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

THE FORMATION OF A VIRUS-SPECIFIC ANTIGEN IN KREBS II ASCITES-TUMOUR CELLS INFECTED WITH ENCEPHALOMYOCARDITIS VIRUS

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1. Krebs II mouse ascites-tumour cells infected with encephalomyocarditis virus were found to contain, in addition to mature virus, a virus-specific protein antigen. An assay, based on the ability of this antigen to block the neutralization of purified virus by its specific antiserum, was developed. 2. This antigen was present both in the culture fluid 17 hr. after the infection of cells with virus and intracellularly, where its titre increased at a time when viral capsid protein was being synthesized. Within the cell, it was mostly localized in the soluble cell sap. 3. In contrast with virus, the antigen did not agglutinate sheep erythrocytes, and its immunological properties were destroyed by digestion with trypsin. Ribonucleic acid was not detected in concentrated preparations of the antigen, nor was the titre of antigen affected by ribonuclease. 4. The antigen had a sedimentation coefficient (20°) of approx. 14s, and its diffusion coefficient, determined by the method of Allison & Humphrey (1960), was 3.2×10^{-7} cm.²sec.⁻¹. The particle weight of the antigen was hence 420000 ± 40000 . 5. The capsid protein from purified encephalomyocarditis virus could be degraded by treatment with ethanolamine into a protein of sedimentation coefficient (20°) of approx. 4s. The 14s antigen, when similarly treated, yielded a protein of similar size. However, no such smaller antigen was detected in virus-infected cells. 6. It is concluded that the non-haemagglutinating antigen represents a polymeric form of the basic viral capsid-protein molecule and that it is synthesized in the cytoplasm of infected cells. It may be either an intermediate or a by-product in the process of viral capsid-protein synthesis.

The EMC virus,* a member of the Columbia SK group of RNA-containing viruses, can be conveniently grown *in vitro* in suspended cultures of Krebs II mouse ascites-tumour cells (Sanders, Huppert & Hoskins, 1958; Martin, Malec, Sved & Work, 1961a). Dalgarno & Martin (1965) have indicated that the synthesis of EMC-virus RNA is confined to the cytoplasm of the infected cell. Martin & Work (1962) showed that the synthesis of viral capsid protein lagged behind that of the viral RNA, and that the mature virus particles accumulated in the cytoplasm shortly after the synthesis of capsid protein. However, no direct evidence for the location of the site of capsid-protein synthesis was obtained.

Experiments are described below in which immunological techniques were employed in an attempt to follow the formation of EMC-virus capsid protein throughout the virus growth cycle.

* Abbreviations: EMC virus, encephalomyocarditis virus; SBP, serum-blocking power.

During these studies an antigen with the immunological specificity of EMC-virus capsid protein and a particle weight of approx. 400000 was detected in infected cells (Kerr, Martin, Hamilton & Work, 1962). Unlike virus it was incapable of agglutinating sheep erythrocytes. This non-haemagglutinating antigen was first detectable in the cytoplasm just before the first detectable increase in mature virus, and its titre increased exponentially throughout the growth cycle. On treatment with ethanolamine it was partially degraded into material of lower molecular weight, probably identical with an antigen obtained by similar treatment of purified virus. The present paper describes the occurrence and properties of this non-haemagglutinating antigen.

MATERIALS AND METHODS

Media. Phosphate-buffered saline, phosphate-buffered glucose-gelatin and Earle's medium were prepared as described by Martin *et al.* (1961a).

Density-gradient markers. The ^{131}I -labelled arachin and ^{125}I -labelled fibrinogen were prepared and supplied by Dr P. A. Charlwood (Charlwood, 1963).

Virus. Crystalline EMC virus was prepared as described by Faulkner, Martin, Sved, Valentine & Work (1961).

Conditions for growth of virus. The preparation of Krebs II mouse ascites-tumour cells and their infection with EMC virus in swirling cultures were as described by Martin *et al.* (1961a). In all experiments the growth of the virus was synchronized by thermal shock (Martin & Work, 1962).

