Particle Counts and some Chemical Properties of Murray Valley Encephalitis Virus

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SUMMARY

Murray Valley encephalitis virus was grown in baby mouse brain and purified. In five out of six experiments, the number of virus particles per chick egg LD 50 was 90 ± 50 . Fifteen preparations of purified virus were pooled and the pool examined chemically. The nucleic acid content was 7·8 %, with bases present in the proportions: adenylic acid, 25·5; guanylic acid, 27·5; cytidylic acid, 21·5; uridylic acid, 25·5. No thymidylic acid was found. The virus preparation contained 11 % lipid; phospholipid and total cholesterol were present to 0·8 % and 1·0 %, respectively, of the virus preparation.

INTRODUCTION

The arthropod-borne viruses are divided into groups on serological grounds; the two most studied are groups A and B. The group A virus, Eastern equine encephalomyelitis, was purified by Taylor, Sharp, Beard & Beard (1948) from extracts of infected chick embryos and found to contain protein, ribonucleic acid, lipid and a small fraction of carbohydrate; the nucleic acid was about 4·4 % of the preparations studied. There was a high lipid content (54 %) of which phospholipid was a major component (35 % of the whole virus); cholesterol and neutral fat were also present.

During a study of methods of purification of group B arthropod-borne viruses from infective baby mouse brains (Ada, Anderson & Abbot, 1961), 15 purified preparations of Murray Valley encephalitis (MVE) virus became available and were pooled. The results of electron-microscope examination of individual preparations and of chemical analyses of the pool are reported here.

METHODS

Purification of virus. Infected baby mouse brains were extracted with phosphate buffered saline and the extract treated with protamine sulphate. This suspension was clarified by brief low-speed centrifugation and the virus sedimented from the clear solution by high-speed centrifugation. The sedimented virus was resuspended, adsorbed on to, and then eluted from, a column of hydroxyl apatite. In the final step the virus in the eluate was sedimented by high speed centrifugation and resuspended in a small portion of the overlying fluid. Thus from an original volume of 90 ml. (60 brains), the virus was obtained in a final volume of about 0·7 ml. In those batches

where it could be estimated, the recovery of haemagglutinin was about 50 %. In contrast, the recovery of infectivity averaged only 15 %; the major loss occurred during the last centrifugation step (Ada *et al.* 1961).

Electron microscopy

Preparation of sample. The virus purified as above was obtained as a suspension in buffered saline (potassium phosphate, 0.25 M; sodium bicarbonate, 0.025 M; cystine, 0.85 mM; sodium chloride, 0.15 M; pH 7.7). Immediately after preparation 0.05 ml. of the virus preparation was applied to a column (4.0 cm. $\times$ 0.8 cm.) of Sephadex G75 (a cross-linked dextran gel, water regain 7.7 g./g. dry gel; from Pharmacia, Sweden) which had previously been equilibrated with ammonium carbonate (10 g./l.) solution; separate drops of effluent were collected. The first 6 drops of effluent (about 0.35 ml.) contained 90 % of the ultraviolet (u.v.) absorbing material (260 m μ) applied to the column; of this amount drops 3 and 4 (0.13 ml.) contained 65 % of the u.v. absorbing material; these were together used for spraying. The gel filtration was carried out at 0–4°, took about 10 min. and yielded a sample of virus in a solution of a volatile salt.

Spraying of virus. The apparatus designed for spraying the infective virus preparation consisted of a desiccator (about 30 cm. high and 25 cm. diameter). The side arm of the spray gun (Backus & Williams, 1950) passed through a stopper and was connected by pressure tubing to a cylinder of medical-grade compressed air. The gauge valve of this cylinder was pre-set to give a pressure of 10 lb./in.² on opening the cylinder valve. Glass tubing fitted with a stop-cock also passed through the stopper and was connected by pressure tubing to a water pump and a mercury manometer. Several carbon-coated electron microscope grids were mounted on the floor of the desiccator directly beneath the spray gun aperture. The distance between gun aperture and grids was usually 12 cm. but this did not appear to be critical. Immediately before final assembly a small beaker containing 10 ml. of a formaldehyde (400 g./l.) solution was taped to the inside wall of the desiccator.

