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THE SEROLOGICAL RELATIONSHIP OF SOUTH AFRICAN BOVINE ENTEROVIRUS STRAINS (ECBO SA-I AND -II) AND THE GROWTH CHARACTERISTICS IN CELL CULTURE OF THE PROTOTYPE STRAIN (ECBO SA-I)

D. W. VERWOERD, R. A. OELLERMANN, J. BROEKMAN and K. E. WEISS,
Veterinary Research Institute, Onderstepoort

INTRODUCTION

A few members of the picornavirus family have been studied in great detail, especially polio- and other human enteroviruses and the Columbia SK-group of mouse enteroviruses (ME-, EMC- and Mengoviruses).

Many bovine enteroviruses have been isolated and a considerable amount of work has been done on their serological relationship and classification (Kalter, 1960; Bürki, 1962; Falk, 1964; La Placa, Portolani & Lamieri, 1965).

Though usually isolated from normal animals and considered as orphan viruses, some indications have been obtained that bovine enteroviruses might play a role in outbreaks of mucosal, respiratory and reproductive disorders (Moll & Finlayson, 1957; Bögel & Mussgay, 1960; Huck & Cartwright, 1964).

Only recently has some attention been paid to physico-chemical characteristics (Polson & Kipps, 1965) and comparative growth characteristics in different cell cultures (Yamada, 1965). No comprehensive study including serological, morphological, physico-chemical and biochemical growth characteristics of any one of the bovine enteroviruses has been reported however.

Although the enteroviruses are known to be very similar in most of their characteristics, regardless of the host from which they are derived, it was nevertheless considered of interest to undertake such a study for comparative purposes. The results of such an investigation of a locally isolated bovine enterovirus, designated ecbo-virus type SA-I, are reported in this and in an accompanying paper (Oellermann, Els & Verwoerd, 1967).

Because of their relatively simple morphology, short replicative cycle, wide range of host cells and their stability, the enteroviruses have been the most commonly used animal virus in replication studies. Ecbovirus SA-I, therefore, promised to be as good a model as any other for investigating the basic biochemical mechanisms involved in viral replication in animal cells.

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Material and Methods

Cell Cultures

(*a*) Monolayer cultures of primary lamb kidney cells in roller tubes were prepared by overnight trypsinization according to the method of Bodian (1956). The cells were cultivated and maintained in a modified Hank's balanced salt solution with 0·5 per cent lactalbumin hydrolysate and 10 per cent bovine serum (Weiss & Geyer, 1959). For virus propagation, serum was omitted from the medium.

Monolayers on coverslips were prepared in a similar manner in 60 mm petri dishes kept in an atmosphere of 5 per cent CO_2 in air.

(*b*) The following cell lines were used during the course of the present investigation:

BHK-21, obtained from Prof. M. P. G. Stoker, Glasgow.

BHK-A, an ascites adapted variant of BHK-21 kindly provided by Dr. F. K. Sanders, Carshalton.

Ehrlich ascites carcinoma of mice, and L-strain mouse fibroblasts kindly supplied by Prof. W. Schäfer, Tübingen.

Monolayers of BHK-21, BHK-A and L-cells were cultivated in Roux flasks using the tryptose-phosphate-broth containing Eagle's medium described by MacPherson & Stoker (1962) with 10 per cent bovine serum. Recently confluent monolayers were used on about the third or fourth day after seeding.

Suspension cultures were prepared in spinner flasks, using the same medium with or without serum.

Ehrlich ascites tumour cells were propagated by weekly passages in mice from a highly inbred colony maintained at Onderstepoort. For experimental use, cells were washed and suspended in Earle's saline according to the procedure described by Martin, Malec, Sved & Work (1961).

Viable cell counts were made after staining with 1 per cent nigrosin in PBS (phosphate buffered saline).