Disruption of cells. After incubation with virus the cell suspensions were chilled and sedimented at 100 g for 10 min. at 4°. (All ensuing operations were carried out at 0–4°.) Cells were washed with 10 mM-magnesium chloride solution, suspended in this medium (10^8 cells/ml.) and centrifuged at 2300 g for 3 min. to obtain the packed-cell volume. They were then disrupted by means of a Dounce homogenizer in 3 times the packed-cell volume of 10 mM-magnesium chloride solution (Martin, Malec, Coote & Work, 1961b) until microscopic examination showed less than 5% of unbroken cells (approx. 15–25 strokes of the homogenizer pestle).

Separation of subcellular fractions. Nuclei and contaminating whole cells were sedimented by centrifugation at 450 g for 10 min. The mitochondrial fraction was sedimented at 10000 g for 10 min. and the microsomal fraction obtained from the supernatant by sedimentation at 105000 g for 90 min. After removal of the fat layer the supernatant from this latter centrifugation was retained as the cell-sap fraction.

Treatment of subcellular fractions before assay for antigen. The cell-sap fractions were made up to a total of 14 ml. with phosphate-buffered saline and centrifuged for 2 hr. at 105000 g to remove whole virus. The fractions containing nuclei and whole cells were disrupted either by osmotic shock and Dounce homogenization (Martin *et al.* 1961b) followed by digestion with deoxyribonuclease (Sigma Chemical Co., St Louis, Mo., U.S.A.) ($0.03 \mu\text{g./ml.}$) for 1 hr. at 32°, or by ultrasonic treatment (60 sec. treatment with an oscillator tuned to 25 kc./sec. at a power of 150 w). In either case the disrupted material was adjusted to a final volume of 14 ml. and a sodium chloride concentration of 0.15 M, and centrifuged at 105000 g for 2 hr. After removal of a layer of fat, the top five-sixths of this supernatant was taken for the assay of antigen. The mitochondrial and microsomal fractions were either treated with ultrasonic vibrations, as described for the nuclear fraction, or were suspended in 12.5 ml. of phosphate-buffered saline, sodium deoxycholate was added to give a concentration of 0.5% and the homogenized suspensions were centrifuged at 105000 g for 2 hr. to remove whole virus.

Virus-free 17 hr.-infected culture fluid. Infected suspensions of cells that had been incubated for 17 hr. for the large-scale production of EMC virus (Faulkner *et al.* 1961) were chilled to 0° and the cell debris was removed by centrifugation at 2000 g for 15 min. Virus was sedimented from the cell-free culture fluid by centrifugation at 105000 g for 90 min. A further centrifugation at 105000 g for 2 hr. yielded a supernatant referred to below as the 'virus-free 17 hr.-infected culture fluid'.

Assay of virus. Haemagglutinin assays were carried out as described by Martin *et al.* (1961a). Assays for viral infectivity *in vivo* were carried out on mice by the method described by Huppert & Sanders (1958) for the assay of

infective viral RNA, and the LD₅₀ titres calculated by the method of Reed & Meunch (1938).

Preparation of rabbit antibody against EMC virus. Antibody to EMC virus was prepared by the intracerebral injection into rabbits of crystalline EMC virus ($1.5 \mu\text{g.}$), followed by three intravenous injections of 100–300 $\mu\text{g.}$ of crystalline virus, at 3-week intervals, as described by Faulkner *et al.* (1961). A $1:10^6$ dilution of this antiserum was able to neutralize 1–2 haemagglutinin units of EMC virus.

Double-diffusion agar-gel precipitation of virus and antigens. Ouchterlony plates were prepared from 1.5% (w/v) washed agar (Martin *et al.* 1961a) dissolved in 0.033 M-sodium phosphate buffer, pH 7.4, containing EDTA (1 mM), according to the method of Pereira, Pereira & Allison (1959). Agar plates with right-angled troughs were prepared as described by Allison & Humphrey (1960).