The filtered virus (0.05 ml.) was mixed with an equal volume of a solution containing latex particles (Dow polystyrene latex, run no. L.S. 0.040 A, particle diameter $0.088\mu \pm 0.008$ s.d.) and bovine plasma albumin (BPA Armour Laboratories, Cohn fraction V) so that the final 0.1 ml. of solution contained 1.5×10^{10} latex particles and 0.17 mg. BPA. A sample (about 2 μ l.) of this solution was loaded by a finely drawn Pasteur pipette into the tip of the gun and the stoppers slipped over the side arm of the gun. This assembly was then placed in the neck of the desiccator and the tubing connexions completed. The desiccator was evacuated to about 40 mm. Hg, the stop-cock closed and the pumping line disconnected. The cylinder valve was then opened for about 2 sec. which was sufficient time for the entire sample to be sprayed. The cylinder valve was closed after 2 sec. and a clamp tightened on the pressure line. The apparatus was left undisturbed for 16 hr., by which time about half the formalin had evaporated. The apparatus was then dismantled, the gun was disinfected in boiling water and the grids removed for examination. Previous experience showed that even after 4 sec. spraying time there was still a pressure of 25 mm. Hg in the desiccator. The apparatus was tested before each spraying by carrying out the procedure with an empty gun.

Examination of droplets

Particle counts. The grids were examined in an electron microscope (Siemens type E4) without shadowing. Pictures of complete droplets were taken at magnifications of $\times 7000$ and photographically enlarged ($\times 3.5$) for counting particles. At least 4000 virus particles/sample were counted. The average ratio of numbers of virus particles to latex particles was 10:1.

Particle size. The electron microscope was calibrated by using Dow Polystyrene Latex, run no. 580 G. The particles in this had been found (Farrant & Hodge, quoted by Gerould, 1950) to have a diameter of $256 \pm 2.5 \text{ m}\mu$; Dow latex, run no. IS040 A was found to have particles of diameter $88 \text{ m}\mu$. The virus particle size was estimated by using a microscope graticule (0.1 mm. divisions) to measure particles on prints which had been photographically enlarged ($\times 5$).

Titration of virus infectivity. The standard method of titration was to inoculate 0.05 ml. volumes of decreasing ten-fold dilutions of virus either on the chorio-allantoic membrane of 12-day-old chick embryos (6 embryos/dilution), or into the peritoneal cavity of 5–8-day mice, with 12 mice/dilution. The diluent for virus was 10% (v/v) unheated Seitz-filtered normal rabbit serum diluted in physiological saline. Infectivity end-points were determined as 50% lethal endpoints at 4 days in chick embryos; the mice were observed during 12 days.

Chemical analysis. To the remaining purified virus was added 1/3 volume of trichloroacetic acid (400 g./l.) and the suspension allowed to stand at $0-4^\circ$ for 5–10 min. It was then centrifuged (2000 *g* for 10 min.), and the sediment resuspended in a small volume of distilled water. The suspension at this stage contained no infective virus; it was stored at -20° . The frozen suspensions from 15 different preparations were pooled and freeze-dried *in vacuo*. The dried virus was transferred to a weighing bottle and dried further over P_2O_5 in a vacuum desiccator; it weighed 12.9 mg.

Extraction and estimation of lipid. Dried virus was extracted thrice at room temperature with a mixture of chloroform + ethanol (2 + 1 by vol.). The extract was evaporated to dryness under nitrogen and the residue extracted with diethyl ether. The ethereal solution was removed by decantation and the insoluble material added to the main virus residue. After evaporation of the samples to dryness under nitrogen, the ether-soluble material was taken up in a known volume of chloroform from which samples were taken for phosphorus, cholesterol and sugar determinations.