The origin of the Virus Strains and their Propagation

(*a*) *Ecbo SA-I*

This virus strain was isolated during the course of experiments to determine the distribution of lumpy skin disease virus in experimentally infected cattle. (Alexander, Weiss & De Lange, 1958.)

In addition to other specimens, rectal swabs were taken from three of the experimental animals at intervals varying from one to three days. The rectal swabs were rinsed in 2·0 ml of tissue culture medium containing sodium penicillin (2000 iu per ml), streptomycin sulphate (2000 μgm per ml), neomycin sulphate (1250 μgm per ml) and amphotericin B (50 μgm per ml). After a contact period of one hour, the suspension was clarified by centrifugation at 3000 rpm for 15 minutes and the supernatant fluid seeded into monolayer cultures of primary lamb kidney cells in the following manner. After removal of the nutrient medium, cultures were infected with an appropriate volume of the virus suspension (0·2 ml for roller tubes and 2·0 ml for coverslips in 60 mm petri dishes). After adsorption for one hour at 37° C the original volume of medium without serum was replaced (1·0 ml for roller tubes and 8·0 ml for petri dishes). The cultures were incubated at 37° C and examined daily for cytopathic effects.

From the rectal specimens of one of the experimental animals (bovine 8582) the ecbovirus SA-I was consistently recovered over a period of 18 days. It was never recovered from the blood, saliva or skin. The animal was sacrificed on the 24th day and the presence of the virus was demonstrated in the caecal contents, but not in the small intestinal and ruminal contents, skeletal muscles, lymph glands or salivary glands.

The cytopathic effect of ecbovirus SA-I in tissue culture has been described by De Lange (1959).

Stocks of the second lamb kidney tissue culture passage of the original ecbovirus SA-I was freezedried and kept in sealed ampoules at —20° C. This material was passaged twice in primary lamb kidney roller tube cultures before being used in the experiments reported in this paper. Initially, bulk production of ecbovirus SA-I was carried out in suspensions of Ehrlich ascites cells at a concentration of 1×10^6 per ml in Earle's saline. Subsequently, virus was produced in monolayers of BHK-21, BHK-A and L cells in Eagle's medium plus tryptose-phosphate-broth but without serum. Cells were infected at an input multiplicity of 10 pfu per cell and harvested 20 to 24 hours after infection.

(*b*) The Mason strain of bovine enterovirus, provisionally named ecbovirus SA-II, was isolated from a semen specimen of a bull in the Heilbron district of the Orange Free State, during the course of investigations into infectious infertility in cattle. The bull was suffering from orchitis at the time, but there is no evidence to suggest that the virus was responsible for the pathological changes in the genitalia of the animal.

The virus was isolated in monolayer cultures of primary calf kidney cells and produced a cytopathic effect similar to that caused by other bovine enteroviruses. The virus was stored, as infected tissue culture fluid, in sealed ampoules at — 70° C. Before being used in the present series of experiments, the virus was passaged twice in primary lamb kidney cultures.

(*c*) The California I and II virus strains were received from Dr. D. G. McKercher, University of California. Both these strains were presumably isolated from the vaginal tract of cattle with vaginitis. The original material, which was stored in sealed ampoules at — 70° C, was passaged twice in primary lamb kidney tissue cultures before being used in the experiments described in this paper.

Antisera

Bovine enterovirus type antisera were kindly supplied by Dr. R. A. Huck, Weybridge, Surrey. Antisera to poliovirus types I, II and III and pools of echovirus types 1–32 were provided by Dr. Malherbe of the Poliomyelitis Research Institute, Johannesburg. Antiserum against ME-virus was obtained from Prof. W. Schäfer, Tübingen.

For the production of an antiserum against ecbovirus SA-I, a rabbit was immunized by intramuscular injections of 1·0 ml of highly purified ecbovirus (Oellermann *et al.*, 1967) mixed with 3·0 ml Freund's incomplete adjuvant. A course of three monthly injections was given and the animal was bled for serum 14 days after the last injection.

