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Arginine metabolism in infected cell cultures as a marker character for the differentiation of orthopoxviruses

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SUMMARY

Arginine has been shown to be essential for the replication of several orthopoxviruses in mouse sarcoma 180 cells and in chick embryo fibroblast cultures. Both host systems are characterized by their inabilities to utilize citrulline for the biosynthesis of arginine due to deficiencies in the requisite cellular enzymes and cell multiplication is absolutely dependent on the availability of exogenous arginine. Virus replication in such cells maintained with citrulline results from the induction of virus-specific enzymes. Significant virus yields in the absence of exogenous arginine or citrulline can arise from the replenishment of intracellular amino acid pools by increased utilization of arginyl residues in cellular proteins. The extent of the phenotypic expression of these characters in infected cells permitted significant discrimination between the viruses examined. Distinctions could be drawn between rabbitpox, ectromelia, cowpox, buffalopox and vaccinia strains. However, cowpox could not be distinguished from other viruses isolated from diseased animals in European zoos.

INTRODUCTION

Viruses belonging to the genus *Orthopoxvirus* have been found in a wide range of animal species and the eradication of smallpox from the human population has lead to an increasing interest in poxvirus infections extant in other hosts. Some animal poxviruses can be transmitted to man and human monkeypox in central Africa is currently under intensive investigation (Bremen *et al.* 1980). In western Europe unresolved questions still surround the epidemiology of cowpox, a zoonosis of more than historical significance. Contrary to the popular view that it is an occupational disease of dairy farmworkers, a recent review of ten human cases in Britain has argued that 'cowpox' is a misnomer (Baxby, 1977). Outbreaks of human cowpox have occurred without direct contact with cattle and in total absence of any evidence that the disease was enzootic in the vicinity. Cowpox-like viruses have also been isolated from diseased animals in European zoos (Baxby *et al.* 1979*b*). In order to understand the natural history of these viruses it is important that their precise identity should be established. Since there is a

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very close serological relationship between all orthopoxviruses the laboratory characterization of virus isolates has been made on the basis of biological properties discerned in experimental animals or *in vitro* cell cultures. Such phenotypic characters are based on complex interactions between the virus and its host which are the product of multigenic functions. The development of other discriminatory criteria based preferably on their genotypic characters could greatly facilitate comparative studies on orthopoxviruses recovered from different hosts. Indeed, variola and 'whitepox' viruses have been shown to be similar to each other but different from other orthopoxviruses on the basis of virus-coded thymidine kinase induced in infected cells (Bedson, 1982).

Previous studies in this laboratory have shown other virus-coded enzymes affect the utilization of citrulline for the biosynthesis of arginine in rabbitpox and in vaccinia-infected cells (Cooke & Williamson, 1973; Williamson & Cooke, 1973). Their expression can be demonstrated by the growth of these viruses in host cells which are defective in the enzymic pathway of arginine anabolism. The replication of rabbitpox virus in mouse sarcoma 180 cells is dependent on the availability of arginine, but this requirement can be met by its substitution with citrulline. Cell multiplication and protein synthesis in uninfected cells, however, was suppressed markedly in the presence of the same concentrations of citrulline compared with similar cultures in medium containing equimolar arginine. Growth of a human citrullinaemia cell line was also inhibited when citrulline replaced arginine yet the replication of vaccinia virus was unaffected. More recent studies have shown that the growth of cowpox virus in chick embryo fibroblasts is also inhibited by the withdrawal of arginine but its white pock variant shows limited growth under such conditions (Williamson & Mackett, 1982). This distinction appears to be based on virus-mediated differences in the replenishment of amino acid pools by increased turnover of cellular proteins in the absence of *de novo* arginine biosynthesis. These observations are extended in the present paper to examine the growth of other orthopoxviruses in mouse sarcoma 180 cells and in chick embryo fibroblasts maintained in complete medium, in arginine-deprived medium and in the presence of citrulline. The taxonomic value of the results obtained is discussed.

