Microelectrophoresis of Enzyme and Chemically Treated Viruses and Cores of Vaccinia, Buffalopox, Variola and Alastrim

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SUMMARY
A microelectrophoresis technique has been used to study purified suspensions of buffalopox, vaccinia, variola and alastrim viruses. The electrophoretic behaviour of purified, but otherwise untreated buffalopox and vaccinia viruses was indistinguishable. Treatment with trypsin, lipase or p-toluene sulphonyl chloride altered the electrophoretic behaviour indicating that the virus surface is lipoprotein, but again, the viruses were indistinguishable. After treatment with 2-mercaptoethanol differences were found in the electrophoretic behaviour of buffalopox and vaccinia viruses. Preliminary experiments showed that cores, extracted from the viruses, also differed in electrophoretic behaviour.

The electrophoretic behaviour of variola virus was indistinguishable from that of alastrim virus even after treatment with 2-mercaptoethanol. However, further experiments indicated that the cores of these two viruses also differed in surface chemical composition.

INTRODUCTION
Douglas, Rondle & Williams (1966) showed that the electrophoretic mobility of purified virus suspensions of vaccinia and cowpox viruses exhibited Gaussian distribution. Reproducible plots of mobility against pH were obtained and from such graphs the isoelectric point of the viruses could be determined. Subsequently they extended their studies to other members of the variola–vaccinia subgroup of viruses (Douglas, Williams & Rondle, 1969). They found that the isoelectric point and overall electrophoretic behaviour of most of the viruses were significantly different. However, variola and alastrim shared the same isoelectric point and overall electrophoretic behaviour. This does not necessarily indicate that their surfaces are identical, merely that the total net charge on the surfaces of the viruses is the same.

We decided to extend these studies on the electrophoretic behaviour of poxviruses by selecting viruses which normally had the same isoelectric point and overall electrophoretic behaviour, and then trying to differentiate them after chemical or enzymic treatment of the virus surface. Douglas (1957; 1959) has shown the value of this approach for investigating the nature of the chemical charge groups on the surface of the bacterial spore, and Mitchener (1969) has recently investigated the morphology of chemical and enzyme treated vaccinia virus.

The viruses we selected were four poxviruses isolated from Indian buffaloes. The biological and serological properties of these viruses have recently been described (Baxby & Hill, 1971); three of the four were strains of vaccinia virus, the fourth was a new poxvirus

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which we call buffalopox virus. In this paper we show that the electrophoretic behaviour of these four viruses is similar but that, after suitable chemical treatment of their surfaces, buffalopox virus can be differentiated from vaccinia virus. In view of the results obtained we then did experiments on variola and alastrim viruses and in this case we were able to show differences in the electrophoretic behaviour of their internal cores.

**METHODS**

*Virus strains.* Two sets of virus strains were used. The first set comprised buffalopox virus and 3 strains of vaccinia virus (b, c and d) isolated in India by Professor I. P. Singh. Their properties and identities have recently been discussed (Baxby & Hill, 1971). They were used in this work when initial electrophoretic studies failed to differentiate them.

The second set comprised the HARVEY strain of variola virus and the BUTLER strain of alastrim virus, both isolated by Professor A. W. Downie. These were also selected because earlier work (Douglas et al. 1969) had not differentiated them electrophoretically.

*Chemicals and enzymes.* The outer coat of poxviruses is believed to consist essentially of lipoprotein (Westwood et al. 1964; Joklik, 1966; Woodson, 1968). Therefore reagents were selected which might be expected to have specific activity against such a surface. The enzymes used were trypsin (3.4.4.4, ex bovine pancreas, Koch-Light) and lipase (3.1.1.3, ex wheat germ, Koch–Light). We also used 2-mercaptoethanol (2-ME) (Koch-Light) and p-toluene sulphonyl chloride (TSCl) (B.D.H.). 2-ME breaks disulphide bonds and has been shown to alter the morphology of vaccinia virus (Easterbrook, 1966). TSCl eliminates NH$_4^+$ from protein by substitution (Cohen, 1945) and alters the electrophoretic behaviour of cowpox virus (Douglas et al. 1969).

