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The inhibition of growth of vaccinia and cowpox viruses in RK13 cells

BY DERRICK BAXBY AND C. J. M. RONDLE*
Department of Bacteriology, Liverpool University, Liverpool 3, *England*

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INTRODUCTION

Many workers have studied the inhibition of growth of vaccinia virus. They have used various virus strains, cell lines and experimental conditions.

Early work (Thompson, 1947) was concerned primarily with the effect of inhibitors on the production of infective virus; later work concerned the effect of growth inhibitors on the production of viral antigens. Using complement fixation, immune fluorescence, and precipitation of radio-active proteins 'vaccinia-specific antigen' has been detected in cultures treated with sodium azide (Easterbrook, 1961), isatin-β-thiosemicarbazone (IBT; Easterbrook, 1962), 5-fluorodeoxyuridine (FUDR; Salzman, Shatkin & Sebring, 1963), 5-bromodeoxyuridine (BUDR; Easterbrook & Davern, 1963) and hydroxyurea (Rosenkranz, Rose, Morgan & Hsu, 1966).

Gel-diffusion techniques have shown the complexity of pox virus-specific antigens (Gispen, 1955; Rondle & Dumbell, 1962; Appleyard & Westwood, 1964; Marquardt, Holme & Lycke, 1965). Such techniques have shown also that the production of some rabbit pox soluble antigens is inhibited by azide and IBT (Appleyard, Westwood & Zwartouw, 1962), by rutilantin A (Hume, Westwood & Appleyard, 1965), but not by BUDR (Appleyard & Westwood, 1964).

Attempts have been made to correlate the varied data available for pox viruses, although Joklik (1966) has emphasized the dangers of comparing results obtained in different ways. This paper compares the effect of several growth inhibitors on the replication of one strain each of vaccinia and cowpox. A standard set of experimental conditions was adopted, and wherever possible one cell line (RK13) was used; exceptions are noted in the text. The criteria of growth studied were infective virus production and the appearance of virus specific soluble antigens as detected by gel diffusion and haemagglutination tests.

MATERIALS AND METHODS

Virus strains

The Lister strain of vaccinia and the Brighton strain of cowpox were used throughout.

* Present address: London School of Hygiene and Tropical Medicine, Keppel Street, London, W.C.1.

Cell lines

The RK13 line of transformed rabbit kidney cells (Beale, Christofinis & Furminger 1963) was used for most of the experiments; in some cases HeLa cells (strain Conway) were used.

Cells were grown as monolayers in 2 in. diam. Petri dishes in Parker's 199 medium. For RK13 cells 5 % calf serum was added, for HeLa cells 5 % inactivated human serum. Incubation was at 35° C. in an atmosphere of 5 % CO_2 in air. Eagle's basal medium was used without serum for maintaining confluent monolayers (Baxby & Rondle, 1967).

Growth inhibitors

These are listed under their most probable mode of action as adjudged from the literature. Most of the compounds have been tested on poxvirus by several workers. Iodacetate was included as it has been overlooked since the work on vaccinia by Thompson (1947). Proflavine was tested because adenovirus replication was inhibited, although adenovirus components were synthesized in its presence (Wilcox & Ginsberg, 1962). Unless otherwise stated, inhibitors were stored at −20° C. as 0·1 M solutions in sterile 0·85 % saline.

Inhibitors of energy-yielding reactions: sodium azide (B.D.H.), sodium iodoacetate (B.D.H.).

Inhibitors of DNA function. Proflavine hydrochloride (K & K Labs.) and actinomycin D (a gift from Mercke, Sharpe & Dohme) which interfere with transcription of RNA from DNA.

Inhibitors of DNA synthesis: FUDR (a gift from Roche Products), BUDR (California Corp. for Biochem. Research), hydroxyurea (Sigma Chemical Co.), mitomycin C (Kyowa Hakko Kogyo Co.): this last compound was kept in the dark in the original ampoules and dissolved in maintenance medium immediately before use.

Inhibitors with specific activity against poxviruses: *n*-methyl-IBT (MIBT) (a gift from Dr D. J. Bauer, Wellcome Labs.). This was dissolved at 2 mg./ml. in dimethylformamide and diluted to 100 μM. in water. The aqueous suspension was autoclaved at 15 lb./in.^{2} for 10 min. to dissolve the compound. Solutions were stored at 37° C. and diluted with maintenance medium when required. Rutilantin A (a gift from Dr G. Appleyard, M.R.E. Porton) was dissolved in acetone at 200 μg./ml., stored at −20° C. and diluted with maintenance medium when required.

Experiments with proflavine, actinomycin D and mitomycin C were done in subdued light.

Antisera

Antisera to vaccinia and cowpox were prepared in rabbits as described by Rondle & Dumbell (1962). Rabbit-grown virus only was used for immunization to avoid production of antibodies not specific for virus.

Virus growth curves

Growth curves were done as described by Baxby & Rondle (1965). Virus multiplicities of 10 and 0·01 plaque-forming units (pl.f.u.) per cell were used with three

dishes per group. Inhibitors were usually added with the inoculum. Cells and supernatant fluids were harvested separately at various intervals after inoculation. Cells were disrupted ultrasonically (M.S.E./Mullard disintegrator) and the virus content of disrupted cells and supernatant fluid determined.

Virus infectivity titrations

These were done either on the chorioallantoic membrane (CAM) of 12-day chick embryos (McCarthy & Dumbell, 1961) or by the RK13 plaque assay (Baxby & Rondle, 1967).