MATERIALS AND METHODS

Virus

The viruses studied were vaccinia (Lister, IHD strains), cowpox (Brighton), rabbitpox (Utrecht), ectromelia (Hampstead), monkeypox (Copenhagen), camelpox (Iran) and buffalopox (Kataria & Singh, 1970). In addition to these accepted species, the study included three viruses related biologically to cowpox but isolated during outbreaks of serious poxvirus infections in captive animals: 'elephant' virus (Baxby & Ghaboosi, 1977). 'cheetah' virus (Baxby *et al.* 1979*a*) and 'anteater' virus (Marrenikova *et al.* 1977). All viruses were grown initially on the chorioallantoic membranes of fertile hens' eggs and these virus stocks were used for all experiments with chick embryo fibroblasts. Since some viruses did not replicate in mouse sarcoma 180 cells, it was not possible to prepare stocks of all viruses in this cell system.

Cell culture

Mouse sarcoma 180 cells and chick embryo fibroblast (CEF) cultures were grown as described previously (Cooke & Williamson, 1973; Williamson & Mackett, 1982). Depletion of intracellular pools of arginine was effected by incubation of cell monolayers for 18 h in an arginine-free maintenance medium (Archard & Williamson, 1971).

Protein synthesis

The incorporation of L-[4,5-^{3}H]leucine (1·0 Ci/mmol) was used to determine protein synthesis in CEF cultures. The procedures used to separate macromolecules and measure radioactivity have been described (Archard & Williamson, 1971).

Virus yields

The growth of various orthopoxviruses in mouse sarcoma 180 cells or in CEF cultures under different nutritional conditions was determined by measurement of virus yield. After maintenance in arginine-free medium for 18 h confluent monolayers of the appropriate cell type were infected using 5 p.f.u./cell. At the end of the adsorption period (zero time) sample cultures were washed thoroughly to remove residual virus and frozen immediately. Replicate infected cultures were maintained for a further 24 h in arginine-free medium, in the presence of 0·5 mM citrulline or with 0·5 mM arginine. At the later time all cell cultures were frozen and thawed twice followed by ultrasonic treatment to release intracellular virus into the maintenance medium. Virus yields were calculated from the infectivity titres of infected cultures taken at zero time and at 24 h post-infection. Each virus was investigated in at least three, separate experiments.

In other experiments to determine virus growth curves, confluent monolayers of arginine-deprived CEF cultures were infected with rabbitpox or cowpox viruses at a multiplicity of infection, based on infective centre determinations, found to ensure one-step growth conditions. Virus was adsorbed from Hanks' balanced salt solution for 1 h before infected cells were washed thoroughly to remove residual virus and maintained subsequently in appropriate media. Samples of infected cultures were frozen after various time intervals of incubation at 36 °C for subsequent recovery of total virus.

Infectivity titrations were made by plaque assays in confluent monolayers of Vero cell cultures.

Virus DNA synthesis

The replication of poxviruses is accompanied by the formation of cytoplasmic inclusions ('factories') containing virus-specific DNA which can be visualized by cytochemical stains (Loh & Riggs, 1961). Radioactively labelled precursors of DNA synthesis supplied to cell cultures after poxvirus infection are incorporated during virus replication and radiolabelled virus DNA can be recovered in the cytoplasmic fractions of infected cells. In the present study the morphology of cytoplasmic, DNA-containing inclusions in infected mouse sarcoma 180 cells was examined by acridine orange staining using methods described previously (Williamson & Archard, 1976). In addition, virus DNA was labelled radioactively by the addition

Table 1. *Protein synthesis in CEF cultures and HeLa cells maintained with arginine or citrulline*

Cell cultures	Incorporated radioactivity* (dpm × 10^{-3}/10^7 cells) in medium containing: 0·5 mM arginine	Arginine-deprived	0·5 mM citrulline
CEF	250	62	70
HeLa	319	99	332

* Cultures were maintained for 18 h in appropriate media supplemented with 0·5 μCi/ml [^{3}H]leucine.