Phosphorus was estimated as described previously (Collins, 1959). Cholesterol was determined by the micromethod of Caraway & Fanger (1955). (We are grateful to Dr J. MacMillan for the use of his Beckman Spinco ultramicro colorimeter.) Samples of the lipid (45 μg .) were digested with 0.5 ml. acid (0.25 N-HCl; 16 hr.; 100°) and the digest evaporated to dryness in a desiccator containing caustic soda. The content of reducing sugar was then estimated by the technique of Park & Johnson (1949). Galactose (3 μg .) added to a sample of virus lipid before digestion was recovered in this test.

Extraction and analysis of nucleic acid. The nucleic acid was isolated from the defatted virus by three extractions (30 min., 100°) with NaCl (100 g./l.) solution. A sample of the extract was kept for phosphorus estimation; 2 vol. ethanol were added to the remainder (0° ; 16 hr.). The precipitated nucleic acid was washed

with 66 (v.) ethanol in water, 95 (v.) ethanol in water and then with diethyl ether.

The nucleic acid content of the 10 % NaCl extract was estimated in two ways: (i) by direct determination of the phosphorus content, assuming this to be all nucleic acid phosphorus; (ii) by determination of the specific absorption at 260 m μ . The formula $Ep = 30.98 E/cl$, where Ep = atomic extinction coefficient at 260 m μ with respect to phosphorus, E = optical density, c = concentration of phosphorus in g./litre and l = thickness of the absorbing layer (Chargaff & Zamenhof, 1948) was used to determine spectrophotometrically the nucleic acid content. A value of 9200 for Ep was assumed (Ada & Perry, 1956). A factor of 10 was assumed for the conversion of the phosphorus value to nucleic acid.

The viral nucleic acid was hydrolysed (M-HCl, 100°, 60 min.) the digest chromatographed and the purine and pyrimidine derivatives detected and estimated. Details of these procedures were given previously (Ada & Perry, 1956).

The virus residue remaining after extraction with hot NaCl solution was washed thrice with distilled water, digested (M-HCl, 100°, 60 min.) and the digest dialysed (24 hr.; 0°) against 3 vol. distilled water. (We are indebted to Dr A. Szenberg for suggesting this procedure.) The content of purine and pyrimidine derivatives in the diffusate was estimated by determining the absorption at 260, 280 and 300 m μ . These values were compared with those given by artificial mixtures of protein and nucleic acid.

RESULTS

The number of particles/infective dose. In five experiments the ratio virus particles/chick embryo infective dose was 90 ± 50 (range 32–160). It may be noted that in these experiments the concentration of virus in the Sephadex column effluent was 25 % as judged by u.v. absorption (260 m μ) and 51 ± 26 % as judged by infectivity titrations of the concentration of virus in deposit 2. In a sixth experiment the infectivity titre of the virus decreased from 6×10^{10} in deposit 2 to 4×10^8 in the Sephadex sample. In this experiment the ratio particle count/chick embryo infective dose was unduly high at 980. On one occasion the stability of a Sephadex effluent was tested. When kept at 20° for 30 min., the infectivity titre decreased from 6×10^9 to 1×10^9 . The corresponding ratios for particle count/chick embryo infective dose were 74 and 290. In three experiments, Sephadex samples were titrated in eggs and in mice. The mouse titre was equal to the egg titre in one experiment, was three times higher in the second experiment and 2.5 times higher in the third experiment.

Virus particle size. Counts were made of the number of virus particles having diameters of 25, 27.5, 30, 32.5 or 35 m μ . Of 378 particles examined: 223 had diameter 25 m μ , 100 diameter 27.5 m μ , 28 diameter 30 m μ , 6 diameter 32.5 m μ , 11 diameter 35 m μ .

Chemical analysis of the virus preparation. The virus obtained from 15 preparations when pooled and dried weighed 12.9 mg. After extraction of lipid, the residual weight was 11.5 mg. and the extracted lipid weighed 1.4 mg. Thus the lipid content was 10.9 % of the total weight. The extracted lipid was estimated from phosphorus analysis to contain 0.10 mg. phospholipid which corresponds to 0.8 % of the virus preparation. Cholesterol was present to the extent of 1.0 % of the virus preparation. The content of reducing substance in an acid digest of virus lipid (45 μ g.) was

equivalent to 2 μg . galactose; if assumed to be galactose and this assumed to derive from cerebroside, then the cerebroside content of the virus lipid would amount to a maximum value of 2.2 % of the total virus.