Plaque inhibition tests

Confluent RK13 monolayers were inoculated with approx. 240 pl.f.u. virus in 1 ml. medium containing inhibitor. After 1 hr. at 35° C. 3 ml. medium containing inhibitor was added and incubation continued. Plaques were counted and measured at the optimum time. This was 46 hr. after inoculation for vaccinia and 50 hr. after inoculation for cowpox (Baxby & Rondle, 1967). The percentage plaque reduction compared with controls was calculated.

Haemagglutination tests

These were done as described by McCarthy & Helbert (1960).

Gel diffusion tests

The Ouchterlony double-diffusion technique was used as modified by Rondle & Dumbell (1962). Fluids and cells from infected cultures were harvested separately, dialysed at 4° C. against daily changes of distilled water until free from salt, and dried from the frozen state. Dried materials were resuspended at suitable concentrations in phosphate-phosphate buffer, pH 7·4 for test. Results of experiments were photographed by dark ground illumination.

Virus antigens

Vaccinia standard antigen (VA) was made from infected rabbit dermis. Cowpox standard antigen (CA) was obtained from infected RK13 cells.

RESULTS

Virus replication in RK13 cells

Virus growth

Growth curves of vaccinia and cowpox in RK13 cells using an input multiplicity of 10 pl.f.u./cell are shown in Fig. 1. Fifty per cent of the virus was adsorbed to the cells by 1 hr. (Table 1). Figure 1 shows that new infective virus was detected at 8–10 hr. for vaccinia and 10–12 hr. for cowpox; and that maximum titres were detected at 20 hr. for vaccinia and at 30 hr. for cowpox.

Even by 48 hr. little virus had been released from the cells into the supernatant fluids. The virus yields for a range of inocula are shown in Table 1. At the end of one growth cycle there was a yield of approximately 100 pl.f.u./pl.f.u. adsorbed

for both viruses. With inocula above 1×10^7 pl.f.u./dish (5 pl.f.u./cell) the yield remained constant at 48 hr. By 48 hr. with lower inocula second cycles of infection gave a higher yield than at 24 hr.

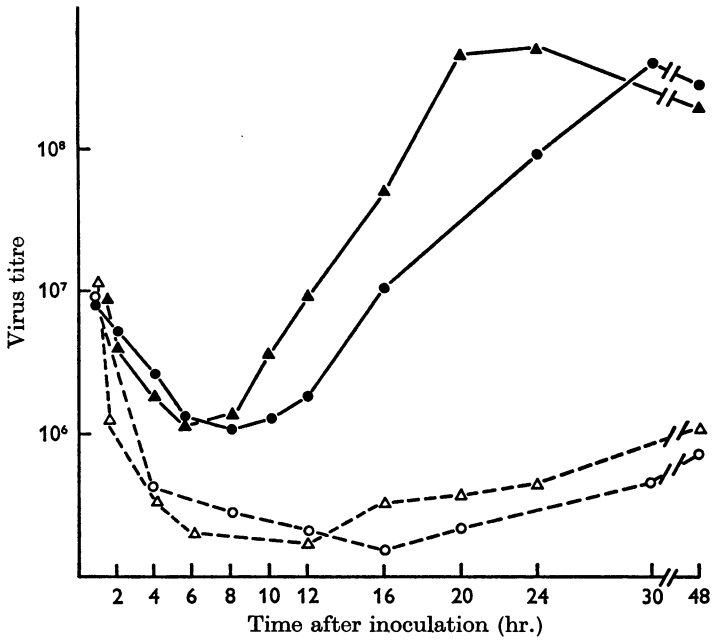

Fig. 1. Growth curves of vaccinia and cowpox viruses in RK 13 cells. ▲——▲, Cell-associated vaccinia virus; △----△, fluid-associated vaccinia virus; ●——●, cell-associated cowpox virus; ○----○, fluid-associated cowpox virus.

Table 1. *Yield of vaccinia and cowpox in RK* 13 *cells*

Inoculum (pl.f.u./dish 10^5)	Virus adsorbed* at 1 hr. (10^5)	Virus yield (10^6)	Virus yield/ virus adsorbed, $\log_{10}$	Virus yield (10^7)	Virus yield/ virus adsorbed, $\log_{10}$
		24 hr.		48 hr.	
A. Vaccinia					
0·1	0·05	0·5	2·0	41	4·9
2·0	1·1	6·2	1·8	32	3·5
10	4·7	67	2·1	36	2·8
100	50	320	1·8	42	1·9
200	91	500	1·8	47	1·7
		30 hr.		48 hr.	
B. Cowpox					
0·1	0·06	0·65	2·1	30	4·7
2·0	1·2	10	1·9	37	3·5
10	6·1	52	1·9	41	2·8
100	51	300	1·8	35	1·9
200	100	590	1·8	53	1·7

* Virus adsorbed at 1 hr. calculated from inoculum virus titre less virus removed in the supernatant fluids at 1 hr.

Virus soluble antigens

Haemagglutinin (HA)

This was detected first at the time new infective virus was produced. Figure 2 shows the relationship between the yields of HA and infective virus at the end of one growth cycle. The particular strain of cowpox used produced HA as readily as vaccinia in RK13 cells. Fenner (1958) obtained the same result on CAM, although other strains of cowpox produced less HA than vaccinia. The mean value for the ratio virus yield (pl.f.u./ml.)/HA (units/ml.) was $10^{5\cdot0}$ for both viruses. At the end of one growth cycle the HA titres in the cells were at least 30 times higher than the titres in the overlaying fluids.

