

THE EFFECT OF ARGININE DEPRIVATION ON
THE REPLICATION OF DIFFERENT ORTHOPOXVIRUSES

BY JOANNA GRACE ELIZABETH OSBORN

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ABSTRACT

Although smallpox has been successfully eradicated, there is still some concern about the possibility of an animal reservoir of smallpox which might cause the disease to reappear. Many poxviruses which closely resemble smallpox in experimental studies have been isolated from mammals and these variola-like viruses have been investigated to determine their relationship to variola virus. Such comparisons have been made using biological characters, antigenic properties and biochemical analysis of the poxvirus genomes and virus induced proteins.

In this thesis certain poxviruses have been compared by the extent to which the phenotypic expression of a virus determined mechanism occurs, resulting in the induction of enzymes involved with arginine biosynthesis, which is essential for poxvirus replication.

Mammalian cells in vivo may synthesise arginine from citrulline via the Krebs-Henseleit urea cycle utilising argininosuccinate synthetase/lyase activity. Many animal cells in vitro can grow normally if citrulline replaces arginine but certain cell lines lack this capacity and exhibit an absolute requirement for arginine. The growth of one such line, mouse sarcoma 180 (S180) is inhibited in the presence of citrulline yet the replication of rabbitpox virus can occur in these cells under such conditions. However when S180 cells are infected with cowpox virus and maintained in the presence of citrulline, no infectious progeny is produced. These results suggest that argininosuccinate synthetase/lyase activity is induced in rabbitpox virus infected cells but this character is not expressed during cowpox virus replication.

In this thesis the replication of twelve different variola-like poxviruses (orthopoxviruses) was compared in the presence of citrulline in four cell types unable to utilise citrulline for cell growth. Two established cell lines, S180 and BHK 21 and two primary cultures derived from the chick embryo, cells from the chorioallantoic membrane and chick embryo fibroblasts were used. Virus yields were determined in all systems and further studies of virus-specific DNA synthesis were made.

The replication of all poxviruses examined was dependant on the availability of arginine. These poxviruses were found not to exhibit an absolute effect in the utilisation of citrulline but gave reduced yields relative to appropriate controls. These ranged from rabbitpox and vaccinia which produced high yields through to cowpox which gave a very low yield if citrulline replaced arginine.

Higher levels of virus-specific DNA synthesis were found during rabbitpox virus replication in the presence of citrulline compared with cowpox virus replication under similar conditions.

Certain viruses gave significant yields in the absence of exogenous arginine or citrulline and further studies were pursued to ascertain the origin of the arginine needed for their replication. Some evidence indicated that an arginine pool was formed from increased protein turnover in the virus infected cells.

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CHAPTER 1

INTRODUCTION

Since the beginning of the smallpox eradication campaign which has now been successfully completed, there has been concern about the possibility of an animal reservoir of smallpox virus which might cause the disease to reappear. One poxvirus in particular, monkeypox has caused 33 cases of clinical smallpox in man, of which six were fatal. Many poxviruses which closely resemble smallpox have been isolated from mammals and these have been studied to try to find methods of differentiating between them. They all possess a common surface antigen 'LS' which has a heat labile 'L' and a heat stable 'S' component (Wilcox & Cohen, 1969). They have been classified in the genus Orthopoxvirus and can be identified by the presence of the 'LS' antigen by complement fixation, by precipitation-in-gel using extracts of infected tissue and an anti-vaccinia serum, or alternatively by a virus neutralisation test on the chorioallantoic membrane (CAM) of the chick embryo, or in tissue culture. All members of the orthopoxvirus group produce characteristic pocks on the CAM and grow in a large number of cell types.

Within the genus, the orthopoxviruses are divided into three sub-groups (Baxby, 1975). The first contains viruses causing smallpox and smallpox-like illness in man, which are; variola major, variola minor, monkeypox and Lenny virus. The latter was isolated from a single outbreak of clinical smallpox in Nigeria, but resembled buffalopox (Bourke & Dumbell, 1972). The second group is of smallpox-like viruses which have been isolated from animals and includes 'whitepox' viruses which have been isolated from the kidneys

of apparently normal cynomolgus monkeys when the disease was absent from the colony, but appeared indistinguishable from smallpox when grown on the CAM. Camelpox is also a member of this group. The third group is of animal poxviruses other than smallpox and smallpox-like viruses and includes vaccinia, cowpox, rabbitpox, ectromelia, buffalopox, elephantpox, cheetahpox and carnivorepox.

Comparison of the viruses within the orthopoxvirus group has been made with limited success by serological techniques. Some specific antigens have been detected using neutralisation tests (Downie & McCarthy, 1950) by indirect complement fixation (Downie & McDonald, 1950) and by immunodiffusion (Gispen, 1955). More recently, Gispen and Brand-Saathof (1974) recognised three precipitable antigens Vc, Va and Mo by immunodiffusion. By identification of the antigens with specific sera, the orthopoxviruses were classified into three groups; variola ($Vc^-Va^+Mo^-$), monkeypox ($Vc^-Va^-Mo^+$) and vaccinia ($Vc^+Va^+Mo^-$). Cowpox tissue extract did not react with any of the specific sera. The variola-like isolates from healthy monkeys were classified serologically as variola. Extracts of tissues infected with rabbitpox, buffalopox and camelpox reacted like vaccinia. White mutants of two monkeypox isolates produced the same antigen as their parent strains.

Maltseva and Marennikova (1976) developed a method for detecting serological differences between closely related orthopoxviruses by a two-step gel diffusion reaction with vaccinia antiserum. Differences in the antigenic structure of vaccinia, variola and monkeypox as well as antigenic identity with cowpox of orthopoxviruses isolated from

carnivora, elephants and okapi were established.

Esposito et al (1977) differentiated closely related human and monkeypoxviruses by a serological technique. Antiviral sera were tested by immunodiffusion for reactivity against six viral antigens prepared from either infected cultures or CAMs. Portions of each antiserum were separately absorbed with heterologous antigens from CAMs to remove common reactivity. The absorbed sera formed immunodiffusion precipitates with both types of antigen preparation and revealed specific character differences that made it possible to classify them as vaccinia, variola or monkeypox. Cross complement fixation tests were also used to examine the immunological activities of antisera to detergent treated purified preparations of three orthopoxviruses. Only common reactivities were detected by this method however and differentiating reactivities were not observed.

Amano et al (1979) examined various strains of vaccinia whitepox, monkeypox and cowpox viruses for their capacity to induce specific early antigen detectable on the surface of infected cells by immunofluorescence. The Elstree strain of vaccinia, two strains of variola minor and white variants of cowpox and monkeypox viruses lacked the capacity to induce the antigen. Variation of the parent cowpox and monkeypox viruses to white variants was always accompanied by loss of antigen inducing capacity.

Since there is a very close serological relationship between the orthopoxviruses, most comparisons until recently have been made on biological characteristics.

The appearance of the pock morphology on the CAM is characteristic for a particular orthopoxvirus. The ceiling

temperature, or the highest temperature at which the virus will produce pocks on the CAM was a phenomenon used by Bedson and Dumbell (1961), particularly to distinguish variola major and variola minor. This varies between 37.5°C and 41°C across the group. The resistance to heating at 56°C for 20 minutes also varies with different orthopoxviruses but is not related to the ceiling temperature on the CAM.

Other biological characters consequent upon infection have been used to identify the orthopoxviruses and are; the relative chick embryo mortality, the mortality of adult mice, the type and intensity of the lesion produced after intradermal inoculation of rabbit skin and the type of cytopathic effect observed in various tissue culture cell lines.

A summary of the biological characters used to distinguish between the orthopoxviruses is given in Table 1. The data for this was obtained from a review by Baxby (1975) with additional information from Baxby and Ghaboosi (1977).

The use of these biological characters for comparison of the orthopoxviruses is based on the product of multigenic functions and usually depends on the response of a secondary host to infection. Thus the observations are sometimes variable.

Recently, more sensitive methods for comparison of the orthopoxviruses have been developed which analyse the virus particle itself or the virus induced products. The poxvirus genome has been studied using restriction endonuclease enzymes, which cleave the DNA into less than 30 fragments which can be separated on an agarose gel by electrophoresis. Each poxvirus has a characteristic fragment migration

Table 1.1. Summary of biological comparisons of orthopoxviruses.

ORTHO- POXVIRUS	loss at 56°C for 20min	CAM ceiling temp (°C)	CAM log titre (35-38)°C	rabbit skin lesion	death of adult mice	relative chick mortality
variola major	5.2	38.5	0.2 - 0.5	TrN	-	low
variola minor	2.4	37.5	2	TrN	-	v.low
monkey- pox	2.4	39.0	0.05	IPC	+	medium
whitepox	-	38.5	0.3	TrN	-	low
camelpox	-	38.5	1.2	TrN	-	v.low/ low
Lenny	-	38.5	0.2	ERN	?	medium
vaccinia	-	41.0	-	V		v.high
cowpox	1.9	40.0	-	IPC		high
ectro- melia	3.2	39.0	-	TrN		high
buffalo- pox	-	38.5	-	ERN		medium
elephant- pox	0.5	39.5	-	IO		-

IPC = indurated lesion with purple centre
 TrN = transient nodule, virus not passaged
 ERN = erythematus red nodule
 V = variable, depending on strain
 IO = inflamed and oedematious

Table 1.1. continued.

ORTHO- POXVIRUS	pock on CAM	HA in tissue culture	CPE in HeLas	'A' type inclusions	diffus- ible 'LS' in CAM	relative CPE in RK 13s
variola major	SOW	high	RC			
variola minor	SOW	low	RC			
monkey- pox	SOH	low-high	SC			
whitepox	SOW	high	RC			
camelpox	SOW	high	GC			
Lenny	PHU/LOW	high	SC	-	+	medium
vaccinia	PHU/LOW			-	+	fast
cowpox	H			+	-	medium
ectro- melia	ISOW			+	-	slow
buffalo- pox	PHU/LOW			-	+	slow
elephant- pox	H			+	-	

SOW = small, opaque, white.

SOH = small, opaque, haemorrhagic.

PHU = pale, haemorrhagic, ulcerated.

LOW = large, opaque, white.

ISOW = irregular, small, opaque, white.

H = haemorrhagic.

RC = round cell

SC = strand cell

GC = giant cell

profile.

Wittek et al (1977) compared the DNAs of rabbitpox and vaccinia, which are very closely related, using the enzymes *Hin dIII* and *Sst I*. Different fragmentation patterns were observed, although both viruses showed identical restriction patterns in an internal region of the genome.

Muller et al (1978) compared five poxvirus genomes; rabbitpox, vaccinia, cowpox, ectromelia and fowlpox, the latter being an avipoxvirus. From similarities and dissimilarities in their restriction patterns, it was again concluded that vaccinia and rabbitpox are genetically the most related although they were clearly distinguishable. Cowpox and ectromelia showed about the same degree of relatedness to each other as to rabbitpox and vaccinia. Fowlpox was found to have the largest genome and was completely different from the others, showing a very low degree of relatedness.

Esposito et al (1978) investigated the fragmentation patterns of twelve closely related orthopoxviruses with the enzymes *Hin dIII*, *Sal I* and *Bam HI*. The viruses were grouped into four species; cowpox, vaccinia, monkeypox and variola. *Hin dIII* best revealed the species groupings and was especially useful in elucidating the variola strain differences. *Bam HI* gave a more complex fragmentation pattern and differences were less easy to see than with *Sal I* or *Hin dIII*.

Thus, using restriction enzymes, discriminating and consistent comparisons of the orthopoxviruses can be made. More recently, the fragments resulting from different enzyme digestions have been mapped using hybridisation techniques, making available even more information about the poxvirus genome for comparison (Mackett & Archard, 1979).

Studies have also been carried out to compare the virus induced proteins synthesised in virus infected cells. Ikuta et al (1978) prepared radioactively labelled virions, free from host cell material and compared the structural proteins of vaccinia, cowpox and Shope fibroma by SDS polyacrylamide gel electrophoresis (PAGE) and autoradiography and were able to distinguish between the viruses, although cowpox and vaccinia were very similar. Arita and Tagaya (1977) compared the structural proteins of variola, monkeypox, cowpox and vaccinia by SDS-PAGE and also observed differences. Harper et al (1979) studied all the proteins produced inside the cell after infection, which includes virus induced enzymes as well as structural proteins, giving a wider spectrum for comparison of the orthopoxviruses; smallpox, whitepox, vaccinia, monkeypox, buffalopox, Lenny, carnivorepox and cheetahpox. The intracellular proteins produced late after infection were very useful for examining the relationship between the orthopoxviruses. This is because there was more variation in the non-structural proteins than in the structural proteins, which form the basic poxvirus unit and are likely to be similar for all orthopoxviruses. Characteristic intracellular protein patterns obtained from four species reinforced earlier groupings based on biological and serological tests, for example carnivorepox was in the cowpox group and whitepox was grouped with smallpox. This Method is useful for comparing the orthopoxviruses as it recognises individual gene products rather than the multi-genic characteristics of biological techniques.

In this thesis the orthopoxviruses were compared by the extent to which the phenotypic expression of a virus

coded mechanism occurred resulting in the production of specific enzymes involved in arginine biosynthesis. Virus yields together with DNA and protein synthesis were measured in certain cell lines deficient in their ability to express these enzyme activities.

Arginine requirement of Mammalian Cells

In the whole animal, cells are metabolically differentiated and each type of tissue has its specific function. A nutrient may be synthesised in one site and then transported to another type of tissue which is unable to synthesise that nutrient, via the blood stream.

When excised tissue is grown in vitro, changes occur in the nutritional requirements of cells. They lose their differential characteristics after passaging a few times and are unable to synthesise certain nutrients which they could in the whole animal.

There are eight amino acids essential for the nitrogen balance in man; isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tyrosine and valine (Rose et al, 1955). Cells in vitro require a further five amino acids; arginine, cysteine, glutamine, histidine and tryptophan (Eagle, 1959).

Although arginine is synthesised in vivo, most of it is directed towards the formation of urea, the means of excretion in ureotelic organisms. For normal growth and development of mammals, supplementary arginine is required in the diet. In vitro arginine is absolutely essential.

De novo synthesis of arginine takes place in the liver

of the whole animal via the Krebs-Henseleit urea cycle.

Initially, α -ketoglutarate, formed from the citric acid cycle is transaminated to form glutamate, which is then converted to ornithine by two pathways, one involving acetylation through N-acetyl glutamate and a second non-acetylated pathway in which glutamate-4-semialdehyde is the intermediate. Both pathways are endergonic and require NAD^+ and H^+ ions.

Ornithine enters the urea cycle by condensing with carbamyl phosphate to form citrulline. This reaction is catalysed by ornithine transcarbamylase. The carbamyl phosphate is formed from the coupling of L-glutamine and CO_2 in the presence of carbamyl phosphate synthetase. Ornithine transcarbamylase occurs in mammalian, but not avian, liver, in higher and lower plants and in certain micro-organisms.

Citrulline is converted to arginine by the next step in the cycle, involving two enzymes. Argininosuccinate synthetase catalyses the ATP dependent formation of an acyl guanidinium linkage between aspartic acid and citrulline, forming argininosuccinate. This is cleaved by argininosuccinate lyase, producing arginine and fumarate.

The remaining step in the urea cycle is the removal of the nitrogen from arginine with concomitant formation of urea and regeneration of ornithine. This is catalysed by the enzyme arginase.

Both enzymes involved in the conversion of citrulline to arginine are subject to repression by arginine both in vivo (Nuzum & Snodgrass, 1971) and in vitro (Schimke, 1964). The presence of arginine however stimulates the activity of arginase.

Figure 1.1 shows the reactions of the Krebs-Henseleit urea cycle. The synthesis of carbamyl phosphate from glutamate and the conversion of ornithine to citrulline take place in the mitochondria of liver cells and the enzymes are not present in the mitochondria of any other tissues. It is probably these enzymes which are absent from mammalian cells in vitro, causing the essential requirement for arginine, since cells in culture have lost their differential characteristics. This is supported by the fact that many cell lines can convert citrulline to arginine, but none have been found to be able to utilise ornithine (Tytell & Neuman, 1960).

Defects in the urea cycle have been demonstrated in vivo by the presence of abnormally high levels of intermediates in patients' urine. Citrullinaemia is a result of accumulation of citrulline in the body fluids and is associated with mental retardation (McMurray et al, 1964). Argininosuccinic acidemia, another rare disease is caused by a deficiency in argininosuccinate lyase activity causing excretion of argininosuccinate in the urine (Westall, 1960).

Role of Arginine as a Growth Factor

Arginine is utilised mainly in protein synthesis, particularly in the synthesis of basic proteins such as histones, which stabilise the highly negative DNA (Smart & Bonner, 1971) and of proteins involved in intracellular transport mechanisms (Ryser, 1968).