*Virus purification.* Virus suspensions were prepared from infected chick chorioallantois by shaking with glass beads followed by two cycles of differential centrifugation at 30,000 g for 30 min. and 1000 g for 10 min. They were treated for 5 min. with trichlorotrifluorethane (Arcton 113, I.C.I. Ltd.) and the aqueous fraction given two more cycles of differential centrifugation. At each stage the virus pellets were resuspended in 0.001 M-NaCl with the aid of the MSE/Mullard ultrasonic disintegrator. Three criteria were used to check the purity of virus: (a) uniform behaviour when tested in the electrophoresis cell (Douglas et al. 1966), (b) absence of visible contamination when examined in the electron microscope and (c) failure of concentrated supernatants, from which the viruses were finally deposited, to fix complement with a potent anti-vaccinia serum.

*Treatment of purified virus.* With the exception of TSCl, reagents were dissolved in 0.01 M-phosphate buffer pH 7.5. Fresh enzyme solutions were made up for each experiment. Viruses were treated with TSCl as described for bacterial spores by Douglas (1959).

Treatments with enzymes were carried out at pH 7.5 rather than their optimum pH 8.0 to obviate the effects of alkaline conditions on the virus (Douglas et al. 1969). Trypsin was used at 0.001 % (Zwartouw, Westwood & Harris, 1965) and lipase at 0.025 % (Mitchener, 1969). Enzyme/virus mixtures were incubated at 37°C for 3 hr, after which they were chilled to 4°C to stop the reaction. Absorption of enzymes without enzymic attack was measured by holding enzyme/virus mixtures at 4°C for 3 hr.

Viruses were treated with 2-ME at a final concentration of 1 % (v/v) for 30 min. at room temperature (Easterbrook, 1966).

After treatment viruses were deposited by centrifuging at 30,000 g for 30 min. and washed by resuspension in 0.001 M-NaCl. The viruses were then deposited as above and resuspended in 1 M-sucrose dissolved in phosphate buffer pH 7.5, ionic strength 0.05 (Douglas et al. 1966). The suspensions were then stored at 4°C and examined within 2 days.
Preparation of cores. The method used was that described by Easterbrook (1966) using 2-ME and the non-ionic detergent NP 40 (Shell Ltd.). However instead of removing the lateral bodies with trypsin we found that they could be removed by brief ultrasonic treatment.

Microelectrophoresis. The final suspensions of viruses or cores were added to Michaelis buffer of ionic strength 0.05 at the required pH and molar to sucrose. The microelectrophoresis technique was that described by Douglas et al. (1966) except that we used the quartz iodine light source introduced subsequently (Douglas et al. 1969).

In all cases the isoelectric point interpolated from the mobility/pH curves was confirmed by direct observation at that pH.

Electron microscopy. Samples of purified virus and cores were negatively stained with 2 % PTA using the method described by Harris & Westwood (1964).

RESULTS

All mobility measurements were made on viruses suspended in buffered molar sucrose of ionic strength 0.05. Histograms were plotted from measurements on a minimum of 128 individual particles at pH 7.0. In all cases the histogram obtained showed a distribution agreeing closely with the Gaussian curve calculated from the experimental standard deviations, as shown for other poxviruses (Douglas et al. 1966; 1969).

The quoted mobility values in this paper are the mean of measurements made on 32 individual particles.