The sera were stored at — 18° C, until used.

Quantitative virus assay

The procedures adopted for cell death titrations and plaque assays were essentially those of Sanders (1957) as described by Martin *et al.* (1961).

Difco agar, washed three times with distilled water, once with acetone and then dried at 70° C, was used at a concentration of 1 per cent in Earle's saline as a base layer (5 ml per 60 mm petri dish). The cell suspension layer consisted of 1·8 ml of 0·8 per cent agarose (Seravac, Cape Town) in Earle's saline, mixed with 1·1 ml ascites cells suspended in Earle's saline (8×10^7 cells/ml) and 0·1 ml virus dilution per plate. Plates were incubated at 37° C in a 5 per cent CO_2 atmosphere for three days, stained with neutral red and the plaques counted one hour later.

Neutralization tests

The 80 per cent plaque depresssion test described by Huck & Cartwright (1964), was employed.

From stock 1: 10 dilutions of the antisera which were inactivated at 56° C for 30 minutes, fourfold dilutions were prepared in Earle's saline. To each serum dilution an equal volume of virus, diluted in Earle's saline to give an estimated 100 to 150 pfu/ml, was added and the mixtures incubated at 37° C for two hours; 0·2 ml of inoculum was used per plate in the subsequent plaque-test. Controls were included in each experiment. Titres were expressed as the reciprocal of the antiserum dilution that depressed the plaque count by 80 per cent.

Histochemical Staining Methods

Acridine orange: the staining method of Dart & Turner (1959) was used, employing the 5-phenyl derivative Acridine Orange R. (Edward Gurr Ltd.—Michrome No. 711.)

The Schiff-Methylene blue reaction: The fixation and staining technique described by Spicer (1961) was used, except that the fixation period in Bouins fluid was prolonged to at least 48 hours to ensure optimal hydrolysis of DNA.

The Feulgen reaction: The staining procedure described by Pearse (1961) was used.

For the fluorescent Feulgen reaction the method of Culling & Vassar (1961) was employed.

Isotope incorporation studies

The procedure followed in labelling experiments was essentially as described previously (Hausen & Verwoerd, 1963). In order to minimize changes in cellular metabolism due to handling, L-cells growing in suspension in a spinner flask were substituted for the monolayers used initially. Cells were washed once in serum-free medium and resuspended at a concentration of 1×10^7 cells per ml.

For infection, a concentrated virus preparation was added to give an input multiplicity of 100 pfu per cell. Adsorption of virus was allowed to take place for 15 minutes at 37° C. The cells were then sedimented at low speed to get rid of excess virus, washed once in serum-free medium and finally suspended in this medium at a concentration of 2×10^6/ml and incubated at 37° C in an atmosphere containing 5 per cent CO_2. This represents time zero.

Every 30 minutes 1·0 ml samples of the cell suspensions were pipetted into a prewarmed test tube containing 0·1 ml of a mixture of labelled uridine and leucine. The concentrations of the labelled precursors giving suitable counts (given in the legends to the figures) were determined in preliminary experiments with normal cells. The tubes were flushed with 5 per cent CO_2, stoppered and incubated in a " roller tube " apparatus. After 15 minutes the cells were precipitated and incorporation stopped by the addition of 5·0 ml ice-cold 4 per cent trichloracetic acid (TCA). After standing for at least 30 minutes at 4° C, the precipitate was collected on " Millipore VC " filters, washed three times with 4 per cent TCA, twice with 4 per cent acetic acid and dried in an oven. The samples were then counted in a toluene-scintillator solution in a Packard scintillation counter.

To reduce the effect of experimental variations, the results, representing the rate of precursor incorporation, are given as the ratios of counts in infected to those in non-infected cells.