None of the other intermediates of the urea cycle have significant roles in protein synthesis. There are

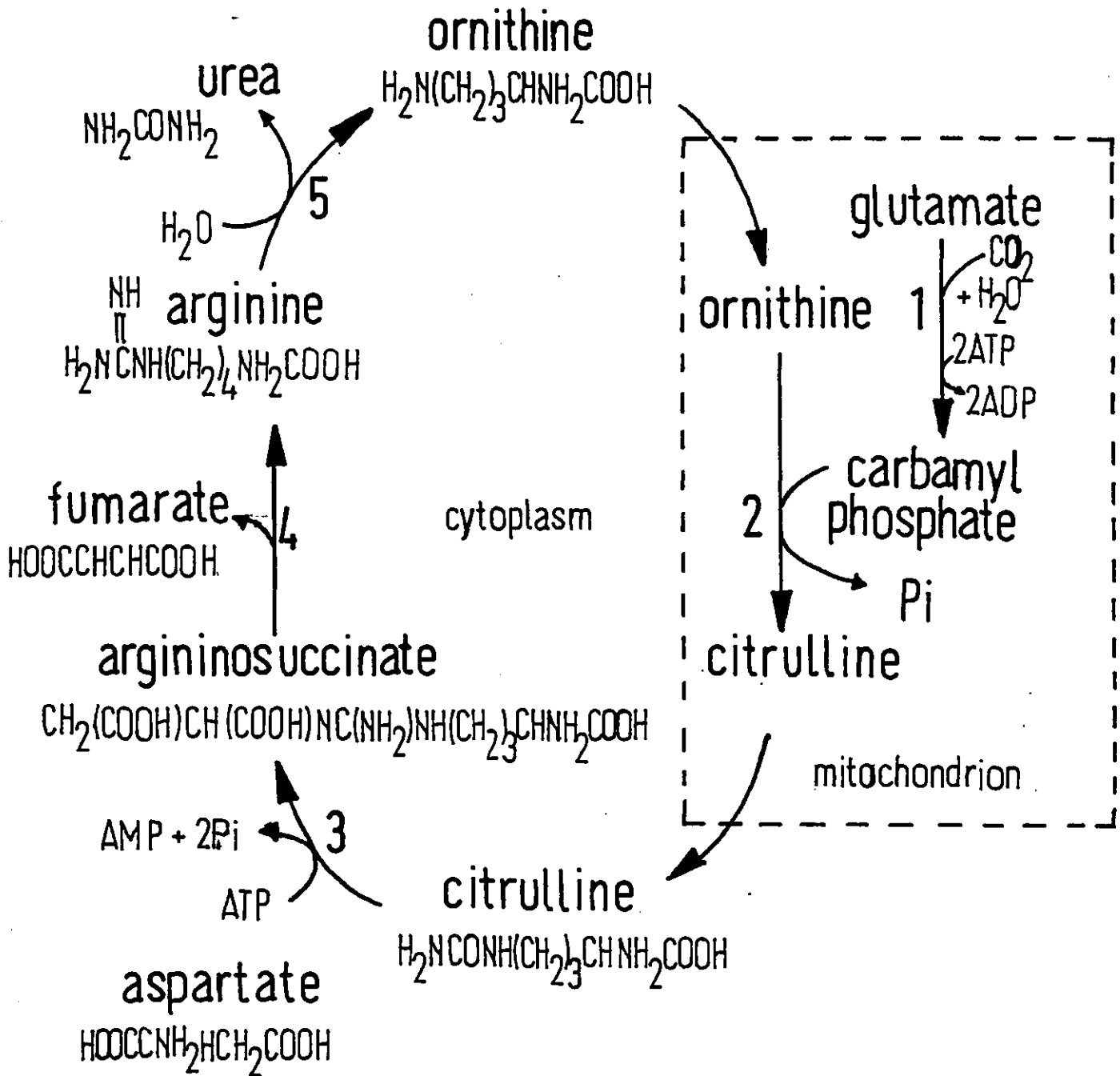


Figure 1.1. Krebs-Henseleit Urea Cycle.

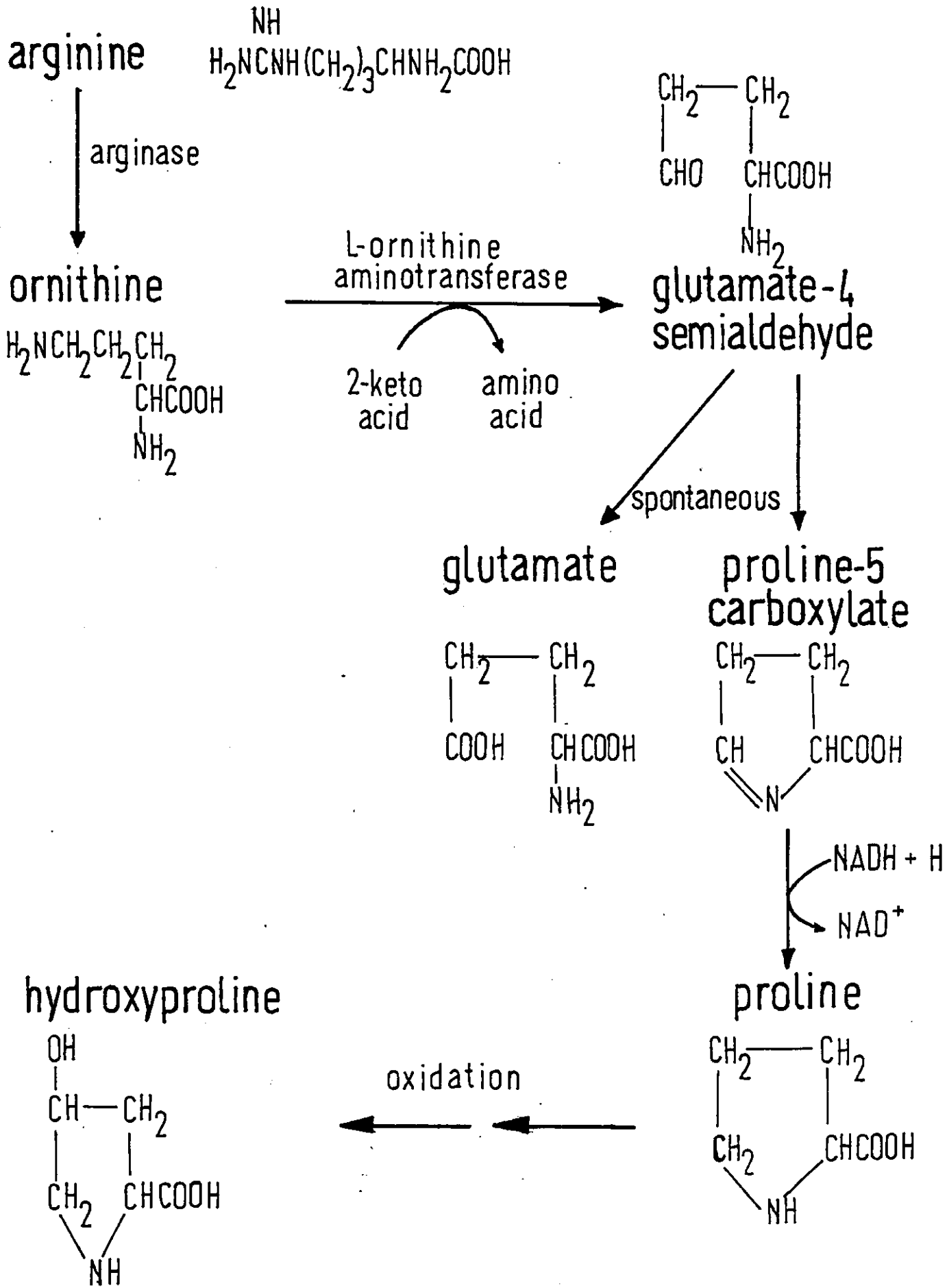
1 = carbamyl phosphate synthetase, 2 = ornithine transcarbamylase, 3 = argininosuccinate synthetase, 4 = argininosuccinate lyase, 5 = arginase.

only two reports of citrulline or ornithine being incorporated into protein. Citrulline has been detected in the medulla of the quill of the african porcupine and in the inner root sheath of the guinea pig (Steinart, Harding & Rogers, 1969) and ornithine has been found in certain α_1 - α_2 globulins (Stetten, Aakesson & Alvsaker, 1971).

Arginine also plays a role in intermediary metabolism, leading to the synthesis of other amino acids, purines and pyrimidines and polyamines. Exogenous arginine, supplied to cell cultures provides a substrate for ornithine production which can be used for the synthesis of two amino acids, proline and hydroxyproline. The pathway is outlined in figure 1.2. Ornithine is deaminated in the presence of a 2-keto acid from the citric acid cycle to yield glutamate-4-semialdehyde and a second amino acid. This is catalysed by L-ornithine 2-keto acid aminotransferase. The product undergoes a spontaneous condensation reaction followed by ring closure to form L-pyrroline-5-carboxylate. Conversion to proline by the enzyme L-pyrroline-5-carboxylate reductase occurs in the presence of NADH and H^+ (Dagley & Nicholson, 1970). Hydroxyproline is formed from oxidation of proline, but this does not occur until the amino acid has been incorporated into a peptide. The ability of cells in vivo to synthesise proline from ornithine is apparently unchanged in vitro as proline is not essential in tissue culture medium.

In addition to the catabolism of arginine by arginase, ornithine can be regenerated from arginine in mammals by reaction with glycine, catalysed by glycine amidinotransferase. The second product is guanidoacetate and is the precursor

Figure 1.2. Proline and hydroxyproline biosynthesis.



to cratine and phosphocreatine which play important roles in the storage and transmission of phosphate bond energy. Guanidoacetate is methylated in the presence of 5-adenosyl methionine by guanidoacetate transmethylase forming creatine and 5-adenosyl homocysteine. The creatine is then phosphorylated by creatine kinase, an ATP dependant reaction, to form phosphocreatine. In a non-enzymic reaction phosphate is released and ring closure occurs to form creatinine which is excreted. (See figure 1.3)

In Lactobacillus leichmanii, the guanido carbon of arginine is a source of 1-carbon units for both purine and pyrimidine synthesis (Hutson & Downing, 1968). The reactions involved in this pathway are initiated by the breakdown of arginine to citrulline by arginine deiminase. Ornithine transcarbamylase cleaves citrulline to ornithine and CO_2 , which is fixed as carbamyl phosphate by carbamyl phosphate synthetase. The carbamyl phosphate condenses with aspartic acid to form ureidosuccinic acid by the enzyme aspartate transcarbamylase. This is then converted to uridine-5-phosphate. (See figure 1.4)

Another important role of arginine and ornithine is in polyamine biosynthesis. The polyamines spermidine and spermine are important growth factors. Elevated polyamine levels have been associated with increased protein synthesis and cell multiplication (Russell & Snyder, 1968) and a requirement for spermidine has been shown for the expression of DNA dependant RNA polymerase activity (Fox & Weiss, 1964). Polyamines are also associated with ribosomes, being necessary for their structural integrity, possibly by association with the negatively charged nucleic acid

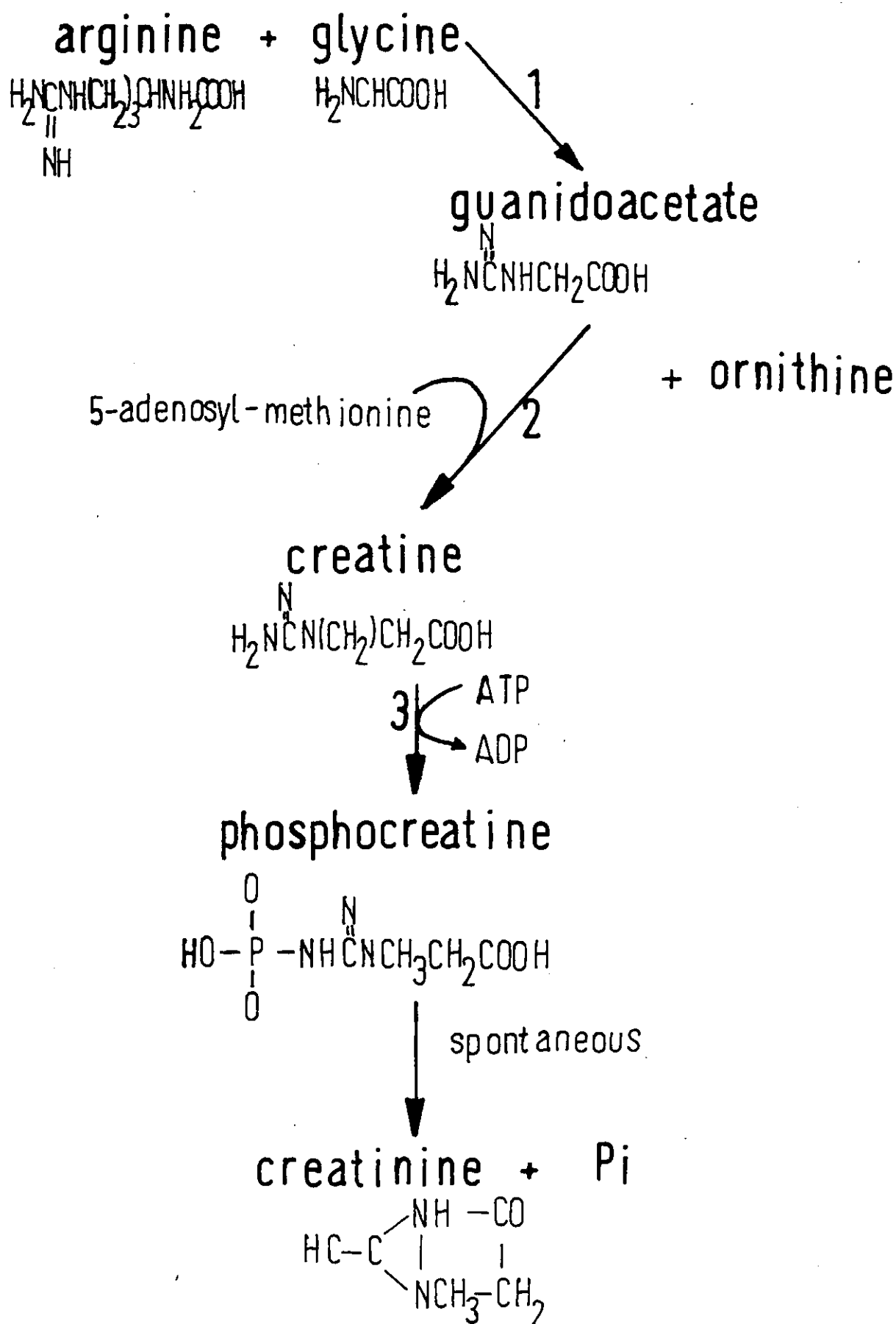


Figure 1.3. Phosphocreatine and creatinine biosynthesis. 1 = glycine amidinotransferase, 2 = guanidoacetate transmethylease, 3 = creatine kinase.

