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Studies on the Cultivation of Influenza Virus *in vitro* *

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Studies have been carried out since 1959 at the Coonoor Influenza Centre to devise a method of cultivating influenza virus in vitro *which would be suitable for large-scale virus production. The authors report the successful cultivation of the virus in tissue cultures of chorioallantoic membrane on glass wool. The method described may be used equally satisfactorily for culture in volumes ranging from 1.0 ml to 350 ml, and is as sensitive as cultivation in eggs for the titration of different strains of influenza virus and their neutralizing antibodies. Relatively pure virus for vaccine production and complement-fixing antigen for diagnostic purposes can be produced in large volumes with ease and economy.*

INTRODUCTION

Attempts have been made by several workers to cultivate the influenza virus *in vitro*. Mogabgab et al. (1955) reported on the isolation and cytopathogenic effect of influenza B viruses in monkey kidney cells. Heath & Tyrrell (1959) found that the WS, Swine and Sendai viruses could multiply in cultures of kidney tissue from man, monkey, beef embryo, guinea-pig, rabbit, hamster, mouse and chick embryo. They observed that serial propagation in monkey kidney cells was readily achieved with mouse-adapted virus strains but not with egg-adapted strains and that the passage of WS and PR8 strains in monkey kidney cultures gave fluids with low infectivity to haemagglutination ratios. Hinz & Syverton (1959) reported that collagenase-dispersed human and porcine lung cells in primary cultures unequivocally propagated human strains A and B and Swine influenza viruses, respectively. They also found that swine kidney cells propagated egg infectious Swine influenza virus and heamagglutinin without cytopathogenic effect. Gostling (1960) demonstrated multiplication of A-prime viruses in whole mouse embryos, the increase in infectivity for eggs in 22 hours ranging from 0.5 to 2.8 log units. Smith & Morgan (1960) reported a lytic effect of PR8 strain influenza virus on mouse fibroblastic (L) cells *in vitro*. They found that the virus replication was limited, resulting in the production of relatively large amounts of virus haemagglutinins but very small quantities of infectious virus.

Studies have been in progress at the Coonoor Influenza Centre since 1959 to evolve a method for the cultivation of influenza virus *in vitro* which would be suitable for growing the virus in large quantities and which would prove as sensitive as the egg for purposes of titration of the virus and its antibodies. An avid Asian strain of influenza virus in its third passage in the egg was selected for this study. Attempts were made to grow the virus in monkey kidney cell cultures but the titres of haemagglutinins and virus obtained were poor. The technique of " allantois-on-shell " developed by Fulton & Armitage (1951) and modified by Fazekas de St Groth & White (1958) were then tried but the haemagglutinin titres obtained were low.

Daniels et al. (1952), in their growth experiments on influenza virus, used minced chorioallantoic membrane and Hanks' solution buffered with ground eggshell to cultivate the virus. Eaton et al. (1953), in their studies on influenza virus growth and cellular energy production, employed minced chorioallantoic membrane and Hanks' balanced salt solution with sodium pyruvate and glucose. The studies of Levine et al. (1956) showed that chorioallantoic membrane tissue culture in balanced salt solution with pyruvate as the sole carbon source supported synthesis of influenza virus (strain WS) but not its neurotropic variant (strain NWS). It was, however, found that membranes maintained in basal

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salt solution containing glucose, xylose or glycerol as the sole carbon source supported virus synthesis of NWS strain. Eaton et al. (1960), in their studies on the formation of non-infectious virus haemagglutinins in ascites tumour cells, used media containing glutamine with lactate or glucose and pyruvate with glucose. Systematic studies were undertaken to determine whether any of these media could be improved so that the results with them would be as good as those obtained in the egg. The results of these investigations are briefly summarized below.

MATERIALS AND METHODS

Tissue culture methods

Glassware. All screw-capped roller tubes, bottles and other glassware used were washed and prepared according to the accepted methods employed in tissue culture.

Glass wool. Thin mats of glass wool (Merck) were used to prevent the clumping of the fragments of minced chorioallantoic membrane used in culture. As the glass wool found was to be alkaline it was soaked in 10% HCl for 3 hours and washed several times in tap water to remove the acid, rinsed repeatedly with single- and double-distilled water, dried in an oven and used.

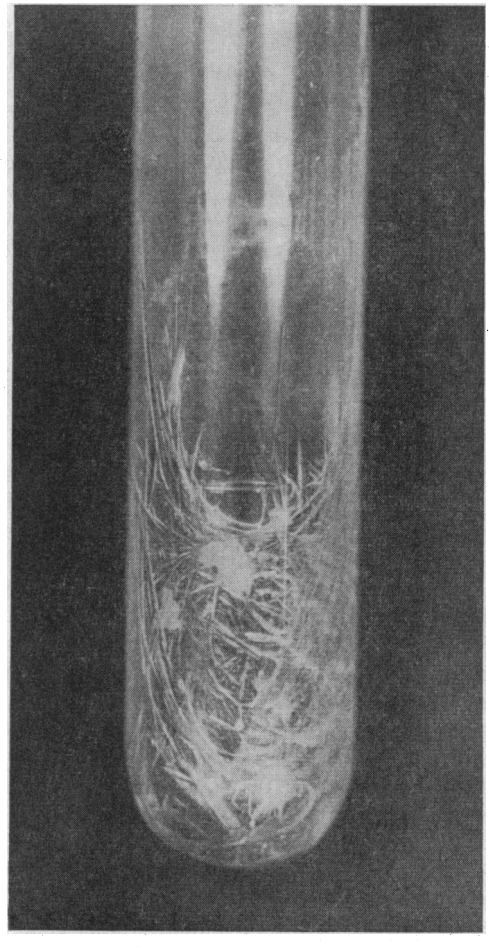
A thin mat 2.5 × 2.5 cm was placed at the bottom of clean roller tubes (Fig. 1), which were then sterilized. When bottles were used, thin mats were spread carefully round the sides of the bottles, leaving the bottom free (Fig. 2).