SBP assay of viral antigen. The ability of an antigen preparation to neutralize the reaction between purified EMC virus and specific anti-viral antibody, as measured by inhibition of haemagglutination, was used as the basis for the following method for titration of antigen. One Medical Research Council standard agglutination plate (consisting of ten rows of eight cups, each of 1.25 ml. capacity) was used for each antigen assay. Cell dilutions were made with phosphate-buffered glucose-gelatin solution. Serial 0.5 ml. twofold dilutions of antiserum, previously diluted 1:250, were made down the left-hand ten-cup side of the agglutination plate (see Fig. 1). Then 0.5 ml. of the antigen solution under test was added to each of the ten cups and the plate allowed to stand overnight at 4°. Control plates, in which the antigen was replaced by diluent, were prepared in the same manner. The next day 0.5 ml. serial twofold dilutions of the antigen-antiserum mixture were made across the plate. Then 0.25 ml. of a solution of crystalline EMC virus

Fig. 1. Examples of SBP titrations. The diagrams illustrate the appearance of agglutination plates at the completion of SBP titration of phosphate-buffered saline control (a) and a high-titre EMC-virus-specific antigen (b). The method of titration is described in the text. Haemagglutination is exhibited in 32 cups in the control and by 59 cups in the test plate (partial haemagglutination is counted as $\frac{1}{2}$ cup). The SBP titre for this antigen is therefore 27. -, No haemagglutination; $\pm$, partial haemagglutination; +, haemagglutination.

containing 2-4 haemagglutinin units was added to each of the 80 cups, followed by 0.25 ml. of a washed sheep-red-cell suspension prepared as described for the haemagglutinin assay. The plates were allowed to stand overnight at 4° and read the next day. The appearance of a typical plate showing a high antigen titre, and that of a control assay containing no antigen, are shown in Fig. 1. The SBP titre of a 'test' antigen was taken to be the increase in number of cups, over the whole test plate, showing red-cell agglutination when compared with the number of cups showing agglutination in the control plate. This titre is referred to below as 'cup shift'.

Methods of analysis for RNA and protein were as described by Martin *et al.* (1961a).

EXPERIMENTAL AND RESULTS

At the outset of this investigation we adopted the working hypothesis that the infected cell should contain, in addition to whole virus, sub-units (capsomers) of the virus capsid protein. It was assumed that antibody to complete virus would also react with such sub-units. However, it seemed useless to attempt any type of direct precipitation reaction, since such reactions were found to be relatively insensitive even for whole virus. The method finally used was a modification of the SBP assay, a technique that DeMars (1955) had shown to be effective for the assay of T2-phage protein. The advantage of this technique, details of which are given in the Materials and Methods section, is that it may be made specific for EMC-virus protein. The specificity of the assay resides in the ability of an antigen to block the reaction between a highly purified virus preparation and its antibody. Non-specific antibodies do not interfere with this reaction, although care was taken to use only highly purified virus for the immunization of the rabbits.

Quantitation of the SBP assay. The relationship between the cup shift in the SBP assay (see the Materials and Methods section) and the antigen concentration was investigated on a number of occasions and found to be non-linear. The results of one such experiment, with both crystalline EMC virus and non-haemagglutinating antigen (see below) as test antigens, are shown in Table 1. A small increase in the quantity of antigen at low initial concentrations produces a large cup shift, whereas at high initial antigen concentrations a much larger increase in quantity of antigen is required to produce the same increase in cup shift; the relationship was more nearly exponential. Hence, in the absence of a purified preparation of antigen, it was not possible to obtain an estimate of the absolute concentration. Variability in the cup shift was noted when the same antigen preparation was titrated from week to week. Nevertheless, the values for cup shifts within a particular experiment were reproducible and, provided that the exponen-

Table 1. *SBP activity of various concentrations of EMC virus and non-haemagglutinating antigen*

Crystalline EMC virus was prepared by the method of Faulkner *et al.* (1961). The antigen preparation was a pelleted and resuspended antigen solution, prepared as described in the text. Cell dilutions were made with phosphate-buffered glucose-gelatin solution. The SBP assay was carried out as described in the text.

EMC virus taken for assay (haemagglutinin units)	SBP titre (cup shift)	Antigen taken for assay (μ l.)	SBP titre (cup shift)
20	4	2	2
60	8	3	6
160	10	9	16
350	13	22.5	20
660	16	100	23
1600	20	400	24
3200	24		

tial relationship is kept in mind, cup shift was a convenient method of expressing the information obtained.