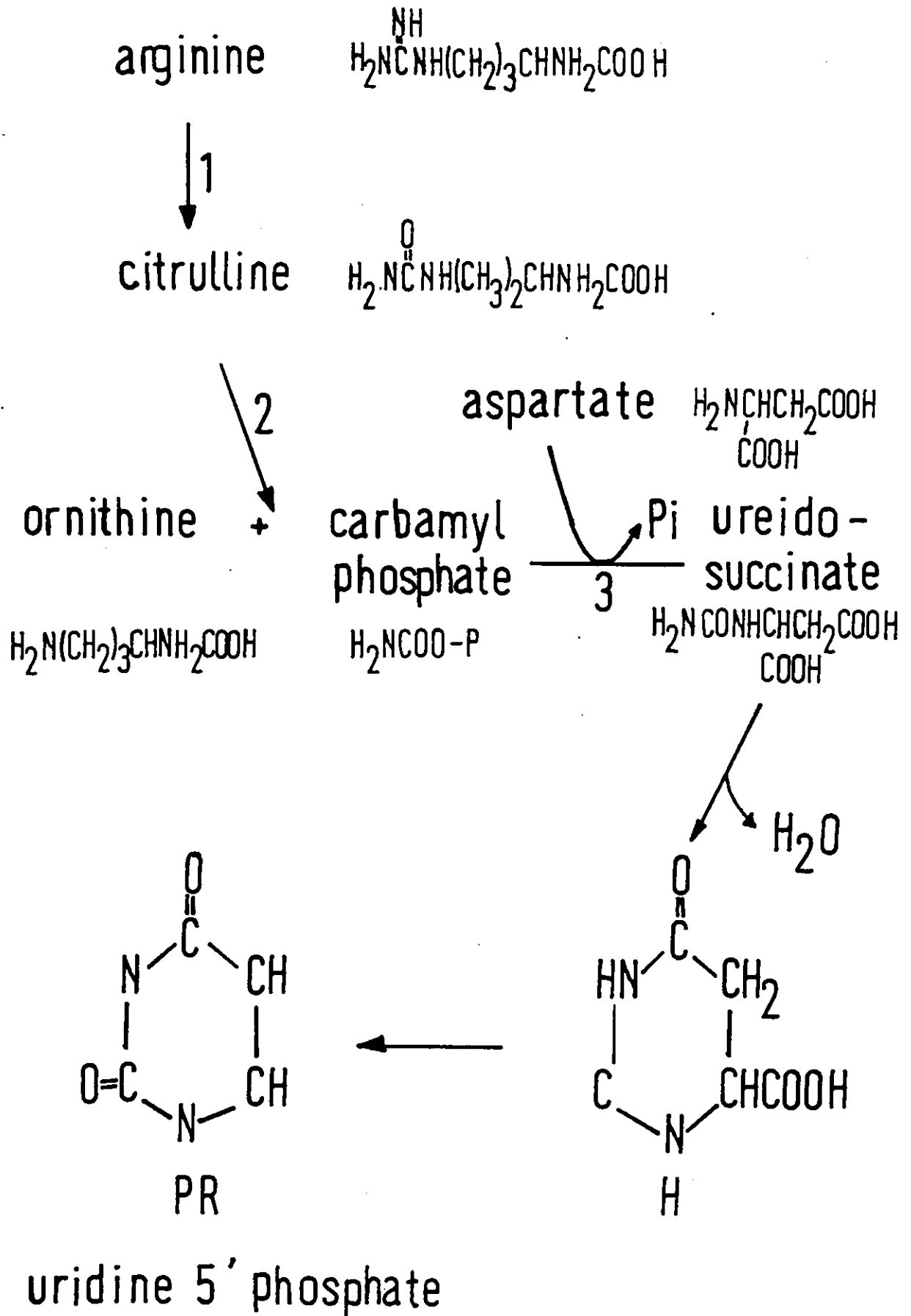


Figure 1.4. Nucleotide synthesis from arginine in Lactobacillus leichmanii. 1 = arginine deiminase, 2 = ornithine transcarbamylase, 3 = aspartate transcarbamylase.

(Cohen & Lichtenstein, 1960).

Polyamines are formed from putrescine and 5-adenosyl methionine. In mammals, putrescine is formed by the decarboxylation of ornithine by ornithine decarboxylase. In Escherichia coli putrescine is derived from arginine, which in the presence of arginine decarboxylase forms agmatine and CO_2 . Agmatine is hydrolysed by agmatase to form putrescine and urea.

5-adenosyl methionine is decarboxylated to 5-adenosyl 3-methylmercaptopyrpylamine by 5-adenosyl methionine decarboxylase, which reacts with putrescine, catalysed by aminopropyl transferase to form spermidine and 5-methylthioadenosine. Spermidine is converted to spermine by a repetition of the last two reactions. (See figure 1.5)

The urea cycle is closely linked to the citric acid cycle, which is responsible for carbohydrate metabolism, by the fact that the amino acids arginine and ornithine can be metabolised to glutamic acid which can enter the cycle. In addition, fumarate, which is formed from the cleavage of argininosuccinate, is one of the intermediates of the citric acid cycle.

The metabolism of arginine thus gives rise to a wide range of products, apart from being used for protein synthesis.

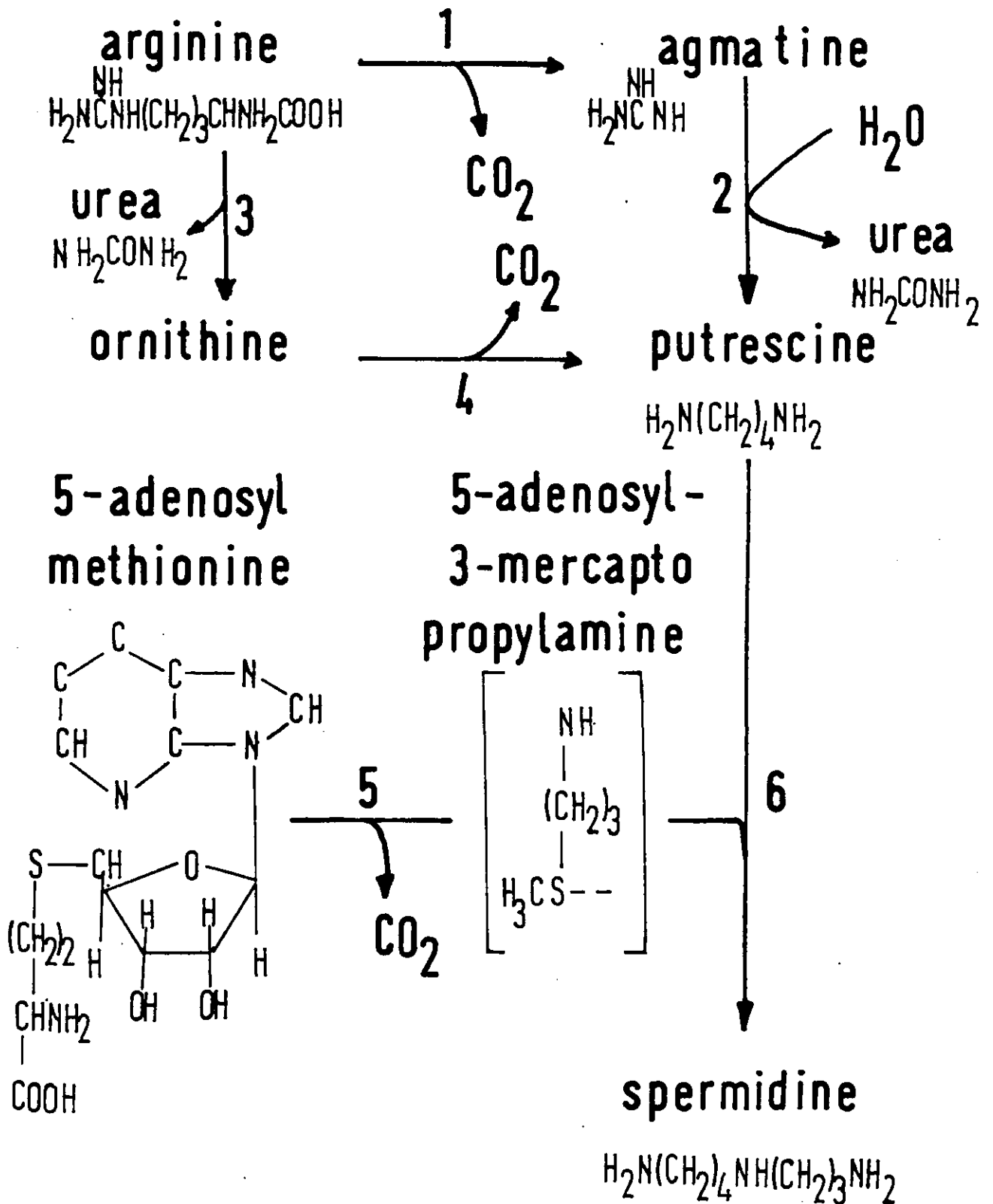


Figure 1.5. Pathways to polyamine biosynthesis.

Reactions 1 + 2, by Escherichia coli, 1 = arginine decarboxylase, 2 = agmatinase. Reactions 3 + 4, by mammalian cells, 3 = arginase, 4 = ornithine decarboxylase. 5 = S-adenosyl-methionine decarboxylase, 6 = aminopropyl transferase.

The Replication of Orthopoxviruses

Orthopoxviruses are large double stranded DNA containing viruses with a capacity for about 150 genes. The DNA is enveloped in a brick shaped capsid about 2000 x 3000 Å.

Adsorption of orthopoxviruses to susceptible cells occurs very quickly and more than half the inoculum may be attached within 15 minutes (Joklik, 1964a). Following penetration of the virus envelope the cell membrane is then broken down releasing the viral DNA and core protein into the cytoplasm of the infected cell at a period of 0.5 - 2 hours post infection. This is an active process and is prevented by inhibitors of RNA and protein synthesis or by UV irradiation of the inoculum (Joklik, 1964b & c). Virus-specific m-RNA is transcribed from the virus genome before DNA replication and is translated to synthesise early viral proteins.

Host macromolecular synthesis is inhibited very soon after infection. Initially, host protein synthesis is reduced, the time required being inversely proportional to the inoculum size. With a high multiplicity of infection, this can occur within 30 minutes (Saltzman & Sebring, 1967; Moss & Saltzman, 1968; Esteban & Metz, 1973). This still occurs when RNA and protein synthesis is inhibited, suggesting that a component of the virus particle is responsible (Shatkin & Moss, 1968).

Synthesis of high molecular weight RNA decreases several hours after infection and is preceded by a block in transport of RNA to the cytoplasm (Becker & Joklik, 1964; Saltzman et al, 1964).

Inhibition of host DNA synthesis is due to the degradation of nascent DNA by a single stranded specific DNase with neutral pH optimum, also derived from the inoculum (Pogo & Dales 1973 & 1974).

In cells synchronously infected with vaccinia virus, virus specific DNA synthesis begins within 1 - 2 hours post infection and is completed by about 5 hours post infection (Saltzman, 1967; Joklik & Becker, 1964). The DNA replication occurs within discrete foci or 'factories' in the cytoplasm of the infected cell (Cairns, 1960; Kato et al, 1960). The host cell nucleus appears not to be required for many virus specific events since vaccinia virus DNA is synthesised in enucleated cells (Prescott et al, 1971 & 1972) However mature virus particles are not formed in enucleated cells (Pennington & Follett, 1974) showing that there is some nuclear involvement in orthopoxvirus replication. Walen (1971) and La Colla & Weissbach (1975) have reported replication of poxvirus DNA in the nuclei of infected cells.

Synthesis and translation of late mRNA occurs from the newly formed DNA. Finally, the components are assembled and progeny virions begin to appear at about 6 hours post infection.

Since the orthopoxviruses replicate in the cytoplasm of infected cells, host cell enzymes found in the nucleus may not be utilised, and certain enzymes, especially associated with DNA replication, are coded by the virus genome. Some virus specific enzymes have been isolated and characterised. The thymidine kinase level rises within 1 - 2 hours post infection. This enzyme is involved with

DNA synthesis and catalyses the phosphorylation of thymidine. The best evidence that the thymidine kinase (TK) activity is virus coded is that it is still induced in TK⁻ cells, and TK⁻ virus has also been isolated (Kit et al, 1963; Dubbs & Kit, 1964). In addition, protein synthesis is required for TK induction (Jungworth & Joklik, 1965; Kit et al, 1963) and host and virus TKs have different enzymic, immunological and electrophoretic properties (McAuslan, 1963; Kit et al, 1974).

DNA polymerase also increases during the early phase of vaccinia infection (Jungworth & Joklik, 1965) and again, the induced polymerase differs from host cell enzyme with regard to chromatographic properties, molecular weight, heat stability and immunological properties.

A deoxyribonuclease, active at alkaline pH, is induced early in infection (McAuslan, 1965; Eron et al, 1966; McAuslan & Kates, 1967). This enzyme has a preference for double stranded DNA and has been characterised as an exonuclease. Induction of this enzyme is not prevented by inhibitors of DNA, but protein synthesis is required.

Later in infection two other DNases are produced, one with a neutral pH optimum (Pogo & Dales, 1969) and one with an acid pH optimum (McAuslan & Kates 1967).

Only about 10% of the total yield of virus is released into the medium (Furness & Younger, 1959; Postlethwaite & Maitland, 1960; Smith & Sharp, 1960; Easterbrook, 1961). With some poxviruses including cowpox and ectromelia, there are dense proteinaceous masses in the infected cell cytoplasm called 'A' type inclusions (Ichihashi et al, 1971; Kato et al, 1963; Ichihashi & Matsumoto, 1966) which contain

mature virus particles and virus induced antigens.

Intercellular fusion (Appleyard et al, 1962; Kaku & Kamahora, 1964) which often occurs after infection with some poxviruses leads to direct cell-to-cell spread of virus. This can result eventually in the formation of plaques in cell monolayers.

Thus in order to measure the total yields of infective virus from infected cells it is necessary to disrupt the cells by techniques such as freezing and thawing and ultrasonic treatment to release the majority of the virus into the medium before accurate infectivity titrations can be carried out.

The Arginine Requirement in the Replication of Orthopoxviruses and other Viruses

The amino acid requirements for vaccinia virus replication in mouse L-cells were studied by Holterman (1969) and all the amino acids necessary for virus growth were those found by Eagle (1959) to be essential for cell growth. A further indication that arginine is necessary for vaccinia virus replication was given by the effects of mycoplasma on the growth of virus (Singer et al, 1970). The presence of M.argininii decreased the yield of vaccinia and the effect could be overcome by addition of extra arginine.

Archard & Williamson (1971) studied the effects of arginine deprivation on vaccinia virus infected HeLa cells. A quantitative relationship was found between the concentration of arginine and the yield of virus, with no yield in the absence of arginine and maximum yield at 0.09mM

arginine. The poxviruses are not unique in their requirement for arginine. It was shown to be essential for adenovirus type 2 replication (Rouse & Schlesinger, 1967) and arginine was shown to be the only amino acid for which there is an absolute requirement in this system. Absence of the other amino acids resulted only in a reduction in the virus yield. Herpes simplex was shown to require arginine (Tankersley, 1964) and also other herpes like viruses, pseudorabies (Mark & Kaplan, 1971), cytomegalovirus (Minamishima & Benyesh Melnick, 1969) and Epstein Barr virus (Henle & Henle, 1968). Arginine is also essential for growth of the papovaviruses SV40 (Goldblum, Ravid & Becker, 1968) and polyoma (Winters & Consigli, 1971). Frog virus 3, a DNA containing virus which replicates in the cytoplasm was similarly found to require arginine for replication in BHK 21 cells (Aubertin, 1975).

The nature of the requirement for arginine in the replication was investigated by Archard & Williamson (1971) with vaccinia virus in HeLa cells. Pulse labelling with 14-C-arginine showed that there was a marked stimulation in the rate of incorporation of arginine within 50 minutes of exposing the cells to virus. This was followed by a rapid decrease in incorporation, but a second peak was observed at 3 hours. Purified vaccinia virus from cell cultures maintained in the presence of 14-C-arginine gave a single peak after density gradient centrifugation that was coincident with a single peak of infectivity.

In the absence of arginine, virus specified DNA synthesis was markedly suppressed. However, production of some early virus specific proteins was detected. Infection

in the presence of sub-optimal concentrations of 0.03mM arginine followed by simultaneous addition of FUdR and 0.06mM arginine at 6 hours post infection allowed maximal production of virus without a lag. These results indicate that arginine is involved in at least two functions in the replication of vaccinia virus in HeLa cells. The first is an early event involved with virus specified DNA synthesis and on which DNA synthesis depends. The second, a quantitatively greater requirement is involved with later events shown to be associated with the formation of virus specified structural proteins. The effect of arginine deprivation is reversible since addition of arginine to cultures previously incubated in its absence results in a yield of virus comparable to appropriate controls. Archard & Williamson (1974) also studied the effects of two arginine derivatives canavanine and canaline on vaccinia virus replication in HeLa cells. Canavanine is an oxyguanidinium analogue of arginine and following hydrolysis with arginase gives canaline, an oxyamino analogue of ornithine. Canavanine was found to inhibit both arginine biosynthesis from citrulline and also replication by competing with arginine forming canavanyl t-RNA.

Canaline also inhibited virus replication by reacting with pyridoxal phosphate, an essential growth factor. Canaline inhibition could be relieved by increasing the concentration of this cofactor.

Obert, Tripier & Guir (1971) have also investigated the arginine requirement of vaccinia virus replication in KB cells. Unlike the HeLa cell system, although there was no virus yield in the absence of arginine, DNA synthesis

was found to occur and was only reduced by 30% of that in the presence of arginine. Simultaneous addition of FUdR and arginine at 8 hours post infection to cell cultures previously deprived of arginine resulted in a yield of virus which was 60% of that produced by cultures infected in the presence of arginine. In the absence of arginine, however, only early virus-specific mRNA was shown to be synthesised. Also electron microscopy revealed that the synthesis of structural components was limited to the formation of virus specific membrane structures in the absence of arginine.