Results

Multiplication of Ecbovirus SA-I in different cell types

In addition to the primary lamb kidney cells used for isolation, the virus was also grown successfully in lamb testis and calf kidney cells (De Lange, 1959), and in the following permanent cell lines: BHK-21, BHK-A, Ehrlich ascites carcinoma and L-strain mouse fibroblasts.

Roughly equivalent amounts of virus, at an average final concentration of 2×10^8 pfu per ml, were obtained on the latter four cell types, corresponding to a yield of about 120 pfu per cell. The use of different cell types for different purposes was dictated by practical considerations. For example, for bulk virus production BHK or BHKA monolayer cultures were used because of their very rapid growth characteristics. Suspension cultures of all the above cell types were prepared, but adaptation to large scale virus production was unsuccessful.

For plaque assays the use of either L-cells or ascites cells in agarose-suspension gave the best results, and the latter was therefore selected for routine use because of the relative ease of production of large numbers of cells.

For isotope labelling experiments, where it is essential to disturb the normal metabolism of the cells as little as possible during handling, suspension cultures of L-cells were found to be most suitable.

Quantitative assay

No hemagglutination was found with the virus strain studied. For rapid results the cell death titration method was therefore used. For more accurate determinations of virus concentration, however, a plaque assay, based on the procedure of Martin *et al.* (1961) and described under methods, was used. Plate 1 illustrates the typical plaque morphology of ecbovirus SA-I.

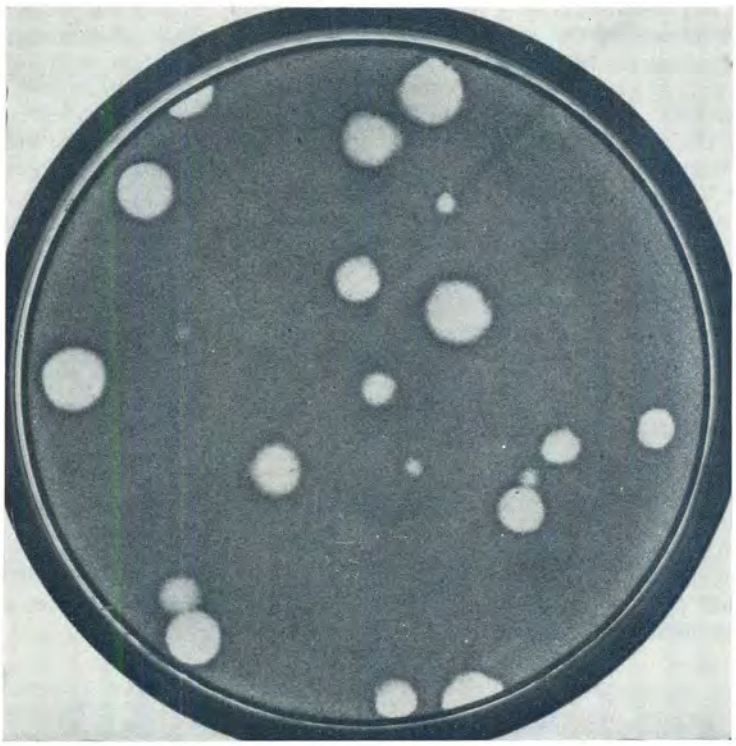

PLATE 1.—Plaque morphology of ecbovirus type SA-I in an agarose suspension of L-strain fibroblasts

Large and small plaques were always present, but all attemps to isolate pure variants failed.

The linear relationship between plaque numbers and relative virus concentration, as well as the small variation between triplicate counts, are evident from Table 1.