Microelectrophoresis of purified buffalopox and vaccinia viruses

Mobility values of buffalopox virus and vaccinia virus strains, B, C and D were determined over the range pH 2.0 to pH 9.0 and the results are shown in Fig. 1. The same mobility/pH
Fig. 2. Mean mobility against pH behaviour for buffalopox (O--O) and vaccinia strain D (A--A) after treatment with 2-mercaptoethanol (2-ME). Accurate measurements at pH 3 could not be made.

curve can be drawn for all the viruses. The shape of the curve is similar to that shown for other vaccinia virus strains (Douglas et al. 1969). The interpolated isoelectric point was pH 4.1. Thus buffalopox virus could not be differentiated electrophoretically from vaccinia virus. However, viruses not differentiated by the above limited measurements cannot be assumed to possess identical surfaces. Also, as we have evidence that there are differences in the surface antigens of these viruses (Baxby & Hill, 1970, we thought that these viruses might be differentiated electrophoretically after enzymic or chemical treatment of the outer coat.

**Enzyme and chemically treated buffalopox and vaccinia viruses**

The four viruses were treated with trypsin, lipase or TSCI with appropriate controls, mobility measurements made over the range pH 2.0 to 8.0 and the isoelectric point determined. These treatments did not enable us to differentiate the viruses electrophoretically, although specific changes in the isoelectric point and the shape of the mobility/pH curves were detected. Thus trypsin, lipase and TSCI reduced the isoelectric point of the viruses to pH 2.8, 3.4 and 2.3 respectively, and increased the negative and decreased the positive mobility values. These changes indicate specific attack on the viruses showing the presence of both protein and lipid at the virus surface.

Following treatment with 2-ME the mobility values of buffalopox virus and vaccinia strain D were determined over the range pH 2.0 to pH 7.0 and the results are illustrated in Fig. 2. In contrast to the results obtained above, treatment with 2-ME differentiated buffalopox virus from vaccinia strain D. The isoelectric point was pH 3.5 for buffalopox virus and pH 2.8 for vaccinia virus. This, along with differences in the shape of the mobility/pH curves indicates that 2-ME-treated buffalopox has a surface which differs in chemical composition from that of 2-ME-treated vaccinia virus.
Microelectrophoresis of poxviruses

Microelectrophoresis of variola and alastrim viruses

Preparations of cores of these viruses were examined in the microelectrophoresis cell. Although less refractile than the intact virus particles, the cores could be easily seen and mobility measurements made. At pH 7.0 buffalopox virus cores had a mean mobility value of $-0.33 \mu m./sec./v./cm.$ whereas those of vaccinia virus had a mobility value of $-0.24$. This difference in mobility values suggests that the surfaces of the cores of the two viruses differ in chemical composition.

Electron microscopy showed no difference between the cores of the two viruses. They were similar to those obtained from vaccinia virus by Easterbrook (1966), and also those prepared from variola and alastrim viruses (Hill, 1971).

Electrophoresis of cores of buffalopox and vaccinia viruses

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Microelectrophoresis of variola and alastrim viruses

Mobility/pH curves of purified variola and alastrim viruses before treatment are shown in Fig. 3. The same curve can be drawn for both viruses and their interpolated isoelectric point was pH 3.3. In view of the results obtained with 2-ME which enabled us to distinguish between buffalopox and vaccinia viruses, we determined the mobility/pH behaviour of variola and alastrim viruses after this treatment. However, as shown in Fig. 3, treatment with 2-ME changed the electrophoretic behaviour of both viruses to the same extent. The negative mobility values were increased and the isoelectric point reduced to pH 2.9.

Cores of variola and alastrim viruses

Cores of variola and alastrim viruses were prepared for electrophoresis as described above except that ultrasonic treatment was reduced to a minimum (5 sec.). The yield of cores was thus substantially higher than that obtained with buffalopox and vaccinia viruses. When examined in the electron microscope the cores of variola and alastrim were indistinguishable.
Mobility measurements were made over the range pH 2.0 to pH 8.0 and the results are shown in Fig. 4. At and below pH 5.0 the cores of variola and alastrim had closely similar mobility values with an isoelectric point in the range pH 4.1 to pH 4.3. However above pH 5.0 cores of variola virus had higher mobility values than cores of alastrim virus, the mobility/pH curves diverging with increasing pH. This experiment was repeated with cores extracted from further preparations of purified variola and alastrim viruses and their similar behaviour at acid pH and increasingly different behaviour at higher pH was confirmed.