Pogo, Katz & Dales (1975) investigated the basic proteins of the poxviruses. Vaccinia DNA was shown to be intimately associated with an arginine enriched protein and analysis by SDS PAGE showed a single component of molecular weight 11 - 12,000. It shared the properties of protamines and histones associated with eukaryotic DNA, but with a higher molecular weight. This protein was found to be present first in the cytoplasmic factories of DNA and later in the cores of mature virus particles. This arginine enriched protein was not synthesised if DNA synthesis did not occur. The role of this protein was suggested to modulate gene activity and control packing of DNA into mature virus particles.

Arginine Biosynthesis in Orthopoxvirus-infected Cells

As previously mentioned, arginine is essential in cell culture, due to the inability of such cells so far examined to convert ornithine to citrulline, the reaction which takes place in liver mitochondria in vivo by the enzyme ornithine transcarbamylase. Many types of cell can grow normally when citrulline replaces arginine, but certain cell lines lack either or both enzymes argininosuccinate synthetase and lyase and exhibit an absolute requirement for arginine (Tytell & Neuman, 1960).

Williamson & Cooke (1973) studied the argininosuccinate synthetase and lyase activities in vaccinia virus infected HeLa cells and mouse L cells, both of which are capable of growing normally if citrulline replaces arginine. The enzyme activities increased markedly following infection. A repression mechanism determines the specific activities in uninfected cells (Schimke, 1964) and a greater activity was obtained in the presence of 0.2mM citrulline than of 0.6mM arginine. However maximum activity was obtained in infected cells in the presence of 0.6mM arginine. The development of increased activity in virus infected cells was inhibited by puromycin or actinomycin D. Treatment of infected cells with FUDR resulted in higher levels of activity than in similar cells without the inhibitor. This phenomenon is characteristic of 'early' virus coded enzymes. The Km values of the enzymes in HeLa and mouse L cells were 0.11×10^{-3} and 0.07×10^{-3} respectively but the same value of 1.0×10^{-3} was obtained for both species of infected cells. Analysis of purified virus, prepared from HeLa cells

in the presence of 14-C-citrulline showed that the radioactivity was associated exclusively with arginine. It was concluded that the anabolism of arginine from citrulline was determined by a virus coded function.

Further evidence for the production of the enzymes argininosuccinate synthetase and lyase by virus directed mechanisms was obtained by Cooke & Williamson (1973) by studying the replication of rabbitpox virus in mouse sarcoma 180 cells, a cell line unable to utilise citrulline in place of arginine. It was shown that rabbitpox virus replication occurred in infected cells maintained in the presence of citrulline or arginine, but no virus was obtained in the absence of either amino acid. It was again concluded that the synthesis of arginine from citrulline was determined by virus directed mechanisms, either by virus coded enzymes or by virus modification of host enzymes.

Human citrullinaemia cells, another cell line unable to utilise citrulline also supported the replication of vaccinia virus in the presence of citrulline.

Chesters (1976) measured the yields of other orthopoxviruses in mouse sarcoma 180 (S180) in the presence and absence of arginine or with citrulline replacing arginine. The viruses examined were; buffalopox, monkeypox, vaccinia (Lister), cowpox and elephantpox. With all viruses except cowpox there was no yield of virus recovered in complete medium, and with cowpox, the yield was low compared with rabbitpox. However in the absence of arginine, or in the presence of citrulline there was no yield, indicating that cowpox, unlike rabbitpox, may not induce the biosynthetic ability to convert citrulline to arginine in S180 cells.

Since this cell system did not support the growth of other orthopoxviruses tested, it was not suitable for further investigations.

Another cell type, reported by Tytell & Neuman (1960) to be unable to grow if citrulline replaced arginine was a primary culture from the chorioallantoic membrane (CAM) of the chick embryo. Chesters (1976) maintained fragments of CAM in growth medium, but was unable to deplete the intracellular pools of arginine sufficiently. During the course of this work a method for the cultivation of cells from the CAM was reported by Cursiefen & Becht (1975). Chesters used this method and confirmed the nature of the metabolic lesion by measuring the incorporation of carbamyl-¹⁴C-citrulline and guanido-¹⁴C-arginine into cell protein. Comparisons of the yields of vaccinia (Lister) and cowpox were made in complete medium, in the absence of arginine and with citrulline or argininosuccinate replacing arginine. It was necessary to add serum to such media since serum factors with molecular weight of less than 700 were essential for the replication of vaccinia and cowpox in CAM cells. Although there was a low yield with both viruses in the absence of arginine, there was a significantly greater yield of vaccinia virus in the presence of citrulline but not with cowpox. Both viruses produced significant yields in the presence of argininosuccinate. However the yield of cowpox virus in the presence of argininosuccinate suggested that either virus-induced argininosuccinate lyase or host specific enzyme activity was utilised.

The work presented in this thesis is an extension of the experiments carried out in this laboratory by Cooke

(1973) and Chesters (1976) and began by comparing the ability of a spectrum of orthopoxviruses to grow in CAM cells in the presence and absence of arginine and with citrulline or argininosuccinate replacing arginine. The yields of virus in this system in complete medium were found to be very low and the relatively high background yields in the absence of arginine made interpretation of the results difficult. Also arginine deprivation was found not to result in a complete inhibition of virus yield as previously described by Cooke (1973) in SL80 cells and Archard (1971) in HeLa cells.

Investigation was made into other cell lines which might lack the capacity to grow if citrulline replaced arginine and two such lines were characterised, namely BHK 21 cells and chick embryo fibroblasts. In the presence of arginine both cell systems supported the growth of all orthopoxviruses tested and experiments were carried out to measure the extent of replication in the presence of citrulline.

Further experiments were also carried out on the growth of other, more recently isolated, poxviruses in SL80 cells.

Measurements of virus yields and the extent of DNA synthesis were made in these four cell systems under different conditions of arginine availability. Some orthopoxviruses were found to produce yields, albeit reduced, in the absence of arginine, even after the intracellular pools had been depleted of the amino acid. Attempts were made to ascertain how these viruses could obtain sufficient arginine for production of progeny virus under these deprived conditions.

CHAPTER 2

MATERIALS AND METHODS

1. Viruses Studied

Vaccinia (Lister)

Vaccinia (International Health Division)

Rabbitpox (Utrecht)

Cowpox (Brighton Red)

Anteaterpox (Moscow carnivore)

Cheetahpox (Whipsnade WV1)

Buffalopox

Elephantpox

Monkeypox (Copenhagen)

Camelpox

Ectromelia (Hampstead)

2. Cell Culture

a) Primary cell culture

i) CAM Cells

Cultivation of cells from the chorioallantoic membrane of chick embryos was carried out by a modification of Cursiefen & Becht (1975).

CAMs were removed from 11 day old chick embryos (Orchards Farm, Surrey) and after washing in phosphate buffered saline 'A' (PBS'A'; Dulbecco, 1954; Oxoid Ltd, London) were finely chopped with scissors. The tissue fragments were washed three times in PBS'A', allowing the pieces to settle by gravity, in order to remove erythrocytes. The washed tissue was resuspended in PBS'A' containing 0.01%

collagenase and 0.001% hyaluronidase (Sigma Ltd, Norbiton, Surrey) and shaken gently in a conical flask at 35°C for 20 minutes.

After allowing the fragments to settle, the supernatant was decanted and the tissue was resuspended in 0.25% trypsin (Flow Laboratories Ltd) in versene buffer (0.2% EDTA, pH=7.2, British Drug Houses, Poole, Dorset) before shaking for a further 45 minutes at 35°C.

The resulting suspension was passed through a sterile strainer to remove the coarse debris and the filtrate centrifuged at low speed to pellet the cells. After washing once with growth medium, the cells were counted using a Neubauer Haemocytometer and trypan blue stain. The cells were seeded at 1×10^6 cells/ml in soda glass tubes (1 ml/tube) in medium 199 (Morgan, Morton & Parker, 1950; Flow Laboratories Ltd) supplemented with 10% foetal calf serum (Sera Lab; Crawley, Sussex). The medium was renewed after 24 hours to remove dead cells and debris and the monolayer incubated for a further 24 hours to grow confluent.

One membrane usually yielded 2×10^7 cells by this treatment. The resulting monolayer consisted of a network of epithelial and fibroblastic cells.

ii) Chick Embryo Fibroblasts (CEFs)

Embryos from 10 day old eggs were removed, the head and limbs cut off, and the contents of the thorax and abdomen dissected out. The remaining skeletal muscle was washed and finely chopped with scissors. The tissue fragments were washed twice with PBS'A' before resuspension in 25 ml PBS'A' containing 1% trypsin and stirred for 30 minutes

at 35°C in a conical flask. The resulting suspension was passed through a sterile strainer to remove debris and the cells spun down at low speed (500g for 5 minutes) on a bench centrifuge.

After washing twice in growth medium (MEM + 10% calf serum; Flow Laboratories Ltd) the cells were resuspended in such medium by vigorous expulsion through a pipette. Viable counts were made in a Neubauer Haemocytometer using trypan blue stain. Cells were seeded at 1×10^6 cells/ml in soda glass test tubes in 1 ml aliquots, and incubated for 24 hours at 35°C after which time the medium was replaced before incubation for a further 24 hours. At this seeding concentration, the tubes usually contained confluent monolayers after 48 hours. One embryo usually yielded about 10^8 cells using this treatment.

b) Continuous Cell Lines

VERO, mouse sarcoma 180 (S180), and Baby Hamster Kidney 21 (BHK 21) cells were used.

Vero cells were obtained from Biocult Laboratories Ltd (Yasamova & Kanakita, 1963). S180 cells (Foley et al, 1960) were obtained from Flow Laboratories Ltd and BHK 21 cells were obtained from the IRCF Laboratories, Lincoln's Inn Fields, London.

The three cell lines were passaged as follows. The spent medium was removed from confluent monolayers in 16 oz. medical flats and the cells washed with PBS'A'. The cell monolayers were treated for 10 minutes with versene (0.02% EDTA in PBS'A') in a sufficient volume to just cover the monolayer. The detached cells were resuspended in 8 ml

fresh medium and 2 ml was placed into each of four 16 oz. medical flat bottles containing 40 ml growth medium.

Vero cells were grown in medium 199 supplemented with 5% foetal calf serum. Sl80 and BHK 21 cells were grown in Eagle's minimal essential medium (MEM, Eagle, 1959; Flow Laboratories Ltd) supplemented with 5% newborn calf serum (Gibco Ltd). BHK 21 cells were also supplemented with 5% tryptose phosphate broth (Difco Laboratories, Detroit, U.S.A.).

After 4 - 5 days' incubation when monolayers had become confluent, the cells were placed on a maintenance medium of appropriate defined medium supplemented with 2% serum.

3. Prevention of Mycoplasma Contamination

All growth media were routinely supplemented with penicillin, streptomycin and kanamycin sulphate. This was to ensure that cell cultures did not become contaminated with mycoplasma or any other bacteria, an extremely important consideration in metabolic studies.

At regular intervals, cell cultures were grown on coverslips and treated with a fluorescent stain (Bisbenzimid H33258, Hoechst, U.K.Ltd, Hounslow, Middlesex) which is very sensitive for cytoplasmic DNA and should thus reveal any intracellular contamination by mycoplasma. None was observed with Vero, Sl80 or BHK 21 cells. Since CAM and CEF cells were primary cultures, there would be less likelihood of contamination by mycoplasma, but other bacterial or fungal contamination would be more likely to

arise during preparation of the cultures.

4. Pirt's Modified Eagle's Medium

Pirt's modified Eagle's medium (PME), (Birch & Pirt, 1969) was used for experimental medium. This was prepared minus arginine. This medium has a high choline content and the serum requirement in some cell systems can be reduced, allowing the total constituents of the medium to be more defined, thus making metabolic studies more meaningful. Table 2.1 shows the constituents of PME.

5. Depletion of Intracellular Pools of Arginine

When confluent monolayers of experimental cells were obtained, it was necessary to deplete the intracellular pools of arginine. This was achieved by leaving the cells for a minimum of 8 hours in arginine free PME. This medium was also serum free for all cell cultures except CAM cells, for which the medium was supplemented with 1% foetal calf serum. Maintenance in arginine-free PME for up to 48 hours had no detrimental cytological effects on the appearance of the monolayers of any cell cultures used in this study.

6. Preparation of Virus Stocks

Orthopoxvirus stocks were prepared by inoculating the CAMs of 12 day old chick embryos with approximately 1000 pock-forming units from a single pock isolate. After incubation for 2 days with vaccinia and rabbitpox viruses,

TABLE 2.1. Pirt's modified Eagle's medium (PME)

<u>Inorganic salts</u>	mg/l	<u>L-amino acids</u>	mg/l
NaCl	8,000	Histidine	100
KCl	400	Lysine	175
CaCl ₂	140	Isoleucine	180
MgSO ₄ ·7H ₂ O	100	Methionine	30
Na ₂ HPO ₄ ·12H ₂ O	60	Leucine	180
KH ₂ PO ₄	60	Phenylalanine	65
MgCl ₂ ·6H ₂ O	100	Threonine	100
NaHCO ₃	1,100	Tryptophan	20
		Valine	150
		Alanine	90
		Serine	20
		Glycine	15
		Tyrosine	70
		Cystine	75
		Glutamine	200
		Glutamic acid	1,530
<u>Vitamins</u>	mg/l	<u>D-sugars</u>	mg/l
i-Inositol	2.0	Glucose	1,000
d-Biotin	0.1		
Choline	10.0		
Nicotinic acid	1.2		
Pantothenic acid	1.2		
Pyridoxal	1.0		
Thiamine	2.0		
Riboflavin	0.2		
Hypoxanthine	10.0		
B ₁₂	1.0		
Folic acid	1.0		

and three days for all the other viruses, the membranes were aseptically dissected out and placed in Universal containers with glass beads. After two cycles of freezing and thawing, McIlvain's buffer, pH=7.4, was added and the tissue agitated vigorously on a Rotamixer for a few minutes, followed by 5 minutes ultrasonic treatment. The gross debris was spun down by low speed centrifugation (1500g for 10 minutes) and the supernatant dispensed in 1 ml aliquots in bijou bottles.

For experiments involving metabolic investigations where a more strictly defined medium was preferred, the virus was purified further to remove any traces of arginine which might be present in the crude virus preparation. The virus was first pelleted at 12,000 rpm for one hour in a 50Ti rotor of a 'Spinco' Ultracentrifuge (Model L, Beckmann) and then banded on a sucrose density gradient in an MSE 65 ultracentrifuge.

The stocks of orthopoxviruses were stored at -50°C . Stock virus suspensions were thawed and treated with ultrasonics to disaggregate the virus particles before use.

7. Measurement of Virus Infectivity

Serial dilutions of experimental samples were prepared in Hank's Balanced Salt Solution (Hank's BSS, Flow Laboratories Ltd.). Appropriate dilutions in 0.25 ml amounts were adsorbed at room temperature on to confluent monolayers of Vero cells, grown in 100 mm^2 dishes divided into 25 compartments (divided dishes)(Sterilin Ltd.). After 1 hour the inocula were removed and the infected cells were

maintained for 48 hours at 35°C in medium containing 1% N-2-hydroxyethyl piperazine-N-2-ethane sulphonic acid (HEPES, Williamson & Cox, 1968, Calbiochem Ltd, London), the latter added to maintain the pH at 7.3.

After incubation, the medium was removed and the monolayers stained with carbol fuchsin (Solmedia Ltd, London). Plaques of about 1 mm in diameter (varying according to the specific orthopoxvirus) were observed with a microfilm reader (Carl Zeiss, Jena, DDR) in order to facilitate counting. (See figure 2.1 for photograph)

The arithmetic mean of the counts from 50 - 500 plaques was used to calculate the total infective virus titre.

8. Infective Centre Titration

In order to determine the optimum multiplicity of infection which ensures one-step growth conditions without cytotoxic effects to the cells, an infective centre titration was carried out with each cell line.

