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Studies on Protein and Nucleic Acid Metabolism in Virus-Infected Mammalian Cells

2. THE ISOLATION, CRYSTALLIZATION AND CHEMICAL CHARACTERIZATION OF MOUSE ENCEPHALOMYOCARDITIS VIRUS

By P. FAULKNER, E. M. MARTIN, S. SVED, R. C. VALENTINE AND T. S. WORK
National Institute for Medical Research, Mill Hill, London, N.W. 7

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Encephalomyocarditis virus is one of the large group of animal viruses, the Col SK group, rather closely related to poliomyelitis virus. They are generally pathogenic to rodents and occasional infection occurs in man (Jungeblut, 1958). The strain that has now been characterized was obtained through the courtesy of Dr Kingsley Sanders (cf. Hoskins & Sanders, 1957). It is highly pathogenic to mice but, after growth for many generations in Krebs II ascites-tumour cells (Martin, Malec, Sved & Work, 1961*b*), it was incapable of causing infection in rabbits even when massive doses of concentrated virus were injected intracerebrally.

Since we had studied the effect of infection on the nucleic acid and protein turnover of mouse-ascites-tumour cells, and had found that virus infection caused striking changes in the pattern of nucleic acid turnover (Martin *et al.* 1961*b*), we wished to extend these studies and to determine, if possible, the site of synthesis of virus nucleic acid and virus protein within the host cell. As part of this project it became necessary to characterize the virus more fully, and in the present paper we describe methods for large-scale culture of the virus and for purification and crystallization of the

infective particle. The virus is, in the dry state, a spheroid of about 27 m μ diameter. Its nucleic acid and protein have been analysed. A convenient method has also been developed for the preparation of a precipitating antibody of high titre which may be used to flocculate or neutralize the virus. A preliminary report of some of this work has been published (Faulkner, Martin, Sved & Work, 1960).

METHODS

Abbreviations. EMC, Encephalomyocarditis; DEAE, diethylaminoethylcellulose.

Protein estimation. The method of Lowry, Rosebrough, Farr & Randall (1951) was scaled down to a total volume of 0.9 ml. and the extinction of the product was measured at 750 m μ ($E_{1\text{ cm.}}$) against a reagent blank. The method was satisfactory over the range 1–20 $\mu\text{g.}$ of protein. Extinction measurements were made on 0.8 ml. of solution in normal 1 cm. cuvettes fitted with Perspex displacers, as described by Martin & Morton (1956). Since no standard preparation of virus protein was available the method was standardized on host-cell protein prepared as already described (Martin *et al.* 1961*b*).

Total nitrogen. The method of Jacobs (1960) was used. In this method, N is converted into NH_3 and estimated by interaction with ninhydrin. It is sensitive to 1 $\mu\text{g.}$

Total phosphorus. The method of Griswold, Humoller & McIntyre (1951) was used. It was satisfactory with 0.5–5 $\mu\text{g.}$ of P in a final volume of 3–5 ml.

Deoxyribonucleic acid. The method of Burton (1956) was scaled down to a final volume of 0.9 ml. Satisfactory results were obtained with 1–10 $\mu\text{g.}$ of DNA.

Ribonucleic acid. The orcinol method of Hurlbert, Schmitz, Brumm & Potter (1954) was used. The nucleic acid sample (0.3 ml.) was mixed with 0.6 ml. of orcinol reagent (0.4% of orcinol and 0.033% of FeCl_3 , w/v, in 12N-HCl) and heated at 100° for 30 min. Extinction was measured at 675 $m\mu$ in microcells (1 cm. path); the satisfactory range was 2–20 $\mu\text{g.}$ of RNA.

Total nucleic acid. Samples were hydrolysed in 0.5N- HClO_4 at 70° for 30 min. Extinction was measured (after centrifuging) at 260 $m\mu$ and ϵ_p was taken as 10 800 (Beaven, Holiday & Johnson, 1955).

Base analysis of ribonucleic acid. Samples were hydrolysed in N-HCl as described by Smith & Markham (1950), and the products, guanine, adenine, cytidylic acid and uridylic acid, separated by chromatography in *tert.*-butyl alcohol-HCl (cf. Markham, 1954). Individual bases were eluted in 0.1N-HCl and the ultraviolet absorptions of the eluates measured at the appropriate wavelengths. The method gave reproducible results with 250 $\mu\text{g.}$ samples of RNA.

