlenmeyer flasks to which was then added 1.8 ml. of Hanks's balanced salt solution containing 0.025 ml. of 1.4 per cent sodium bicarbonate per ml., subsequently referred to as BSS. This initial fluid was completely removed after 24 hours' incubation at 36° to 37°C. and renewed with fresh BSS. Thereafter, the fluids were changed every 4 days.

Flask cultures were infected with virus either at the time they were prepared or on the 13th day. In both cases, the amount of virus in the initial infecting fluids and the amount remaining in the fluids after 24 hours' contact with tissue was determined separately by pooling the initial fluids and subsequently the fluids removed after 24 hours and injecting both undiluted into twelve 7-day-old embryonated eggs each, as described below. Thereafter, at 4 day intervals, the amount of virus in the fluids from each individual culture was determined. The cultures infected on the 13th day were maintained in the nutritionally deficient BSS alone prior to this time, with changes of fluids on the 1st, 5th, 9th, and 13th days. The various materials tested on cultures infected on the 13th day were added both on the 13th and 14th days, unless otherwise specifically noted, and were diluted in BSS for use.

At the close of an experiment, the tissue in the cultures was fixed by adding a mixture of equal parts of 95 per cent alcohol and ether to the flasks. The discs with the adhering cells were removed, stained by the Papanicolaou procedure, as modified by the Cytology Laboratory of Strong Memorial Hospital (7), and mounted on microscopic slides. By examining the stained discs, a rough estimate of the degree of cellular proliferation was made by observing the numbers of fibroblasts growing out from tissue fragments. The condition of those cells present on the discs was evaluated by observing the state of the nucleus and cytoplasm, including the degree of vacuolation and granulation. The number of cells containing viral inclusion bodies was also noted.

Virus Titration.—The single-dilution method of Golub (8) was used for determining the amount of virus in culture fluids. The virus-containing fluids from each flask were diluted 10^{-1} in broth and 0.25 ml. of this was injected into the yolk sac of each of twelve 7-day-old embryonated eggs. The virus titers found were expressed as the $\log_{10}$ of the LD_{50} for embryonated eggs.

Toxicity Test.—In order to determine a concentration of colchicine which would inhibit the fibroblastic proliferation induced by embryo extract, but would possess minimal toxic effects for chick embryo tissue, several concentrations of colchicine (Parke, Davis & Co.), in a nutrient solution consisting of 23 per cent Simms's (9) serum ultrafiltrate (SUF), a 7 per cent beef embryo extract (BEE), and 70 per cent BSS, were tested on minced 11-day-old chick embryo heart fragments on the walls of roller tubes in clotted rooster plasma. The nutrient solution without colchicine was the control. Each duplicate tube was inspected daily and a record kept of the average distance the fibroblasts migrated from the fragments, the shape and degree of vacuolation of the cells, and the number of fragments still beating.

Materials Used.—The embryo extract used throughout this work was prepared from the muscle tissues of fresh beef embryos of varying ages, which were homogenized in a Waring

blendor and extracted with an equal volume of BSS without bicarbonate for 1 hour in the refrigerator. The mixture was then centrifuged at about 2000 R.P.M. for 10 minutes, the supernatant fluid removed and sealed in 2 or 4 ml. amounts in sterile Wassermann tubes, quick frozen in an alcohol–dry ice mixture and stored at −40°C. This 50 per cent beef embryo extract (BEE) was used at a final concentration of 10 per cent in BSS.

BEE was dialyzed in a cellophane bag against BSS without bicarbonate under sterile conditions in the refrigerator. In the first dialysis, 7.5 ml. of BEE, lot 1, was dialyzed for 24 hours against 36 ml. of BSS without bicarbonate. The dialysate was then tested undiluted after the pH had been adjusted to 7.2 using bicarbonate (1.4 per cent) and the residue tested as a 10 per cent solution in BSS. In the second dialysis, 7.3 ml. of BEE, lot 4, was dialyzed against 3 consecutive changes for 24 hours each of 36 ml. of BSS without bicarbonate and each dialysate and the final residue tested as above.

BEE ultrafiltrate and a sample of the whole extract from which this was prepared were obtained from Microbiological Associates, Inc., and both were tested as 10 per cent solutions in BSS. Simms's serum ultrafiltrate (SUF) from Microbiological Associates, Inc., was tested as a 22.5 per cent solution in BSS. Lactalbumin hydrolysate (enzymatic) from Nutritional Biochemicals Corp. was made up as a stock 5 per cent solution in BSS, sterilized by autoclaving at 10 pounds' pressure for 10 minutes, and tested as a 10 per cent solution of the stock solution in BSS. An assay of the lactalbumin hydrolysate supplied by the manufacturer is presented here:—

General components		Amino acids				Vitamins	
per cent		*per cent*		*per cent*		*mg./gm.*	
Moisture	3.1	Lysine	7.6	Methionine	2.5	Riboflavin	4.25
Total N	12.8	Isoleucine	6.4	Cystine	0.3	Thiamine	0.11
Amino N	6.8	Leucine	10.5	Phenylalanine	4.5	Niacin	4.50
NaCl	1.0	Valine	7.0	Histidine	2.4	Pantothenate	2.19
Total ash	5.2	Arginine	3.3	Tryptophane	0.9	Biotin	0.51
		Threonine	4.0	Glutamic	20.4	Pyridoxine	1.29

Synthetic medium 199 (10), kindly supplied by Dr. R. C. Parker at the Connaught Medical Research Laboratory of the University of Toronto, was used undiluted after the pH had been adjusted with carbon dioxide immediately prior to use.