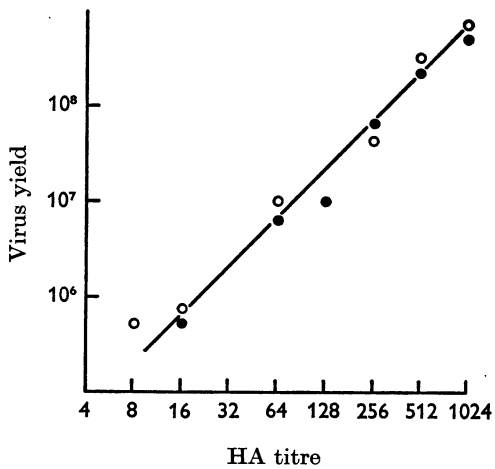

Fig. 2. Relationship between yield of infective virus and yield of HA at the end of one growth cycle. ●, Vaccinia virus; ○ cowpox virus.

Antigens detected by gel diffusion

Plates 1A and B show the 'standard system' used for the analysis of vaccinia and cowpox soluble antigens. By suitable tests thirteen different lines were regularly detected in gel diffusion patterns. Numbers were allotted to the lines and used to designate the antigens or haptens which were line pattern components (lpc). Numbers 1 and 2 do not appear in this paper; they were used in the course of the work to designate line pattern components which were not detected regularly. Lpc 3, 5 and 7 were not normally found in cowpox soluble antigens prepared from CAM (cf. lpc *f* in Rondle & Dumbell, 1962). Lpc 3 is heat labile and lpc 5 and 7 heat stable; they are serologically related to the 'L' and 'S' antigens of vaccinia (Smadel & Shedlovsky, 1942; Williamson, 1963; Williamson & Rondle, 1964). Lpc *d* was the material normally present in cowpox soluble antigens, but absent from antigens prepared from vaccinia and the white pock variant of cowpox (Rondle & Dumbell, 1962).

Plate 2A shows that all the components present in *VA* were present in vaccinia soluble antigens produced in RK13 cells with the possible exception of lpc 1 and 2, which were not regularly detected in standard antigen *VA*. Plate 2A also shows that

the water-insoluble buffer-soluble fraction (*VARK bs*) deposited during dialysis contains a full complement of lpc, whereas the water-soluble fraction (*VARK ws*) contained only lpc 10, 11 and 12 with traces of lpc 8 and 9. Dialysis thus presented a convenient method of concentrating vaccinia soluble antigens. Cowpox soluble antigens shared many components with *VA* and *VARK*, but lpc *d* was not present in the vaccinia-infected tissues and lpc 11 was never demonstrated in cowpox soluble antigens. Although cowpox soluble antigens from CAM lack lpc 3, 5 and 7 (Plate 1A) cowpox soluble antigens from RK13 cells contained lpc 3 and small amounts of lpc 5, 7 (Plate 1B). Rondle & Dumbell (1962) extracted lpc 3 and 5 from cowpox-infected CAM with trypsin, and it is possible that these components may be present in a diffusible form in cowpox-infected RK13 cells.

The distribution of lpc between cells and culture fluids was determined. All components were detected in cells, but lpc 3, 4, 5, 6, 7 and 11 were not detected in culture fluids. This may be a general feature of poxvirus-infected tissue cultures since similar results were obtained with chick embryo fibroblasts, HeLa and GMK cells. The qualitative difference in lpc distribution between cells and fluids permitted recognition of two further components, lpc 13 and 14, which were readily detected in the fluids but obscured in tests on cell extracts by other components (Plate 2B).

Tables 4 and 5 (controls) show the times after inoculation at which each lpc could be detected in infected RK13 cells. The common components appeared approximately 2 hr. later in cowpox-infected RK13 cells than in vaccinia-infected RK13 cells. Lpc *d* was the first component detected in cowpox-infected tissues.

EVENTS FOLLOWING INFECTION OF RK13 CELLS TREATED WITH INHIBITORS OF VIRUS GROWTH

Virus production

Sodium azide

This compound was cytotoxic, but comparison of infected and uninfected monolayers in the presence of inhibitor suggested that virus-specific cytopathic effect occurred in cultures treated with 2·5 mM. but not 5 mM. azide. No evidence of infective virus synthesis was obtained from growth curves done in the presence of the latter concentration. Results obtained with vaccinia and cowpox were identical and similar to those described for rabbit pox in HeLa cells (Appleyard *et al.* 1962) and for vaccinia in KB cells (Easterbrook, 1961).

Sodium iodoacetate

This compound was cytotoxic and plaque inhibition tests could not be done. However, growth curves showed that cowpox virus replication was completely inhibited by 50 μM. iodoacetate (Fig. 3B), although this concentration permitted approximately 5% vaccinia virus synthesis (Fig. 3A). Such a difference in the effect of iodoacetate on closely related viruses has not previously been reported.

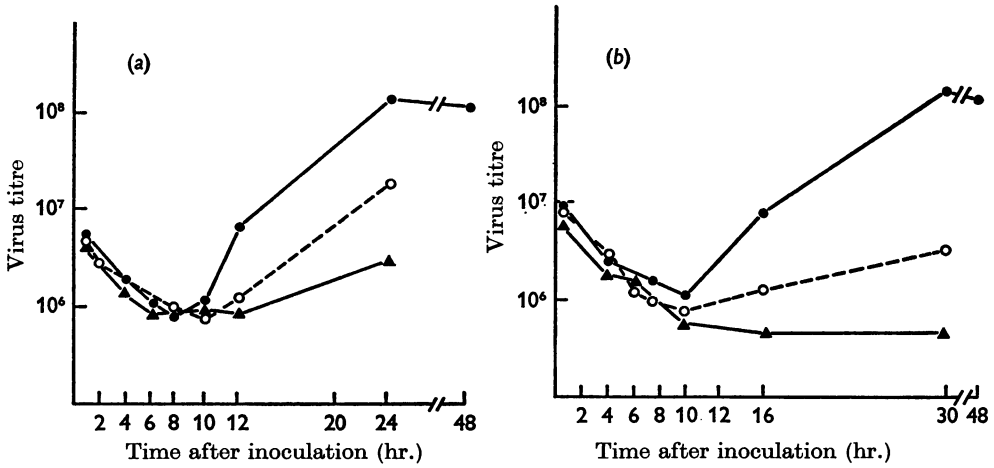

Fig. 3. Effect of iodoacetate on growth curves of (*a*) vaccinia and (*b*) cowpox in RK 13 cells. ●——●, Control; ○----○, 20μM.; ▲——▲, 50μM. iodoacetate.