Basal salt solution. Hanks' balanced salt solution without glucose and sodium bicarbonate but containing adequate concentrations of penicillin and streptomycin (BSS) was used in the preparation of the media. BSS adjusted with 1% $NaHCO_3$ to pH 7.1 was employed for virus dilutions, etc.

Media. A variety of media were tried. They were prepared in BSS, adjusted to pH 7.1 with 1% or 5% $NaHCO_3$ depending on the volume, and distributed into roller tubes or bottles with glass wool. With bottles, the medium was poured through a sterile glass funnel directly to the bottom. The bottle was then slowly tilted, placed on its side and rolled to make the mat of glass wool stick to the sides of the bottle.

The volume of media used varied. For purposes of titration of the virus and its neutralizing antibody, 0.9 ml was dispensed into screw-capped roller tubes. In experiments designed for determining the values of different compounds 1.4 ml was used. Volumes of 10 ml, 20 ml, 150 ml and 350 ml were used in bottles for studies on large-scale cultivation of the virus.

FIG. 1
LOWER SECTION OF A ROLLER TUBE CULTURE WITH GLASS WOOL



Pieces of membrane can be seen sticking to the glass wool.

Chorioallantoic membrane. Chorioallantoic membrane (CAM) from 11- to 13-day-old chick embryos were washed and finely minced to a size of about 1 mm with curved scissors. The minced membrane was suspended in BSS, stirred with a magnetic stirrer for 5 minutes to remove the blood, and then centrifuged at 1000 r.p.m. for 5 minutes. The membrane was washed two more times with BSS,

after which it was resuspended in an equal volume of BSS to give a 50% suspension. This suspension was added in the proportion of 0.07 ml per ml of final volume with an automatic pipette. This proportion of membrane was found to give the best results with the different media employed and was used throughout. The tubes were shaken immediately after adding the membrane to make the membrane adhere to the glass wool. With bottles, this was achieved by rolling the bottle on its side. The pieces of membrane stuck to the glass wool, resulting in almost a sheet. After an hour in the roller drum it was found that all the fragments of membrane had adhered to the glass wool, leaving the medium clear.

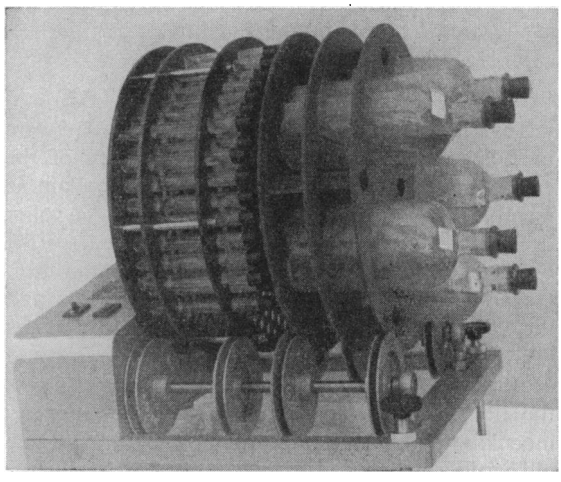
FIG. 2

350-ml INFLUENZA VIRUS CULTURE IN 1000-ml BOTTLE

Pieces of membrane can be seen sticking to the glass wool.

FIG. 3

ROLLER DRUM WITH TUBES AND 1000-ml BOTTLES



Though the eggs available were small, it was possible to obtain at least 1.75 ml of a 50% membrane suspension per egg.

Inoculum. For virus titration, 0.1 ml of tenfold virus dilutions and for antibody titrations 0.2 ml of the serum virus mixture were used. The inoculum used in studies on the growth-promoting effect of different compounds was 0.1 ml of virus adjusted to contain 2-8 haemagglutinating (HA) units per 0.25 ml. In attempts to grow the virus in large quantities the haemagglutination titre of the inoculum was further reduced. The inoculum in these cases was based on virus titre.

Incubation. The inoculated cultures in tubes and bottles were set in a Matburn roller drum (Fig. 3) revolving at 9 revolutions per hour and incubated at 37°C. The rollers to take bottles of different sizes were made locally. The cultures used for titration of the virus and its antibody were incubated for 4 days. In experiments for the cultivation of the virus in large volumes the period of incubation varied depending on when the highest virus titres were reached.

Haemagglutinin titration

The method was the same as that described by Veeraraghavan.[1] All titrations were done in tubes. The cultures used for titration of virus or antibody

[1] See the article on page 679 of this issue.

were tested for the presence of haemagglutinins in 2, 4 and 8 dilutions at the end of 96 hours. In all other experiments regular titrations were carried out at the end of 24, 48, 72 and 96 hours.