Detection of a non-haemagglutinating EMC-virus-specific antigen in virus-free 17 hr.-infected cell material. When Krebs II ascites-tumour cells are incubated with EMC virus, death of the cells and concomitant release of progeny virus begins at about 7 hr. and is complete by about 11 hr. after infection. It seemed probable that any virus protein that had failed to form complete virus would also be released on the death of the cells. Accordingly, initial attempts to detect such a protein were made on samples of the 17 hr.-infected culture fluid produced during the large-scale preparation of virus. Virus was removed from these culture fluids by two periods of centrifugation at 105 000 *g*, each for 2 hr. Such 'virus-free' culture fluids consistently showed SBP activity when subjected to the SBP assay. Further investigations (see below) also showed that such activity could be found in the cell sap of virus-infected cells (but not in uninfected cells) that had been freed of virus by similar means. These results indicated the presence in infected cultures of a non-haemagglutinating EMC-virus-specific antigen that was smaller than whole virus.

Although these 'virus-free' preparations exhibited no haemagglutinating activity they did, in fact, contain a small quantity of virus when assayed *in vivo* in mice, usually about 10^3 LD₅₀ units/ml. As 1 haemagglutinin unit is equivalent to about 10^5 LD₅₀ units it is not surprising that such preparations showed no haemagglutinating activity. This residual virus could not be completely removed by further centrifugation, nor by adsorption on erythrocytes or ascites cells. However, as shown by

Table 2. *Effect of antiserum neutralized with EMC virus on the SBP titre of non-haemagglutinating antigen*

The antigen used was a 'virus-free' 17 hr.-infected culture fluid. The antiserum was prepared with partially purified EMC virus (Faulkner *et al.* 1961). This antiserum was neutralized by incubating a 1:4000 dilution of the serum at 4° for 18 hr. with 3.8 µg. of crystalline EMC suspension. Un-neutralized serum was prepared by a similar treatment except that the EMC virus was omitted. Preparations were centrifuged for 90 min. at 105 000 g to remove precipitated and free excess virus. The neutralized antiserum did not haemagglutinate sheep erythrocytes, nor did it reduce the haemagglutinin titre of crystalline EMC virus. A 1:50 dilution of the un-neutralized serum prevented haemagglutination by 2 haemagglutinin units of EMC virus. SBP titrations were carried out with these supernatants as described in the Materials and Methods section. All dilutions were made in phosphate-buffered glucose-gelatin diluent (Martin *et al.* 1961a). Equal volumes of antigen and antisera were used in the assay.

Solution assayed	SBP titre (cup shift)
Diluent	0
Un-neutralized antiserum, diluent	-10*
Neutralized antiserum, diluent	0
Antigen, diluent	11
Antigen, un-neutralized antiserum	4
Antigen, neutralized antiserum	12
EMC virus,† un-neutralized antiserum	4
EMC virus,† neutralized antiserum	10

* A negative titre indicates a decrease in the number of cups showing haemagglutination when compared with control.

† 160 haemagglutinin units of crystalline virus were used in this assay.

the results given below, this residual virus did not interfere with the detection and assay of the antigen.

Characterization of non-haemagglutinating antigen. That the non-haemagglutinating antigen had the immunological specificity of EMC-virus capsid protein was shown in the following experiments, summarized in Tables 2 and 3. The addition of EMC-virus-specific antiserum, from which all antibodies to whole virus had been removed by precipitation with crystalline EMC virus, to a solution of non-haemagglutinating antigen had no effect on its SBP titre. The antigen was prepared from 'virus-free' 17 hr.-infected culture fluid. The addition of control EMC-virus-specific antiserum that had not been neutralized with EMC virus caused a marked fall in SBP titre (Table 2). The antigen also had the power to neutralize the protective effect of EMC-virus-specific antiserum. Table 3 shows the results of one such experiment in which mice were injected either with EMC virus plus antiserum or with virus,

Table 3. *Ability of non-haemagglutinating antigen to block protective action of anti-EMC antiserum in mice*

The antigen used was a 'virus-free' 7.5 hr.-infected cell sap that had been concentrated tenfold by pressure dialysis. It was free of haemagglutinating activity, and had an SBP titre of 95/ml. Samples (0.25 ml.) of serial dilutions of EMC-virus-specific antiserum were incubated for 18 hr. at 4° with 0.25 ml. of antigen or phosphate-buffered gelatin-glucose (control). Each of the mixtures was then incubated for 5 hr. at 4° with 0.5 ml. of a suspension of EMC virus containing 100 LD₅₀ units/ml. Then 0.2 ml. portions of each sample were injected into each of four mice (strain T.O. males, 16–18 g. wt.), and deaths between second and fifth days after the injection were noted. Percentage protection indicates the percentage of mice surviving beyond 5 days.