EXPERIMENTAL

The Stimulation of Virus Growth in Tissue Cultures Maintained in Nutritionally Deficient Media.—The medium which had been used in the previous experiments (1–4) was the relatively deficient nutrient consisting of Hanks's BSS with Simms's serum ultrafiltrate (SUF). It was felt that perhaps the lack of one or more substances in this nutrient fluid might have rendered the cells in cultures which had exhibited decreases in virus multiplication unsuitable for sustained virus growth. To test this possibility, a relatively rich source of nutrient materials, beef embryo extract (BEE), was added to the cultures at a time when virus multiplication had virtually ceased and in a number of instances there resulted a marked increase in the quantity of virus appearing in culture fluids.

Since with this Hanks-Simms medium a considerable period of time (*i.e.* 26 days) was required before virus multiplication had decreased sufficiently to test various substances for stimulating activity as described above, an even more deficient medium, BSS alone, was used to cultivate the chick embryo tissues. In this case, the virus grew remarkably well and had reached insignificant levels only by the 21st day (Fig. 1). Nevertheless, BEE added on the 9th, 13th, or 17th day, when virus multiplication had reached a maximum and was decreasing, definitely enhanced its growth in comparison with cultures maintained in BSS throughout (Table I, Fig. 1).

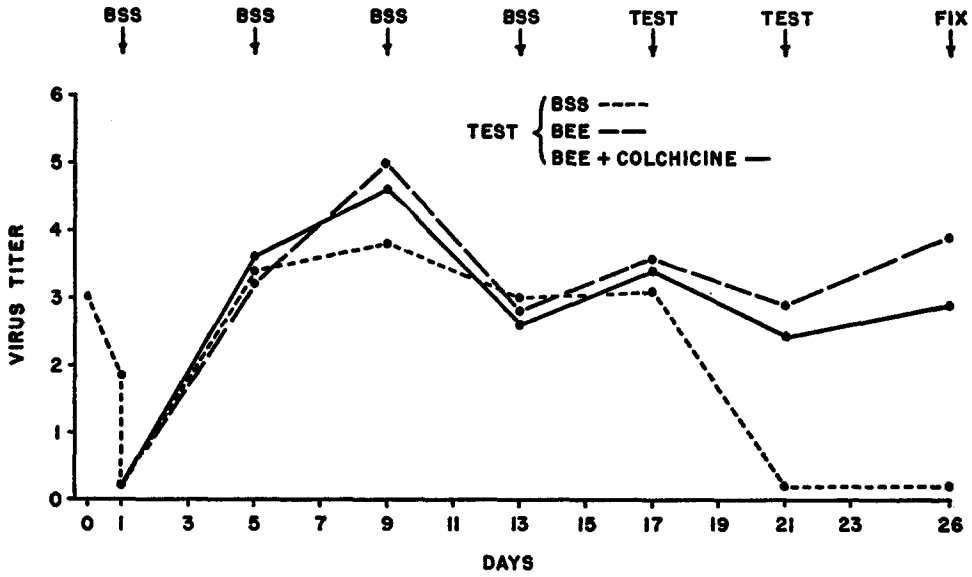

FIG. 1. Stimulation of growth of psittacosis virus (6BC) in chick embryo tissues by BEE after long term maintenance in BSS.

In cultures maintained in BSS throughout the culture period, there was microscopic evidence of early fibroblastic proliferation, and the cellular outgrowth from fragments on culture discs reached a maximum around the 5th to 9th days and then steadily decreased from then on until the 21st day. With the addition of BEE on the 9th, 13th, or 17th day, there was evidence of considerable fibroblastic proliferation when the tissues were fixed, although due to the destruction of cells by virus growth there were undoubtedly fewer cells left on the culture discs than would have been present had no virus multiplied. Because the cells on the cellophane discs represented only a variable proportion of the total number of cells in the whole culture and, furthermore, since during virus growth the host cells are destroyed, this method of appraising the extent of cellular proliferation in cultures was only an approximation. Microscopic examination of the stained discs has been interpreted only as a rough indication of the numbers and state of the cells. It should be mentioned that cells stained by the Papanicolaou method were well preserved and the viral inclusion bodies were easily differentiated from cytoplasmic material. This staining method appears to be highly satisfactory for investigations of both cellular and viral morphology.

Since the action of BEE on viral growth might have been a consequence of its stimulatory effect on cellular multiplication, as well as resulting from its acceleration of the metabolic activity of the cells already present in culture, it was decided to attempt to inhibit the cellular proliferation induced by BEE through the antimitotic activity of colchicine. Table II shows the results of an experiment carried out to find a concentration of colchicine which would

TABLE I

The Effect of the Addition of Beef Embryo Extract on the Growth of Psittacosis Virus in Cultures Maintained in a Nutritionally Deficient Salt Solution

Treatment	Virus titers*							Microscopic examination	
	Day of cultivation							Proliferation‡	Remarks
	0	1	5	9	13	17	21		
Balanced salt solution	—	<0.1	1.7	4.0	2.1	—	—	2+	Few infected cells.
			0.1	2.1	0.9	—	—	2+	
Beef embryo extract, 10 per cent added on 9th day	2.3	<0.1	3.8	3.1§	5.3	—	—	3+	Few infected cells.
			0.5	2.6	5.4	—	—	4+	
Balanced salt solution	—	1.9	3.4	3.8	3.0	1.9	<0.1	0	Few infected cells.
			3.6	4.8	2.9	3.1	0.6	0	
Beef embryo extract, 10 per cent added on 13th day	4.0	3.6	4.7	3.9	3.9§	6.0	4.2	4+	Many infected cells.
			4.8	6.0	4.6	5.7	3.6	2+	Few infected cells.

* Virus titers are expressed as the $\log_{10}$ of the lethal dose for 50% of embryonated eggs.