Haemagglutinin assay. The method has been described by Martin *et al.* (1961*b*). In addition to the plate technique, the rapid haemagglutinin assay was particularly useful in following individual steps during the purification of the virus.

Plaque counts. The method of Sanders (1957) was used as already described (Martin *et al.* 1961*b*).

Particle counts. The number of virus particles/ml. was determined by the method of Backus & Williams (1950). The virus suspension was mixed with an equal volume of a suspension of polystyrene latex in the form of spherical particles of a very uniform diameter (0.188 μ); such suspensions were generously supplied by the Dow Chemical Co., Midland, Mich., U.S.A. (Bradford & Vanderhoff, 1955). The latex-particle count was known from the particle size and density and the dry weight of the suspension. The electron microscope was then used to determine the ratio of latex to virus-particle count in the mixed suspension, and the count of virus particles/ml. thus obtained. Sufficient particles were counted to give an accuracy of about 25% (Breese & Trautman, 1960).

Electron microscopy. The virus preparations were examined by the negative-staining method of Brenner & Horne (1959) by a slightly modified technique (Valentine, 1959).

Chromatography on calcium phosphate. Calcium phosphate was prepared by the method of Taverne, Marshall & Fulton (1958). When examined microscopically, freshly prepared calcium phosphate consisted predominantly of spherical microcrystalline aggregates with lesser amounts of relatively larger rhombic crystals. Preparations containing a higher proportion of this latter crystalline material had poorer packing properties and columns prepared from them tended to contract, thus slowing down the eluent-flow rate. The proportion and size of these crystals increased with storage. Therefore, the calcium phosphate was stored at 0° and used within 3 weeks of preparation.

The calcium phosphate was suspended in 0.02M-sodium phosphate buffer (pH 7) and poured into a column (diameter 25 mm.) fitted with a glass-sintered bottom. Sufficient

was added to give a final packed column of 8 cm. The solid was packed under pressure (5 cm. Hg) and washed with 0.02M-buffer (100 ml.). Trypsin-treated virus suspension (10 ml.), and containing 100–200 mg. of protein, was diluted with an equal volume of 0.02M-buffer and added to the column. The flow rate was maintained at about 2 ml./min. and the virus preparation was washed in with 10–20 ml. of 0.02M-buffer. The elution gradient was developed by siphoning 0.5M-sodium phosphate buffer (pH 7) from a 175 ml. conical flask into an open cylindrical mixing chamber of 5 cm. diameter (cf. Boman, 1955). Mixing was started with 90 ml. of 0.02M-buffer in the chamber and the liquid level equal in the two vessels. The first 30 ml. of effluent was collected in one lot and thereafter 30 fractions of 5 ml. were collected. Rapid haemagglutinin assays were performed in each fraction (diluted 1:1000) and those fractions that were obviously rich in virus were assayed more accurately by the standard haemagglutinin method (Martin *et al.* 1961*b*). Fractions were stored at 0° overnight.

Those fractions with a haemagglutinin titre above 25 000 were pooled and dialysed against 0.02M-phosphate on a rocking dialyser for 3 hr., and the dialysed material (usually about 35 ml.) was applied to a second calcium phosphate column and washed in with 50 ml. of 0.02M-buffer. The second column (diameter 15 mm.; length 10 cm.) had a flow rate of 0.5 ml./min. A steep elution gradient of phosphate was established by adding 60 ml. of 0.02M-phosphate (pH 7) to the cylindrical mixing chamber and by siphoning into this 90 ml. of 0.5M-sodium phosphate (pH 7) from a 100 ml. standard volumetric flask. The first 35 ml. of effluent was collected as one batch and the remainder in 2.5 ml. fractions. The rapid haemagglutinin assay was again used to locate those fractions rich in virus.