Proflavine and actinomycin D

Plaque inhibition tests and growth curve experiments showed that replication of vaccinia and cowpox viruses in RK13 cells was completely inhibited by 20 μM. proflavine. A similar result was obtained for vaccinia in KB cells by Bubel & Wolff (1965). Replication of both viruses was completely inhibited by 0·5 μg/ml. actinomycin D.

FUDR

It is difficult to inhibit poxvirus replication completely in RK13 monolayers with FUDR (Baxby & Rondle, 1965). However, results of plaque inhibition and growth curve experiments indicated that vaccinia and cowpox virus replication was inhibited completely by 80 μM. FUDR.

BUDR

Replication of both viruses was completely inhibited by 25 μM. BUDR. This was of interest since Easterbrook & Davern (1963) showed that different poxviruses may differ in susceptibility to this reagent.

Hydroxyurea

Cowpox virus was completely inhibited by 10 mM. hydroxyurea (Fig. 4B), whereas a 50 mM. concentration was required to inhibit vaccinia (Fig. 4A). The result with cowpox was similar to that obtained with vaccinia in human amnion cells by Rosenkranz *et al.* (1966), but as far as is known the results reported here represent the first finding that this compound has different effects on closely related viruses.

Mitomycin C

Both viruses were completely inhibited in RK13 cells by 10 μg./ml. mitomycin C. This compares well with the result obtained for vaccinia in HeLa cells by Oda (1963).

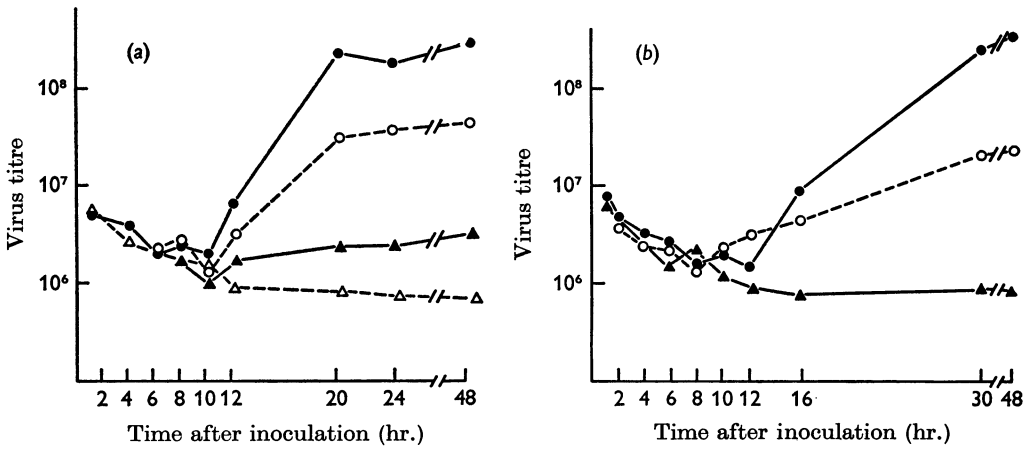

Fig. 4. Effect of hydroxyurea on growth curves of (*a*) vaccinia and (*b*) cowpox in RK 13 cells. ●——●, Control; ○----○, 1 mM; ▲——▲, 10 mM; △----△, 50 mM. hydroxyurea

Table 2. *Plaque inhibition tests with MIBT in HeLa cells*

MIBT (μM.) ...	0	10	20	30	40
Vaccinia count	100*	16	0	0	NT†
Plaque diam. (mm.)	0·7	0·1	0	0	NT†
Cowpox count	100*	36	9	1·5	0
Plaque diam. (mm.)	0·6	0·4	0·2	0·1	0

* Control counts adjusted to 100: other counts expressed as % of this.
† Not tested.

Thiosemicarbazone

Thiosemicarbazones are relatively inefficient in RK13 cells (Appleyard, Hume & Westwood, 1965; Baxby, 1967). Experiments were done therefore in HeLa cells. Using these cells cowpox proved to be less susceptible to IBT than vaccinia (Table 2.). This finding corresponds to that of Bauer (1961) who studied the effect of IBT on the growth of these viruses in mice.

Rutilantin A

The minimum inhibitory concentration of this compound for both cowpox and vaccinia in RK13 cells was 0·6 μg./ml. Hume *et al.* (1965) obtained a similar result for rabbit pox virus in these cells.

Haemagglutinin

Production of vaccinia and cowpox HA was inhibited by azide, iodoacetate, proflavine, actinomycin D, hydroxyurea, FUDR, MIBT and rutilantin A. Concen-

trations of these inhibitors which completely inhibited virus replication completely inhibited HA production. In cultures where virus replication was not inhibited completely the amount of HA produced was proportional to the virus yield. Vaccinia virus HA has been reported previously to be sensitive to inhibition in tissue culture by actinomycin D (Fujio, 1963), FUDR (Loh & Payne, 1965) and proflavine (Bubel & Wolff, 1965). Results with the other compounds are novel.

Oda (1963) reported that vaccinia virus HA was produced in HeLa cells in concentrations of mitomycin C (MC) which completely inhibited virus replication. We obtained a similar result using RK13 cells (Table 3). No virus was produced at inhibitor concentrations of 10 μg/ml. and above, although significant amounts of HA were produced in cowpox infections at 15 μg./ml. and in vaccinia infections at 30 μg./ml. inhibitor. It is interesting that increasing concentrations of inhibitor affected cowpox HA more than vaccinia HA.