‡ Proliferation: 0 indicating no fibroblastic outgrowth from tissue fragments, to 4+ indicating entire disc covered with typical long, thin, closely packed fibroblasts.

§ Stimulating material added.

—, no test.

inhibit cellular proliferation, as measured by the restriction of fibroblastic growth from the edge of the fragments, and yet have minimal toxic effects on chick embryo cells, as indicated by the ability of embryo heart fragments to continue to contract and by the degree of vacuolation of the fibroblasts.

Since there was considerable evidence of an escape from the antimitotic effect of colchicine at the lowest concentration, it was felt that by employing a concentration of 0.001 mg. per ml., at which there was complete inhibition of cellular proliferation initially with only a slight escape from the antimitotic effect after 6 days, the toxicity

manifested by the colchicine would be minimized and the inhibition of proliferation retained.

The stimulating effect of BEE, added on the 17th day and tested under the influence of 0.001 mg. of colchicine per ml., was very little reduced, as seen in Fig. 1. Microscopically, there was little evidence of the proliferation of fibroblasts seen with BEE alone. This was an indication that the cellular multiplication induced by BEE, resulting in a larger population of cells in which more virus could multiply, was not a crucial factor in its stimulatory action on viral growth.

TABLE II

Colchicine Toxicity Test on Beating Chick Embryo Heart Fragments in Roller Tubes

Treatment		Day of cultivation						Microscopic examination of cells at end of experiment
		1	2	3	4	6	7	
Nutrient control	A	2/16	0/16	5/16	4/16	3/16	2/16	Many cells present—long, thin, finely granular.
	B	210	700–840	840	840	840	840	
Colchicine, 0.01 mg./ml.	A	4/12	4/12	3/12	0/12	2/12	2/12	Few cells—all rounded and vacuolated.
	B	0–70	70	70	70	70	70	
Colchicine, 0.001 mg./ml.	A	3/12	5/12	4/12	1/12	2/12	3/12	New outgrowths of fibroblasts—all vacuolated and thin.
	B	0–70	70	70	70	140	140	
Colchicine, 0.0001 mg./ml.	A	7/12	7/12	7/12	2/12	1/12	3/12	New outgrowths of fibroblasts—all vacuolated.
	B	35–70	70–120	70–120	70–120	420	840	

A, $\frac{\text{Number of fragments beating}}{\text{Total number of fragments}}$.

B, average distance fibroblasts had grown out from fragments, measured in micra with an ocular micrometer.

Other experiments (11) had indicated that colchicine had little direct effect on the multiplication of the virus in chick embryo cells cultured in Hanks-Simms solution when applied in the early part of the cultivation period. However, there was a delayed decrease in virus growth after the colchicine had been removed, due probably to the late consequences of an exhaustion of the restricted cellular population through destruction of cells by virus growth (11).

Based on this information, it was postulated that the virus-stimulating action of BEE must be due to activation of the metabolism of the cells already present and only secondarily to the production of a larger cell population. Therefore, a new series of experiments was performed to investigate both BEE and various fractions thereof, as well as some other nutrient materials in order to more fully define the manner in which virus multiplication was increased by BEE in tissue no longer able to support its growth.

The Growth of Virus in Cells Made Deficient Prior to Infection.—A somewhat different approach to the problem of defining more clearly the mode of action of BEE made use of the observation that tissue which had been maintained in BSS for 13 days and then infected was unable to support appreciable virus growth if it remained in BSS. On the other hand, if BEE was added at the time of infection, there was a significantly greater multiplication of virus during the following 9 days (Fig. 2), even though the tissue was returned to BSS during the last 4 days.

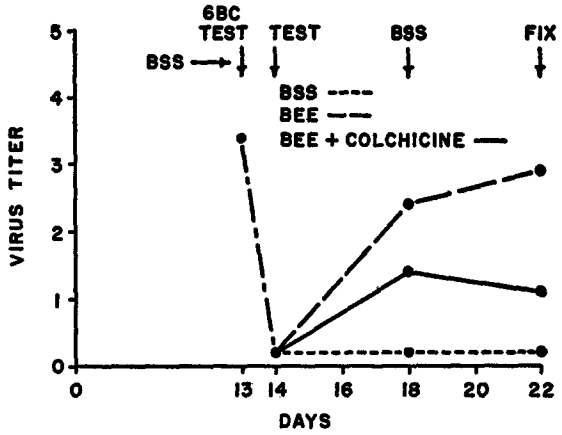

FIG. 2. Infection of chick embryo tissues cultivated in BSS for 13 days with psittacosis virus (6BC) and stimulation of viral growth with BEE.

Under the mitotic inhibitory influence of colchicine, BEE retained its stimulative capacity, although the increase in multiplication of virus which resulted was variable and typically not as great as with BEE alone (Fig. 2). In a number of experiments (Table III), however, the growth in the presence of BEE with colchicine was just as great as with BEE alone, though during the second 4 day period virus growth declined in cultures with colchicine, when stimulating materials were applied on the 13th and 14th days.

Microscopically, discs with tissue maintained in BSS for the 22 days of culture showed little cellular outgrowth from the tissue fragments, while those with BEE added usually showed some evidence of typical fibroblastic proliferation, of which cells only a small proportion showed visible evidence of infection; *i.e.*, the presence of viral elementary bodies. In those treated with BEE and colchicine, no typical fibroblastic proliferation was seen, although many sparsely situated, small, round cells with scanty cytoplasm were present but not nearly equal to the numbers of cells observed with BEE alone. With colchicine and BEE visibly infected cells were rarely seen.