Cell monolayers grown to confluence in soda glass tubes, were infected with varying inocula from 2×10^4 to 10^7 pfu/tube in 0.2 ml aliquots in Hank's BSS. After adsorption for 1 hour at room temperature, the infected monolayers were washed in BSS and incubated further in maintenance medium to allow the virus to penetrate the cytoplasm of the infected and begin the replication cycle. At 2 hours post infection, the monolayers were thoroughly washed and resuspended as described previously. After counting in a Neubauer haemocytometer, the cells were diluted to 5000/ml and 0.2 ml added in triplicate to 1 ml

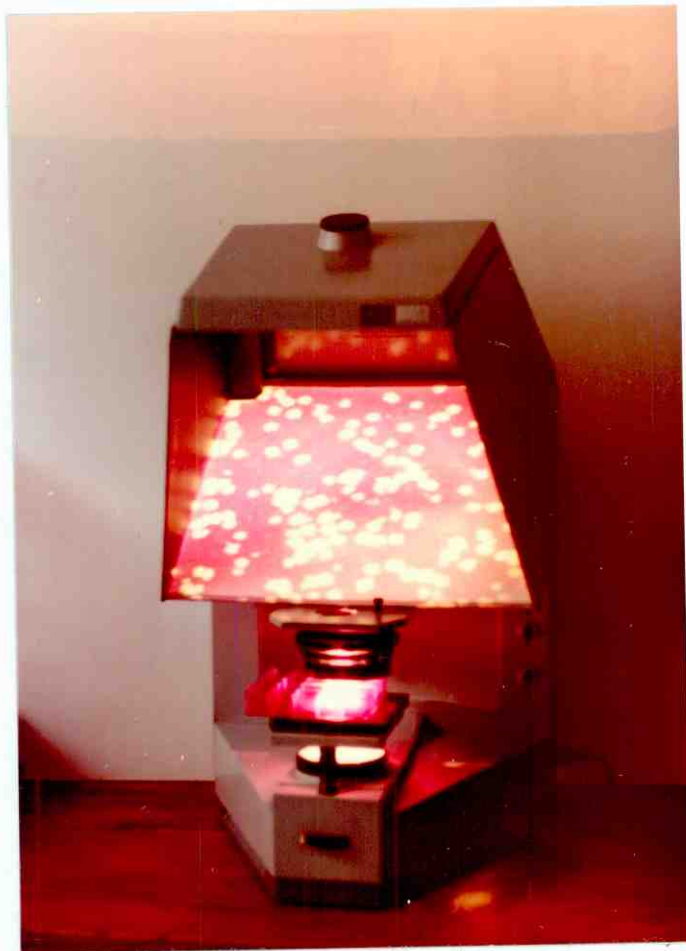


Figure 2.1. Photograph of microfilm reader showing
plaques from one well of a divided petri
dish.

suspension of Vero cells in the wells of a divided dish at a confluent density, thus producing a maximum of 1000 infective centres per well if every cell is infected.

After incubation for 48 hours, the monolayers were stained with carbol fuchsin and the plaques counted. Each plaque is equivalent to one infective centre.

9. Measurement of Virus Yields

Confluent monolayers of the appropriate cell type were grown in 6 x 5/8" test tubes and after depletion of the intracellular pools of arginine, the cells were infected. The multiplicity of infection used varied according to the particular experiment and appropriate details will be presented in the results section. Virus was adsorbed from 0.2 ml Hank's BSS for one hour at room temperature using 5 tubes per sample. After adsorption, the inoculum was removed and the infected cells washed thoroughly with Hank's BSS to remove any residual virus, before the addition of appropriate media in 1 ml aliquots.

Samples of infected cultures were frozen immediately after the adsorption period for subsequent titration of infective virus to determine the inoculum recovery. Further samples were frozen at appropriate time intervals after incubation at 37°C for measuring the virus yield.

After two cycles of freezing and thawing, cells from replicate samples were washed or scraped into the medium using a Pasteur pipette, and resultant suspensions pooled into sterile 10 ml centrifuge tubes. Cell disruption was completed with ultrasonics for 2 minutes before titration

of the infective virus as previously described.

10. Use of Radiochemicals

All radiochemicals were obtained from the Radiochemical Centre, Amersham.

a) Incorporation of labelled amino acids into protein

Experiments involving the use of radiochemicals were carried out in 6 x 5/8" test tubes or in 4 oz medical flat bottles.

Protein synthesis was followed by incorporation of ^3H -leucine, an amino acid common to all proteins. The incorporation of arginine was followed using guanido- ^{14}C -arginine and citrulline using carbamyl- ^{14}C -citrulline. Radioactive argininosuccinate was not available from Amersham, so the ability of the cells to utilise it was assessed by the level of protein synthesis occurring, measured by the incorporation of ^3H -leucine. A small amount of ^{14}C -argininosuccinate was prepared enzymatically from guanido- ^{14}C -arginine in this laboratory and a limited number of experiments were carried out with this preparation.

All radiolabelled compounds were supplied to cell cultures in media containing known concentrations of the unlabelled compound. ^3H -leucine was normally used at a final concentration of $0.5\mu\text{Ci/ml}$ and the ^{14}C -labelled amino acids at $0.1\mu\text{Ci/ml}$. Cell cultures were depleted of their intracellular pools of arginine as described previously before exposure to the radiochemicals.

After incubation of infected or uninfected monolayers with labelled media for the appropriate time intervals,

the cells were rapidly frozen to -50°C and incorporation of the radiolabel into protein was determined as follows. After two cycles of freezing and thawing, the replicate tubes from each sample were pooled into a 10 ml centrifuge tube, or in the case of a 4 oz bottle, the cells were resuspended in 5 ml PBS'A' in a 10 ml centrifuge tube. Disruption was completed by treatment with ultrasonics for two minutes.

The resulting cell extracts were treated with trichloroacetic acid (TCA) to a final concentration of 5% (w/v) and flocculation of the resulting precipitate was facilitated by immersion in a 56°C water bath for 5 minutes. The precipitates were centrifuged at 1500g for 10 minutes in an MSE Superminor centrifuge. After washing twice in 2% TCA and once in distilled water the precipitate was dissolved in 0.5 ml Soluene (Packard Instruments Ltd, Wembley, Middlesex) and scintillation liquid added. This was based on metapol : toluene : water (3:6:1) containing 4g PPO and 50 mg dimethyl POPOP per litre (Packard Instruments Ltd).

Radioactivity was measured in a Packard three channel liquid scintillation spectrophotometer, model 3310. Appropriate quench corrections were made using a channels ratio method and the radioactivity was expressed as disintegrations per minute per sample.

b) Incorporation of labelled nucleotides into nuclear and cytoplasmic DNA

Confluent monolayers in 4 oz medical flat bottles were placed on maintenance medium containing $2\text{-}^{14}\text{C}$ -thymidine at

0.1 μ Ci/ml for 24 hours to prelabel the host cell DNA, which would be used subsequently as a marker for nuclear DNA. After this time the cells were placed on an arginine free PME without the radiolabel for 8 hours which both chased the ^{14}C label and depleted the intracellular pools of arginine.

The arginine starved cells were infected or sham-infected and, after 1 hour adsorption at room temperature, were maintained further using appropriate medium, containing 6- ^3H -thymidine at 0.5 μ Ci/ml. Such media were supplemented with unlabelled 20 μM thymidine.

After appropriate times the cells were fractionated as follows. The medium was poured off, the cells resuspended with 5 ml versene and pelleted by low speed centrifugation. After one wash in isotonic sucrose buffer (8.6% sucrose in 0.01M phosphate/phosphate buffer, pH=7.4), the cells were resuspended in 5 ml 0.25% Nonidet P40 (NP40, British Drug Houses) in sucrose buffer and agitated vigorously. After standing for 5 minutes the tubes were agitated again before centrifugation at low speed to pellet the crude nuclei. The supernatant was carefully removed and retained as the cytoplasmic fraction. The pellet was resuspended in 4.5 ml sucrose buffer with 0.5 ml 0.67% NP40; 0.33% sodium deoxycholate and, after mixing well, was left for a further 5 minutes. The tubes were centrifuged again and the second supernatant, the perinuclear wash fraction, was carefully removed and retained leaving the gelatinous nuclear pellet, the final fraction, at the bottom of the tube.

The three fractions were then treated with cold (4°C) TCA to a final concentration of 10% and the radioactivity

associated with the high molecular weight precipitates was determined as described previously (10a).

The extent of DNA synthesis was assessed by the level of incorporation of ^3H -thymidine into high molecular weight material recovered in each fraction.

11. Acridine Orange Staining

Cells were seeded at $5 \times 10^5/\text{ml}$ on to circular coverslips in plastic wells and incubated for 24 hours at 35°C . After depleting the intracellular pools of arginine, the cells were infected or sham-infected for 1 hour at room temperature before maintenance in appropriate media. At various times post infection the cells were fixed and stained as follows. The medium was removed and the cells fixed by treatment for 10 minutes with 0.5 ml absolute ethanol, before the addition 1 ml 0.1% acridine orange in McIlvain's buffer, $\text{pH}=3$.

After 20 minutes, the coverslips were removed from the stain, washed twice in McIlvain's buffer, $\text{pH}=3$, and twice in PBS'A' before mounting in 10% glycerol in PBS'A' on microscope slides.

12. Protein Estimations

Protein estimations were carried out by the Biuret-Phenol method (Sutherland et al, 1949). The following solutions were prepared:

Solution 1 2% Na_2CO_3 in 0.1M NaOH

Solution 2a 1% copper sulphate

Solution 2b 2% sodium potassium tartrate

Solution 3 50 ml solution 1 + 0.5 ml 2a and 2b

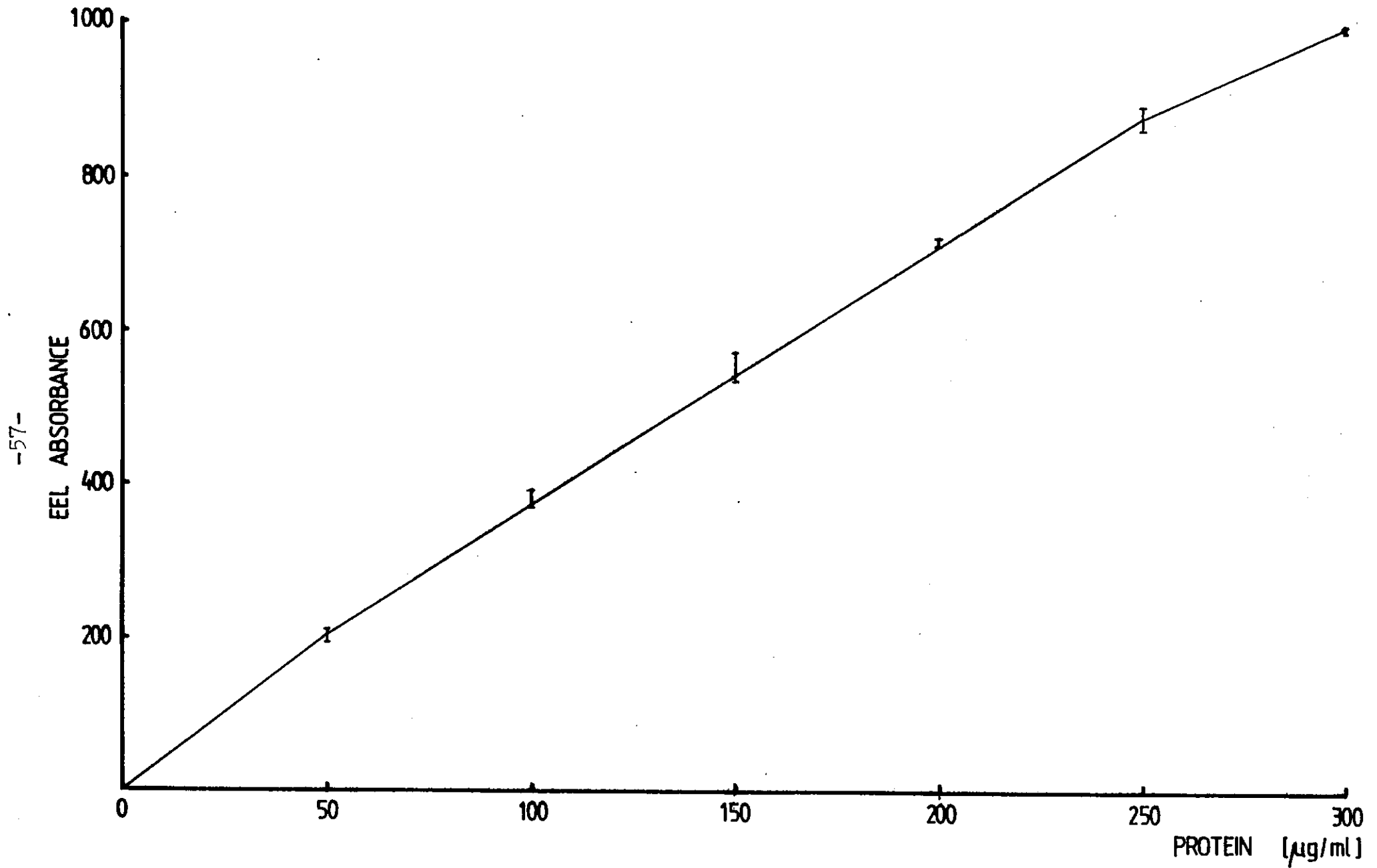
Solution 4 Folin Ciocalteu Reagent (BDH)

Samples of cell extract were dissolved in 1M NaOH and 5 ml of solution 3 was added to 1 ml extract. After standing for 10 minutes, 0.5 ml solution 4 was added and mixed well.

After a further 30 minutes the absorbance of each sample was read with reference to an appropriate blank on an EEL Colorimeter (Evans Electroselenium Ltd, Halstead, Essex) with a red filter 607.

A standard curve was set up using bovine serum albumen (BDH) covering a range of concentrations between 30 - 300 $\mu\text{g/ml}$ in 1M NaOH. There was a linear relationship between the concentration of protein and the optical density obtained from the EEL colorimeter. The concentration of experimental samples was determined using the standard curve, an example of which is figure 2.2.

Figure 2.2. Protein standard curve using bovine serum albumen by Biuret-Phenol method.



13. Preparation of Immunoabsorbent

The globulin fraction of a sheep antivaccinia serum was salted out by addition of solid Na_2SO_4 to 10 ml serum to give a final concentration of 20% (w/v). After incubation at 37°C for 30 minutes, the resulting precipitate was pelleted, dissolved in 2 ml distilled water and dialysed for 16 hours against 1 litre PBS'A' to remove any remaining Na_2SO_4 . The volume increased to 5 ml during dialysis. A protein estimation was carried out on the resulting preparation of the concentrated globulin fraction.

Microcrystalline cellulose (500 mg) was suspended in 10 ml distilled water and 10 ml 5% (w/v) cyanogen bromide in distilled water was added carefully. All manipulations with cyanogen bromide were made in a fume cupboard. Activation was achieved by adding 1M NaOH dropwise, one drop every 3 seconds for about 10 minutes, until the pH of the solution was maintained at 10 - 11. The activated cellulose was collected on Whatman filter paper in a Buchner funnel and washed with 2.5 l 0.5M NaHCO_3 followed by 1 litre coupling buffer (76.8 ml 1M NaHCO_3 and 7.74 ml 1M Na_2CO_3 in 1 litre distilled water). Both buffers were freshly prepared and maintained at 4°C .

After washing, the cellulose was scraped off the filter paper and added to the concentrated globulin fraction together with 8 ml coupling buffer. (The ratio of protein to cellulose was 1:1 for optimal binding).

Before use the immunoabsorbent was washed with the following buffers, again freshly prepared and at 4°C . Firstly, three washes with 10 ml 0.5M NaHCO_3 followed by

three washes with coupling buffer containing 0.1M ethanol-amine, the first two washes for 2 hours and the third wash for 24 hours, before a further two washes with 0.5M NaHCO_3 . These washes were followed by two washes with 10 ml Walpole's acetate buffer (23 ml glacial acetic acid and 6.56 g anhydrous CH_3COONa in 4.8 l distilled water adjusted to pH=4 with NaOH). Finally the serum bound cellulose was washed twice with 0.5M NaHCO_3 and stored at 4°C.

CHAPTER 3 THE REPLICATION OF ORTHOPOXVIRUSES IN CAM CELLS

Cells from the chorioallantoic membrane (CAM) of the chick embryo were reported by Tytell & Neuman (1960) to be unable to grow in media containing citrulline in place of arginine. Chesters (1976) was unable to obtain monolayers of CAM cells using trypsin alone as described previously, but followed another method for their cultivation reported by Cursiefen & Becht (1975) using hyaluronidase and collagenase before treatment with trypsin. The success of Tytell & Neuman in 1960 could have been due to the earlier crude preparations of trypsin being contaminated with other enzymes.

Since all orthopoxviruses are known to grow on the CAM, this provided scope for a cell system to measure the capacity of orthopoxvirus-infected cells to induce the enzymes argininosuccinate synthetase and lyase activity.

1. Characterisation of the Cell System

Initially, experiments were carried out to confirm the nature of the metabolic defect in CAM cells. The ability of CAM cells to utilise citrulline or argininosuccinate in place of arginine was determined by the incorporation of radioactively labelled amino acids into cell protein. cell cultures were prepared as previously described. After depletion of the intracellular pools of arginine the cells were maintained in PME minus arginine containing ^3H -leucine at $0.5\mu\text{Ci/ml}$ and citrulline, argininosuccinate or arginine at 0.5 mM concentrations with the respective ^{14}C -labelled

amino acid at $0.1\mu\text{Ci/ml}$. Cell cultures were incubated for 24 hours at 35°C and the total amounts of radioactivity incorporated into TCA-precipitable material were determined by methods previously described. The experiment was carried out in serum free medium and with medium supplemented with 2% foetal calf serum.