Virus titration

Tenfold dilutions of the material to be tested were inoculated in 0.1 ml amounts into 0.9 ml of the culture medium containing minced chorroallantoic membrane. The cultures were rotated in a roller drum at 37°C for 96 hours and then tested for the presence of virus by the haemagglutination test. Generally 5 tubes were used for each dilution and the virus titres—i.e., tissue culture infective doses ($TCID_{50}$) per ml—were calculated by the method of Reed & Muench (1938). In several experiments the titres were compared with those obtained by inoculating 0.1 ml amounts allantoically into 10-day old fertile eggs.

Neutralizing antibody titration

The method used for the titration of poliomyelitis antibodies was modified. The test and control sera were inactivated at 56°C for 30 minutes or treated with potassium periodate to remove the inhibitors and suitable dilutions made in BSS. An equal amount of virus, diluted to contain 100 $TCID_{50}$ per ml on the basis of previous titration, was added to each serum dilution, mixed and incubated for 60 minutes at room temperature, after which 0.2 ml of each mixture was inoculated into each of 5 tissue culture tubes. The tubes were set in a roller drum, incubated at 37°C for 96 hours and then tested for the presence of haemagglutinin. Antibody levels were calculated by the method of Reed & Muench (1938). In some experiments the results were compared with parallel tests done in the egg by inoculating the mixtures into groups of five 10-day-old eggs allantoically.

Complement-fixation (CF) test

The method was the same as that described by Hoyle (1948) but using the culture virus or the infected allantoic fluid as the antigen instead of mouse lungs.

Human convalescent serum was used with the type A viruses, and pooled guinea-pig antiserum raised against the soluble antigen of B/Denmark/53 according to the method described by the WHO Expert Committee on Respiratory Virus Diseases (1959) with the type B virus.

Virus

As stated earlier an avid Asian strain in its third passage in the egg (Gilbert, LEP) was used mainly in this study. The results obtained were confirmed with the following antigenically representative strains: (*a*) PR8 (type A), (*b*) FM1 (type A1), (*c*) PAR (non-avid A2), (*d*) Gilbert (avid A2) at high (13th) egg passage (HEP), and (*e*) Crawley (type B).

Antisera

The antisera raised against PR8, FM1, Gilbert (LEP), PAR and Crawley in fowls were used in the neutralization tests. The non-specific inhibitor contents of the sera were removed by potassium periodate.

RESULTS

Experiments with media described by Eaton et al. (1960)

Using CAM tissue culture, preliminary trials were carried out with the media described by Eaton et al. (1960). The media tried are given in Table 1.

The results with Gilbert (LEP) virus may be summarized as follows:

(1) The HA titres obtained with medium C were poor.

(2) The HA titres obtained with media A and B were not high.

(3) Although the HA titres with both media A and B were about the same, the virus titres with medium B were higher.

(4) The virus titres, employing medium B, were about 2 log values less than the titre in the egg.

TABLE 1
GROWTH MEDIA TESTED

Constituents	Medium		
	A	B	C
Glutamine	100 mg		100 mg
Sodium lactate	100 mg		
Sodium pyruvate		100 mg	
Glucose		20 mg	100 mg
BSS	100 ml	100 ml	100 ml
pH adjusted to 7.1 with	N/10 NaOH	1% $NaHCO_3$	1% $NaHCO_3$

TABLE 2
EFFECT ON HAEMAGGLUTINATION OF USING GLASS WOOL

Culture in	Quantity (ml)	Glass wool	HA titres at hours:				
			0	24	48	72	96
Roller tube	1.5	without	4	16	24	24	16
Roller tube	1.5	with	4	32	32	32	48
Bottle	20	without	4	12	24	16	8
Bottle	20	with	4	24	48	64	48

It was found that batches of cultures inoculated with the same inoculum showed variations in HA titre. On investigation it was noticed that the low titres were generally associated with cultures where the CAM fragments had clumped into a mass. Attempts were made to prevent clumping by coating the bottom of the roller tube with a thin film of agar or by introducing small glass beads. Both methods proved unsatisfactory. Then the introduction of a thin mat of glass wool at the bottom of the tube or on the sides of a bottle was tried. When glass wool was used all the CAM fragments adhered to it. In bottles they formed almost a sheet. After an hour in the roller drum the medium became clear. The results of an experiment using Gilbert (LEP) virus with and without glass wool are given in Table 2.

The HA titres obtained with glass wool were better. It was also possible to grow the virus in larger volumes. Consistent results were obtained when glass wool was used and the method was therefore employed in all subsequent work.

Effect of addition of different compounds on growth

Experiments were undertaken to see whether any of the three media shown in Table 1 could be improved by adding different substances to give results as good as or better than those obtained in the egg. For this purpose the medium with and without the substance to be tested was prepared in bulk and distributed in 1.4-ml amounts into roller tubes. 0.1 ml of 50% CAM in BSS was added, followed by 0.1 ml of the appropriate dilution of Gilbert (LEP) virus. Four tubes were used for testing each substance. The HA titres of the cultures with and without the substance, 24, 48, 72 and 96 hours after incubation were determined and the mean values calculated. If the haemagglutinins appeared earlier and the titres were higher than in the controls the substance was considered to be growth-promoting. The effects of the addition of some compounds to the media A, B and C in certain concentrations are given in Table 3. It was found that the effect of the same compound varied when used with the different media.