	Maximum dilution of antiserum showing:		
	100% protection	50% protection	Zero protection
Control antiserum	1:20 000	1:50 000	1:160 000
Antiserum incubated with antigen*	< 1:5 000	< 1:5 000	< 1:5 000

* 1:5 000 was the lowest dilution of antiserum tested.

antigen and antiserum. As shown in Table 3, reaction between non-haemagglutinating antigen and antibody destroyed the protective capacity of the antiserum.

In contrast with crystalline EMC virus, the antigenicity of the antigen was completely destroyed by incubation at 37° with trypsin (Table 4). Digestion with pancreatic ribonuclease under similar conditions had no effect on the cup-shift titre.

With highly concentrated solutions of the non-haemagglutinating antigen it was possible to obtain an antibody-antigen precipitate after incubation with EMC-virus-specific antiserum. The washed precipitate was analysed for RNA and protein. No RNA was detected in the precipitate. However, insufficient material was available to exclude the possibility that RNA may have been present to the extent of less than 2% of the weight of the precipitate.

Sedimentation properties of the non-haemagglutinating antigen. The supernatant after centrifugation of infected cell culture fluid for 4 hr. at 105 000 g contained high titres of non-haemagglutinating antigen. When this supernatant was, in turn, centrifuged for a further 17 hr. at 105 000 g, all the SBP activity was found to have sedimented (Table 5). This, in fact, provided a convenient method for the concentration of the antigen. The 'virus-free' 17 hr.-infected culture fluid was centrifuged overnight at 105 000 g and the sedimented material

Table 4. *Effect of ribonuclease and trypsin on non-haemagglutinating antigen*

The source of the antigen was the 'virus-free' cell sap from cultures infected for 7.5 hr. The pH adjustments were made with 4.4% (w/v) sodium bicarbonate solutions. After incubation with 1 vol. of 0.01% trypsin (Armour Pharmaceutical Co., Eastbourne, Sussex), the solutions were cooled to room temperature and the trypsin was neutralized with 1 vol. of 0.02% soya-bean trypsin inhibitor (Worthington Biochemical Corp., Freehold, N.J., U.S.A.) before carrying out the SBP assay. Ribonuclease (C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany) was used as a 0.01% solution. All incubations with trypsin or ribonuclease were for 1 hr. at pH 7.7 at the temperatures indicated.

Antigen	Treatment before SBP assay	SBP titre (cup shift)
Sucrose (0.25 M)-KCl (0.1 M) solution	Trypsin (37°)	0
	Ribonuclease (37°)	1
Non-haemagglutinating antigen	Brought to pH 7.7 and stored at 4°	19
	Trypsin (room temperature)	15
	Trypsin (37°)	1
	Ribonuclease (37°)	19
EMC virus*	Brought to pH 7.7 and stored at 4°	18
	Trypsin (37°)	21

* 600 haemagglutinin units/ml.

Table 5. *Effect of prolonged centrifugation on non-haemagglutinating antigen*

Antigen was prepared either from a 'virus-free' 17 hr.-infected culture fluid ('extracellular'), or from the cell sap obtained from washed cells disrupted after incubating with EMC virus for 7.5 hr. and centrifuged for 2 hr. at 105 000 *g* to remove infective virus ('intracellular'). These preparations were then centrifuged at 105 000 *g* for 17 hr. at 4°. SBP titrations were carried out, as described in the Materials and Methods section, on the supernatant and on the pellets after suspension in one-eighth of the original volume of phosphate-buffered saline.

Source of antigen	Fraction assayed after centrifugation at 105 000 <i>g</i> for 17 hr.	SBP titre (cup shift)
Extracellular	Supernatant	0
	Pellet	18
Intracellular	Supernatant	4
	Pellet	28

resuspended in about 5 ml. of phosphate-buffered saline. After centrifugation at 2300 *g* for 10 min. to remove undissolved material, this solution provided the 'pelleted and resuspended' antigen preparation.