The Effect on Virus Growth of Extending the Period of Stimulation by BEE Alone and with Colchicine.—A modification of the basic experimental procedure was carried out in order to accentuate the difference of the effect on virus

TABLE III

The Enhancement of Virus Growth by Beef Embryo Extract, Alone and with Colchicine, in Tissue Made Deficient Prior to Infection

Treatment	Virus titers*				Microscopic examination
	Day of cultivation				
	13	14	18	22	
Balanced salt solution	2.1	<0.1	<0.1 <0.1	<0.1 <0.1	Very few cells, scattered and small.
Beef embryo extract, 10 per cent	3.4‡	<0.1‡	2.3 3.6	3.9 4.2	Excellent proliferation. Closely packed, large, thin fibroblasts. Very few visibly infected.
Beef embryo extract, 10 per cent plus colchicine, 0.001 mg./ml.	3.6‡	<0.1‡	3.2 4.7	2.2 2.3	No typical fibroblastic proliferation. Many rounded cells. Very few visibly infected cells.

* Virus titers are expressed as the $\log_{10}$ of the LD_{50} for embyronated eggs.

‡ Stimulating material added.

TABLE IV

The Effect on Virus Multiplication of the Presence of Beef Embryo Extract, Alone and with Colchicine, during 9 Days of Incubation after Infection on the 13th Day

Treatment	Virus titers*				
	Day of cultivation				
	13	14	18	22	26
Balanced salt solution	3.8	0.9	<0.1 <0.1	<0.1 0.3	<0.1 <0.1
Beef embryo extract, 10 per cent	3.8‡	0.9‡	5.4‡ 6.3	4.6 5.0	5.0 5.0
Beef embryo extract, 10 per cent plus colchicine, 0.001 mg./ml.	3.8‡	0.9‡	3.9‡ 4.0	2.3 2.3	2.3 2.4

* Virus titers are expressed as the $\log_{10}$ of the LD_{50} for embryonated eggs.

‡ Stimulating material added.

growth played by the stimulation of the metabolism of cells already present (BEE plus colchicine) as compared to that played by the emergence of a larger population of cells which were also more active metabolically (BEE alone).

Thus BEE was applied, alone and with colchicine, on the 18th day as well as on the 13th and 14th days, extending the period of their respective influences from 5 to 9 days. As seen in Table IV, with BEE alone there was excellent growth of virus ini-

tially which decreased somewhat during the subsequent 4 day periods. Under the influence of colchicine, however, there was a more marked decrease in virus growth subsequent to the initial growth response which also was not as great as with BEE alone. With BEE, alone and with colchicine, virus multiplication was maintained at a higher rate than the controls after the 18th day, even though both sets of cultures were returned to BSS alone on the 22nd day. This experiment would indicate that the greater decline in virus multiplication in cultures treated with BEE plus colchicine was due to the constant destruction of cells by virus growth which were not replenished by new cells resulting from the increased cellular proliferation which would take place with BEE alone. Here the initial burst of viral multiplication in BEE plus colchicine was not as great as with BEE alone, probably owing to the fact that virus could multiply only in those cells present in the cultures at the time of infection. Thus, viral

TABLE V

The Growth of Psittacosis Virus in the Chorioallantoic Membrane of 9-Day-Old Chick Embryos

Treatment	Virus titers*			
	Day of cultivation			
	13	14	18	22
Balanced salt solution	3.1	1.9	3.4	3.8
			4.0	4.8
Beef embryo extract, 10 per cent	3.1‡	1.9‡	5.8	6.4
			6.6	6.5
Beef embryo extract, 10 per cent plus colchicine, 0.001 mg./ml.	3.1‡	1.9‡	5.0	4.2
			5.2	3.4

* Virus titers are expressed at the $\log_{10}$ of the LD_{50} for embryonated eggs.

‡ Stimulating material added.

proliferation was not further enhanced by the increase in the population of cells resulting from host cell proliferation induced by BEE.

The Growth of Psittacosis Virus in Chorioallantoic Membrane.—An interesting variation from results obtained with whole minced embryo tissue was found when chorioallantoic membrane tissue from 9-day-old chick embryos was employed after maintenance in BSS for 13 days before infection with virus. As seen in Table V, this tissue supported good virus growth while maintained in BSS alone for the entire experimental period of 22 days. Better growth of virus resulted when BEE plus colchicine was added, but when BEE was added alone, virus proliferation was even more active. Apparently chorioallantoic tissue is less affected in its capacity to support growth of psittacosis virus than is whole embryonic tissue when maintained for 13 days in the deficient BSS culture medium.

The Stimulating Activity of Fractions of BEE and Serum.—In an attempt to

characterize the factors present in BEE responsible for its stimulating action on viral proliferation, an ultrafiltrate and a dialysate of BEE were obtained and found to possess a considerable capacity to stimulate virus growth when tested in cultures made deficient prior to infection (Table VI).

TABLE VI

The Enhancement of Virus Multiplication in Deficient Cultures by the Ultrafiltrate of Beef Embryo Extract

Treatment	Virus titers*				Microscopic examination	
	Day of cultivation				Proliferation‡	Remarks
	13	14	18	22		
Balanced salt solution	—	3.4	<0.1 <0.1	<0.1 <0.1	±	Rare fibroblasts.
Beef embryo extract, 10 per cent	—§	3.4§	1.2 1.4	2.1 3.1	+2	Typical fibroblasts.
Beef embryo extract ultrafiltrate, 10 per cent	—§	3.4§	4.0 4.4	2.3 3.0	+4	Typical fibroblasts.
Beef embryo extract ultrafiltrate, 10 per cent plus colchicine, 0.001 mg./ml.	—§	3.4§	2.2 3.4	2.1 0.1	+2	Very few typical fibroblasts, mostly rounded cells.

* Virus titers are expressed as the $\log_{10}$ of the LD_{50} for embryonated eggs.