Table 3. *Effect of mitomycin C on HA production*

Mitomycin C (μg./ml.) ...	0	5	10*	15	20	25	30
Vaccinia HA	1024†	1024	1024	1024	512	64	16
Cowpox HA	1024	1024	1024	128	4	4	2
No virus	4	—	—	4	—	—	4

* No new virus produced with 10 μg./ml. mitomycin C or above.
† Figures are reciprocals of HA endpoint.

Vaccinia and cowpox HA were also produced in cultures treated with sufficient BUDR to inhibit virus replication completely. At BUDR concentrations of 25 μM. or above no new virus was produced. However, HA production by infected cells occurred even in the presence of 80 μM. BUDR. The titre of HA detected (1/1024) did not differ from that of controls with no inhibitor added.

Antigens detected by gel diffusion

The effects of inhibitors on the production of vaccinia and cowpox soluble antigens are shown in Tables 4 and 5. Results with both viruses are similar. Compounds which probably disrupted energy utilization in host cells (azide, iodoacetate) severely curtailed the production of detectable soluble antigens. This is despite the finding that new vaccinia virus was produced in the presence of 50 μM. iodoacetate. Compounds which inhibit DNA function (proflavine, actinomycin D) had a similar effect, although results obtained with actinomycin D depended upon amount of inhibitor used and time of application to the test system. Probable inhibitors of DNA synthesis (FUDR, BUDR, HU and MC) allowed production of most of the virus-specific substances found in infected RK13 cells, although no new virus was produced. Indeed with BUDR and MC virus-specific HA was detected.

MIBT could not be used in RK13 cells and experiments were done in HeLa cells. At the concentrations used the result obtained was similar to that given by MC in RK13 cells except that HA was not produced. With rutilantin A a dose-response effect was found similar to that found with actinomycin D.

Table 4. *Effects of inhibitors on production of vaccinia soluble antigens in RK* 13 *cells*

Inhibitor	Components detected after inoculation at (hr.) 4	5	6	8–10	10	12
None (control)	10, 12	8, 9	6	4, 3, 5, 7	HA	14*, 11
Components detected 24 hr. after inoculation						
Azide, 5 mM.	10, 12	8, 9	—	—	—	—
Iodoacetate, 50 μM.†	10, 12	8, 9	—	—	—	—
Proflavine, 20 μM.	10, 12	—	—	—	—	—
Actinomycin D, 3 μg./ml.	10, 12	—	—	—	—	—
Actinomycin D, 0·5–2 μg./ml.	10, 12	—	—	—	—	14
Actinomycin D, 2 μg./ml.‡	10, 12	—	—	4	—	14
Actinomycin D, 0·5–1·5 μg./ml.‡	10, 12	8, 9	6	4	—	14
FUDR, 80 μM.	10, 12	8, 9	6	4, 3, 5, 7	—	14, 11
BUDR, 25–80 μM.	10, 12	8, 9	6	4, 3, 5, 7	HA	14, 11
Hydroxyurea, 50 mM.	10, 12	8, 9	6	4, 3, 5, 7	—	14, 11
Mitomycin C, 10–20 μg./ml.	10, 12	8, 9	6	4	HA	14, 11
MIBT, 20 μM.§	10, 12	8, 9	6	4	—	14
Rutilantin A, 1·2 μg./ml.	10, 12	—	—	—	—	14
Rutilantin A, 1·0 μg./ml.	10, 12	—	—	4	—	14
Rutilantin A, 0·6–0·8 μg./ml.	10, 12	8, 9	6	4	—	14

* Lpc 14 obscured in cells by other materials. Recognized in fluids at 12 hr.
† Virus synthesis detected.
‡ Inhibitor added 1 hr. after inoculation.
§ Experiments done in HeLa cells.

Table 5. *Effect of inhibitors on production of cowpox soluble antigens in RK* 13 *cells*

Inhibitor	Components detected after inoculation at (hr.) 4	6	8	10–12	12	16
None (control)	*d*	10, 12	8, 9, 6	4, 3, 5, 7	HA	13*, 14*
Components detected 30 hr. after infection						
Azide, 5 mM.	*d*	10, 12	8, 9	—	—	—
Iodoacetate, 50 μM.†	*d*	10, 12	8, 9	—	—	—
Proflavine, 20 μM.	*d*	10, 12	—	—	—	—
Actinomycin D, 3 μg./ml.	—	10, 12	—	—	—	13
Actinomycin D, 2 μg./ml.	*d*	10, 12	—	—	—	13, 14
Actinomycin D, 2 μg./ml.‡	*d*	10, 12	—	4	—	13, 14
Actinomycin D, 1·5 μg./ml.‡	*d*	10, 12	8, 9, 6	4	—	13, 14
FUDR, 80 μM.	*d*	10, 12	8, 9, 6	4, 3, 5, 7	—	13, 14
BUDR, 25–80 μM.	*d*	10, 12	8, 9, 6	4, 3, 5, 7	HA	13, 14
Hydroxyurea, 10 mM.	*d*	10, 12	8, 9, 6	4, 3, 5, 7	—	13, 14
Mitomycin C, 10–20 μg./ml.	*d*	10, 12	8, 9, 6	4	HA	13, 14
MIBT, 40 μM.§	*d*	10, 12	8, 9, 6	4	—	13, 14
Rutilantin A, 1·5 μg./ml.	*d*	10, 12	—	4	—	13, 14
Rutilantin A, 1·2 μg./ml.	*d*	10, 12	8, 9, 6	4	—	13, 14

* Lpc 13 and 14 obscured in cells by other lpc; detected in fluids at 16 hr.
† No virus synthesis detected.
‡ Inhibitor added 1 hr. after inoculation.
§ Experiments done in HeLa cells.