Additive effect of some compounds on growth

All the compounds which were found useful with each medium were tested with that medium in different concentrations and combinations. Among the several combinations tested the following were found to give consistently good results.

1. Glutamine (0.1%), sodium lactate (0.1%) and valine (0.005%) in BSS.
2. Sodium pyruvate (0.1%), glycine (0.005%), sodium molybdate (0.005%), folic acid (0.0004%) and glycerol (0.4%) in BSS.
3. Medium 2 with glucose (0.1%).
4. Medium 2 with twice the concentration of sodium pyruvate (0.2%).
5. Medium 4 with glucose (0.1%).
6. Sodium pyruvate (0.2%), glycine (0.005%), glycerol (0.4%), pimelic acid (0.005%) and putrescine hydrochloride (0.002%).

It was found that the pH of media 2 and 4, to which no glucose was added, could be adjusted with NaOH.

In order to determine which of the media was the most suitable comparative titrations of a pool of Gilbert (LEP) virus were carried out in CAM tissue cultures using the different media with parallel titration in the egg. The results are given in Table 4.

The results with medium 1, containing glutamine, sodium lactate and valine, were poor. The virus titres obtained with media 2, 3 and 5 were almost similar. Good results were obtained with medium 4

TABLE 3

EFFECT OF ADDITION OF VARIOUS COMPOUNDS TO MEDIA A, B AND C

Compounds tested	Final concentration per 100 ml	Medium A	Medium B	Medium C
Acids and their salts				
cis-Aconitic acid	2 mg	—	—	—
Ammonium butyrate	10 mg	—	—	—
Iminodiacetic acid	2-10 mg	—	—	—
Isocitric acid	2 mg	—	—	—
α-Ketoglutaric acid	2-5 mg	+	+	—
Oxalacetic acid	2 mg	—	—	—
Pimelic acid	2-5 mg	—	+	—
Sodium acetate	50 mg	—	+	—
Sodium formate	100 mg	—	—	—
Sodium glycero-phosphate	2-10 mg	+	+	+
Sodium glyoxalate	10 mg	—	—	—
Sodium malate	50 mg	—	—	—
Sodium succinate	20-100 mg	+	+	—
Stearic acid	10 mg	—	—	—
Amides, amines and amino alcohols				
Cadaverine	2 mg	—	—	—
Choline	4 mg	—	—	—
Ethanolamine in glyoxylic acid	1 ml	—	—	—
Neurine	2 mg	—	—	—
Putrescine	2 mg	+	+	—
Soluscepticine	2 mg	—	+	—
Taurine	5 mg	—	—	—
Thioacetamide	10 mg	—	—	—
Amino acids				
Alanine	5 mg	—	—	+
p-Aminobenzoyl-glutamic acid	2 mg	—	—	—
α-Aminobutyric acid	5 mg	—	+	—
γ-Aminobutyric acid	5 mg	—	+	—
Arginine	5 mg	—	—	—
Asparagine	5 mg	—	—	—
Betaine	2-10 mg	—	—	—
Creatine	2 mg	+	—	—

Compounds tested	Final concentration per 100 ml	Medium A	Medium B	Medium C
Amino acids (continued)				
Glutamic acid	5 mg	—	+	—
Glycine	5 mg	+	+	—
Histidine	5 mg	—	+	—
Hydroxyproline	5 mg	+	—	—
Isoleucine	5 mg	+	+	—
Leucine	5 mg	—	+	—
Lysine	5 mg	—	+	—
Methionine	5 mg	—	—	—
Ornithine	2 mg	+	—	—
Phenylalanine	5 mg	—	+	—
Potassium glutamate	2 mg	+	—	—
Proline	5 mg	—	+	—
Threonine	5 mg	—	—	—
Tryptophane	2 mg	—	—	—
Tyrosine	5 mg	—	—	+
Sarcosine	5 mg	—	—	—
Sarcosine anhydride	5 mg	—	—	—
Valine	5 mg	+	+	—
Carbohydrates (including sugars, alcohols and glycosides)				
Adonite	100 mg	—	—	—
Arabinose	100 mg	+	—	—
Calcium hexosedi-phosphate	5 mg	+	+	—
Dextrin	100 mg	—	—	—
Dulcite	100 mg	+	—	—
Galactose	100 mg	—	+	—
Glucose	20-100 mg	—	—	—
Glycerol	0.2-0.6 ml	—	+	—
Glycogen	100 mg	—	—	—
Inosite	100 mg	—	—	—
Inulin	100 mg	—	+	—
Lactose	100 mg	—	—	—
Levulose	100 mg	—	—	—
Maltose	100 mg	—	—	—
Mannite	100 mg	—	—	—
Mannose	100 mg	—	—	—
Mucin	100 mg	—	—	—

+ = HA titres increased.
— = no effect.

(containing twice the concentration of sodium pyruvate with glycine, sodium molybdate, folic acid and glycerol) and with medium 6 (containing twice the concentration of sodium pyruvate with glycine, glycerol, pimelic acid and putrescine), the latter medium being a shade better. The titres with these media were similar to those obtained in the egg. It was also found that, although NaOH could be used for adjusting the pH of the media containing no glucose, the results were better when $NaHCO_3$ was employed.