of 0·5 μCi/ml [6-^{3}H]thymidine (23 Ci/mmol) to maintenance medium supplemented with 20 μM thymidine which was supplied to confluent monolayers (5×10^6 cells) immediately after infection. At 24 h post infection the cultures were washed, the cells re-suspended with 0·02 % EDTA in phosphate-buffered saline and cytoplasmic fractions prepared by detergent treatment with Nonidet P40 (Shell U.K. Ltd) and sodium deoxycholate (Archard, 1983). The amounts of radioactivity incorporated into these fractions were determined by precipitation with trichloroacetic acid followed by liquid scintillation spectrometry (Archard & Williamson, 1971).

RESULTS

Growth responses of host cells to arginine or citrulline

The inhibitory effect of the substitution of citrulline for arginine on the growth of mouse sarcoma 180 cells has been demonstrated in earlier studies (Tytell & Neuman, 1960; Cooke & Williamson, 1973). The growth of CEF cultures in media containing 0·5 mM arginine, 0·5 mM citrulline or neither amino acid was determined by viable cell counts using trypan blue. Cultures seeded initially with $5{\cdot}0 \times 10^6$ cells yielded viable counts of $9{\cdot}2 \times 10^6$ cells after 4 days in medium containing arginine. Although cell viability was unaffected during the same period in the presence of citrulline and in the absence of arginine, no increase in cell numbers was measured. In further experiments the incorporation of [^{3}H]leucine into CEF cultures during 18 h was determined in the presence of 0·5 mM citrulline or equimolar arginine. There was a marked reduction in the level of incorporation following the withdrawal of arginine which was not restored in the presence of citrulline (Table 1). Protein synthesis in CEF cultures was reduced by 72 % after substitution of citrulline for arginine in the maintenance medium; similar results were obtained with mouse sarcoma 180 cells (Cooke & Williamson, 1973). These effects reflect the inability of CEF cultures and mouse sarcoma 180 cells to utilize citrulline for the anabolism of arginine. Optimal growth of each cell type was obtained in media containing 0·5 mM arginine. By comparison, protein synthesis in HeLa cell cultures is unaffected by maintenance in the presence of 0·5 mM citrulline (Table 1).

Growth of various orthopoxviruses in mouse sarcoma 180 cells deprived of arginine or maintained with citrulline

Preliminary experiments established that maximum yields of rabbitpox and cowpox viruses were obtained with infected mouse sarcoma 180 cells under one-step

Table 2. *Growth of various orthopoxviruses in mouse sarcoma* 180 *cells maintained in medium with arginine or citrulline and in arginine-deprived medium*

Virus	Infectivity titres at 24 h.p.i.* (p.f.u./ml × 10^{-5}) in medium containing: 0·5 mM arginine	0·5 mM citrulline	Arginine-deprived
Rabbitpox	100	10	1
Vaccinia (IHD)	232	19	14
Ectromelia	237	102	73
Cowpox	26	1	1
Anteater virus	33	1	1
Cheetah virus	10	1	1
Elephant virus	6·7	1	1
Camelpox	3·5	1	1
Buffalopox	1	1	1
Vaccinia (Lister)	1	1	1

* Replicate cultures were infected with each virus using 5 p.f.u./cell.

Table 3. *Incorporation of* [^3H]*thymidine into cytoplasmic fractions of mouse sarcoma* 180 *cells infected with various orthopoxviruses and maintained in medium containing arginine or citrulline and in arginine-deprived medium*

Virus	Incorporated radioactivity (dpm × 10^{-3}/culture) in medium containing: 0·5 mM arginine	0·5 mM citrulline	Arginine-deprived
None	41	37	32
Rabbitpox	423	197	84
Vaccinia (IHD)	74	50	48
Cowpox	277	74	72
Buffalopox	106	44	60

growth conditions in the presence of 0·1 mM arginine. Further experiments proceeded to determine the abilities of various orthopoxviruses to replicate in cell cultures with intracellular pools depleted by maintenance in arginine-free medium for 18 h before infection. At the end of the adsorption period the infected cultures were maintained subsequently in the absence of arginine, in the presence of 0·5 mM citrulline or an equimolar concentration of arginine and the yields of infectious, progeny virus were assayed at 24 h post-infection.