These results indicate that the cores prepared from variola and alastrim viruses differ in their surface chemical composition.

**DISCUSSION**

Earlier work on the microelectrophoresis of poxviruses has shown that, with the exception of variola and alastrim, members of the variola–vaccinia subgroup have characteristic electrophoretic behaviour and isoelectric points (Douglas et al. 1969). The work reported here has shown that microelectrophoresis cannot differentiate highly purified but otherwise untreated suspensions of buffalopox and vaccinia viruses, although biological and serological studies have shown their separate identities (Baxby & Hill, 1971).

The shapes of the mobility/pH curves are similar to those obtained for other poxviruses, and are consistent with the view that the surface of the viruses consists of lipoprotein. In addition treatment with lipase, trypsin and TSCI altered the electrophoretic behaviour of the viruses thus confirming the presence of both protein and lipid at the virus surface although both viruses behaved similarly in these tests.

Mitchener (1969) has recently suggested that the outer coat of vaccinia virus is a proteolipid, with the actual outer layer being lipid only. This suggestion was based on changes in virus morphology after enzyme or solvent treatment. However, the electrophoretic technique used here is sensitive to even partial changes in the composition of surface monolayers which
would not be detected by electron microscopy. Hence we regard our results with lipase, trypsin and TSCI as indicating that the outer coat of these viruses is lipoprotein in nature with both protein and lipid present at the surface with their characteristic bonds exposed.

After treatment with 2-ME we were able to distinguish between buffalopox and vaccinia viruses. The effects of 2-ME are more drastic than those of the other reagents, and lead to swelling and loosening of the outer coat (Easterbrook, 1966). Thus 2-ME treatment will lead to exposure of previously hidden groups and it is not possible to say with certainty where the differences we detected originate. However, despite this uncertainty, it is clear that there are chemical differences between the outer coats of buffalopox and vaccinia viruses which may reflect the serological differences detected earlier (Baxby & Hill, 1971).

In contrast to the results discussed immediately above, treatment with 2-ME did not enable us to differentiate the viruses of variola and alastrim.

It was of interest to find that cores, readily prepared as described by Easterbrook (1966) could be examined by microelectrophoresis. At pH 7.0 the cores of buffalopox virus had a different mobility from those of vaccinia virus. This indicates that the surfaces of the cores differ in surface chemical composition. However, measurements at other pH values are required before any speculations can be made about the nature of such differences.

Full mobility/pH curves were made on the cores of variola and alastrim viruses. In general these are characteristic of the curves expected from studies on protein and nucleoprotein surfaces (Douglas, unpublished). The results clearly showed that the surfaces of variola and alastrim cores differ in chemical composition.

Other workers have extracted cores by this method and have shown that they have pox-virus enzymic (Kates & McAuslan, 1967) and serological (Fenner & Sambrook, 1966; Cohen & Wilcox, 1968) activity, so it is possible that the core surface we have studied is the true surface and not a degraded or modified surface produced during extraction of the core.

Thus microelectrophoresis has proved a valuable tool for studying the chemical structure of poxviruses. It has confirmed the lipoprotein nature of the outer coat and has enabled us to differentiate the outer coats of vaccinia and buffalopox viruses, although we could not differentiate the outer coat of variola and alastrim viruses. The results also show that not only can cores be studied but that the cores of vaccinia, buffalopox, variola and alastrim viruses differ in surface chemical composition.

The difference between the cores of variola and alastrim viruses is perhaps particularly interesting. Apart from differences in virulence, the only other differences so far detected between these viruses are their ceiling temperatures and heat resistance (Bedson & Dumbell, 1961). Only the last character can be considered as a property of the virus particles themselves.

Further work is required to extend the electrophoretic studies to other strains of variola and alastrim viruses, and to establish the chemical nature of the differences found between poxvirus cores. Studies correlating the microelectrophoretic behaviour of the cores of different poxviruses with their serological and enzymatic properties might provide valuable information on the differences that exist between these closely related viruses.

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