The sedimentation behaviour of this preparation was studied by the equilibrium-centrifugation method of Charlwood (1963). The antigen solution was mixed with ¹³¹I-labelled arachin (*S*_{20,w} 13.5 s) and ¹²⁵I-labelled fibrinogen (*S*_{20,w} 7.8 s), and 0.8 ml. of this mixture layered on to 12 ml. of a 10–50% sucrose gradient dissolved in phosphate-buffered saline, containing trisodium citrate (0.03 M). The

gradient was centrifuged at 105 000 *g* for 17 hr. at 20° and 0.6 ml. fractions were collected from the bottom of the centrifuge tube. The fractions were dialysed to remove sucrose and assayed for SBP titre and radioactivity. The results are shown in Fig. 2. The main peak of SBP activity, peak *A*, corresponds almost exactly with that of the arachin marker, and we conclude that the non-haemagglutinating antigen has a sedimentation coefficient at 20° of approx. 14s. The main peak exhibited a shoulder, peak *B*, of *S*_{20,w} 6.5 s, that may represent a smaller antigen, possibly a breakdown product of the 14s antigen.

Diffusion coefficient of the non-haemagglutinating antigen. When the pelleted and resuspended antigen preparation was placed in a peripheral cup of an Ouchterlony plate, and EMC-virus-specific antiserum placed in the central cup, a clear line of precipitation was observed. It was therefore possible to obtain an estimate of the diffusion coefficient of the antigen. This was done by utilizing the technique of Allison & Humphrey (1960). Agar plates were prepared and two troughs (20 mm. long × 2 mm. wide) cut at right angles into each plate. Antiserum was placed into one trough and the other trough filled with pelleted and resuspended antigen. After 48 hr. at room temperature, a clear precipitation line between the troughs was observed (Kerr *et al.* 1962). In three experiments the angles between the line and the antigen trough were 42°, 42.5° and 43°. The diffusion coefficient of the antigen is the product of the diffusion coefficient of the antibody and the tangent of this angle (Allison & Humphrey, 1960). As the coefficient for rabbit γ-globulin is 3.8 × 10⁻⁷ cm.²sec.⁻¹ (Kabat & Meyer,

Fig. 2. Equilibrium-density-gradient centrifugation of non-haemagglutinating antigen. Pelleted and resuspended antigen from 17 hr.-infected culture fluid (0.4 ml.), together with a solution containing ^{131}I -labelled arachin and ^{125}I -labelled fibrinogen (0.4 ml.), was layered on top of 12 ml. of a graded 10–50% (w/v) solution of sucrose and centrifuged for 17 hr. at 105 000 g; 18 0.6 ml. fractions were then collected from each of five centrifuge tubes and the corresponding fractions from each tube pooled. Radioactivity and SBP titres were assayed as described in the Materials and Methods section. —, SBP titre of non-haemagglutinating antigen; ----, radioactivity of ^{131}I -labelled arachin; ·····, radioactivity of ^{125}I -labelled fibrinogen.

1948), this gives an estimate of the diffusion coefficient of the non-haemagglutinating antigen of $3.2 \times 10^{-7} \text{ cm}^2 \text{ sec}^{-1}$. By combining this value with the sedimentation coefficient (13.5s) we obtain an estimate of the particle weight of the antigen of $420\,000 \pm 40\,000$.

Comparison of the non-haemagglutinating antigen with antigens obtained by degradation of crystalline EMC virus. When crystalline EMC virus is dialysed at 4° against 0.15 M-sodium chloride, pH 10.5, containing ethanolamine (0.03 M), an antigen of sedimentation coefficient 3–4s is obtained (Hamilton, Kerr & Work, 1962). It was decided therefore to ascertain whether the 14s antigen could be similarly degraded by ethanolamine treatment. Non-haemagglutinating antigen was prepared from 'virus-free' 17 hr.-infected culture fluid by centrifugation at 105 000 g for 17 hr. A portion of the pelleted material was resuspended in phosphate-buffered saline and the remainder dissolved in 0.15 M-sodium chloride, pH 10.5, containing ethanolamine (0.03 M). Undissolved

material was removed by low-speed centrifugation and the solutions were kept for 2 days at 4° . The solution was then examined for low-particle-weight antigen by examination on Ouchterlony plates. It was found that treatment of the non-haemagglutinating antigen with ethanolamine gave rise to a smaller antigen of a size similar to that of the antigen obtained by ethanolamine treatment of crystalline virus. Absolute identity between these two antigens could not be demonstrated by the Ouchterlony technique; nevertheless, it seems likely that the proteins derived from the degradation of the non-haemagglutinating antigen are identical with that obtained by similar treatment of the purified virus.