DISCUSSION

The results showed that in RK13 cells new virus was detected 8–10 hr. after inoculation with vaccinia and 10–12 hr. after inoculation with cowpox. Virus-specific soluble antigens were detected 4 hr. after inoculation with both viruses. Antigens shared by the viruses however were detected earlier in vaccinia-infected tissues than in cowpox-infected tissues. Baxby & Rondle (1967) observed that cowpox plaques developed more slowly than vaccinia plaques in RK13 cells, but reasons for this phenomenon are not known.

In this work thirteen line pattern components were regularly detected in gel diffusion tests. Ten were shared by both viruses but lpc 11 was found only in vaccinia-infected tissues and lpc *d* and 13 in cowpox-infected tissues. The result with lpc *d* agrees with the observations of Rondle & Dumbell (1962) using antigens prepared from infected CAM: the other results are novel and require further investigation to establish whether or not lpc 11 and 13 are respectively vaccinia and cowpox-specific.

The times at which each lpc was first detected are given in Tables 4 and 5. These might also indicate the order in which virus-specific materials were made. Detection of materials however depends on the antibody composition of the antiserum used and the concentration of antigen or hapten present in the preparation. Identical results were obtained using several antisera. Order of manufacture could be confused however if an 'early' antigen was produced so slowly that its detection was delayed and a 'late' antigen was produced so fast that it was detected quickly.

Survey of the effect of inhibitors of virus growth showed that compounds of similar probable activity had similar effects upon the replication of both viruses. Concentrations of inhibitor which prevented production of infective virus did not necessarily inhibit production of soluble antigens. Compounds which appear to interfere with DNA function had the most dramatic effect. Lpc 10 and 12 were the only virus-specific materials found in vaccinia-infected cells treated with proflavine. In cowpox-infected cells lpc *d* was detected in addition to lpc 10 and 12. With actinomycin D additional materials were detected (lpc 14 with vaccinia; lpc 13 and 14 with cowpox). Lpc 10, 12 and *d* were the first virus-specific materials detected in infected cultures not treated with inhibitors. It is possible that in cultures treated with inhibitors the materials found were breakdown products of the infecting virus. This is, however, unlikely as low virus doses were used and few specific materials were detected and it seems reasonable to suppose that virus breakdown would furnish more than two or three of the thirteen soluble antigens known.

The dose-response effect with actinomycin D suggests that the steps involved in virus replication are not uniformly sensitive to inhibition. The effect of delaying application of this inhibitor until 1 hr. after inoculation was also of interest. At 2 μg./ml. one additional virus-specific substance was detected (lpc 4); with lower concentrations of inhibitor a further three substances were found (lpc 8, 9 and 6). This suggests that pathways leading to the production of nine or more line pattern components (lpc 10, 12, 4, 8, 9, 6, 14, and in addition in cowpox lpc 13 and *d*) were

established quickly after infection. After 1 hr. these pathways were not susceptible to blockage by an inhibitor of RNA synthesis. Since not all the pathways required for virus synthesis were present it suggests further that manufacture of virus-specific material is a sequential process.

It is tempting to suggest that some of the materials produced early in the replication cycle might be enzymes necessary for producing virus structural components and DNA rather than being virus structural components themselves. New enzymes are known to be produced in pox virus-infected cells (reviewed by Joklik, 1966) and the 'new' DNA polymerase found in herpes-infected cells has virus specificity (Keir *et al.* 1966).

Inhibitors of energy-yielding reactions also had a marked effect on the production of pox virus soluble antigens. As with proflavine lpc 10 and 12 were detected in vaccinia-infected cells, and lpc *d*, 10 and 12 in cowpox-infected cells. In cultures infected with either virus, however, lpc 13 and 14 could not be found, although lpc 8 and 9 were regularly present. The materials synthesized were those recognized early in the time study of antigen production. This supports the view that virus synthesis in infected RK13 cells treated with azide or iodoacetate proceeds sequentially until energy supplies are exhausted.

The effects of inhibitors of DNA synthesis are of interest because inhibitor-treated infected cells produced most, if not all the virus-specific substances found in uninhibited infected RK13 cells. Moreover, three different results were obtained with the four inhibitors tested suggesting that different methods of inhibition were involved. It was not possible to demonstrate cessation of DNA synthesis in treated RK13 cells, but provided this was achieved the results showed that in the presence of BUDR, inoculum virus DNA could code for all the virus-specific materials, detected by gel diffusion and haemagglutination, normally associated with virus replication. In the presence of FUDR and HU the only virus-specific substance not detected was HA. With mitomycin C, HA was produced, but lpc 3, 5 and 7 were not detected in cowpox-infected cells, nor lpc 3, 5, 7 and 11 in vaccinia-infected cells. Failure of inoculum virus DNA to code for virus-specific material could be understood if the DNA were degraded. It is known that in other systems mitomycin C both inhibits DNA synthesis and potentiates degradation of DNA by DNA-ase (Kersten, 1962; Kersten *et al.* 1964; Pricer & Weissbach, 1964). If MC did not immediately affect the input DNA it would accord with the finding that it inhibited only the manufacture of the materials detected late in the time-study of antigen production.

Results with specific inhibitors of poxvirus growth require little comment apart from the relative inefficiency of thiosemicarbazones in RK13 cells. Rutilantin A showed a dose-response effect and results obtained with the smallest dose were identical with the results given by MIBT in HeLa cells.