TABLE 1.—*Typical plaque count*

Dilution	Counts in triplicate			Average count	Titre
5×10^{-5}	112	106	109	109	$5{\cdot}5 \times 10^{8}$
2×10^{-6}	21	21	29	24	$4{\cdot}8 \times 10^{8}$
1×10^{-7}	6	3	4	4·3	$4{\cdot}3 \times 10^{8}$
5×10^{-7}	1	2	1	1·3	$6{\cdot}5 \times 10^{8}$

Serological Relationship

The serological relationship between ecbovirus SA-I, ecbovirus SA-II, the two Californian strains, Cal. I and Cal. II, and the bovine enterovirus type strains of Huck & Cartwright (1964) were investigated by means of the 80 per cent plaque depresssion technique. A pooled poliovirus antiserum (types I, II and III), as well as pools of all echovirus antisera were included in the series to detect any possible cross reaction with related human viruses.

The results are summarized in Table 2. Ecbovirus SA-I shows a cross reaction only with the Weybridge type 134, whereas ecbovirus SA-II, Cal. I and Cal. II strains are related to the serotype M63 and its subtypes FS and VG(5) 27. No relationship to any human type or to ME-virus, representative of the Columbia SK-group, could be detected. Subcutaneous injection of virus concentrates into baby mice produced no symptoms of disease.

TABLE 2.—*Serological relationship between the enteroviruses investigated*

Antiserum	Virus			
	Ecbo SA-I	Ecbo SA-II	Cal. I	Cal. II
Ecbo SA-I	*5120*	40	10	<10
F 226 A	<10	40	40	<10
M 80	10	40	10	10
M 6	10	<40	10	10
T 10	10	40	10	10
M 153	40	40	<40	<40
VG(5)27	10	*640*	*640*	*2560*
M 63	10	*160*	*160*	*160*
M 134	*160*	40	<10	10
F 46	<10	40	10	10
FS	10	40	*160*	*640*
T11F	40	40	<40	10
Polio types I, II & III	40	—	—	—
Ecbo types	<10	—	—	—
ME (Columbia SK)	<10	—	—	—

Intracellular site of virus formation

The localization of the site of virus replication in the cell was attempted by histochemical methods. Infected monolayer cultures, stained with acridine orange and examined microscopically with ultraviolet light, showed intracytoplasmic masses with intensive orange-red RNA fluorescence which was absent in control cells. Staining with Schiff's methylene blue demonstrated that infected cells develop blue-staining masses in the cytoplasm, which eventually displace the nucleus to one side of the cell where it diminishes in size until only a dark staining crescent is left. No increase in DNA synthesis could be detected by Feulgen or fluorescent Feulgen staining. There is little doubt, therefore, that the virus contains RNA and multiplies in the cytoplasm of its host cell.

Growth characteristics

Fig. 1 summarizes the results of a single step growth curve experiment, using the cell-death titration method for the assay of virus production in L-cells. A lag phase of four to five hours is followed by a logarithmic growth phase until about 90 per cent of the cells are dead at 14 hours after infection.