The results in Table 3.1 show that the level of incorporation of citrulline into high molecular weight material in CAM cells was reduced by 91% with 2% serum and 97% without serum compared with similar cell cultures maintained in the presence of an equimolar concentration of arginine. The level of incorporation of argininosuccinate was reduced by 99% of that of arginine in the absence of serum. Overall protein synthesis in the presence of serum, as assessed by the concomitant incorporation of ^3H -leucine, was reduced by 51% in the arginine minus condition and with citrulline and argininosuccinate was reduced by 43% and 31% respectively. The latter result may have been slightly lower due to some suspected argininosuccinate lyase activity in the serum. In the absence of serum there were similar reductions in the levels of protein synthesis.

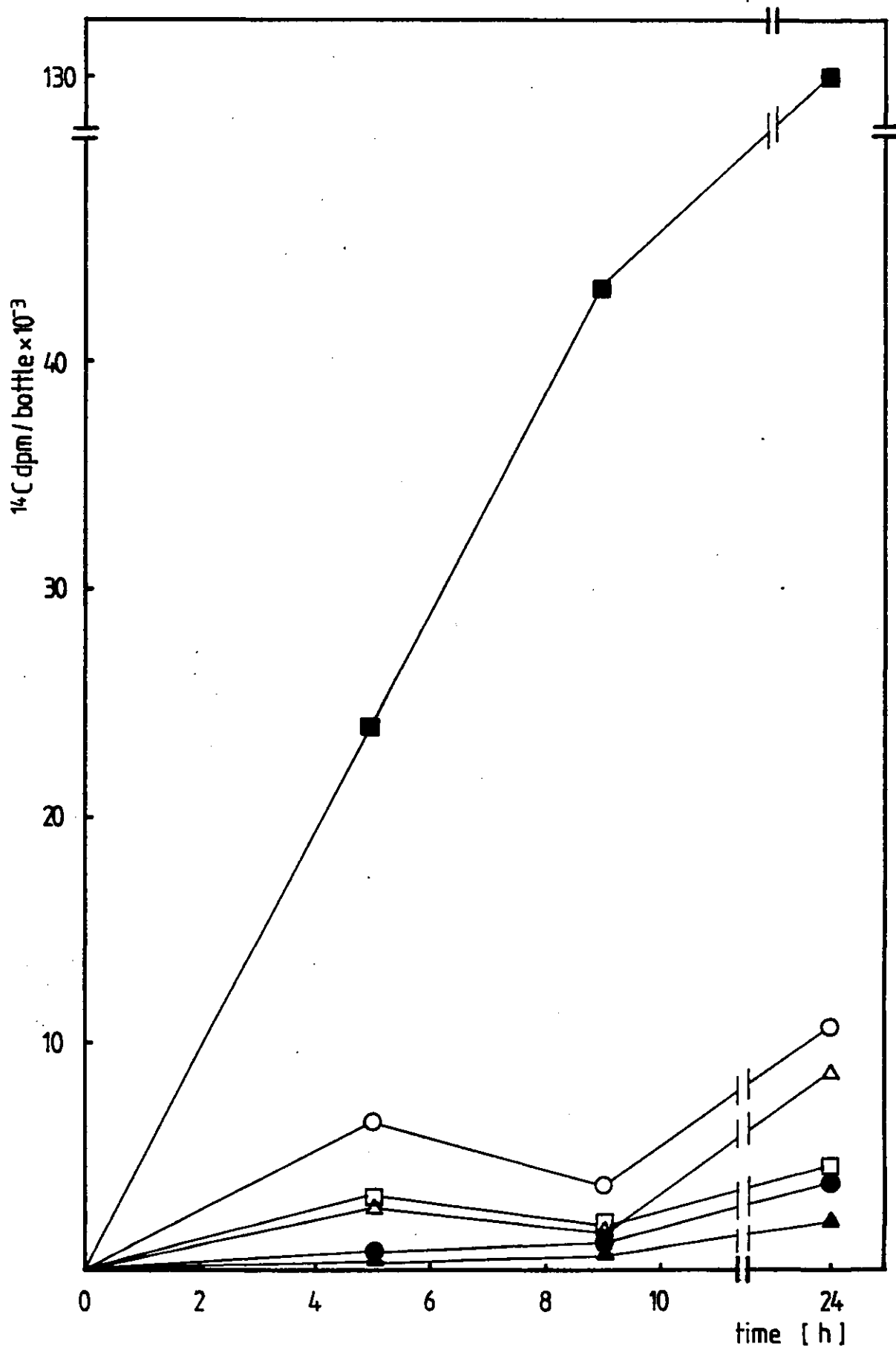
Figure 3.1 shows the rates of incorporation of ^{14}C -labelled citrulline, argininosuccinate and arginine into both TCA-soluble and TCA-precipitable fractions of CAM cells over 24 hours. Since incorporation into TCA-precipitable material increases linearly, this shows that all three amino acids are being taken up by the cells, albeit at different rates, and that the metabolic deficiency is not one of transport across the cytoplasmic membrane. Measurements of the intracellular levels of the amino acids

TABLE 3.1. Incorporation of ^3H -leucine and guanido- ^{14}C -arginine, guanido- ^{14}C -argininosuccinate and carbamyl- ^{14}C -citrulline into high molecular weight material of CAM cells.

	- SERUM		+ 2% SERUM	
	^{14}C -dpm	^3H -dpm	^{14}C -dpm	^3H -dpm
- ARGININE	-	611,781	-	28,849
+ CITRULLINE (0.5 mM)	3,781	617,600	1,492	33,098
+ ARGININOSUCCINATE (0.5 mM)	2,028	484,775	-	39,984
+ ARGININE (0.5 mM)	130,221	1,179,026	16,769	57,745

Figure 3.1. Rates of incorporation of ^{14}C labelled citrulline, argininosuccinate and arginine into TCA precipitable and soluble fractions of CAM cells.

TCA precipitable; ●—● + citrulline, ▲—▲ + argininosuccinate, ■—■ + arginine. TCA soluble: ○—○ + citrulline, △—△ + argininosuccinate, □—□ + arginine.



were also made after incubation at various time intervals by washing the monolayers 5 times quickly with appropriate maintenance medium at 4°C but without the radiolabel, before determination of the radioactivity associated with the TCA-soluble fraction. The levels of all three amino acids were found to be similar, giving supporting evidence for comparable membrane transport. The intracellular levels of citrulline and argininosuccinate were higher than the levels incorporated into protein, showing that although these amino acids are taken up, very little is assimilated compared with arginine.

Figure 3.2 shows the rates of protein synthesis as measured by the incorporation of ³H-leucine into high molecular weight material and also shows the levels of label taken up into the intracellular pools of CAM cells. The results show that protein synthesis occurs continually in the limiting conditions but at a slower rate. These results confirm the work of Tytell & Neuman (1960) and show that CAM cells are unable to utilise citrulline in place of arginine. They also indicate that the cells are unable to utilise argininosuccinate but that both citrulline and argininosuccinate are taken up by the cells.

In order to determine whether the enzyme deficiency is a substrate affinity effect, cell cultures were exposed to different concentrations of citrulline or arginine containing the respective ¹⁴C-label, the specific activity remaining constant. Table 3.2 shows that increasing the concentration of citrulline from 1 mM to 4 mM had no effect on the amount of carbamyl-¹⁴C-citrulline which was incorporated into high molecular weight material,

Figure 3.2. Incorporation of ^3H -leucine into TCA precipitable and soluble fractions of CAM cells, minus arginine, with citrulline, argininosuccinate, and with arginine.

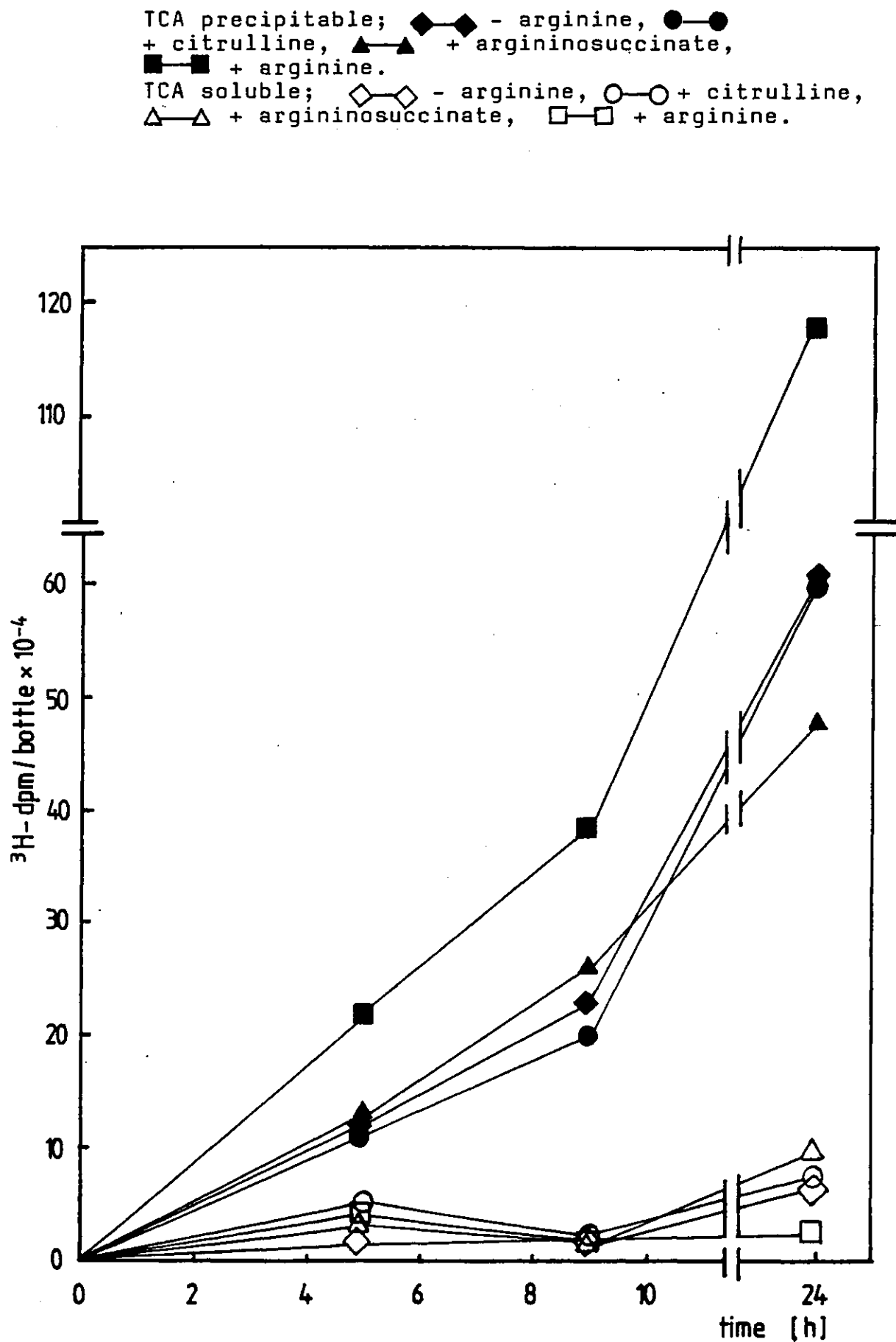


TABLE 3.2. Incorporation of guanido-¹⁴C-arginine and carbamyl-¹⁴C-citrulline at different concentrations but with the same specific activity, into TCA precipitable material from CAM cells after 24 hours' incubation.

CONCENTRATION	¹⁴ C-CITRULLINE incorporated (dpm)	¹⁴ C-ARGININE incorporated (dpm)
1 mM	2,695	42,137
2 mM	2,993	50,013
3 mM	2,921	46,348
4 mM	3,439	42,971

indicating that there is no substrate affinity effect causing the enzyme deficiency. The control cultures exposed to higher concentrations of arginine incorporated similar amounts of radioactivity, showing that maximal incorporation could take place at 1 mM arginine, and that higher doses of radioactivity did not cause cytological damage to the cell monolayers.

2. Virus Replication in CAM Cells

a) Serum Requirement

When studying the metabolic requirements of uninfected or virus-infected cell systems, it is preferable to use a completely defined medium. Serum contains a multitude of undefined growth factors and could contain free arginine or enzymes which might affect the results of arginine deprivation experiments. However, some growth factors, as yet uncharacterised, are essential for orthopoxvirus replication in CAM cells (Chesters, 1976). Consequently, it was necessary to ascertain the lowest concentration of serum which would permit significant virus growth, and whether dialysed serum would support virus replication.

A 2 ml volume of foetal calf serum was dialysed for 24 hours into 100 ml PME minus arginine. The dialysed serum was used subsequently at 2% and the serum dialysate used undiluted, after both had been sterilised by passage through 450 nm Millipore filters.

CAM cells were grown in tubes and after depletion of the intracellular pools of arginine, were infected with vaccinia (Lister) virus at a low inoculum of 10^4 plaque

forming units (p.f.u.) per tube, to magnify any differences in results by multiple cycles of virus infection. After adsorption for 1 hour, the cells were incubated for 48 and 72 hours in PME with and without arginine, supplemented with whole serum at 2%, dialysed serum, serum dialysate or without serum.

The results shown in table 3.3 confirm the serum requirement for vaccinia virus in CAM cells, although there was a small yield of virus with no serum present in the maintenance medium. The dialysed serum and serum dialysate both caused a reduction in the yield of about 60%, although the summation of these yields was only a little less than that of the reconstituted serum. This shows that growth factors of both high and low molecular weight are required for optimal virus replication in vaccinia infected CAM cells.

In order to determine the minimum concentration which would give a significant yield of vaccinia virus, CAM monolayers were again infected with a low inoculum and maintained in PME with and without 0.5 mM arginine, supplemented with various concentrations of serum from 0 - 2%. Serum was also heated to 56°C for 12 hours to inactivate any enzymes and this was also used at 2%. The yields of virus were measured at 48 and 72 hours post infection.

Table 3.3_a and figure 3.3 show that the yield of vaccinia virus increased with increasing serum concentration, but that the yields both with and without arginine at 1% serum were only slightly less than at 2%, and were much higher than at 0.5% or in the absence of serum. The heat inactivated serum at 2% gave yields similar to those with no serum at all, thus showing that an essential serum factor is heat

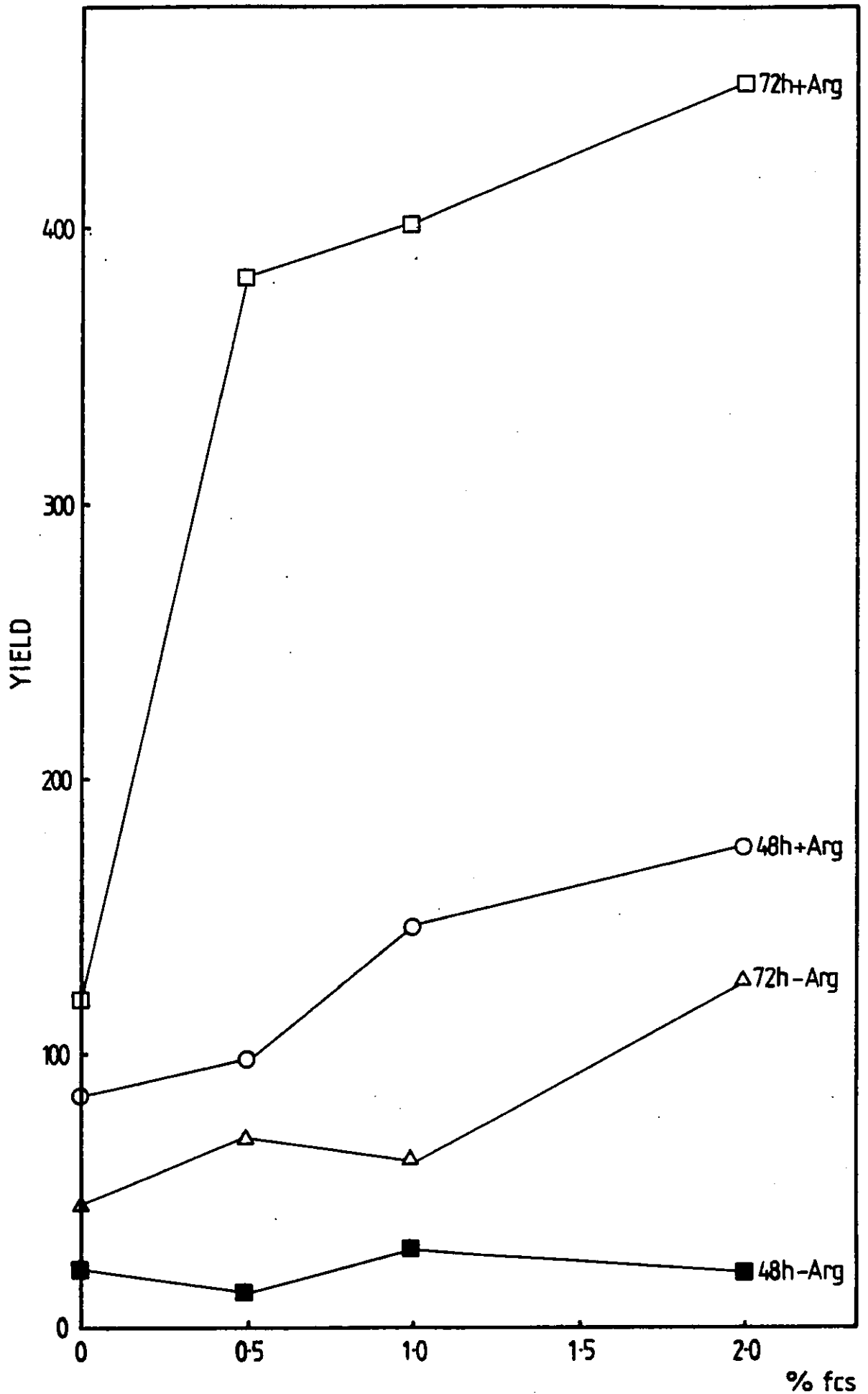
TABLE 3.3. Yields of vaccinia virus with dialysed and undialysed serum, and with serum dialysate in CAM cells at 48 and 72 hours post infection.