In view of the possibility of putrescine being toxic to human beings, medium 4 was selected. This medium, evolved by Veeraraghavan and referred to as " medium V " (for Veeraraghavan), was used in all further work.

TABLE 3 (continued)

Compounds tested	Final concentration per 100 ml	Medium A	Medium B	Medium C
Carbohydrates (continued)				
Raffinose	100 mg	—	—	—
D-Ribose	2-10 mg	—	+	—
Saccharose	100 mg	—	—	—
Salicin	100 mg	—	—	—
Sorbitol	100 mg	+	—	—
Starch	100 mg	+	—	—
Tween 80	5 mg	—	—	—
Xylose	100 mg	+	—	—
Growth factors				
Biotin	200 γ	+	+	—
Casein hydrolysate	50-100 mg	—	—	—
Gibberellic acid	200 γ	—	—	—
Indole-3-acetic acid	2 mg	—	—	—
Indole-3-propionic acid	2 mg	—	—	—
α-Lipoic acid	200 γ	+	+	—
Yeast extract	50 mg	—	—	—
Nucleic acids				
Adenylic acid	2 mg	+	—	—
Protamine sulfate	2 mg	—	—	—
Sodium DNA	5 mg	—	+	—
Sodium RNA	5 mg	+	—	+
Polypeptides				
Chloroacetylglycyl-glycine	5 mg	—	—	—
Diglycylglycine	5 mg	—	—	—
Glutathione	5 mg	—	—	+
Glycylglycine	5 mg	—	—	—
Glycylglycine ethyl ester hydrochloride	5 mg	—	—	—
Glycylglycylglycine	5 mg	—	—	—
Leucylglycine	5 mg	—	+	—

Compounds tested	Final concentration per 100 ml	Medium A	Medium B	Medium C
Purines and pyrimidines				
Adenosine	2 mg	—	—	—
Cytosine	2 mg	+	—	+
Guanine	2 mg	+	—	—
Hypoxanthine	2 mg	+	—	—
Inosine	5 mg	—	—	—
5-Methylcytosine	2 mg	+	+	—
5-Methylorotic acid	2 mg	+	+	—
Orotic acid	2 mg	+	+	—
Thymine	2 mg	+	+	—
Uracil	2 mg	—	—	—
Xanthine	2 mg	—	+	—
Trace elements				
Boron	20 γ	—	—	—
Cobalt	20 γ	—	—	—
Copper	500 γ	—	—	—
Iron	420 γ	+	—	—
Manganese	1 mg	—	—	—
Molybdenum	500 γ	—	+	—
Nickel	500 γ	—	—	—
Zinc	500 γ	—	—	—
Vitamins				
p-Aminobenzoic acid	2 mg	+	—	—
Calcium pantothenate	2 mg	—	—	—
Folic acid	2 mg	+	+	—
Inositol	20 mg	+	—	+
Nicotinamide	2 mg	—	+	—
Nicotinic acid	2 mg	—	—	—
Pyridoxine	2 mg	—	—	—
Riboflavine	2 mg	—	—	—
Thiamine	2 mg	+	—	—
Vitamin A	400 γ	—	—	—
Vitamin B_{12}	200 γ	—	+	—
Vitamin D	1750 IU	—	—	—
Vitamin E	2 mg	—	—	—

+ = HA titres increased.
— = no effect.

With the above medium the addition of 0.07 ml of 50% minced CAM suspension per ml of the final volume gave the best results. Although the virus could grow in a pH range of 6.8 to 7.6, it was found that an initial pH of 7.1 adjusted with $NaHCO_3$ gave the best results. There was no advantage in adding Tris (tris(hydroxymethyl)-aminomethane) as buffer.

Effect of storage on medium V

In the earlier part of the work the medium was freshly prepared and used. The effect of storage on the medium was investigated. All the ingredients in tenfold concentration were dissolved in double-distilled water, filtered through a Ford's Sterimat GS pad, and stored at —20°C in screw-capped tubes or bottles with ground-glass stoppers. When

TABLE 4

COMPARISON OF SENSITIVITY OF DIFFERENT MEDIA [a]

Medium	Log $TCID_{50}$/ml of virus with medium, pH being adjusted with:	
	$NaHCO_3$	NaOH
1	5.5	
2	6.5	6.5
3	6.5	
4	7.0	6.5
5	6.8	
6	7.2	

[a] The log ID_{50}/ml of the virus titrated in egg was 7.2.

required, the concentrated medium was diluted tenfold with BSS, adjusted to pH 7.1 with sodium bicarbonate and used. The sensitivity of a sample stored for a month was compared with freshly prepared medium by titrating pools of FM1 and Gilbert (LEP) viruses. Titration of the viruses was also done in the egg. The results are presented in Table 5.

It was found that the results with the medium after one month's storage were similar to those obtained when fresh medium was used.

Preparation of medium V

The concentrated medium was prepared as follows:

Sodium pyruvate	2.0 g
Glycine	50.0 mg
Sodium molybdate	50.0 mg
Folic acid (dissolved in minimum quantity of 1% $NaHCO_3$)	4.0 mg
Glycerol (analytical reagent grade)	4.0 ml
Double-distilled water to	100.0 ml

The medium was sterilized by filtration through a Ford's Sterimat GS pad. As the pads were generally alkaline they were washed with double-distilled water before sterilization. The medium was distributed into screw-capped tubes or Pyrex bottles with ground-glass stoppers and stored at −20°C.