Consideration of Tables 4 and 5 *in toto* shows that the suggested sequential synthesis of virus-specific materials cannot be simple. For example, lpc 4 was on occasion detected in inhibitor-treated, infected RK13 cells in the absence of lpc 8 and 9; it was never detected in the absence of lpc 10, 12 and 14. In other experiments lpc 8 and 9 were found in the absence of both lpc 4 and 14, and lpc 14 was

found in the absence of lpc 8, 9 and 4. A possible interpretation of these results is that following synthesis of lpc 10 and 12 one pathway leads to production of lpc 8 and 9 and another independent pathway to production of lpc 14 followed by lpc 4.

Similar reasoning suggests that lpc 6 is formed next, followed by lpc 3, 5 and 7. The materials which are possibly specific to vaccinia or cowpox, lpc *d*, 11 and 13, have been omitted from this sequence. It can be seen that the possible order of sequential synthesis deduced from studies with inhibitors is similar to the results of the time-study of antigen production. For complete agreement it is necessary only to postulate that lpc 4 is produced before lpc 6 and that lpc 14 is produced in the cells 7–9 hr. before its detection in the supernatant fluids.

The work described emphasizes the close relationship between vaccinia and cowpox viruses. New differences have been detected however; these are the possible qualitative differences in soluble antigens discussed previously, and the quantitative differences in the presence of various inhibitors: thus vaccinia virus production was less sensitive than cowpox to iodoacetate and hydroxyurea, and production of vaccinia HA was affected less by MC than was production of cowpox HA. On the other hand, cowpox virus production was less sensitive than vaccinia virus to MIBT and more rutilantin A was required to inhibit production of cowpox antigens than was required to inhibit the serologically identical materials in vaccinia soluble antigen.

It is not known if these results apply to other strains of these viruses as Easterbrook & Davern (1963) showed that different strains of vaccinia had different susceptibilities to BUDR. It is intended to compare the properties of other strains of vaccinia and cowpox viruses with those reported here for the Lister and Brighton strains.

SUMMARY

The growth of vaccinia and cowpox in RK 13 cells was studied by measurement of infective virus production, gel diffusion and haemagglutination. The effects of virus growth inhibitors on the normal course of infection were then followed. Although the results with the two viruses were essentially similar some differences were detected between them. Inhibitors of DNA synthesis permitted production of many virus-specific soluble antigens. Compounds which inhibit DNA function and compounds that affected energy-yielding reactions had more dramatic effects. The different results obtained suggested that the synthesis of virus-specific materials was sequential and a possible part of the sequence is suggested.