Absence of antigens smaller than 14s. As purified virus could be readily degraded into antigens of molecular weight much less than that of the 14s non-haemagglutinating antigen (M. G. Hamilton, I. M. Kerr, E. M. Martin & T. S. Work, unpublished work), it seemed possible that such smaller antigens may exist either intracellularly or extracellularly in virus-infected cell cultures. Cell-free culture fluids from cultures infected for 17 hr. and cell homogenates from 7.5 hr.-infected cultures were centrifuged for 17 hr. at 105 000 g to remove all 14s antigen. The supernatants were concentrated 10–16-fold by pressure dialysis and the preparations examined for the presence of EMC-virus-specific antigens by the Ouchterlony technique. This technique had previously been shown to be more sensitive in detecting low-molecular-weight antigens from degradation of the virus than had the SBP assay. Only small amounts of the 14s antigen could be detected, and no antigen of smaller size was observed.

Presence of 14s antigen within infected cells. Infected ascites cells were examined for the presence of the 14s antigen intracellularly in the following manner. Cells were harvested 7.5 hr. after infection with EMC virus, washed and disrupted. Cell debris and nuclei were removed by low-speed centrifugation and cytoplasmic particles and infective virus sedimented at 105 000 g for 2 hr. The supernatant cell sap was assayed for its SBP titre and this was found to be quite high (Table 4). When this cell sap was subjected to centrifugation at 105 000 g for 17 hr. all of the SBP titre was found to have sedimented. When this pellet was redissolved and tested for antigenicity by the Ouchterlony technique it gave a single precipitation line. The SBP titre of the cell sap was abolished by trypsin, but not by ribonuclease (Table 4). It was concluded from these results that the sap of infected cells contained a virus-specific antigen that was probably identical with the 14s non-haemagglutinating antigen obtained from the extracellular culture fluids.

Time-course of synthesis of the non-haemagglutin-

ating antigen. As the presence of the non-haemagglutinating antigen could be demonstrated within the infected cell it was decided to examine the time and site of its synthesis. Washed Krebs cells were infected with EMC virus and divided into ten portions each of 5×10^8 cells. At appropriate intervals after the beginning of incubation a batch of cells was chilled to 0° and the cells were disrupted in the Dounce homogenizer. Subcellular fractions were separated by differential centrifugation and the fractions treated to remove infective virus as described in the Materials and Methods section. These fractions were then assayed by the SBP technique and the results are summarized in Fig. 3. The non-haemagglutinating antigen was first detected in the cell sap 3.5 hr. after infection and its titre in this fraction increased throughout the growth cycle. The antigen appears to be synthesized slightly before, or concurrent with, the rise in titre of mature virus. From 5.5 hr. onwards increasing amounts of antigen were also found in the nuclear fraction. It seems unlikely that this association is due to antigen being synthesized in the nucleus; it is more probable that during the progressive degeneration of the nucleus (which is obvious even in low-power electron microphotographs of 6 hr.-infected cells) this organelle would

physically entrap antigen present in the cytoplasm, particularly during the terminal stages of infection.

In Fig. 3, the results are given for subcellular fractions pretreated with deoxycholate to release any adsorbed antigen; negligible antigen was found in the cytoplasmic particulate fractions. However, in an experiment in which release was attempted by using ultrasonic vibrations, considerable SBP titres were exhibited by a mixed mitochondrial and microsomal fraction. The antigen in this fraction appeared at the same time as that in the cell sap, and the titres of antigen almost equalled those in the soluble fraction. The explanation for this discrepancy is not clear, but it may represent immunologically reactive products released by the ultrasonic disruption of the mature virus particles contained in these fractions. However, it seems reasonable to conclude that the cytoplasm is the first site of accumulation of the non-haemagglutinating antigen.