The results obtained with different viruses can be divided qualitatively into four main categories (Table 2). Rabbitpox did not grow in arginine-deprived cultures but a low yield was obtained with citrulline. Vaccinia (IHD) and ectromelia also produced significant yields in the presence of citrulline but, unlike rabbitpox, virus growth also occurred in the absence of arginine. Ectromelia is distinguished quantitatively by the high virus yields obtained under all nutritional conditions. Camelpox, cowpox and the related elephant, cheetah and anteater viruses grew

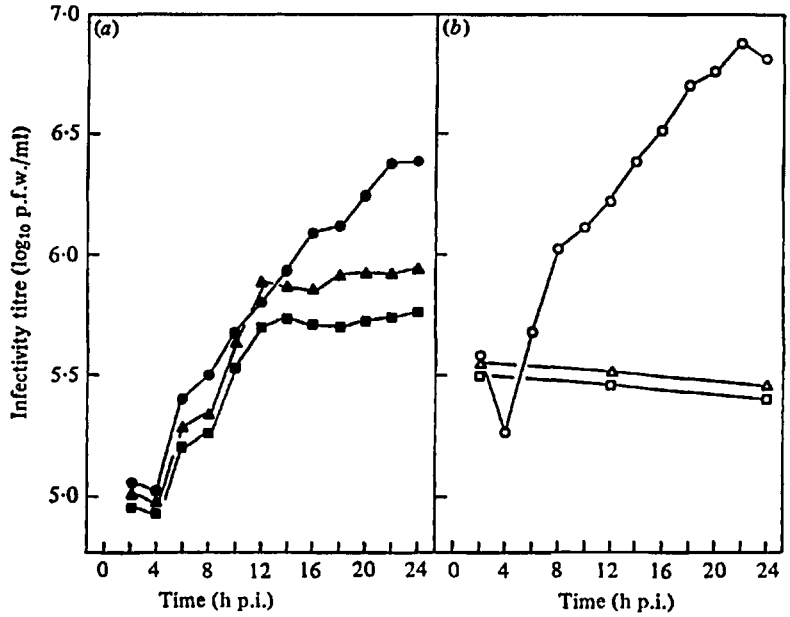

Fig. 1. One-step growth curves of (*a*) rabbitpox virus (closed symbols) and (*b*) cowpox virus (open symbols) in CEF cultures maintained in media containing 0·5 mM arginine (●, ○) or 0·5 mM citrulline (▲, △) and in arginine-deprived medium (■, □).

in complete medium only but with lower yields than rabbitpox, vaccinia (IHD) or ectromelia. Both vaccinia (Lister) and buffalopox failed to produce virus even with arginine and mouse sarcoma 180 cells, therefore, are non-permissive to these viruses.

Effect of arginine and citrulline on virus DNA synthesis in mouse sarcoma 180 *cells infected with different orthopoxviruses*

Incorporation of [^{3}H]thymidine into the cytoplasmic fractions of mouse sarcoma 180 cells after infection with rabbitpox, vaccinia (IHD), cowpox or buffalopox viruses is shown in Table 3. Radioactivity in the cytoplasm of uninfected cells is due to mitochondrial DNA synthesis but the increased levels of incorporation into infected cells can be ascribed to the synthesis of virus-specific DNA. In the presence of arginine there was a 10-fold increase after rabbitpox infection but 7-fold, 2·5-fold and 1·8-fold increases were obtained with cowpox, buffalopox and vaccinia (IHD)-infected cultures, respectively. These results show that, although mouse sarcoma 180 cells are non-permissive to buffalopox virus, there was evidence of virus DNA synthesis. The incorporation of [^{3}H]thymidine into all infected cells was reduced markedly in the absence of arginine and similar reductions were obtained with vaccinia (IHD), cowpox and buffalopox viruses if citrulline replaced arginine. In rabbitpox-infected cultures, however, higher levels of radioactivity were measured in the presence of citrulline compared with arginine-deprived cultures but about 50% lower than with complete medium. These results are consistent with the utilization of citrulline for the production of infectious, progeny virus in mouse sarcoma 180 cells infected with rabbitpox virus.