RESULTS

Large-scale growth of virus

The conditions adopted were essentially those given by Martin *et al.* (1961*b*). For good virus growth in the Krebs II mouse-ascites-tumour cells, it was best to suspend the cells in Earle's medium at a concentration of 2×10^7 cells/ml. at room temperature and to add 3 plaque-forming units of virus/cell. After 30 min. the flasks were transferred to a rotary shaker (70 cyc./min.) and incubated for 18 hr. at 36°. After this time the number of live cells was below 5% and (after removal of the cells) the haemagglutinin titre of the supernatant was about 25 000. It was necessary to maintain a fixed ratio of liquid volume to gas space in the culture flasks and 'penicillin culture flasks' (Laboratory Glassblowers Co. Ltd., High Wycombe, Bucks.) gave excellent results. These flasks are cylindrical, of 17 cm. in diameter and 9 cm. in height, and each flask held 100–150 ml. of Earle's medium.

Partial purification with Arcton 63

Several viruses have been purified by extraction with a fluorocarbon (Gessler, Bender & Parkinson,

1956; Manson, Rothstein & Rake, 1957; Hummeler & Hamparian, 1957). The method depends upon the resistance of virus nucleoprotein to surface denaturation. A suspension of EMC virus prepared as described above was separated from dead cells by centrifuging for 10 min. at 8000g and the supernatant from this recentrifuged at 100 000g (Spinco) for 1 hr. The Spinco pellet was resuspended in phosphate-buffered saline (Martin *et al.* 1961b) and this crude virus preparation was homogenized with the fluorohydrocarbon (Arcton 63; Imperial Chemical Industries Ltd.) as described in Table 1. At each extraction, a large amount of denatured protein was discarded but there was also a considerable loss of haemagglutinin. Moreover, the method did not distinguish between virus particles and ribosomes (the RNA-rich particles of the microsomal fraction of ascites-tumour cells). A

batch of uninfected cells, disrupted by ultrasonics, gave a microsome fraction, which was extracted with Arcton in the same way as the virus culture. The product had virtually the same analytical composition as the Arcton-purified virus (Table 2). Thus Arcton treatment will readily purify the virus about 25-fold, but it will also carry along with the virus non-viral ribonucleoproteins from the host cell.

Partial purification of virus by combined proteolysis and centrifuging

Weil, Warren, Breese, Russ & Jeffries (1952) reported that EMC virus, partially purified from mouse brain, was resistant to digestion with either trypsin or chymotrypsin. Since proteolytic digestion would be expected to remove at least some of the contaminating protein the effect of these two enzymes on EMC virus from ascites-tumour cells was examined. The supernatant from an 18 hr. infected culture, such as that described in Table 1, was spun first at 8000g for 10 min. and then at 100 000g for 1 hr. The pellet collected after high-speed centrifuging was resuspended in sodium phosphate buffer (pH 7.1; 0.02M; 10 ml.), divided into two lots and incubated with either chymotrypsin or trypsin (0.01% of crystalline bovine pancreatic enzyme) for 1 hr. at 37°. The chymotrypsin-treated preparation lost 97% of its haemagglutinating activity whereas there was no measurable loss during incubation with trypsin. Treatment with trypsin was therefore adopted as the first step in large-scale purification of the virus.

The yield of virus and the nitrogen content of the trypsin-treated concentrate varied considerably in successive cultures. The mean specific virus concentration of these concentrates (nine preparations) was 2.0×10^4 haemagglutinin units/mg. of protein. This represented an eightfold purification of the virus from the original infected 8000g supernatant. The yields varied between 7×10^3 and 2.4×10^4

Table 1. *Purification of encephalomyocarditis virus by extraction with Arcton 63*

The virus culture (80 ml.; 1×10^8 cells) was prepared as described in the text. Cells were removed at 8000g (10 min.) and the virus was spun down at 100 000g (1 hr.). The pellet was homogenized with 10 ml. of phosphate-buffered saline (Martin *et al.* 1961b) in a Potter homogenizer and then with an equal volume of Arcton 63 in a sealed blender at 20 000 rev./min. for 15 sec. Denatured protein separated at the interface and was removed; the virus was spun down and resuspended, and Arcton treatment was then repeated. The virus concentration in each fraction is expressed as haemagglutinin units/ml.