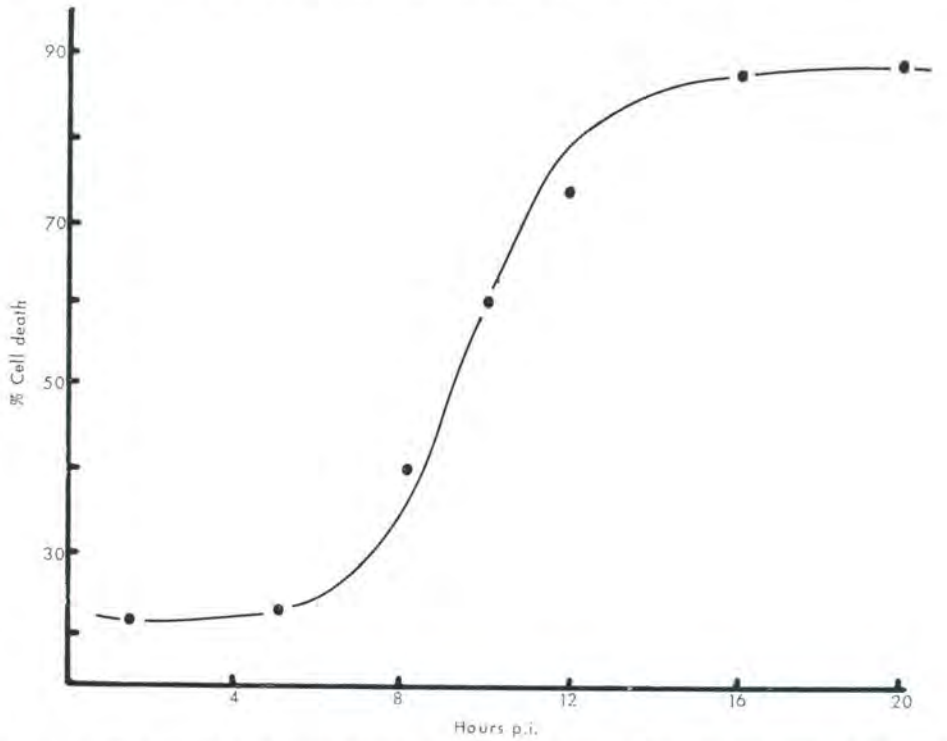

FIG. 1.—Growth curve of ecbovirus SA-1 in L-strain fibroblasts, determined by means of cell death titration

The effect of some known inhibitors of protein and nucleic acid synthesis on virus replication is shown in Table 3. Washed ascites cells at a concentration of 2×10^5/ml were infected with ecbovirus at an input multiplicity of 10 pfu/cell. The infected cells were incubated for 10 minutes at 37° C, sedimented by low speed centrifugation and resuspended in Earle's balanced salt solution containing the inhibitors at the final concentrations indicated.

TABLE 3.—*Effect of inhibitors on ecbovirus multiplication*

Inhibitor	Concentration	Virus yield (% of control)
Control	—	100·0
Actinomycin D	20 μgm/ml	65·0
FPA	20 μgm/ml	7·5
Puromycin	100 μgm/ml	2·5
Guanidine-HCl	100 μgm/ml	3·5

Actinomycin-D, known to inhibit DNA-dependent RNA synthesis but not single-stranded RNA-dependent RNA synthesis (as in the case of enterovirus-induced nucleic acid synthesis) has only a slight effect on ecbovirus SA-I production. Puromycin inhibits protein synthesis and therefore also the production of complete virus particles. Fluorophenylalanine is incorporated into proteins instead of the normal analogue, leading to the formation of inactive virus-induced enzymes (Scholtissek & Rott, 1961) and preventing viral replication in this way.

Guanidine has been found to inhibit the growth of some enteroviruses while others, in some instances mutants of the same virus, are either insensitive or even dependent on the substance for replication (Tamm & Eggers, 1963). The experimental results presented indicate that the ecbovirus, as used in these experiments, is sensitive to guanidine.

Synthesis of viral components

The rates of RNA and protein synthesis in virus-infected cells were determined by the double pulse-labelling technique previously described (Hausen & Verwoerd 1963). The rate of formation of free infective particles was determined simultaneously by means of plaque-titration.

These experiments yielded curves similar to those obtained for other enteroviruses (Fig. 2). Immediately after infection the rate of both C^{14}-uridine and H^3-leucine incorporation decreases dramatically. Previous work on ME-virus has shown this decrease to be the result of an inhibition of cellular synthesis mediated by virus-induced inhibitors. There is no reason to doubt the existence of a similar mechanism in the case of ecbovirus-infected cells.

Between two and four hours after infection, apparently depending on both the type of cell used and the physiological condition of the culture, there is a sharp rise in the rate of uridine incorporation. This is followed half to one hour later by a similar, though less apparent increase in the rate of amino acid incorporation. The appearance of infective virus immediately afterwards strongly suggests that these peaks represent the synthesis of virus-specific RNA and protein respectively.