Table 4. *Incorporation of [^3H]thymidine into cytoplasmic fractions of CEF cultures infected with rabbitpox or cowpox virus and maintained in medium with arginine or citrulline and in arginine-deprived medium*

Virus	Incorporated radioactivity (dpm $\times 10^{-3}$/culture) in medium containing: 0·5 mM arginine	0·5 mM citrulline	Arginine-deprived
None	156	134	125
Rabbitpox	474	360	220
Cowpox	688	314	333

Table 5. *Virus yields in CEF cultures infected with different orthopoxviruses and maintained in medium with arginine or citrulline and in arginine-deprived medium*

Virus	Virus yields at 24 h post-infection in medium containing: 0·5 mM arginine	0·5 mM citrulline	Arginine-deprived
Rabbitpox	65	13	9
Ectromelia	45	8	8
Vaccinia (Lister)	75	6	4
Buffalopox	75	9	2
Camelpox	177	8	3
Monkeypox	210	1	1
Cowpox	29	1	1
Elephant virus	28	2	2
Cheetah virus	25	1	1
Anteater virus	80	1	1

Although the radiolabelling experiments demonstrate virus-specific DNA synthesis, any quantitative evaluations must be made cautiously since intracellular nucleoside pool sizes are not known. Consequently, qualitative evaluations have also been made by acridine orange staining. With this cytochemical technique small, discrete inclusions containing virus DNA can be visualized in the cytoplasm of poxvirus-infected cells at about 4 h post-infection. As the virus replication cycle proceeds the inclusions become larger and more diffuse until they occupy large areas of the cytoplasm by 24 h post-infection (Loh & Riggs, 1961; Williamson & Archard, 1976). In the present study similar cytological changes were seen with rabbitpox-infected mouse sarcoma 180 cells under all nutritional conditions but the large, diffuse inclusions produced 24 h after infection with cowpox virus were seen only in the presence of arginine. In cowpox-infected cultures deprived of arginine or maintained with citrulline there was a reduced frequency of inclusions which remained both small and discrete in appearance even at the later time. These observations are consistent with the inhibition of cowpox virus replication in mouse sarcoma 180 cells under such conditions.

Growth curves of rabbitpox and cowpox viruses in CEF cultures maintained with arginine or citrulline

The requirement for arginine in the replication of rabbitpox and cowpox viruses in CEF cultures is shown clearly by one-step growth curves (Fig. 1). In the presence of 0·5 mM arginine (maximum virus yields required 0·1 mM arginine) infectious, progeny virus was first detected at 6 h after infection and similar kinetics of virus replication continued in rabbitpox or cowpox-infected cells until maximum yields were obtained at 24 h post-infection. Following the withdrawal of arginine or its substitution by citrulline the replication of cowpox virus was inhibited completely. However, rabbitpox virus replication continued in the absence of exogenous arginine to produce an eventual yield 80% lower than in the presence of arginine. The yields of rabbitpox virus obtained with 0·5 mM citrulline were higher than with arginine-deprived medium but 60% lower than virus yields with complete medium.

Effect of arginine and citrulline on virus DNA synthesis in CEF cultures infected with rabbitpox or cowpox viruses

In radiolabelling experiments similar to those described with mouse sarcoma 180 cells the incorporation of [^{3}H]thymidine into the cytoplasmic fractions of rabbitpox or cowpox virus-infected CEF cultures was found to be dependent on the availability of arginine (Table 4). Withdrawal of arginine resulted in a 50% reduction in cultures infected with either virus and a similar reduction occurred with cowpox virus-infected CEF cultures in the presence of citrulline. By contrast, increased thymidine incorporation was measured in the cytoplasmic fraction of rabbitpox virus-infected CEF cultures maintained with citrulline to reach a level only 25% lower than similar fractions from infected cells in medium with an equimolar concentration of arginine.