When required, concentrated medium was diluted tenfold with BSS, adjusted to pH 7.1 with sterile $NaHCO_3$ and used.

TABLE 5

COMPARISON OF SENSITIVITY OF FRESH AND STORED MEDIUM

Virus	Log $TCID_{50}$/ml using:		Log EID_{50}/ml
	Fresh medium	Stored medium	
FM1	5.2	5.2	4.8
Gilbert (LEP)	6.0	5.8	5.8

TABLE 6

SENSITIVITY OF CAM CULTURE COMPARED WITH THAT OF EGG

Gilbert (LEP) virus pool	Log $TCID_{50}$/ml	Log EID_{50}/ml
1	7.8	7.7
2	7.2	7.5
3	7.7	7.5
4	7.2	7.2

Sensitivity of medium V for virus titration

With a view to confirming the earlier findings the sensitivity to virus of CAM tissue cultures using medium V was compared with that of the egg. Three pools of Gilbert (LEP) virus were titrated in tissue culture and in the egg. The results are given in Table 6.

The experiment was repeated twice using different pools of PR8, FM1, PAR, Gilbert (HEP) and Crawley viruses. The results are presented in Table 7.

These results indicated that the CAM tissue culture method using medium V was comparable to the egg

TABLE 7

SENSITIVITY OF CAM CULTURES TO DIFFERENT STRAINS OF INFLUENZA VIRUS COMPARED WITH THAT OF EGG

Experiment	Virus	Log $TCID_{50}$/ml	Log EID_5/ml_0
1	PR8	6.5	6.3
	FM1	5.2	5.4
	Gilbert (HEP)	8.4	8.2
	Crawley	7.2	6.5
2	PR8	7.2	7.5
	FM1	5.6	5.5
	PAR	8.5	8.2
	Crawley	6.5	7.2

TABLE 8
COMPARISON OF NEUTRALIZATION TITRES IN CAM CULTURE AND EGG

Virus	Antiserum	Antibody titre[a] in egg	Antibody titre[a] in culture	Log $TCID_{50}$ used
PR8	PR8	3.2	3.2	2.1
FM1	FM1	2.8	2.9	1.9
PAR	PAR	2.9	2.9	2.1
Gilbert (LEP)	Gilbert (LEP)	3.3	3.4	2.2
Crawley	Crawley	2.6	3.1	1.9

[a] Expressed as log of the reciprocal of the highest dilution of the serum which neutralized the virus.

in sensitivity for purposes of virus titration of the different strains tested.

Suitability of medium V for neutralizing antibody titration

Since the tissue culture method employing medium V was found to be as good as the egg for titration of influenza virus, experiments were undertaken to determine whether it would be suitable for titration of neutralizing antibody against the virus. For this purpose the method used for the titration of poliomyelitis antibody in tissue culture was adopted and the antibody titres of animal sera immunized against antigenically different strains were determined in tissue culture as well as in the egg. The sera were treated with M/90 potassium periodate to remove the non-specific inhibitors. The results with five antigenically different strains are given in Table 8.

The data indicated that the method was comparable to the egg for purposes of titration of the neutralizing antibody level in sera and that it should prove useful in the determination of antibody levels in vaccine trials.

Serial passage of virus

The Gilbert (LEP) virus was serially passaged in CAM tissue culture using medium V. The initial titre of the virus was $10^{5.2}$ per ml and after 15 serial passages its titre was found to be $10^{6.2}$, indicating that the virus could be propagated serially.

Cultivation of influenza virus in large volumes

The results of an experiment in which an attempt was made to cultivate Gilbert (LEP) virus in different volumes are presented in Table 9. The proportions of the medium and CAM used were kept constant.

The data indicated that as long as the proportion of the ingredients used was maintained good results could be obtained even when the virus was grown in large amounts.

The experiment was repeated by trying to grow PR8, FM1, PAR, Gilbert (LEP) and (HEP) and Crawley viruses in 150-ml quantities. The results are given in Table 10.

It will be seen that with the method described equally good results could be obtained with all the strains tested.

Haemagglutination and virus titres of influenza virus strains cultivated in 350-ml amounts

The PR8, FM1, PAR, Gilbert (LEP) and (HEP) and Crawley viruses were cultivated in 350-ml quantities in 1000-ml bottles containing glass wool. The proportion of the various ingredients used was maintained. Specimens from each bottle were collected at 0, 24, 48, 72 and 96 hours and titrated for haemagglutinin and virus content. The $TCID_{50}$/HA

TABLE 9
HAEMAGGLUTINATION TITRES OF VIRUS GROWN IN DIFFERENT VOLUMES

Virus	Culture in	Quantity (ml)	HA titre at hours:				
			0	24	48	72	96
Gilbert (LEP)	Roller tube	1.5	2	32	32	32	48
	Bottle	10.0	2	48	48	64	48
	Bottle	20.0	2	64	64	64	48
	Bottle	150.0	2	24	48	48	64

ratios were calculated. The results are presented in Table 11.

With 350 ml the results were comparable to those obtained with smaller amounts.

Generally there was a steady rise in the HA and virus titres and $TCID_{50}$/HA ratios in the first 48-72 hours and then there was a tendency to fall. The high values obtained for the $TCID_{50}$/HA ratios with all the strains used indicated that in each case complete virus was being produced.