Nucleic acid was estimated in two ways which gave similar results. As estimated by u.v. absorption the preparation contained 7.8 % nucleic acid of which 92 % (920 μg .) was present in the extract made with 10 g./l. sodium chloride solution. The sodium chloride extract contained 2.93 μM phosphorus; this would correspond to about 910 μg . nucleic acid. Paper chromatography of an acid digest of the nucleic acid in the sodium chloride extract showed four components, corresponding in R_F values to adenine, guanine, cytidylic acid and uridylic acid; no component corresponding in R_F value to thymidylic acid was observed. The components were present in the following proportions: adenylic acid, 25.5; guanylic acid, 27.5; cytidylic acid, 21.5 and uridylic acid, 25.5.

DISCUSSION

When the infectivity of purified Murray Valley encephalitis virus (deposit 2) was titrated in chick embryos, the average infective dose contained about 100 virus particles. There was some evidence that mouse infectivity titration would have yielded a lower figure perhaps 40 virus particles/mouse infective dose. Previous work had indicated that there was a greater decrease in infectivity than in haemagglutinin titre or in u.v. absorption during the last stage of purification; the relative change was about four-fold. Thus, perhaps there were 10 virus particles/mouse infective dose in the original brain extract. This recalls the figure of 10 virus particles/chick embryo infective dose for many strains of influenza virus (Donald & Isaacs, 1954). The distribution curve of virus particle size was very skew. One possibility is that the undamaged particles had a narrow distribution of size and were about 25 m μ diameter. Particles with a greater diameter may be damaged and flattened.

No confidence can be placed on the results of chemical analysis of a virus preparation unless the purity of the preparation has been demonstrated; this requirement has here been met only partially. The main evidence of purity was the fact that the electron microscope picture of deposit 2 (Ada *et al.* 1961) showed little but virus particles. Because of the inadequacy of this evidence and the fact that the results were from a single chemical analysis, the values obtained must be regarded as preliminary. However, if the results are taken at their face value, three comments are worth making. Earlier work showed that phospholipase A destroyed viral infectivity (Anderson & Ada, 1961); this was believed to mean that intact phospholipid was necessary for the viability of this virus. The present findings show that the virus particles contained only a small amount of lipid, particularly of phospholipid, in an amount which might all be carried on the viral surface. Phospholipid, cholesterol and cerebroside account for a maximum of 38 % of the viral lipid. If it be assumed that lipid components (particularly phospholipid phosphorus) have not been lost from the virus during isolation, it seems possible that the remaining lipid is a triglyceride. The relative proportions of lipid, phospholipid and cholesterol in the virus preparation (10:0.7:0.9) are different from those in 7-day mouse brain (10:7.1:1.2; Folch-Pi, 1955). In contrast the ratio of phospholipid:cholesterol:triglyceride in influenza virus is similar to that found in the chick embryo chorio-

allantoic membrane (Frommhagen, Freeman & Knight, 1958). It would be of interest to know the composition of Murray Valley encephalitis virus derived from other hosts. With this idea in mind, virus grown on chick embryo chorioallantoic membrane was treated with phospholipase A and found to be fully inactivated (Anderson & Ada, unpublished).

The failure to find thymidylic acid indicated that the viral nucleic acid was largely if not entirely RNA. This agrees with the earlier finding (Anderson & Ada, 1959) that pancreatic ribonuclease destroyed the infectivity of Murray Valley encephalitis virus nucleic acid. Lack of material prevented an attempt to identify the sugar component of this nucleic acid. As might be expected, the base ratio found for the viral nucleic acid differed from that of mammalian cell RNA. It is not yet known whether the Murray Valley encephalitis virus contains carbohydrate other than that associated with the nucleic acid or lipid fractions.

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