‡ See Table I.

§ Stimulating material added.

—, no test.

Both BEE ultrafiltrate and the BEE from which the ultrafiltrate was obtained were tested at the same concentration, the ultrafiltrate producing somewhat better growth than the whole extract. The stimulation produced by the ultrafiltrate plus colchicine was variable but not as good as with the ultrafiltrate alone. Microscopically there was evidence of good fibroblastic proliferation with the ultrafiltrate alone, while with ultrafiltrate plus colchicine there were fewer cells and many were rounded.

A 24 hour dialysate of BEE was shown to have excellent virus-stimulating capacity when tested undiluted, although the response with colchicine was relatively not as good (Table VII). There was evidence of some cellular proliferation in flasks with the dialysate alone, although not nearly as heavy as with whole BEE. A high proportion of the cells in the dialysate were infected. With colchicine, there were only a few rounded cells.

Since the residue of BEE remaining after dialysis for 24 hours retained considerable stimulating capacity (Table VII), a comparable sample was dialyzed for 72 hours against 3 consecutive 36 ml. amounts of BSS changed every 24 hours. The 3 dialysates were tested undiluted and the residue was examined as a 10 per cent solution in BSS.

Each dialysate had a significant stimulating activity, as shown in Table VIII, which diminished somewhat with successive dialysates, but the final residue was still relatively active, indicating that although a high proportion of the active materials were

TABLE VII

The Virus-Stimulating Capacity of a 24 Hour Dialysate of Beef Embryo Extract

Treatment	Virus titers*				Microscopic examination	
	Day of cultivation					
	13	14	18	22	Proliferation‡	Remarks
Balanced salt solution	3.4	<0.1	<0.1 <0.1	<0.1 <0.1	0	Rare cells.
Beef embryo extract, 10 per cent	3.4§	<0.1§	3.6 2.6	4.2 3.9		Many typical fibroblasts. Excellent proliferation. Many infected.
Beef embryo extract, 10 per cent plus colchicine, 0.001 mg./ml.	3.4§	<0.1§	1.4 1.6	<0.1 0.6	0 to +1	Few rounded cells.
24 hr. dialysate of beef embryo extract	3.4§	<0.1§	4.7 3.0	4.8 3.6	+2	Large scattered fibroblasts. Many infected.
24 hr. dialysate of beef embryo extract plus colchicine, 0.001 mg./ml.	3.4§	<0.1§	0.7 1.7	1.2 1.4	0 to +1	No typical fibroblasts. Few round cells. Some infected.
Residue after dialysis, 10 per cent	3.4§	<0.1§	1.2 1.9	1.6 1.2	+3	Typical fibroblasts, closely packed.

* Virus titers are expressed as the $\log_{10}$ of the LD_{50} for embryonated eggs.

‡ See Table I.

§ Stimulating material added.

in the diffusible fractions, some remained behind, either bound to or part of the larger components.

Simms's serum ultrafiltrate, tested at a concentration of 22.5 per cent in BSS, showed very little virus-stimulating activity as compared to the ultrafiltrate or dialysate of BEE.

The Stimulation of Virus Growth by Lactalbumin Hydrolysate.—An enzymatic digest of lactalbumin had been shown (12) to be useful as a replacement for

embryo extract and horse serum in the cultivation of poliomyelitis virus in tissue cultures. This material was tested and shown to have very good virus-stimulating activity on tissue infected on the 13th day (Table IX). Furthermore, it was sterilized by autoclaving at 10 pounds' pressure for 10 minutes, indicating that the materials responsible were relatively heat-stable.

TABLE VIII

The Effect of Repeated Dialysis on the Virus-Stimulating Capacity of Beef Embryo Extract

Treatment	Virus titers*			
	Day of cultivation			
	13	14	18	22
Balanced salt solution	3.2	<0.1	<0.1	<0.1
Beef embryo extract, 10 per cent	3.2‡	<0.1‡	1.6 2.2	3.6 2.8
Beef embryo extract, 10 per cent plus colchicine, 0.001 mg./ml.	3.2‡	<0.1‡	1.3 1.7	3.1 2.3
1st 24 hr. dialysate	3.2‡	<0.1‡	3.2 4.0	3.8 4.2
2nd 24 hr. dialysate	3.2‡	<0.1‡	2.9 3.6	3.6 3.4
3rd 24 hr. dialysate	3.2‡	<0.1‡	2.6 3.2	1.7 2.6
72 hr. residue, 10 per cent	3.2‡	<0.1‡	1.2 3.2	4.0 2.4

* Virus titers are expressed as the $\log_{10}$ of the LD_{50} for embryonated eggs.

‡ Stimulating material added.

Observations on the Length of Time Virus Can Remain in Deficient Cultures and Still Multiply.—Preliminary observations were made to determine the length of time virus could remain in deficient tissue without the addition of stimulatory nutrients and still be induced to multiply.

With tissues infected on the 13th day, no virus growth could be induced by the addition of BEE or lactalbumin hydrolysate on the 22nd day; *i.e.*, 9 days after infection. However, when lactalbumin hydrolysate was added on the 18th day, *i.e.* 5 days postinfection, and the 14th day, *i.e.* 1 day postinfection, virus growth took place. However, the growth resulting from the stimulation on the 14th or 18th day was not as great as when the stimulators were added on the 13th day, possibly indicating that

the materials essential to the initiation and maintenance of virus multiplication in deficient tissue were especially effective during the early part of the intracellular existence of the virus. Another partial explanation for this phenomenon may be that after the 13th day there was a decrease in numbers of cells present until very few remained. Further work is in progress to define more clearly the importance of the action of the stimulating agents during the first 24 hours after infection of the cells with virus.