Production of complement-fixing antigen

The materials collected in the previous experiment, in which 350-ml quantities of different viruses were grown, were tested for complement-fixing antigen. As mentioned earlier human convalescent serum was used with type A viruses and guinea-pig serum against type B virus. These were compared with the corresponding infected allantoic fluids. The results are given in Table 12.

The results indicated that the culture virus compared favourably with the virus produced in the egg as CF antigen.

TABLE 10

HAEMAGGLUTINATION TITRES OF DIFFERENT STRAINS OF INFLUENZA VIRUS GROWN IN 150-ml AMOUNTS

Virus	Total HA units in inoculum	HA titre of culture at hours:				
		0	24	48	72	96
PR8	118	0 [a]	96	128	128	96
FM1	38	0	16	24	32	16
PAR	118	0	196	196	256	196
Gilbert (LEP)	38	0	24	196	128	96
Gilbert (HEP)	230	0	32	256	196	196
Crawley	154	0	12	64	96	96

[a] No haemagglutination with undiluted material.

TABLE 11. HAEMAGGLUTININ AND VIRUS TITRES OF DIFFERENT STRAINS OF INFLUENZA VIRUS CULTIVATED IN 350-ml AMOUNTS

Virus	Total HA units in inoculum	0 hour		24 hours			48 hours			72 hours			96 hours		
		HA/ml	log $TCID_{50}$/ml	log HA/ml	log $TCID_{50}$/ml	log $TCID_{50}$/HA	log HA/ml	log $TCID_{50}$/ml	log $TCID_{50}$/HA	log HA/ml	log $TCID_{50}$/ml	log $TCID_{50}$/HA	log HA/ml	log $TCID_{50}$/ml	log $TCID_{50}$/HA
PR8	274	0 [a]	4.6	2.1	6.6	4.5	2.4	7.2	4.8	2.7	6.6	3.9	2.4	5.8	3.4
FM1	90	0	3.5	1.8	5.6	3.8	2.1	6.2	4.1	2.1	6.2	4.1	2.0	5.6	3.6
PAR	274	0	4.5	2.3	6.8	4.5	2.7	7.2	4.5	2.7	6.8	4.1	2.7	6.6	3.9
Gilbert (LEP)	90	0	3.4	2.1	7.2	5.1	2.4	7.7	5.6	2.6	8.2	5.6	2.9	7.2	4.3
Gilbert (HEP)	538	0	3.63	2.1	6.6	4.5	2.4	7.2	4.8	2.6	7.2	4.6	2.4	6.6	4.2
Crawley	358	0	3.63	1.5	5.5	4.0	2.1	5.8	3.7	2.3	6.2	3.9	2.2	5.5	3.2

[a] No haemagglutination with undiluted material.

TABLE 12. COMPLEMENT-FIXATION TITRES OF INFLUENZA VIRUS STRAINS CULTIVATED IN 350-ml AMOUNTS

Virus	CF titre [a] with culture virus at					CF titre [a] with infected allantoic fluid
	0	24	48	72	96	
PR8	< 4	32	32	32	16	32
FM1	< 4	32	32	64	32	48
PAR	< 4	32	32	32	8	32
Gilbert (LEP)	< 4	8	8	16	16	8
Gilbert (HEP)	< 4	8	16	16	16	8
Crawley	< 4	8	16	16	16	16

[a] Expressed as reciprocal of serum dilution.

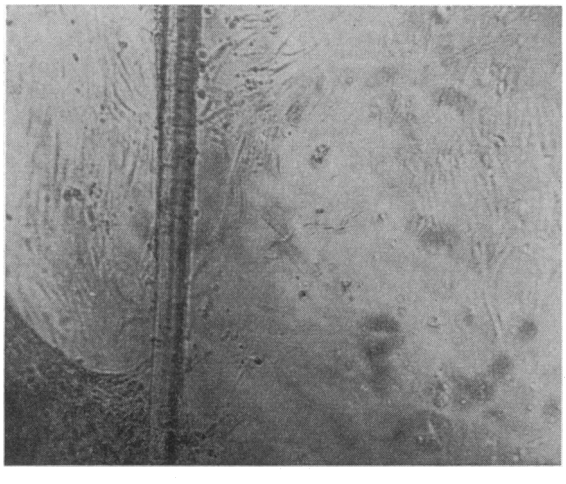
DISCUSSION

Eaton et al. (1960) reported that with minced CAM tissue cultures using the sodium-pyruvate glucose medium described by them, the virus titres were about one log value less than the egg titres. Our studies with the low egg passage Asian virus indicated that the titres in tissue cultures with the above medium were about 2 log values less. System-

atic studies were undertaken to improve the medium so that the results with CAM tissue cultures were as good as those obtained in the egg.

Early in these investigations it was observed that low haemagglutination titres were associated with clumping of the CAM fragments. Attempts were made to prevent the clumping by coating the bottom of the roller tubes with agar and by the addition of small sterile beads. The results with both the methods were unsatisfactory. Glass wool was tried next and gave excellent results. The fragments of membrane adhered to the glass wool and in bottles they formed almost a sheet. After an hour in the roller drum all the pieces of membrane were found adhered to the glass wool, leaving the medium clear. Microscopic studies indicated that the fragments of membrane attached to the glass wool showed a certain degree of fibroblastic and epithelial cell proliferation (Fig. 4). The success of the method reported was in a large measure due to the use of glass wool. On going through the literature it was found that the use of glass wool as a matrix for supporting growing tissue fragments in place of the standard clot method has been reported by Frisch (1952).