Fraction	Concn. of virus
Culture fluid (80 ml.)	6 400
100 000g supernatant (80 ml.)	500
Resuspended pellet (10 ml.)	25 000
Arcton treated (10 ml.)	50 000*
Resuspended pellet (2 ml.)	128 000
Arcton treated (2 ml.)	100 000

* The apparent increase in haemagglutinin after the first Arcton treatment is probably due to removal of phospholipid (Porterfield & Rowe, 1960).

Table 2. *Analytical composition of virus concentrate and of ribosomes after homogenizing with Arcton*

The crude virus preparation was a culture supernatant from 30×10^8 infected ascites-tumour cells incubated as described in text. The virus concentrate was prepared as in Table 1. The crude microsome fraction was from 34×10^8 tumour cells disrupted by sonic disintegration and was prepared by the method of Martin, Malec, Coots & Work (1961a).

	Protein (mg.)	RNA (mg.)	Protein/RNA
Crude virus	300	—	—
Crude microsomes	185	—	—
Concentrated sample of virus* ($\times 4$ Arcton extracted)	7.7	5.6	1.37
Ribosome concentrate ($\times 4$ Arcton extracted)	6.9	4.4	1.57

* The crude virus contained 0.8×10^4 haemagglutinin units/mg. of protein and the virus concentrate 2.0×10^5 haemagglutinin units/mg. of protein (haemagglutinin units = haemagglutinin titre $\times$ vol. in ml.).

haemagglutinin units/ml. of culture fluid and the specific virus concentration varied between 1.2×10^4 and 2.8×10^4 haemagglutinin units/mg. of protein.

Use of modified cellulose ion-exchange columns

The capacity of the two resins DEAE-SF and ECTEOLA-SF (Peterson & Sober, 1956) to adsorb virus was measured by adding successive samples of trypsin-treated virus to small columns of the ion-exchange material previously equilibrated with phosphate buffer. Levintow & Darnell (1960) have reported that poliomyelitis virus is not held in ECTEOLA columns and that the resin may be used to remove contaminating protein from the virus. ECTEOLA also appears to have a low capacity for EMC virus but the hold-up of virus was sufficient to render the method unsatisfactory for purification. Attention was devoted instead to the possibility of stepwise or gradient elution of virus from DEAE columns. Columns were equilibrated with phosphate buffer, loaded with trypsin-treated virus and eluted with phosphate containing increasing concentrations of sodium chloride.

The best results were obtained when the virus was eluted by a steeply rising concentration gradient of sodium chloride (Fig. 1), but even under these conditions the virus was eluted over rather a broad concentration range of sodium chloride.

Fig. 1. Partial purification of EMC virus on DEAE. The column (0.8 cm. diameter) contained 1 g. of DEAE in sodium phosphate buffer (pH 7.0, 0.02 M). The column load was 30–60 mg. of trypsin-treated virus. Elution was achieved with a rising concentration gradient of NaCl in sodium phosphate buffer (pH 7.0; 0.02 M). The gradient was established by a method similar to that described for the elution of calcium phosphate columns (see text). The concentration of chloride in the effluents was measured by the method of Schales & Schales (1941). ▲, Haemagglutinin; ●, chloride.

The best preparations contained about 8×10^5 haemagglutinin units/mg. of protein; thus the degree of purification over the crude trypsin-treated preparations was about 40-fold but the virus could not be crystallized.

Purification of virus on calcium phosphate

Calcium phosphate columns showed two important advantages over DEAE: firstly the recovery of virus was usually quantitative and secondly the bulk of the non-viral material failed to adsorb on the column and was immediately separated from the virus. For complete purification of the virus, it was necessary to use two successive columns but as each column run, and the associated virus assays, could be completed in a few hours this was no serious disadvantage. The behaviour of the crude trypsin-treated virus on the first column is given in Fig. 2 and the behaviour of the partially purified virus on a second, smaller, column is shown in Fig. 3. Virus was usually eluted at phosphate concentrations between 0.2 and 0.3 M. These columns also showed some capacity to fractionate nucleic acids present in the crude virus preparation (Vizoso & Burness, 1960).

The mean specific virus concentration (three preparations) in the virus-containing fractions eluted from the first column was 8×10^5 haemagglutinin units/mg. of protein (i.e. a 40-fold purification from the trypsin-treated concentrate), and, from the second column, was 1.5×10^6 haemagglutinin units/mg. of protein (i.e. a further three-fold purification).