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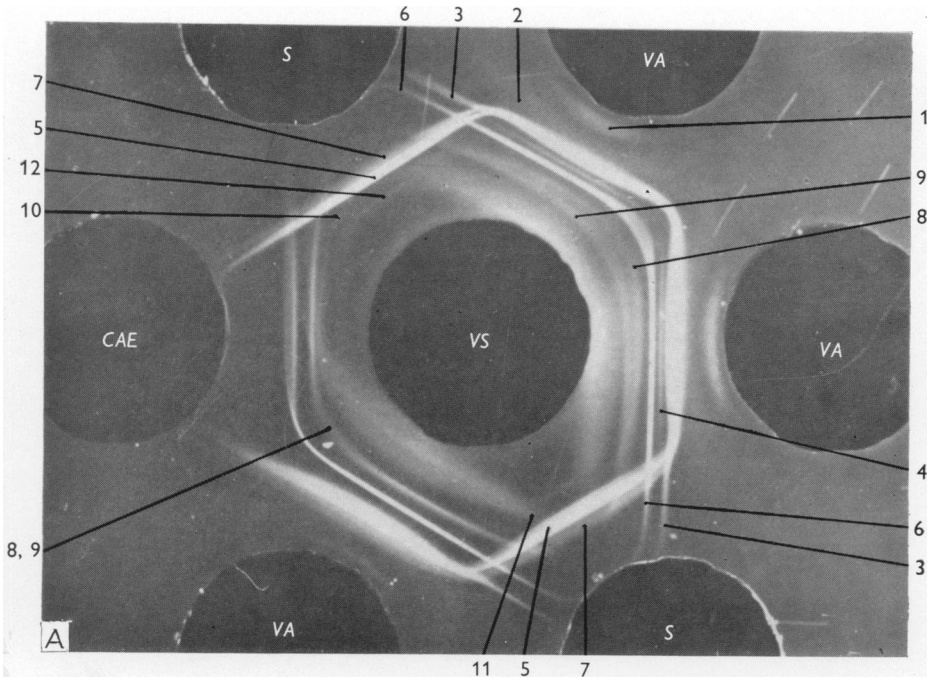
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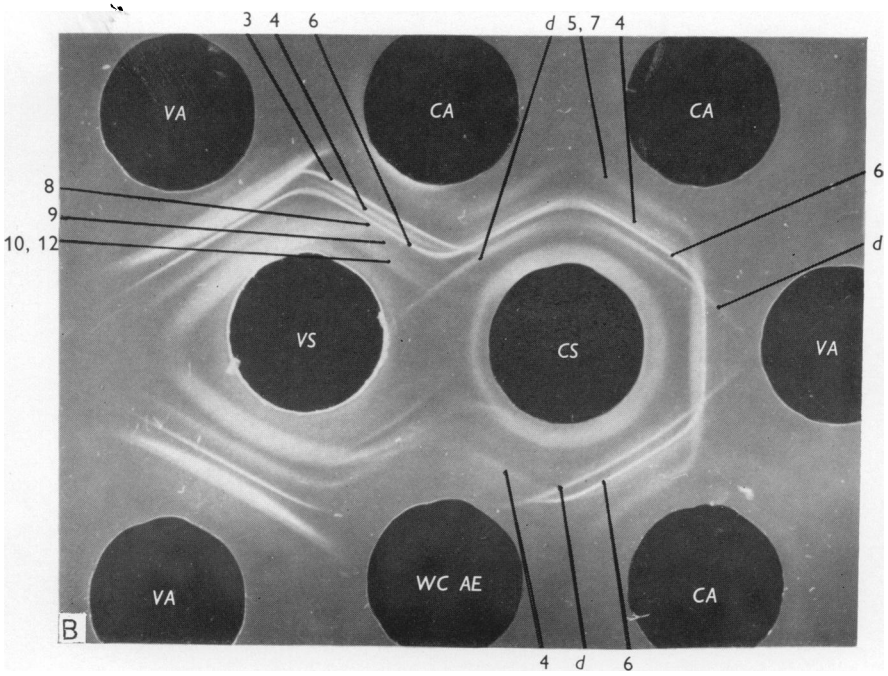
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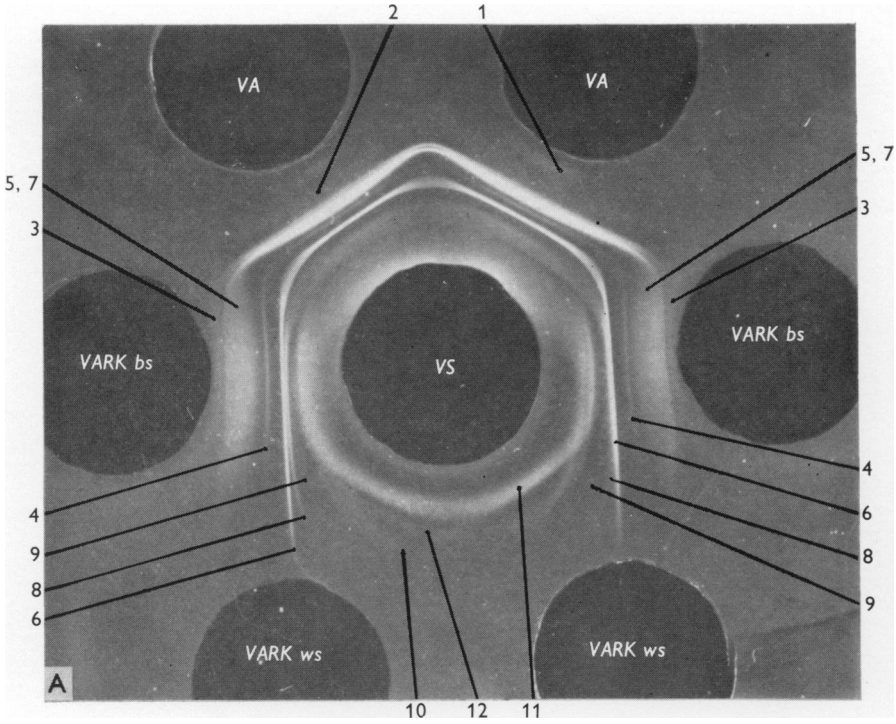
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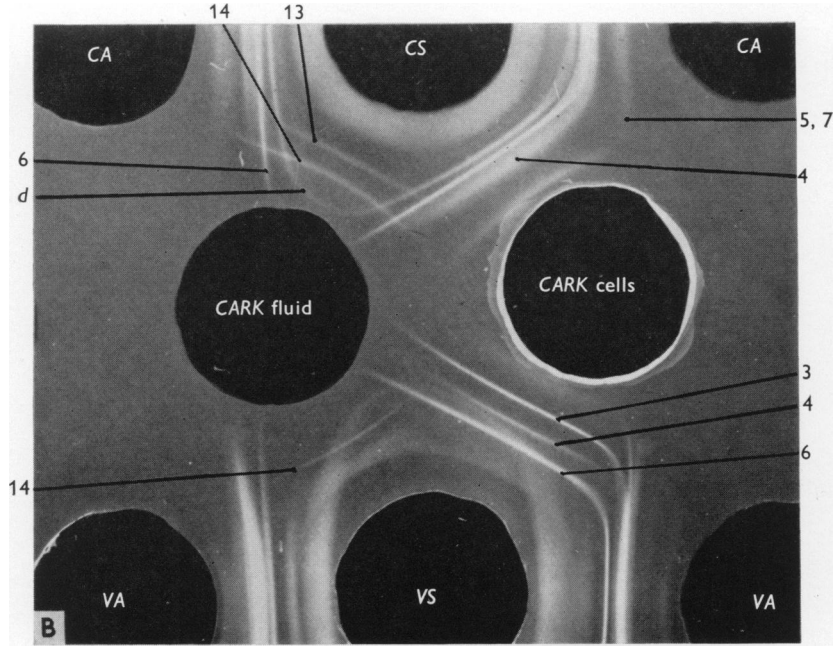
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EXPLANATION OF PLATES

PLATE 1

A. Labelling of vaccinia soluble antigen produced on rabbit skin (*VA*) using antivaccinia serum (*VS*), egg-grown cowpox antigen (*CAE*) and heated preparation of vaccinia soluble antigen (*S*).

B. Labelling of cowpox soluble antigen produced in RK 13 cells (*CA*) using *VA*, *VS*, anti-cowpox serum (*CS*), and egg-grown white cowpox antigen (*WCAE*).

PLATE 2

A. Vaccinia soluble antigens produced in RK 13 cells, labelled with the aid of *VA* and *VS*. RK 13 antigens have been separated by centrifugation after dialysis into buffer soluble (*VARK bs*) and water soluble (*VARK ws*) fractions.

B. Recognition of lpc 13 and 14 in the supernatant fluids (*CARK* fluids) from cowpox-infected RK 13 cells (*CARK* cells).