The Adsorption of Virus to Deficient Tissue in BSS Alone.—In order to determine whether the absence of viral multiplication in BSS alone was due to the failure of virus to adsorb to host tissue in this medium, an experiment was carried out to measure the amount of viral adsorption to chick embryo

TABLE IX

The Virus-Stimulating Capacity of Lactalbumin Hydrolysate (Enzymatic)

Treatment	Virus titers*			
	Day of cultivation			
	13	14	18	22
Balanced salt solution	4.0	1.1	<0.1 <0.1	<0.1 <0.1
Beef embryo extract, 10 per cent	4.0‡	1.1‡	1.3 1.8	3.6 3.2
Lactalbumin hydrolysate, 0.5 per cent	4.0‡	1.1‡	1.9 2.6	4.2 4.6

* Virus titers are expressed as the $\log_{10}$ of the LD_{50} for embryonated eggs.

‡ Stimulating material added.

tissue maintained in BSS for 13 days during 6 hours' incubation at 34° to 36°C. in BSS alone.

The tissue in duplicate flasks was removed after 6 hours, washed thoroughly, and ground in 6 ml. of BSS, and 0.25 ml. of the suspension from each flask was injected into each of a dozen eggs. The amount of virus in the initial infecting fluids and the amount remaining in the fluid from each flask after 6 hours' incubation was also determined. Representative results are presented in Table X.

Virus was adsorbed to tissue in BSS alone, since virus was shown to be present in the tissue after incubation; furthermore, the virus in the extracellular fluids declined during this time an amount greater than could be accounted for by thermal inactivation. This is evidence that the lack of virus multiplication in BSS was not due to a failure of virus to adsorb to and invade the cells.

The Stimulating Action of a Synthetic Medium for Tissue Culture.—Further work is in progress to determine the nature of the substances responsible for the ability of a number of complex materials to enhance the growth of virus

in deficient tissues. The problem has been greatly simplified since it has been found that synthetic medium 199 of Parker (10) can replace or supplant the natural materials, with their complex and largely undefined composition, producing virus growth in deficient tissue that is almost as good as that ob-

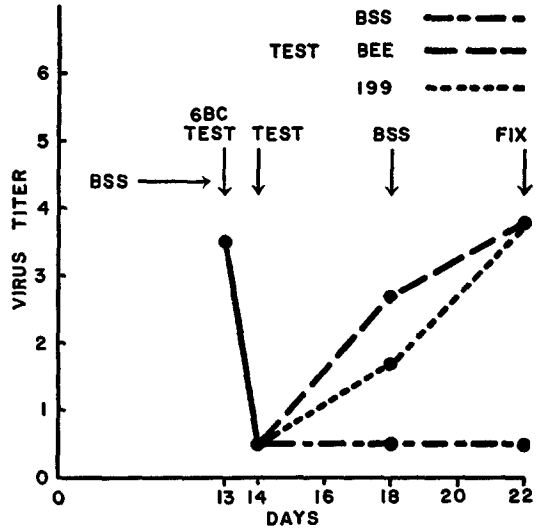

FIG. 3. Stimulating action of a synthetic medium 199 on growth of psittacosis virus (6BC) in chick embryo tissues cultivated in BSS.

TABLE X

Adsorption of Virus to Deficient Chick Embryo Tissue Suspended in Balanced Salt Solution

Amount of extracellular virus in BSS:	Initial	−5.7*
	After 6 hrs.	−3.0
Amount of virus in the tissue after 6 hrs.' incubation in BSS		−2.0
Thermal inactivation of virus during 24 hrs.' incubation at 35°C. in BSS without tissue:	Initial	−4.7
	After 24 hrs.	−3.7

* Virus titers are expressed as $\log_{10}$ of the LD_{50} for embryonated eggs.

tained using BEE (Fig. 3). A systematic evaluation of the virus-stimulating capacity of the compounds present in 199 is in progress at the present time and will be reported in a subsequent publication.

DISCUSSION

The results of this study indicate quite clearly that cultures of chick embryo cells may be obtained experimentally which are incapable of supporting the

growth of psittacosis virus, but which can be induced to support its growth once again by the addition of certain materials rich in essential growth factors. This indicates that the original inability to support viral proliferation was probably due to a deficiency of one or more substances essential to the initiation and maintenance of virus multiplication. Furthermore, the results of these experiments partially explain why virus growth fell off in cultures maintained in BSS-SUF or BSS alone over a period of days at a time when a considerable number of cells were still present. These remaining cells were probably deficient in one or more essential metabolites whose presence was of critical importance in the growth of this virus and which could not be synthesized by the cell and were not supplied by the medium.

In other studies on viral growth (13), it has been shown that the growth of influenza virus is reversibly slowed down in chorioallantoic membrane which has been depleted of carbohydrate stores by cultivation in a glucose-free medium. The addition of glucose to these depleted tissues restored their ability to support the growth of this virus. In the present study, however, the salt solution used to deplete the tissues contained glucose, thereby eliminating the possibility that it was a deficiency of this energy source that was responsible for the failure of virus growth in deficient tissues. It is possible, though, that the cells used in these investigations had been depleted of one or more vitamins known to be essential to the proper utilization of glucose supplied to them in the medium.

Because the synthetic medium 199, a complex yet chemically defined nutritional mixture, enhanced virus growth in a manner analogous to BEE, it should be possible to characterize eventually the substance or, more likely, the substances responsible. Whatever their nature, they must be relatively heat-stable, since they are present in autoclaved lactalbumin hydrolysate, and must be of low molecular weight, since they are dialyzable and are also present in synthetic medium 199. It remains to be established whether the factors responsible for virus stimulation in BEE, lactalbumin hydrolysate and synthetic medium 199, are the same or whether these substances are actually different, merely having reactions in common, resulting in the same effect; *i.e.*, stimulation of viral growth.