Fig. 2. Partial purification of EMC virus on calcium phosphate (column 1 of Methods). ●, Phosphate; ▲, haemagglutinin; ■, nucleic acid.

Crystallization of encephalomyocarditis virus

By the use of a steep phosphate gradient as described under Methods, it was possible to elute virus from the second calcium phosphate column in a concentrated and nearly pure form. Those fractions which had a haemagglutinin titre above 50 000 were pooled and centrifuged at 100 000g for 60 min. The gelatinous pellets from 10 ml. of the original trypsin-treated virus were combined and resuspended in about 0.2 ml. of 0.9% sodium chloride together with 0.02 ml. of penicillin (50 000 i.u./ml.). After 2 days at 0° crystals had been deposited. The average yield of crystals was about 1 mg./100 ml. of the original Earle's culture medium. The crystals were washed repeatedly with cold water (2 ml./mg. of crystals) and dissolved either in phosphate buffer (pH 8.5; 1 ml.) or in water (1 ml.) to which about 2 drops of 0.06M-sodium carbonate were added. On readjusting to pH 7 and leaving at 0° crystalline virus separated again.

Characterization of crystalline encephalomyocarditis virus

The virus crystallized in the form of extremely thin rectangular wafers. Photographs were taken at wavelengths of 257, 275 and 300 mμ. These photographs (Plate 2) showed clearly that the crystalline material absorbed strongly in the nucleoprotein region and this was confirmed by measuring the absorption spectrum of crystals dissolved in sodium carbonate as described above (Fig. 4). X-ray photographs of crystals have been taken both by Mrs Kennard of this Institute and by

Dr Klug and Dr Finch of Birkbeck College. The crystals are extremely fragile and entirely satisfactory photographs have not been obtained, but two conclusions can be drawn from the available photographs: first, the individual particles of virus are almost spherical; and secondly, the particle diameter in the wet preparation is 30 ± 1 mμ.

Electron-microscope photographs of spray preparations of the crystalline material were taken after negative staining with phosphotungstate (see

Fig. 4. Absorption spectrum of a solution of purified EMC virus in dilute Na_2CO_3 , pH 8.5.

Fig. 3. Preparation of pure EMC virus on calcium phosphate (column 2 of Methods). Symbols as in Fig. 2.

Table 3. Analysis of crystalline encephalomyocarditis virus for nitrogen, phosphorus and ribonucleic acid

For methods of analysis see text. Analyses are expressed as μg. of the appropriate component in each standard solution of crystalline virus.

Prep.	N	P	RNA*	RNA† (%)
43	42.5	—	90	33
46	9.2	—	20.7	35
48	220	—	398	28.5
49	273	—	522	30.1
57	160	30	—	32.4

* Spectrophotometric estimation.

† The percentage of RNA was calculated by making the following assumptions: RNA P = 8.75%, RNA N = 15%, protein N = 6.25%. It was also assumed that the virus contained no DNA and no phospholipid since these components were not detected; hence, the estimates represent percentages of total dry weight of the virus.

Table 4. *Haemagglutinin, particle count and infectivity for various preparations of encephalomyocarditis virus*

Where estimations were not made this is indicated by the symbol —. HA, Haemagglutinin units.

Prep.	HA/ ml.	HA/ mg. of N	HA/ mg. of RNA	Particles/ml. (electron microscope)	Infectivity/ml.		Remarks
					Plaque	Cell death	
F30-2	2.4×10^6	8.8×10^6	—	6×10^{11}	3.3×10^{10}	—	Purified in DEAE
F39-2	5.0×10^6	4.13×10^6	2.02×10^6	—	1×10^{11}	—	Calcium phosphate effluent
F40-2	5.0×10^6	4.74×10^6	2.32×10^6	1×10^{15}	1.2×10^{14}	—	Crystals
F64	0.6×10^4	2.05×10^6	1×10^6	—	1.05×10^9	1.5×10^9	Crystals stored at -70° for 1 week in Earle's medium
F66	2.5×10^4	5.0×10^6	2.45×10^6	4×10^{11}	3.1×10^9	3.5×10^9	Calcium phosphate effluent, centrifuged twice at 100 000g for 1 hr.
F66A	1.2×10^4	—	—	—	3.3×10^9	—	F66 stored at -70° for 1 week in 10% glucose-phosphate