DISCUSSION

In the above studies no conclusive evidence was obtained for the presence in infected cell material of an EMC-virus-specific antigen smaller than the non-haemagglutinating 14s antigen. However, though the SBP assay is capable of detecting whole EMC virus at a concentration of approx. $2 \mu\text{g./ml.}$, neither the SBP assay nor the Ouchterlony-plate technique could detect the 4s antigen at concentrations of less than approx. $40 \mu\text{g./ml.}$ In view of this, the presence of very small amounts of the 4s or similar antigens in the infected cell cannot be completely excluded. Further, Maizel (1963) has reported that a sub-unit of poliovirus capsid protein of particle weight about 27000 is incapable of reacting with antiserum to whole poliovirus. However, by using antiserum to this sub-unit, it has been detected in the poliovirus-infected HeLa cell (Scharff, Shatkin & Levintow, 1963). In degradation studies on molecular EMC virus, a particle weight about 29000 has been detected (M. G. Hamilton, I. M. Kerr, E. M. Martin & T. S. Work, unpublished work); however, as this material has only been detected in concentrated solutions of guanidinium chloride, immunological studies have not been possible. The possibility remains, therefore, that this EMC-virus sub-unit will also be incapable of reacting with antiserum to whole virus. If this is in fact the case, even if present in the infected cell, clearly it would not have been detected in the above studies. The question whether the ultimate sub-unit of EMC-virus capsid protein exists as such in the infected cell must remain open.

Results obtained by M. G. Hamilton, I. M. Kerr, E. M. Martin & T. S. Work (unpublished work) show that the sub-units of EMC-virus capsid protein are

Fig. 3. Course of appearance of non-haemagglutinating antigen in various subcellular fractions. Portions (25 ml.) of EMC-virus-infected (3 plaque-forming units/cell) suspensions of ascites-tumour cells (2×10^7 cells/ml.) were kept overnight at 4° , then incubated for the various times indicated. The cells were washed and disrupted and separated into the various subcellular fractions as described in the Materials and Methods section. The fractions were assayed for whole virus (haemagglutinin titration) and for SBP activity after prior treatment with deoxycholate to release bound antigen and centrifugation to remove whole virus (see the Materials and Methods section). No antigen was detectable in either the mitochondrial or microsomal fractions up to 7.5 hr. after infection. At 8.5 hr. after infection, the mitochondrial fraction exhibited an SBP titre of 2 and the microsomal fraction a titre of 7. ●, SBP titre of cell sap; ▲, SBP titre of nuclear fraction; ○, haemagglutinin titre of whole virus.

capable of existing in a number of polymeric forms (the smaller units tending to polymerize and precipitate at physiological pH values). It seems most probable, therefore, that the 14s antigen represents an extremely stable polymer of this type. Whether it represents a fragment of a normal virus particle, or a stable aggregate of sub-units that is incapable of further polymerization into whole virus, is not yet clear. However, the protein of tobacco-mosaic virus consists of 5 of the ultimate sub-units (Knight, 1961; Takahashi & Gold, 1960), and a poliovirus-specific antigen of sedimentation coefficient 10s and a virus-specific antigen of sedimentation coefficient 8s have been detected in poliovirus- (Watanabe, Watanabe & Hinuma, 1962) and mouse-encephalomyelitis-virus- (W. Schäfer, personal communication) infected cells. It seems possible, therefore, that aggregates of the ultimate protein sub-units may be common features of cells infected with picornaviruses.

With the exponential nature of the SBP assay in mind (Table 1), it is clear from the results presented in Fig. 3 that the amount of antigen increases exponentially throughout the virus growth cycle. However, in the absence of any absolute quantitation of the SBP assay in terms of weight of 14s antigen, the relative amounts of this antigen and whole virus present during the course of infection remain uncertain. Nevertheless, the titre of antigen increases at a somewhat earlier time than that of the whole virus. Its time of synthesis corresponds more closely to that of the synthesis of the protein that is incorporated into the mature viral capsid (Martin & Work, 1962). A similar situation has been reported for mouse fibroblasts infected with mouse encephalomyelitis virus by Scholtissek, Rott, Hausen, Hausen & Schäfer (1962), who found that the increase in titre of a complement-fixing antigen during the infection cycle corresponded to the increase in viral precursor protein. However, insufficient information is available to decide in

either of these cases whether the non-haemagglutinating or complement-fixing antigens are true precursors of the capsid protein, or merely by-products of the infectious process.

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