	Time Post Infection (h)	Titre	Yield
Inoculum Recovery	0	7.90×10^3	-
- Serum	48	1.07×10^5	13
- Arginine	72	3.30×10^5	33
- Serum	48	4.80×10^5	60
+ Arginine	72	1.20×10^6	152
2% Serum	48	1.40×10^6	177
+ Arginine	72	2.70×10^6	341
2% Dialysed Serum	48	5.88×10^5	74
+ Arginine	72	8.70×10^5	110
Serum Dialysate	48	4.40×10^5	55
+ Arginine	72	8.60×10^5	108
Reconstituted Serum*	48	1.10×10^6	139
+ Arginine	72	2.00×10^6	253

TABLE 3.3a. Yields of vaccinia virus in CAM cells with different concentrations of serum in PME plus and minus arginine, at 48 and 72 hours post infection.

	Time Post Infection (h)	Titre	Yield
Inoculum Recovery	0	4.08×10^3	-
- Serum	48	8.92×10^4	22
- Arginine	72	1.39×10^5	46
- Serum	48	3.50×10^5	85
+ Arginine	72	4.92×10^5	120
0.5% Serum	48	5.44×10^4	13
- Arginine	72	2.82×10^5	69
0.5% Serum	48	4.00×10^5	98
+ Arginine	72	1.56×10^6	382
1% Serum	48	1.15×10^5	28
- Arginine	72	2.50×10^5	61
1% Serum	48	6.00×10^5	147
+ Arginine	72	1.66×10^6	406
2% Serum	48	7.96×10^4	19
- Arginine	72	5.20×10^5	127
2% Serum	48	7.20×10^5	176
+ Arginine	72	1.85×10^6	453
2% Heat Inactivated Serum + Arginine	48	1.37×10^5	33
	72	3.24×10^5	79
2% Heat Inactivated Serum + Arginine	48	3.50×10^5	85
	72	6.96×10^5	170

Figure 3.3. Yields of vaccinia virus with varying concentrations of foetal calf serum, + or - arginine in CAM cells.



sensitive.

Serum was used for all subsequent experiments at 1% to minimise the effect of any enzymes or free arginine present.

b) Infective Centre Titration for Vaccinia (Lister) in CAM Cells

In order to determine the appropriate multiplicity of infection for one step growth conditions, an infective centre titration was carried out as previously described. Figure 3.4 shows the percentage of CAM cells infected with varying multiplicities of infection. The number of infective centres plateaus off at about 10 p.f.u./cell inoculum, although only 56% of the cells appear to be infected.

c) Yields of Orthopoxviruses in CAM Cells

The yields of 11 orthopoxviruses in CAM cells were compared in the presence and absence of arginine and with citrulline or argininosuccinate replacing arginine. Some of the orthopoxvirus yields obtained in preliminary experiments were very low and so rather than use one step growth conditions, the cells were exposed to lower inocula and were incubated for 48 hours to magnify any differences.

After depleting the intracellular pools of arginine, CAM cells were infected with different orthopoxviruses at approximately 10^5 p.f.u. per tube in order to infect 10% of the monolayer.

The yields of the 11 orthopoxviruses were measured in two experiments each with six viruses, rabbitpox being repeated as a reference for comparison. A histogram of the

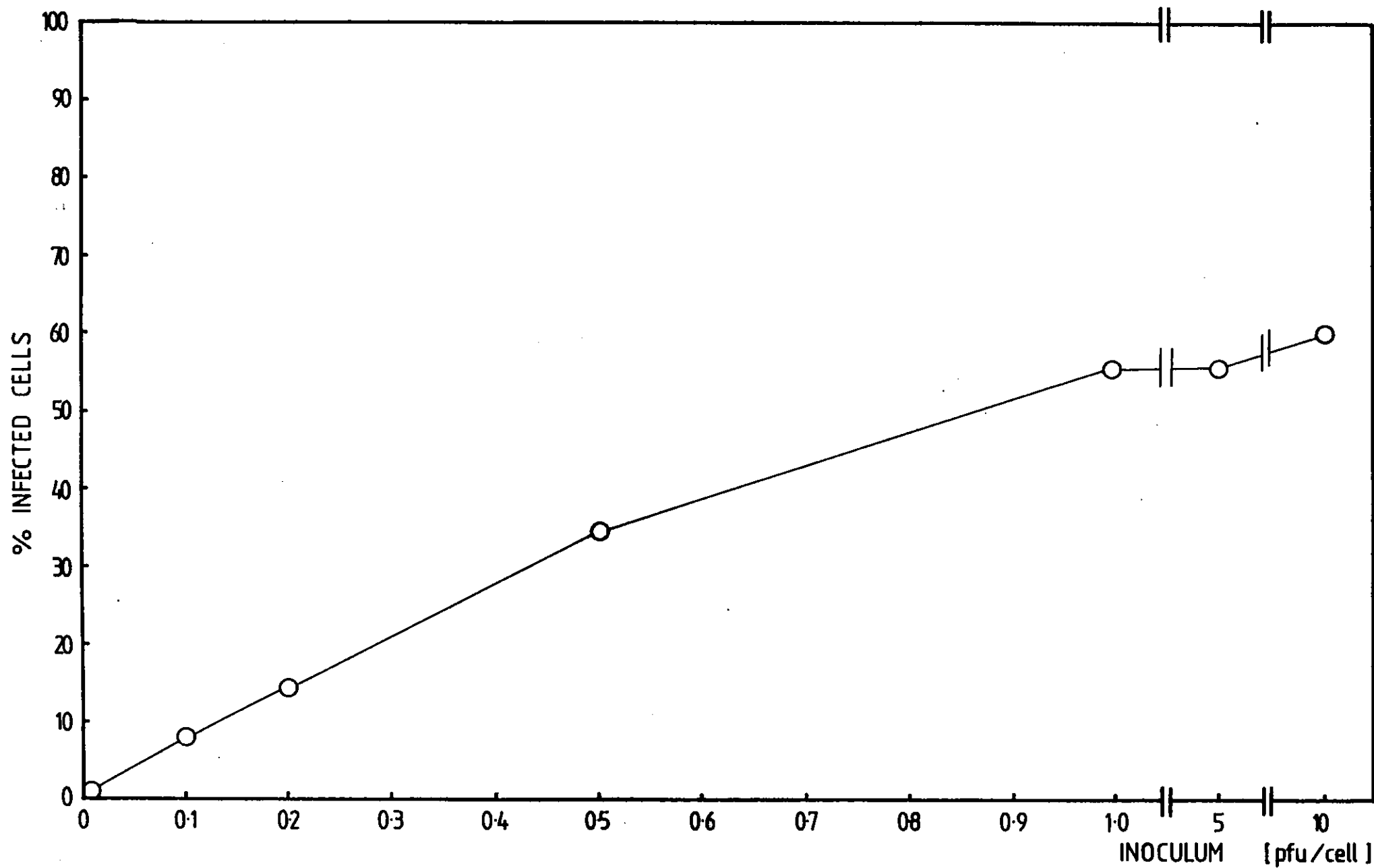
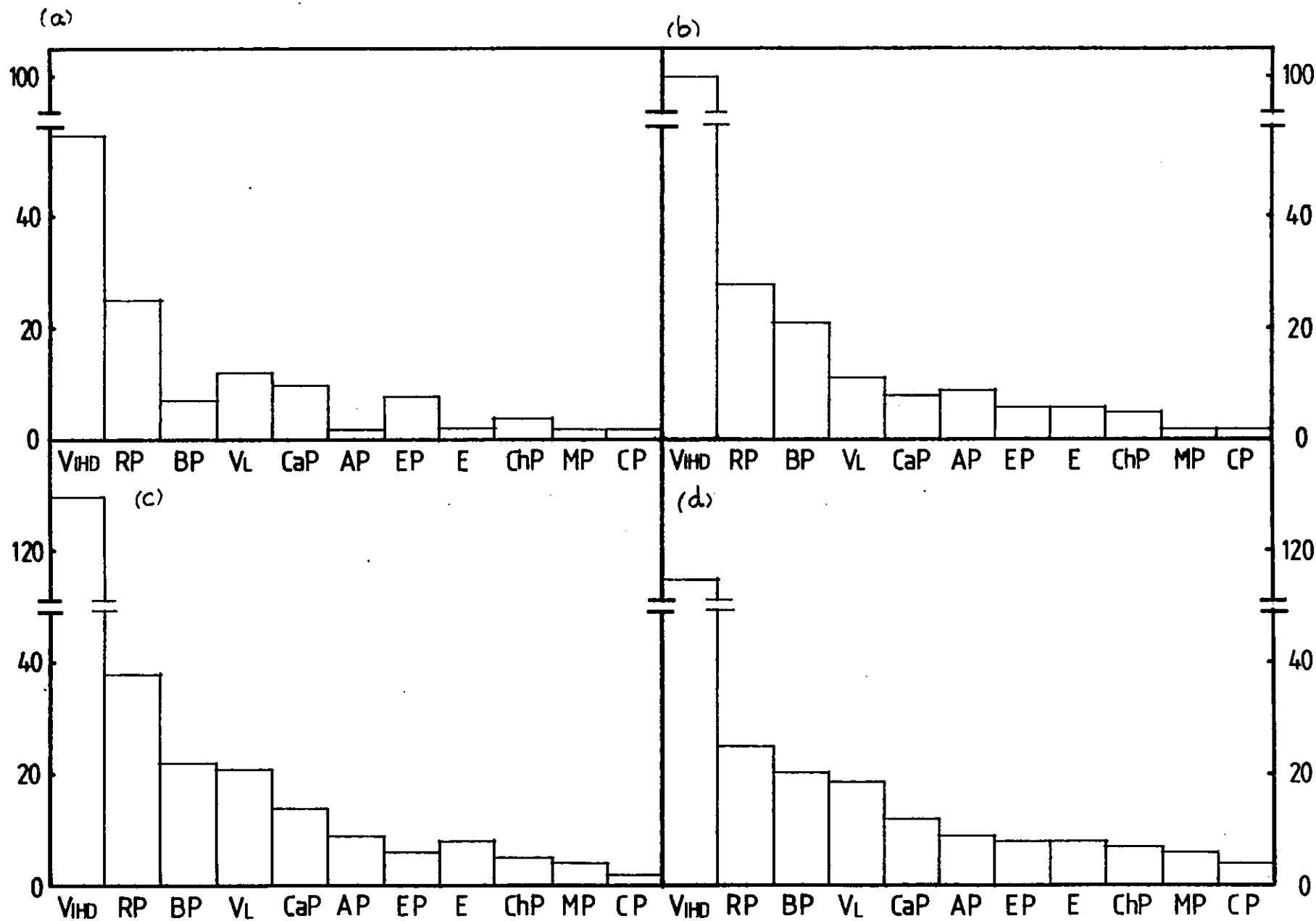


Figure 3.4. Infective centre titration for vaccinia (Lister) in CAM cells.

Figure 3.5. Histograms of 48h yields of 11
orthopox viruses in CAM cells;
(a) minus arginine, (b) plus citrulline,
(c) plus argininosuccinate (d) plus
arginine.

V_{IHD} = vaccinia (IHD), RP = rabbitpox,
BP = buffalopox, V_L = vaccinia (Lister),
CaP = camelpox, AP = anteaterpox,
EP = elephantpox, ChP = cheetahpox,
E = ectromelia, MP = monkeypox,
CP = cowpox.



results is shown in figure 3.5. The yields of the viruses were very low in CAM cells with the exception of vaccinia (IHD). There were also relatively high background yields in the absence of arginine, and no significant differences in the ability to utilise citrulline or argininosuccinate were resolved. The results showed that there were differences in the ability of the different orthopoxviruses to grow in CAM cells and that rabbitpox virus was able to grow equally well in the absence of arginine as in its presence.

Table 3.4 shows the virus yields expressed as a percentage of the yields with arginine. There appears to be a spectrum of ability to utilise citrulline, but since the yields are so low, there are no significant differences. The yields in the presence of argininosuccinate may have been affected by some suspected argininosuccinate lyase activity present in the serum, which although was only used at 1%, may have affected the results.

d) Growth of Orthopoxviruses in CAM Fragments

Since all orthopoxviruses grow well on the CAM, fragments of CAM were prepared and infected to determine whether a greater yield of virus could be obtained from tissue fragments compared with cell monolayers.

CAMs were removed and minced with scissors. The resulting tissue fragments were suspended in 10 ml PME minus arginine with 2% foetal calf serum in order to deplete the intracellular pools of arginine and incubated for 24 hours in a shaking water bath at 35°C. The fragments were then concentrated to 2 ml, and 10^6 p.f.u. vaccinia, cowpox or rabbitpox viruses introduced and allowed to adsorb for 30 minutes

TABLE 3.4. 48 hour yields of 11 orthopoxviruses in CAM cells in the absence of arginine and with citrulline or argininosuccinate replacing arginine as a percentage of the yield with arginine.

ORTHOPOXVIRUS	- ARGININE	+ CITRULLINE	+ ARGININO-SUCCINATE
Vaccinia (IHD)	50	95	131
Vaccinia (Lister)	66	62	116
Rabbitpox	100	112	160
Buffalopox	36	102	110
Camelpox	88	61	144
Anteaterpox	27	110	106
Elephantpox	120	75	75
Cheetahpox	50	67	79
Monkeypox	32	33	65
Ectromelia	24	100	114
Cowpox	51	49	44

with the tissue fragments stirred continually. After washing the infected fragments were incubated for 72 hours in experimental media, samples being removed for titration immediately and every 24 hours.

The virus yields obtained with CAM fragments were much higher than from experiments with CAM cell monolayers as shown in table 3.5. However, there were again very high background yields in the absence of arginine, especially at 72 hours post infection. This could be due to incomplete depletion of the intracellular pools as the virus spread to the centre of the fragment, or alternatively to autolysis of the cells, releasing free arginine.

However, the general trend of the results suggests that vaccinia is able to utilise citrulline in CAM cells but the yield is not as high as with arginine. The yield of cowpox at 24 hours and 48 hours post infection on the other hand is very low in the absence of arginine or with citrulline, whereas there is a significant yield in the presence of arginine. Rabbitpox produced the same high yield at 48 hours post infection under all conditions and the yields at 72 hours were reduced, probably due to thermal inactivation.

These results however, are not necessarily very accurate as it was difficult to ensure that all the samples removed from the flasks were the same size since the fragment suspension was not homogenous.