Methods). Some of these were taken in this Institute and some by Mr Hills (A.R.C. Virus Research Institute, Cambridge). The conclusion from the two sets of micrographs was that the virus particle (Plate 2) resembles turnip yellow virus both in size and in appearance and that, moreover, the virus particle shows a sub-unit structure closely similar to that reported by Nixon & Gibbs (1960). A particle count was also made (see Methods) and this was compared with infectivity measurement by the plaque technique. Three determinations on three separate preparations of crystalline material were made; the best results indicated that about 1 particle in 10 produced a focus of infection. This compares favourably with the ratios (1:30) reported for partially purified poliomyelitis virus by Schwerdt & Schaffer (1956) and is much higher than the ratio (1:2000) found for crystalline poliomyelitis virus.

Analysis of crystalline encephalomyocarditis virus

Four separate preparations of crystalline virus have been analysed by the methods already described, and the results are summarized in Table 3. No virus preparation contained measurable quantities of DNA and all contained around 30% of RNA. Since the RNA accounts for all the phosphorus of the virus (Table 3) the virus must be substantially free of phospholipid.

The relationship between haemagglutinin, infectivity, total RNA and particle count is set out in Table 4.

From the plaque assays made on solutions of crystals that were also analysed for RNA it was calculated that there were about 5×10^{13} infective particles/mg. of viral RNA. Since the virus contains about 30% RNA it may be calculated that

1 g. of virus should contain about 1.6×10^{16} infective particles or, if allowance is made for the ratio between plaque counts and particle counts, a total of about 1.6×10^{17} particles. This agrees reasonably well with the figures arrived at by measurement of particle weight in the ultracentrifuge (6×10^{16} , see below) and by measurement of the diameter of the spherical particle with the electron microscope (7×10^{16}).

The base ratios for the RNA of crystalline EMC virus were determined by quantitative paper chromatography. Three successive batches of crystals were analysed. The analyses are quite consistent and differ considerably from similar analyses made on host-cell RNA (Table 5). Poliomyelitis-virus RNA is, however, closely similar in base composition to that of EMC virus.

A sample of crystalline virus was also hydrolysed with 6N-HCl at 108° and the amino acid composition of the hydrolysates was determined by our colleague, Dr S. Jacobs, by ion-exchange chromatographic analysis (Moore, Spackman & Stein, 1958). Since the method requires rather large quantities of material only a single analysis has been carried out. Our analysis for EMC virus is compared in Table 6 with a similar analysis of poliomyelitis virus recently published by Levintow & Darnell (1960).

EXPLANATION OF PLATE 2

A. Electron-microscope photograph of a spray preparation of EMC virus immediately after elution from second calcium phosphate column (negative-staining technique; magnification $\times 140\ 000$).

B, C and D. Ultraviolet-microscope photographs of EMC virus crystals taken at 300 m μ (B), 275 m μ (C) and 257 m μ (D). Magnification $\times 2100$.

Table 5. *Base composition of crystalline encephalomyocarditis-virus ribonucleic acid compared with that of various other types of ribonucleic acid*

Figures for EMC virus are for three successive preparations of crystalline material. Figures for poliomyelitis virus are taken from Schaffer, Moore & Schwerdt (1960). Results are expressed as moles of purine or pyrimidine in 100 moles of base recovered after hydrolysis.

Base	EMC virus			Source of material		
	54	55	56	Krebs II tumour-cell nuclei	Total RNA of Krebs cells	Polio virus
Guanine	23.7	23.6	23.4	22.4	32.3	24.0
Adenine	27.8	27.3	27.0	24.9	21.4	28.5
Cytosine	23.9	23.2	23.3	28.4	29.1	22.0
Uracil	24.5	26.0	26.4	23.5	17.2	25.4
A + G						
C + U	1.06	1.03	1.01	0.91	1.16	1.11

Table 6. *Amino acid composition of crystalline encephalomyocarditis virus compared with that of poliomyelitis virus*

The figures for poliomyelitis virus are taken from Levintow & Darnell (1960). Results are expressed as g. of amino acid/100 g. of amino acid recovered.