Growth of different orthopoxviruses in CEF cultures deprived of arginine or maintained with citrulline

The growth of various orthopoxviruses in CEF cultures under different nutritional conditions was investigated by determination of the magnitude of virus yields. The results presented in Table 5 show the quotients calculated from the infectivity titres measured at 24 h post-infection and at zero time. Although virus yields could be affected by the multiplicity of infection, the overall pattern of results was reproducible. Unlike mouse sarcoma 180 cells, each virus grew well in CEF cultures maintained in complete medium, including vaccinia (Lister) and buffalopox, with monkeypox and camelpox producing maximum virus yields. Following the withdrawal of arginine the production of infectious, progeny virus was inhibited markedly in all infected cultures. Only rabbitpox and ectromelia gave significant yields in the absence of exogenous arginine but they were 86% and 82% lower, respectively, than in complete medium. In parallel experiments to determine citrulline utilization the growth of ectromelia, unlike rabbitpox, was not enhanced. Vaccinia (Lister) and camelpox also produces a higher yield with citrulline than in the absence of arginine but such increases were not statistically significant. Buffalopox, however, showed a 4-fold increase (standard deviation $\pm 0{\cdot}5$ in five, separate experiments) in the virus yield from infected CEF cultures maintained

with citrulline compared with arginine-deprived cultures. Very low yields were obtained with elephant virus following the withdrawal of arginine or its substitution with citrulline but such results do not permit a significant distinction from the other cowpox-related viruses. Monkeypox, cowpox, cheetah virus and anteater virus grew in CEF cultures only in the presence of arginine.

DISCUSSION

Arginine has been found to be essential for the growth of each orthopoxvirus included in the present study. In terms of the molecular biology of these viruses, the nature of this requirement bears comparison with previous investigations. The yields of infectious virus were dependent on the concentration of arginine in the maintenance medium supplied to infected cells and it is pertinent that maximum yields of both rabbitpox and cowpox virus were obtained with the same arginine concentration in either mouse sarcoma 180 cells or in CEF cultures. This points towards qualitative distinctions between these viruses as the basis for the different effects on their replication seen after arginine deprivation. The growth of vaccinia virus in HeLa cells has been shown to have requirements for arginine which are demonstrated at both early and late stages in the replication cycle (Archard & Williamson, 1971). An early requirement leading to virus DNA synthesis is satisfied by a lower concentration of exogenous arginine than the later event associated with the formation of mature vaccinia virus particles. Although the withdrawal of arginine from vaccinia-infected HeLa cells affected a 93% reduction in virus DNA synthesis, a lesser, 40% reduction occurred in KB cells under the same conditions (Obert, Tripier & Guir, 1971). In both HeLa and KB cells arginine deprivation resulted in complete inhibition of the production of infectious progeny virus. Similar observations have been made in the present study with some viruses but significant virus yields were obtained with ectromelia or rabbitpox in CEF cultures maintained in the absence of arginine. Since protein synthesis is inhibited markedly in the uninfected cells maintained in medium without arginine, virus growth in these host systems under the same conditions is unlikely to be supported by *de novo* arginine biosynthesis determined by host-specific enzymes. Consequently, it is more likely that arginyl residues must be made available from cellular proteins to replenish intracellular amino acid pools in ectromelia and rabbitpox-infected cells without exogenous arginine. This mechanism appears to be variously affected by different viruses since camelpox, cowpox, cheetah virus, anteater virus and elephant virus did not produce any measurable amounts of infectious progeny in mouse sarcoma 180 cells and very reduced yields in CEF cultures after the withdrawal of arginine from medium supplied to the infected cultures. Since these distinctions between the orthopoxviruses tested could be made in either host cell system, it seems they are due to virus-determined mechanisms.