The demonstration that virus can associate *in vitro* with host cells in such a manner that this agent cannot multiply until some as yet undefined materials are added seems to present a controllable experimental model in which to study the phenomenon of latency in virus infections of cells. Although the virus probably does not remain in these deficient cells indefinitely, it has already been demonstrated to be present for as long as 5 days. Experiments directed toward elucidating the conditions under which virus will remain in cells for longer periods in the absence of any demonstrable multiplication are in progress. It may be possible to develop a medium deficient only in the substances essential for virus growth which would allow longer cell survival without virus multiplication. Latent infection, however, is a phenomenon occurring in the intact host and other mechanisms probably affect this state *in vivo*.

The problem of latent infection is of particular interest in the case of psittacosis virus, since it has been clearly demonstrated that the virus tends to persist for varying lengths of time without producing demonstrable evidence of infection in several hosts including the chick embryo, psittacine birds, mice, and man (14–17).

The extent of the role played by cellular proliferation in the increased viral multiplication resulting from the addition of nutrients has not been completely evaluated. Virus growth was not as good when colchicine was used in conjunction with the various stimulators. At the concentration used, *i.e.* 0.001 mg. of colchicine per ml. or 2.5×10^{-6} M, proliferation was markedly reduced, as shown in the roller tube toxicity tests and from the microscopic examination of stained discs from the tissue cultures. This is consistent with other investigations which have shown that 4×10^{-7} M colchicine completely inhibited spindle formation in chick embryo tissues *in vitro* (18). It would be expected that at least a portion of the difference between virus growth with and without colchicine would be due to the difference in the number of cells in which virus could grow. The importance of cellular proliferation would vary depending on the number of cells present, being relatively greater when the cultures contained only a few viable cells at the time when stimulating agents were added. Thus, in the presence of colchicine, when stimulating nutrients were added no new cells could grow and virus growth would be restricted to the few cells present. Without colchicine, a great new population of cells would develop and viral proliferation could reach high levels. However, when a large population of cells was present at the time colchicine and the stimulator were added, the virus would have a larger number of cells in which to grow from the time of infection, and increments to this host cell population would have less effect on virus yields. The experiments carried out here began with a large population of cells, so that by the end of the 13 day starvation period, a significantly large number of cells would remain in which virus could be stimulated to grow. Under these conditions, it was hoped that the number of cells present would not become the limiting factor. Since some of the cultures treated with the stimulators and colchicine showed very poor virus growth as compared with those without colchicine, this ideal situation was not always provided. Nevertheless, in a number of instances virus growth with the stimulator plus colchicine was as good as without colchicine, so that the cell population did not always become the limiting factor. Owing to the destruction of cells during virus multiplication, however, sustained, long term growth of virus could only be obtained under conditions in which free cellular proliferation could take place (*i.e.* without colchicine) to replenish the supply of susceptible cells as has been shown in other investigations (11).

A similar situation has been described by Scherer (19) who found decreased proliferation of herpes simplex and pseudorabies viruses in mouse fibroblasts maintained *in vitro* under conditions in which the cells ceased to multiply and he stated that the rates of cellular proliferation and of cellular destruction were important factors which determined the yields of these viruses.

A second factor which should be considered in evaluating the difference in the amount of virus resulting in cultures treated with stimulator, with and without colchicine, is that colchicine is a toxic substance. In these investigations, it was used at concentrations which did not influence the contractility of chick embryo heart fragments and it was shown previously to have no immediate influence on the pro-

liferation of virus in fresh chick embryo tissue (11). Yet, cellular vacuolation, which would indicate some degree of cellular toxicity, was observed quite regularly in its presence. Furthermore, since colchicine was administered, in most cases, to tissue which had been maintained in deficient media for considerable periods, it could be expected that it might have a higher degree of toxicity for these deficient cells.

It might be concluded, therefore, that part of the difference between virus growth with and without colchicine was due to a difference in the number of cells available for virus multiplication and part due to the toxic influence of the agent on deficient cells, both factors tending to reduce virus growth in cultures treated with colchicine. In this work, it is impossible to evaluate the exact extent of the role played by either of these factors. Yet, in certain experiments these effects of colchicine did not seriously interfere with the stimulating action of a number of materials on viral growth, indicating that their importance is relative only.

From a consideration of the foregoing discussion and the experimental results presented, it may be postulated that the major effect of the various stimulating agents was to influence the metabolism of the deficient cells by providing them with essential metabolites which they lacked as a result of cultivation in deficient media and which were necessary for the multiplication of psittacosis virus. Likewise, it may be postulated that the role played by cellular proliferation in the increased viral growth seen on the addition of the stimulators was secondary and became the limiting factor solely in cases in which but a few viable cells were present to begin with and diminished in importance as the number of cells present at the time of viral infection was increased.

The results of a preliminary experiment, indicating that virus growth in deficient cells was greater when the stimulating substances were present during the first 24 hours after the addition of virus, are interesting in light of work demonstrating the existence of a non-infectious stage early in the intracellular growth of the meningopneumonitis virus, an agent closely related to psittacosis virus (20, 21). The subsequent demonstration by the same group (22) that aureomycin must be present in this early postinfection period in order for it to be completely virustatic indicates the importance of the initial 24 hour period after infection for the growth of this group of viruses. Further work is in progress to define better the phenomenon reported here as it relates to the intracellular growth of virus in the immediate postinfection period.