	EMC	Poliomyelitis
Aspartic acid	11.4	12.2
Threonine	8.1	8.5
Serine	6.6	5.3
Glutamic acid	10.2	8.7
Proline	6.8	7.0
Glycine	5.2	3.9
Alanine	5.0	5.3
Cystine ($\frac{1}{2}$)	1.4	0.7
Valine	6.3	6.6
Methionine	2.3	2.4
Isoleucine	4.1	4.8
Leucine	8.7	8.3
Tyrosine	5.4	6.0
Phenylalanine	6.7	5.7
Lysine	5.2	5.0
Histidine	1.7	2.9
Arginine	4.9	6.6

Sedimentation of purified virus in the analytical ultracentrifuge

Two preparations of crystalline virus were kindly analysed for us by Dr Charlwood of this Institute. Both preparations showed a single component and the two sedimentation constants ($S_{20,w}$) were 160 and 156. For $S_{20,w}$ 160 and a density 1.37 the calculated particle (molecular) weight is 1×10^7 .

Stability of crystalline encephalomyocarditis virus

The crystalline virus retains its infectivity for considerable periods if stored as the moist pellet at 4°. For experimental use, however, it was necessary to store the virus as a suspension of known infectivity and methods for the preservation of viral suspensions were therefore investigated. A preparation of crystalline virus was dissolved in 0.02M-phosphate buffer by careful addition of

sodium carbonate solution, and the clear suspension of particles diluted with various media (5 and 50 % sucrose, 5 and 50 % glucose, 5 and 50 % glycerol, 0.1 and 1.0 % bovine serum albumin, and Earle's medium) until the haemagglutinin titre of the diluted material was approx. 30 000 haemagglutinin units/ml. Haemagglutinin titre and plaque assay were carried out before and after storage at -70° for 6 months in sealed ampoules. A portion of the suspension diluted in Earle's medium was also freeze-dried and kept at room temperature.

The infectivity and haemagglutinating properties of the virus were almost completely preserved in the presence of either concentration of serum albumin, and only a 50 % loss was obtained with the preparations stored in sucrose or glucose solutions. Glycerol, particularly at the higher concentration, caused a marked loss in infectivity, whereas freeze-drying resulted in an almost complete loss of biological properties. The results with Earle's medium were variable, but usually no more than 25 % of infectivity was retained. The method of preservation now in use is to prepare ampoules of a clear suspension of the virus containing about 40 µg. of RNA/ml. in a sterile solution of 0.02M-phosphate buffer containing 0.2 % of bovine serum albumin or 10 % (w/v) of glucose. The ampoules are stored at -70° and diluted with 3 vol. of Earle's medium after thawing before use. Storage under these conditions has resulted in excellent preservation of infectivity.

Ratio of infectivity to haemagglutinin during purification

In three preparations F44, F64 and F66 (cf. Table 5) the haemagglutinin and plaque-forming activities were estimated on the crude preparation as well as on the purified virus. The ratios of plaque-forming units to haemagglutinin units were 1.2×10^5 , 1.2×10^5 and 1.9×10^5 before purification and 2.5×10^5 , 1.1×10^5 and 1.2×10^5 after purification.

*Precipitating antibody against
encephalomyocarditis virus*

Several attempts were made to immunize rabbits against EMC virus by intramuscular and intravenous injection of virus but no precipitating antibody was formed. In a further series of experiments rabbits were injected intracerebrally. Five animals received 1×10^7 plaque-forming units each and survived with no apparent symptoms. After 17 days these animals each received an intravenous injection of 1×10^{10} plaque-forming units and after 120 days a further 1×10^{11} units of purified virus. Trial bleedings were made 10, 13, 16 and 20 days after the last injection, and a steady rise in the titre of precipitating antibody was observed. In further experiments it was found that the best antiserum was produced by two intracerebral injections followed by two intravenous injections. In this way antisera were produced which precipitated virus at 1/640. Non-specific antibody against ascites-tumour-cell protein could not be detected. Several animals were injected with the microsomal fraction of tumour cells and failed to develop antibodies against this protein.