The expression of virus-specific enzymes active in the anabolism of arginine from citrulline has been demonstrated in rabbitpox-infected mouse sarcoma 180 cells (Cooke & Williamson, 1973; Williamson & Cooke, 1973). Similar observations have been made with rabbitpox virus in the present study although the virus yields with citrulline were lower than those obtained in the earlier investigations. Exhaustive efforts failed to resolve this discrepancy, which may be due to differences between

the batches of calf serum used in the cell culture media. However, additional evidence for the utilization of citrulline in rabbitpox-infected cells has been obtained by studies of virus DNA synthesis. There were significant increases in the incorporation of radiolabelled precursors into acid-precipitable material in the cytoplasmic fractions of rabbitpox-infected mouse sarcoma 180 cells or CEF cultures maintained with citrulline compared with arginine-deprived cultures. Such changes were not seen with cowpox-infected cultures of the murine or avian cells, results consistent with the markedly suppressed virus yield obtained in either host system in the presence of citrulline. Although low yields in the CEF cultures in the absence of arginine may have masked similar activity by other orthopoxviruses, only buffalopox in addition to rabbitpox showed significant ability to utilize citrulline. Such evidence was not obtained in buffalopox-infected mouse sarcoma 180 cells but this system failed to produce infectious progeny in the presence of arginine.

It is apparent that these different aspects of arginine metabolism in infected mouse sarcoma 180 cells and CEF cultures can permit some discrimination between the orthopoxviruses studied. Ectromelia showed significant growth in the absence of arginine but only rabbitpox and buffalopox were able to utilize citrulline although the murine cells were non-permissive to buffalopox. Monkeypox, camelpox, cowpox, elephant virus, cheetah virus and anteater virus failed to grow in mouse sarcoma 180 cells in the absence of arginine or in the presence of citrulline and these viruses were also distinguished by their low yields in CEF cultures under similar conditions. The discrimination between vaccinia (IHD) and vaccinia (Lister) achieved in mouse sarcoma 180 cells is interesting but only one strain of the other viruses was included in the present survey and further strain differences may emerge when others are tested. The categories obtained in the present study should be compared with the distinctions seen with other genotypic characters utilized recently as marker characters for different orthopoxviruses. Four groups can be resolved by electrophoretic analysis of their late intracellular polypeptides: cowpox (including cheetah virus and anteater virus), vaccinia (including buffalopox), monkeypox and variola-related viruses (Harper, Bedson & Buchan, 1979). Similar physical studies on structural polypeptides divided the viruses into a vaccinia group (including buffalopox), an ectromelia group (including elephant virus and anteater virus), cowpox, camelpox and monkeypox (Turner & Baxby, 1979). Restriction endonuclease analysis distinguished variola, monkeypox, vaccinia (including rabbitpox), cowpox and ectromelia viruses (Mackett & Archard, 1979).

The primary objective of most comparative studies on orthopoxviruses has been to seek any differences between viruses isolated from various diseased hosts. The application of biological properties fails to separate rabbitpox and vaccinia viruses, camelpox is difficult to distinguish from smallpox and buffalopox is serologically related to vaccinia and cowpox (Baxby, 1975). Although a number of these viruses can now be identified specifically on the basis of their genotypic characters, there are many parallels in broader terms with the biological classification. In particular, the 'cowpox-like' viruses isolated from captive carnivores in Russia, from elephants in Germany and from cheetahs in England are known to be closely related by either molecular or biological criteria. The poxvirus isolated from infected carnivores has been shown to have the polypeptide pattern of elephant virus and it could not be

distinguished from cowpox virus by immunological analysis (Baxby *et al.* 1979*b*). Elephant virus is very closely related to cowpox virus although they can be differentiated by biological and serological methods (Baxby & Ghaboosi, 1977). All available evidence indicates that the infection in cheetahs was due to cowpox virus (Baxby *et al.* 1979*a*). Close relationships between these viruses have also been established in the present study.

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