FIG. 4. PHOTOMICROGRAPH OF CHORIOALLANTOIC MEMBRANE ATTACHED TO A FIBRE OF GLASS WOOL



Fibroblastic proliferation can be seen around the glass wool.

A large number of compounds singly and in different concentrations were tested for their growth-promoting properties. It was found that a medium containing sodium pyruvate, glycine, sodium molybdate, folic acid and glycerol and another containing sodium pyruvate, glycine, glycerol, pimelic acid and putrescine gave very good results. In view of the possibility of putrescine being toxic to human beings, the former medium was selected. The observation that glycerol could replace glucose in the medium was similar to that reported by Levine et al. (1956) in their studies with NWS strain of influenza virus.

The method described for the cultivation of the influenza virus is simple and elegant. Medium V is easy to prepare and gives good results with the different strains of virus used in this study.

The results indicate that the CAM tissue culture method using medium V is comparable to the egg in its sensitivity for purposes of titration of different strains of influenza virus and their neutralizing antibodies. It is economical inasmuch as about 25 culture tubes can be put up with CAM obtained from one egg. The saving in the number of eggs required, in incubator space and in the labour involved in handling a large number of eggs before and after inoculation is obvious. For instance, in a small roller drum it is possible to produce about 1750 ml of virus, which would otherwise mean inoculation, incubation and collection of allantoic fluid from about 500 eggs. Further, the variations due to differing susceptibilities of eggs can be avoided by employing this method.

The greatest advantage of this method is the possibility of growing different strains of influenza virus in large quantities in a short time. This should prove useful in the large-scale production of influenza vaccine.

Our preliminary studies show that the protein content of the culture material is low compared with that of allantoic fluid, indicating that the culture virus is relatively pure. This would mean that the difficulties usually encountered in the concentration and purification of the infected allantoic fluids could be greatly reduced. It should also be possible to use a relatively pure antigen like the culture virus for purposes of live virus immunization against influenza.

The data presented indicate that this method could be used for the production of good complement-fixing antigens for diagnostic purposes.

It is obvious that with suitable alterations in the medium this method developed by Veeraraghavan could also be used for the cultivation of other viruses which grow in the chorioallantoic membrane or allantoic cavity of the egg. Veeraraghavan (unpublished data) has found that it is possible to cultivate vaccinia virus by this method.

An interesting observation has been that the recent Asian virus isolates could be cultivated readily,

immediately after amniotic isolation. But primary isolation of Asian virus from throat gargles has not been possible so far. Studies to improve the medium to make primary isolation possible are in progress.

An important finding has been that the mouse-adapted Asian strains could be readily cultivated by this method.

The growth cycle of different strains of influenza virus cultivated in large volumes under these conditions and the immunizing value of the culture virus will be reported.

Investigations are also in progress to determine whether the method could be used for the production of interferon.

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RÉSUMÉ

Depuis 1959, des essais sont en cours à l'Institut Pasteur de Coonoor (Centre de la Grippe pour l'Inde), en vue de mettre au point une méthode de multiplication du virus grippal *in vitro*, qui donne une abondante récolte d'un virus facile à titrer et aussi sensible aux anticorps que le virus cultivé sur œuf, compte tenu des expériences d'autres chercheurs dans ce domaine.

Les recherches ont abouti à l'adoption d'une culture de tissu de membrane chorio-allantoïdienne, nourrie par un milieu contenant du pyruvate de sodium, de la glycine, du molybdate de sodium, de l'acide folique et de la glycérine (remplaçant le glucose). Le virus obtenu est d'une sensibilité égale à celle du virus cultivé sur œuf. Cette méthode est économique: la membrane chorio-allantoïdienne d'un seul œuf permet de préparer 25 tubes de culture. La variable provenant de la différence de sensibilité individuelle des œufs est ainsi éliminée. Il est aussi possible de préparer simultanément de grandes quantités de virus de diverses souches en peu de temps, ce qui est un grand avantage pour la préparation massive de vaccin antigrippal. La préparation d'environ 1750 ml de virus dans les tubes de culture d'un seul tambour rotatif remplace l'inoculation, l'incubation et la récolte des membranes de quelque 500 œufs. La culture du virus ainsi obtenue est relativement pure. Les manipulations en vue de la concentration et de la purification du liquide allantoïdien infecté peuvent être considérablement réduites. L'emploi d'un virus préparé par cette méthode pour la vaccination par virus vivant présenterait des avantages. Le virus se prête fort bien également à la réaction de fixation du complément. Il est vraisemblable que moyennant des modifications appropriées du milieu, on pourra cultiver d'autres virus selon ce procédé. La souche asiatique isolée au cours de la dernière pandémie a pu être cultivée facilement sur ce milieu après isolement sur amnios, mais on n'a pu réussir encore de culture primaire à partir de lavages de gorge. On cherche à y parvenir en modifiant la composition du milieu. Les souches asiatiques du virus grippal adaptées à la souris ont pu, elles aussi, être facilement cultivées sur culture de tissu de membrane chorio-allantoïdienne.

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