Agar-gel precipitation of encephalomyocarditis virus

Ouchterlony plates were prepared by the method of Pereira, Pereira & Allison (1959). The circumferential cups were filled with crude virus-culture supernatant, purified virus or an ultrasonic homogenate of uninfected cells. To one set of plates antiserum was added immediately (centre cup) and the plates were stored at 0°. The other set of plates were left 3 days at 0° without antiserum and the circumferential cups were refilled with 0.9% sodium chloride. After another 3 days this process was repeated and the centre cup was then filled with antiserum. The first set of plates showed slight precipitation on the edge of the cup containing purified virus but no precipitation elsewhere. This result is explicable since the large size of the virus will result in extremely slow diffusion through agar. The second set of plates showed clearly defined precipitation zones around the cups containing purified virus but no lines around either the cell protein cups or the crude virus-culture supernatant. From these results it was evident that intracerebral injection followed by intravenous injection had resulted in the production of a rather specific anti-EMC serum.

DISCUSSION

The smaller animal viruses are rather stable nucleoprotein complexes and their purification presents much the same problems as are presented by any similar group of macromolecules. The main

impediment to progress has probably been inability to obtain virus in sufficient quantity and in this respect we were fortunate in that the methods of Hoskins & Sanders (1957) for growth of EMC virus in Krebs cells could be scaled upwards without difficulty. The problems involved in purification and in identification of a particular type of particle as an infective unit have been fully reviewed by Sharp (1953) and need not be repeated here. The conclusion that our crystalline preparation represents pure EMC virus depends on the several complementary lines of evidence set out in our Results. No one line of evidence is conclusive when considered alone but taken together, they amount to a fairly rigid proof of our assumption that the crystalline material is indeed pure EMC virus.

The ratio of about 1:10 between infective units and particle counts is about the same as that achieved with other animal virus preparations. The problem of the relationship between particle count and infectivity has been fully discussed by Isaacs (1957). Although the total count and the infective particle count are roughly equal for some bacteriophages (Luria, Williams & Backus, 1951), the failure to reach a 1:1 ratio does not necessarily indicate that the preparation is impure. There is no precise knowledge of the number of particles required to infect a single cell and in the absence of this knowledge no definite conclusion can be reached regarding the infectivity of individual particles. The analytical ultracentrifuge and the electron microscope have indicated that our preparation consists of physically uniform particles and ready crystallization of the purified virus reinforces this conclusion.

In size, shape, protein:nucleic acid ratio, amino acid composition and purine-pyrimidine composition, EMC virus resembles poliomyelitis virus. Yčas (1960) has suggested that the base composition of the RNA of a virus may be calculated from the amino acid composition of its protein. Such a calculation suggests that the base ratios should be guanine 23.3, adenine 29.7, cytosine 22.0, uracil 25.0. These calculated figures agree moderately well with the analytical figures (Table 5) except for adenine. Unfortunately we have not been able to prepare sufficient crystalline virus to permit repetitive analyses and determination of the standard error, but we consider it unlikely that the analytical figures for the amino acids would be in error to a sufficient degree to account for the discrepancy. This is, however, obviously a point upon which additional information is required.

Since the ratio of haemagglutinin to infectivity remained constant throughout the purification we conclude that the haemagglutinin is the protein of the virus. This is an important conclusion since it

has enabled us to follow synthesis of virus protein by following the incorporation of radioactive amino acids into haemagglutinin.

SUMMARY

1. A method for large-scale production of encephalomyocarditis virus has been developed.

2. The behaviour of the virus on columns of diethylaminoethylcellulose, ECTEOLA-cellulose and calcium phosphate has been studied.

3. Crystalline virus has been obtained from effluents after chromatography on calcium phosphate columns.

4. The virus has been shown to be a ribonucleo-protein containing about 30 % of ribonucleic acid.

5. Electron-microscope photographs show that the virus is a spheroid with a diameter of about 27 m μ (dry).

6. Particle counts indicate that about 10 % of the particles in a crystalline preparation are inactive (as shown by plaque counts). This compares very favourably with crystalline preparations of poliomyelitis virus.

7. The base composition of the viral ribonucleic acid has been determined and also the amino acid composition of the viral protein.

8. Virus haemagglutinin is associated with and is purified along with the virus. It is concluded that the haemagglutinin is the virus protein.

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