An approach to the study of tissue tropism is possible, using the experimental method described here, and work of previous investigators suggests its application. During studies on the growth of poliomyelitis virus in various tissues, it was found (23) that this agent would not multiply in human tonsillar tissue maintained in Simms's salt solution with serum ultrafiltrate. However, it was subsequently shown (24) that this virus would proliferate quite readily in human tonsillar tissue if it were cultivated in synthetic medium 199. The present work shows that serum ultrafiltrate is a poor source of nutrients for the growth in tissue *in vitro* of psittacosis virus as it ap-

parently is for poliomyelitis virus. On the other hand, synthetic medium 199 is a ready source of nutrients for both viruses.

It would appear then that the problem of tissue tropisms may be due, at least in part, to a deficiency in certain cells of substances essential to the growth of some viruses and not others. A study of the nutritional requirements of the various viruses in deficient cells produced in a manner such as that described here would provide valuable information not only about general growth requirements of viruses but also regarding the factors which determine whether a tissue will or will not support the growth of a given virus; *i.e.*, tissue tropisms.

The method of studying virus growth outlined here is in direct contrast to the method used most frequently in the past by most workers, in which the growth requirements of viruses are usually arrived at indirectly by determining what metabolic analogues inhibit virus growth. By knowing what substances or metabolic reactions are interfered with by the appropriate metabolic analogue, it is deduced that these reactions or substances are important in the synthesis of virus components. It is suggested that the more direct approach to the problem of virus multiplication outlined here may have an advantage which will aid in a better understanding of the peculiar and intimate association between the animal virus and its host cell.

SUMMARY

The growth of psittacosis virus (6BC) was studied in cultures of minced whole chick embryo tissue maintained in either Hanks-Simms solution or Hanks's balanced salt solution (BSS), and in neither medium could sustained, long-term virus growth take place. Addition of beef embryo extract (BEE) to cultures at a time when virus multiplication was declining reversed this general trend and resulted in greater virus growth. This virus-stimulating action of BEE was only partially diminished by colchicine, a mitotic inhibitor, indicating that the action of BEE was not due entirely to the development of a larger population of cells as a result of its enhancement of cell proliferation.

Chick embryo tissue cultivated for 13 days in BSS prior to infection lost its ability to support the growth of psittacosis virus, but this capacity could be restored by the addition of BEE, alone or with colchicine, at the time of infection. A significant amount of virus was adsorbed to tissue in BSS alone, indicating that the failure of virus to grow in depleted tissue maintained only in BSS after infection was not due entirely to failure of virus to attach to and invade the cells.

It was found that an ultrafiltrate and a dialysate of BEE contained the major part of the stimulating capacity of the whole extract, indicating that the active materials were substances of low molecular weights. Autoclaved lactalbumin hydrolysate was an active stimulator, suggesting that the materials responsible for its activity were relatively heat-stable.

Since a chemically defined medium (Parker 199) was equally effective in stimulating viral growth, it should be possible eventually to define the chemical nature of the virus stimulators.

The implications of the findings are discussed with special reference to their application in the study of tissue tropisms and of latency in viral infections of cells.

BIBLIOGRAPHY

1. Morgan, H. R., *Proc. Soc. Exp. Biol. and Med.*, 1948, **67,** 29.
2. Morgan, H. R., *J. Exp. Med.*, 1948, **88,** 285.
3. Morgan, H. R., *J. Exp. Med.*, 1952, **95,** 269.
4. Morgan, H. R., *J. Exp. Med.*, 1952, **95,** 277.
5. Hanks, J. H., and Wallace, R. E., *Proc. Soc. Exp. Biol. and Med.*, 1949, **71,** 196.
6. Evans, V. J., and Earle, W. R. J., *Nat. Cancer Inst.*, 1947, **8,** 103.
7. Peters, H., and Benjamin, J. A., *Surg., Gynec., and Obst.*, 1950, **91,** 660.
8. Golub, O. J., *J. Immunol.*, 1948, **59,** 71.
9. Simms, H. S., and Sanders, M., *Arch. Path.*, 1942, **33,** 619.
10. Morgan, J. F., Morton, H. J., and Parker, R. C., *Proc. Soc. Exp. Biol. and Med.*, 1950, **73,** 1.
11. Morgan, H. R., *J. Exp. Med.*, 1954, **99,** 451.
12. Melnick, J. L., and Riordan, J. T., *Proc. Soc. Exp. Biol. and Med.*, 1952, **81,** 208.
13. Eaton, M. D., *Arch. ges. Virusforsch.*, 1952, **5,** 53.
14. Bedson, S. P., *Brit. J. Exp. Path.*, 1938, **19,** 353.
15. Early, R. L., and Morgan, H. R., *J. Immunol.*, 1946, **53,** 251.
16. Davis, D. J., and Vogel, J. E., *Proc. Soc. Exp. Biol. and Med.*, 1949, **70,** 585.
17. Meyer, K. F., and Eddie, B., *J. Infect. Dis.*, 1951, **88,** 109.
18. Hughes, A. F. W., *Quart. J. Micr. Sc.*, 1950, **91,** 251.
19. Scherer, W. F., *Am. J. Path.*, 1953, **29,** 113.
20. Sigel, M. M., Girardi, A. J., and Allen, E. G., *J. Exp. Med.*, 1951, **94,** 401.
21. Girardi, A. J., Allen, E. S., and Sigel, M. M., *J. Exp. Med.*, 1952, **96,** 233.
22. Allen, E. G., Girardi, A. J., Sigel, M. M., and Klein, M., *J. Exp. Med.*, 1953, **97,** 783.
23. Smith, W. M., Chambers, V. C., and Evans, C. A., *Northwest Med.*, 1950, **49,** 368.
24. Franklin, A. E., Duncan, D., Wood, W., and Rhodes, A. J., *Canad. J. Med. Sc.*, 1953, **31,** 64.