Effect of guanidine on the inhibition of cellular synthesis

Guanidine-HCl at a concentration of 100 mg/ml inhibits ecbovirus SA-I replication almost completely. According to Tamm & Eggers (1963) this effect is mainly due to an inhibition of viral RNA synthesis. Furthermore, the substance has no direct effect on cellular RNA or protein synthesis. Guanidine is therefore a suitable reagent to determine whether or not viral replication is a prerequisite for the inhibition of cellular synthesis of RNA and protein.

Guanidine-HCl at a final concentration of 100 μgm/ml was therefore added to a suspension culture of L-cells at the time of infection and the rate of C^{14}-leucine and H^3-uridine incorporation determined.

The results, shown in Fig. 3, indicate that both protein and RNA synthesis are still inhibited under these conditions. Replication of the infecting virus is therefore not necessary for the formation of the virus-specific inhibitors of cellular processes.

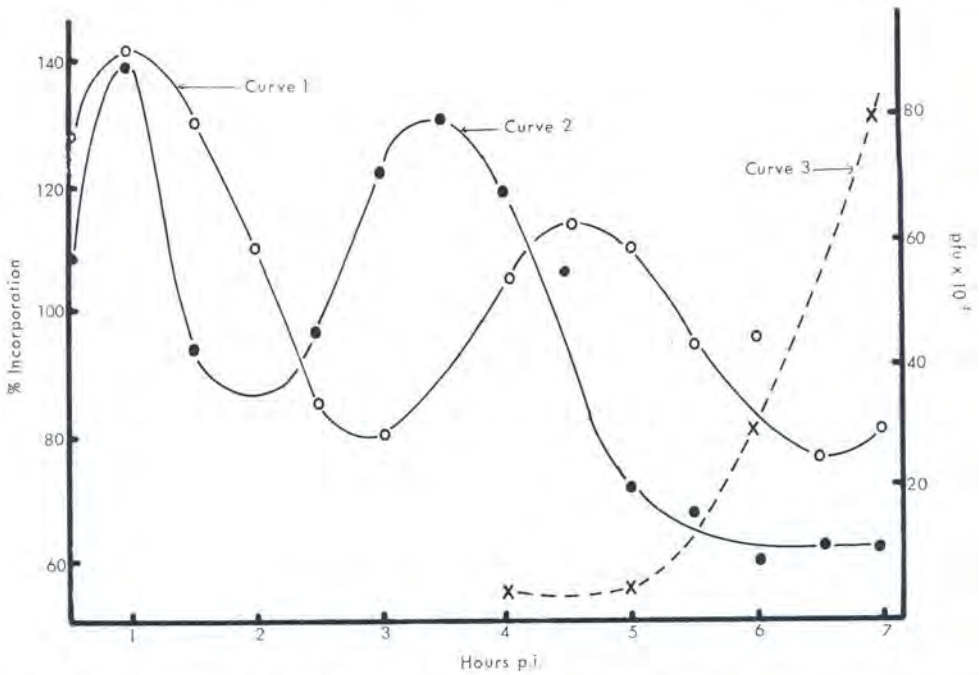

FIG. 2.—Rate of C^{14}-uridine and H^3-leucine incorporation in ecbo-infected L-strain fibroblasts. Rates are expressed as the percentage incorporation (during a 15 minute pulse) of the incorporation in uninfected control cells. C^{14}-uridine was used at a concentration of 0·1 μc/ml and H^3-leucine at 5 μc/ml cell suspension. Curve 1: Rate of H^3-leucine incorporation. Curve 2: Rate of C^{14}-uridine incorporation. Curve 3: Formation of infective virus particles (plaque-forming units)

DISCUSSION

Ecbovirus SA-I, an enterovirus isolated from a normal bovine, was found to be serologically related to the bovine serotype 134 of Huck & Cartwright (1964) but no relationship to any other enterovirus type could be demonstrated. In all other respects, however, the virus is very similar to those members of the enterovirus family that have been studied extensively, i.e. poliovirus and the encephalomyocarditis-, ME- and Mengo-viruses.

A very wide range of cultured cell types are susceptible to the virus, all showing marked cytopathic effects. Like all enteroviruses, ecbo SA-I is produced in high titre, and has a relatively short replicative cycle. Isotope incorporation studies indicate that the growth cycle is perhaps somewhat slower than that of polio- and ME-viruses, but variations due to cell type used might have been responsible for the differences found.

As in the case of the other enteroviruses, ecbovirus SA-I has a marked effect on cellular RNA and protein synthesis, inhibiting both reactions immediately after infection. It has been shown in the case of ME-virus that this inhibition is caused by protein inhibitors synthesized within 30 minutes after infection of the cell. One problem that remained unsolved was whether the virus genome, after entering the

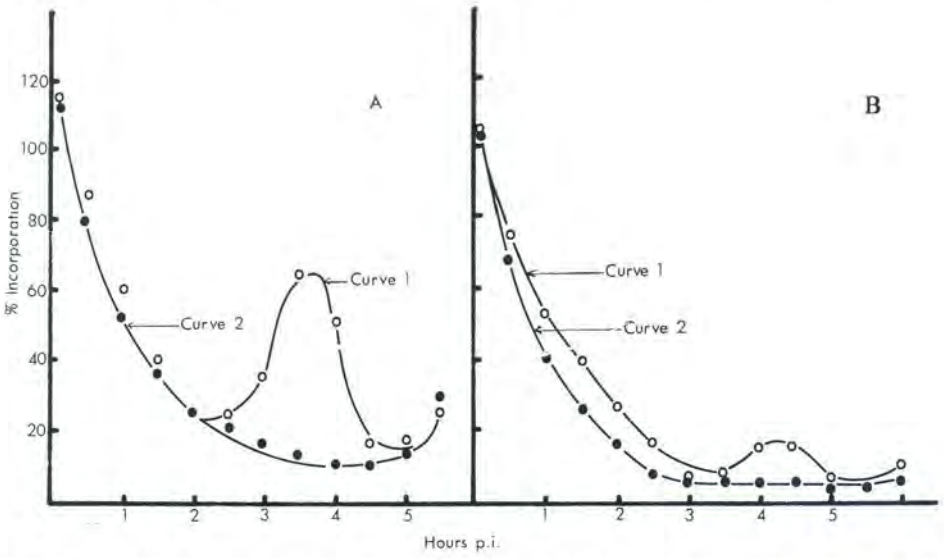

FIG. 3.—A: Rate of C^{14}-uridine incorporation, B: rate of H^3-leucine incorporation in ecbo-infected L-strain fibroblasts. Curve 1: In the absence of guanidine. Curve 2: In the presence of guanidine-HCl at a concentration of 100 μgm/ml cell suspension. For isotope concentrations see Fig. 2.

cell, can code directly for the inhibitors, or has to replicate first. An answer to this question, at least in the case of ecbovirus, was obtained by using guanidine. This substance inhibits viral RNA synthesis without affecting the cellular metabolism in any way. In the presence of guanidine, ecbo-infected L-cells do not produce any virus, but the inhibition of cellular RNA and protein synthesis is as complete as in normally infected cells. Replication of the infecting virus is, therefore, not a prerequisite for the formation of the inhibitors.

Since completion of these experiments similar results obtained with poliovirus have been published (Holland, 1964; Penman & Summers, 1965).

SUMMARY

A locally isolated bovine enterovirus designated ecbovirus SA-I was found to belong to the Weybridge serotype 134. The growth characteristics of the virus in a number of cell types were studied. Its replicative cycle and its effect on the metabolism of the host cell, studied by means of isotope-labelling techniques, closely resemble those of other enteroviruses. It was shown that the inhibitors of cellular RNA and protein synthesis are produced normally even if replication of the infecting virus is blocked by guanidine.

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