TABLE 3.5. Yields of vaccinia (Lister), cowpox and rabbitpox in CAM fragments in PME plus and minus arginine, with citrulline and with Medium 199. (All media contained 2% foetal calf serum)

VACCINIA	24h.p.i.	48h.p.i.	72h.p.i.
PME - Arginine	6	54	55
PME + Citrulline	19	60	80
PME + Arginine	73	143	160
Medium 199	35	266	154
COWPOX			
PME - Arginine	<1	2	60
PME + Citrulline	3	6	28
PME + Arginine	9	55	171
Medium 199	7	71	75
RABBITPOX			
PME - Arginine	-	150	>150
PME + Citrulline	-	150	>150
PME + Arginine	-	150	>150
Medium 199	-	150	>150

e) Effect of Trypsin in the Medium

Brown & Kiehn (1977) and Carney & Cunningham (1977) both reported that in the presence of low concentrations of proteolytic enzymes, certain cells had a faster growth rate, suggesting that their presence made the cells metabolically more active. The mode of action is not clear, but it could possibly be due to the enzymes breaking down the cell membrane proteins and thus stimulating the cell repair mechanisms.

Since the yields of the orthopoxviruses in CAM cells were normally very low, experiments were carried out to determine whether there would be any increase in the yield of cowpox and rabbitpox when trypsin was present in the medium.

CAM monolayers were prepared in tubes and infected at a low multiplicity of infection, and incubated for 48 hours in PME in the presence and absence of arginine or with citrulline, both amino acids at 0.5 mM concentration. This was carried out in the absence or presence of trypsin at 10 μ g/ml concentration. Serum was used at 2%.

Table 3.6 shows the yields resulting. Although there were increased yields of the viruses, the background yields in the absence of arginine were also higher. This could have been due to trypsin breaking down the cell protein and forming a pool of free arginine which allowed virus replication to take place.

TABLE 3.6. The effect of trypsin at $10\mu\text{g/ml}$ on the yields of rabbitpox and cowpox in CAM cells at 48 hours post infection. (All media contained 2% foetal calf serum)

	- TRYPSIN		+ TRYPSIN	
	Titre	Yield	Titre	Yield
RABBITPOX Inoculum Recovery	3.00×10^4	-	-	-
PME - Arginine	1.90×10^6	63	1.20×10^6	40
PME + Citrulline	9.34×10^5	31	3.00×10^6	100
PME + Arginine	1.54×10^6	51	2.40×10^6	80
COWPOX Inoculum Recovery	6.24×10^4	-	-	-
PME - Arginine	1.30×10^5	2.1	1.90×10^5	3.0
PME + Citrulline	5.08×10^4	0.8	1.20×10^5	1.9
PME + Arginine	8.36×10^4	1.3	4.76×10^5	7.6

3. Incorporation of ^{14}C -labelled arginine and citrulline together with ^3H -leucine into infected and sham-infected CAM cells.

Monolayers of CAM cells were prepared in tubes and after the intracellular pools of arginine had been depleted, the cells were infected with vaccinia virus at 10 p.f.u. per cell. Infected cultures were subsequently maintained in PME minus arginine supplemented with 1% foetal calf serum containing ^3H -leucine at $0.5\mu\text{Ci/ml}$ and with carbamyl- ^{14}C -citrulline or guanido- ^{14}C -arginine at $0.1\mu\text{Ci/ml}$, each amino acid at 0.5 mM concentration. After 24 hours incubation at 35°C the amount of radioactivity associated with TCA precipitable material was determined. Vaccinia (Lister) was chosen for this experiment because it gives a significant yield in CAM cells. Sham-infected cultures were also maintained under the same conditions.

Table 3.7 shows that the level of incorporation of the ^{14}C -labelled citrulline and arginine is similar for both infected and sham-infected cells, the level of ^{14}C -citrulline being about 3% of ^{14}C -arginine. The level of incorporation of ^3H -leucine however is higher in infected cells than in sham-infected cells under all conditions lacking exogenous arginine. In the presence of arginine the levels of incorporation of ^3H -leucine are similar in infected and sham-infected cells.

The increased level of protein synthesis in infected cells compared with sham-infected cells could be the result of virus specified protein synthesis which occurs in the absence of arginine.

TABLE 3.7. Incorporation of carbamyl-¹⁴C-citrulline and guanido-¹⁴C-arginine together with ³H-leucine in vaccinia-infected and sham-infected CAM cells at 24 hours post infection in the presense of 2% serum.

	Vaccinia-infected		Sham-infected	
	³ Hdpm/mg protein	¹⁴ Cdpm/mg protein	³ Hdpm/mg protein	¹⁴ Cdpm/mg protein
PME - ARGININE	29,315	-	12,098	-
PME + CITRULLINE	21,443	218	11,743	173
PME + ARGININO-SUCCINATE	21,846	-	11,791	-
PME + ARGININE	24,381	5,983	26,394	7,892

4. Discussion

Cells were cultivated from the CAM and produced monolayers of a mixed population of epithelial and fibroblastic cells. These were shown to lack the ability to utilise citrulline or argininosuccinate in place of arginine, even though the amino acids were shown to be taken up by the cells at the same rate as arginine. Increasing the concentration of citrulline had no effect on the amount incorporated, showing that it was not a substrate affinity change of the enzymes.

Vaccinia (Lister) was found to be unable to replicate optimally in CAM cells unless foetal calf serum was present. Growth factors were found to be necessary from both high and low molecular weight fractions of serum separated by dialysis. Since the presence of serum in the medium means that it is no longer chemically defined, it is preferable to minimise the concentration used for investigations of cell metabolism. A concentration of 1% foetal calf serum was found to be suitable for a significant yield of vaccinia virus in CAM cells and this was used in all further experiments.

The appropriate multiplicity of infection for one step growth conditions was found to be 10 p.f.u. per cell. However, even with this inoculum, only 56% of the monolayer was infected. This could have been due to the cells tending to grow over each other, thus masking some from exposure to the virus. Alternatively, since the monolayer is heterogeneous, only some of the cells may be susceptible to virus infection.

The yields of eleven different orthopoxviruses were measured in CAM cells in the presence and absence of arginine and with citrulline replacing arginine. All the virus yields were found to be low with high relative background yields in the absence of arginine and with citrulline replacing arginine.

They did not exhibit an "all or nothing" effect in the presence of citrulline but gave a cross section of reduced yields. There was wide variation between the yields obtained in consecutive experiments, which was probably due to the differences in the preparation of the monolayers. Since each was a primary culture, there were factors such as the condition of the embryos used, the activity of the enzyme preparations and the manipulations carried out in the mechanical harvesting of the cells, which could result in differences in the metabolic condition of the monolayers. However, with comparisons across consecutive experiments, general trends were found to be consistent. To obtain the best comparison of the yields of the orthopoxviruses, the results were taken from two experiments each with six orthopoxviruses but with rabbitpox used in both as a cross reference. Rabbitpox and vaccinia (IHD) were found to grow equally well in the presence as in the absence of arginine, or if citrulline replaced arginine. The other viruses gave reduced yields with citrulline but there were no significant differences in their ability to utilise the amino acid. The only differences observed were in the actual yields of each orthopoxvirus in CAM cells in complete medium, which are shown in figure 3.5 (d) in

descending order.

The high yields of some of the orthopoxviruses in the absence of any exogenous arginine were unexpected since arginine has been shown to be essential for vaccinia replication in HeLa cells (Archard & Williamson, 1971). It is unlikely that de novo synthesis of arginine is occurring as this does not happen in vaccinia-infected HeLa cells and the CAM cells cannot synthesise arginine. An alternative explanation is that host cell protein is being broken down either by autolysis or virus directed, resulting in the formation of a pool of arginine which can be used for replication. The incorporation of ^{14}C -labelled arginine and citrulline together with ^3H -leucine was measured in vaccinia (Lister) infected and sham-infected CAM cells. The levels of ^{14}C -labelled arginine and citrulline were comparable in infected and sham-infected cells. The level of ^3H -leucine incorporation however was higher in infected cells than in uninfected cells under all conditions lacking exogenous arginine. This could be a result of increased protein turnover stimulated by virus infection in the absence of arginine. This would allow a pool of arginine to appear inside the cell and sufficient for a limited amount of virus replication to occur.

Since the yields of poxviruses in CAM cells were very low, experiments were carried out in CAM fragments which were incubated in suspension. The yields of orthopoxviruses from both ends of the spectrum were compared, rabbitpox and cowpox. Similar trends were observed as with the CAM cell monolayers but the yields were higher. However, again there were high background levels of replication in arginine

minus conditions. This could have been due to inadequate depletion of the intracellular pools of arginine in the fragments.

The effect of a low concentration of trypsin in the medium on the orthopoxvirus yields in CAM cells was investigated but, again, although this resulted in higher yields in complete medium, the background yield in the absence of arginine was similarly affected.

All the results tended to suggest that rabbitpox and vaccinia (Lister) viruses are able to replicate in CAM cells if citrulline replaces arginine and in the absence of arginine compared with growth in complete medium.

The CAM system was thus found to have very limited use in comparing the orthopoxviruses and so a search began for other cell types which lack the ability to utilise citrulline in place of arginine but support the replication of orthopoxviruses.

CHAPTER 4 THE REPLICATION OF ORTHOPOXVIRUSES IN BHK 21 CELLS

In view of the limitations experienced with CAM cultures, alternative cell systems were sought which are unable to grow in the presence of citrulline, and which support the growth of a broad spectrum of orthopoxviruses.

BHK 21 cells were found to be unable to incorporate carbamyl-¹⁴C-citrulline into cell protein, and this cell line was studied as a potential cell system for measuring the capacity of different orthopoxviruses to induce argininosuccinate synthetase/lyase activity.

1. Characterisation of the Cell System

Experiments were carried out to study the metabolic lesion of BHK 21 cells. Cell cultures were prepared as previously described and incubated in PME minus arginine, supplemented with 2% foetal calf serum and containing carbamyl-¹⁴C-citrulline or guanido-¹⁴C-arginine, both at 0.1 μ Ci/ml and 0.5 mM concentrations, together with ³H-leucine at 0.5 μ Ci/ml. After incubation at 35°C for 24 hours, the total amounts of radioactivity incorporated into TCA precipitable high molecular weight material were determined as previously described.

Table 4.1 shows that the level of incorporation of carbamyl-¹⁴C-citrulline into high molecular weight material of BHK 21 cells was reduced by 98% compared with similar cell cultures maintained in the presence of an equimolar concentration of labelled arginine. Overall protein synthesis as assessed by the concomitant incorporation of ³H-leucine

TABLE 4.1. Incorporation of ^3H -leucine with guanido- ^{14}C -arginine, carbamyl- ^{14}C -citrulline or unlabelled argininosuccinate into high molecular weight material of BHK 21 cells.

	^{14}C -dpm	^3H -dpm
- ARGININE	-	15,559
+ CITRULLINE (0.5 mM)	256	21,796
+ ARGININOSUCCINATE (0.5 mM)	-	17,926
+ ARGININE (0.5 mM)	16,105	678,248

was reduced by 77% in the absence of arginine and by 68% when citrulline replaced arginine. When argininosuccinate replaced arginine at an equimolar concentration, the amount of ^3H -leucine incorporated was also reduced by 73%, suggesting that BHK 21 cells are unable to utilise this intermediate for arginine biosynthesis.

The inability of BHK 21 cells to utilise citrulline could be due either to the lack of the enzymes necessary for conversion to arginine, or alternatively the amino acid is not taken up by the cells, although the biosynthetic enzymes may be present. Another explanation could be a substrate affinity effect which could be overcome by using higher concentrations of the substrate to effect more efficient conversion of citrulline to arginine.

In the next experiment, monolayers of BHK 21 cells were prepared and, after depleting their intracellular pools of arginine, were maintained in PME minus arginine supplemented with 2% calf serum and with citrulline or arginine at increasing concentrations from 0 to 5 mM all with the respective ^{14}C radiolabel at the same specific activity of $100\mu\text{Ci/mMol}$ for guanido- ^{14}C -arginine and $200\mu\text{Ci/mMol}$ for carbamyl- ^{14}C -citrulline. All media also contained ^3H -leucine at $0.65\mu\text{Ci/ml}$.

After 24 hours incubation the amounts of radioactivity associated with the TCA precipitable material was determined. Table 4.2 shows that although increasing the concentration of citrulline increases the incorporation of the ^{14}C label linearly, the overall incorporation still remains very low. (This might be due to contamination of the carbamyl- ^{14}C -citrulline preparation with ^{14}C -urea, which could be up to

TABLE 4.2. Incorporation of carbamyl- ^{14}C -citrulline and guanido- ^{14}C -arginine together with ^3H -leucine into high molecular weight material of BHK21 cells with increasing concentrations of the amino acids but keeping the specific activities constant.

CONCENTRATION (mM)	CITRULLINE		ARGININE	
	^{14}C -dpm	^3H -dpm	^{14}C -dpm	^3H -dpm
0	-	1,431,300	-	-
0.5	16,976	1,454,600	502,660	3,231,400
1	31,581	1,568,000	548,750	3,595,900
2	33,867	1,225,587	553,070	3,491,600
3	45,938	1,392,700	597,380	3,674,600
4	62,300	1,701,200	554,140	3,329,000
5	69,249	1,668,800	610,550	3,703,300

1%). There is only a slight increase in overall protein synthesis as shown by the incorporation of ^3H -leucine at the higher concentrations of citrulline. Under such conditions, protein synthesis is markedly lower than in the presence of equimolar arginine.

There is also a slight increase in the incorporation of guanido- ^{14}C -arginine as the concentration is increased, but over a 10-fold increase in arginine concentration, there is only a 20% rise in the level of ^{14}C incorporation, thus showing that a 0.5 mM concentration is at or above the optimum concentration of arginine for maximal cell growth.

2. Orthopoxvirus Replication in BHK 21 Cells

The yields of eight orthopoxviruses were measured in BHK 21 cells in the presence and absence of arginine and with citrulline replacing arginine, both amino acids being at 0.5 mM concentration. After depleting the intracellular pools of arginine, confluent cell cultures in 4 oz medical flat bottles were infected with eight different orthopoxviruses at 10^6 p.f.u. per bottle (multiplicity of infection of 0.5). The yields were measured at 36 hours post infection and the results obtained are shown in table 4.3. All the viruses gave significant yields in complete medium. Vaccinia (Lister) and rabbitpox gave lower yields if citrulline replaced arginine and also in the absence of arginine. None of the other viruses tested produced yields of infective virus unless arginine was present.

TABLE 4.3. Replication of orthopoxviruses in BHK 21 cells in the presence and absence of arginine and where citrulline replaces arginine.

ORTHOPOX- VIRUS	PME - ARGININE	PME + CITRULLINE	PME + ARGININE
Vaccinia (Lister)	24	30	130
Rabbitpox	4.5	5.5	60
Cowpox	1	1	50
Buffalopox	1	1	31
Elephantpox	1	1	20
Anteaterpox	1	1	60
Cheetahpox	1	1	28
Camelpox	1	1	50

3. Investigation of the intracellular pools of BHK 21 cells and the effect of arginine deprivation and virus infection.

Since the replication of rabbitpox and vaccinia viruses appears to occur in BHK 21 cells to a small but significant degree in the absence of exogenous arginine, there must be a supply of arginine being made available endogenously. The threshold level of arginine resulting in production of progeny virus may vary for different poxviruses. This low level may permit replication of vaccinia and rabbitpox but is not sufficient for the other viruses.

The virus infected cells may autolyse if deprived of arginine, or alternatively this could be a virus directed mechanism which occurs to a greater extent with vaccinia and rabbitpox.

Protein turnover in BHK 21 cells was investigated under arginine deprived conditions by prelabelling cells with guanido-¹⁴C-arginine and ³H-leucine and monitoring the radioactivity remaining in the cell protein or appearing in the medium during the subsequent 72 hours in arginine free medium.

Semiconfluent cultures of BHK 21 cells were incubated for 24 hours in MEM supplemented with 5% calf serum and containing ³H-leucine at 0.5 μ Ci/ml and guanido-¹⁴C-arginine at 0.1 μ Ci/ml. After further incubation in unlabelled complete medium for 4 hours to reduce the radioactivity in the intracellular pools, the cells were subsequently incubated in complete PME or arginine deprived medium containing no radiolabel. At 12 hour intervals, the medium

was recovered and, after washing the cells quickly at 4°C, the radioactivity associated with the TCA precipitable material was determined. In these studies, further experiments were made with prelabelled cells infected with cowpox or rabbitpox viruses.

Figure 4.1 shows the loss of radioactivity from cell material into the medium in uninfected cells (a) in complete medium and (b) in PME minus arginine. When arginine is present, both leucine and arginine are lost at similar rates. However in the absence of arginine, the leucine is lost at a similar rate to that in complete medium but the arginine is preferentially retained and is lost to the medium at a much slower rate.

Loss of radioactivity from infected cells incubated in the absence is shown in figure 4.2. Both rabbitpox (a) and cowpox (b) infected cells gave a similar pattern of results. The loss of both amino acids was at a slower rate than in uninfected cells, showing inhibition of protein synthesis/turnover following virus infection. Again, arginine was conserved more than leucine in the absence of exogenous arginine.

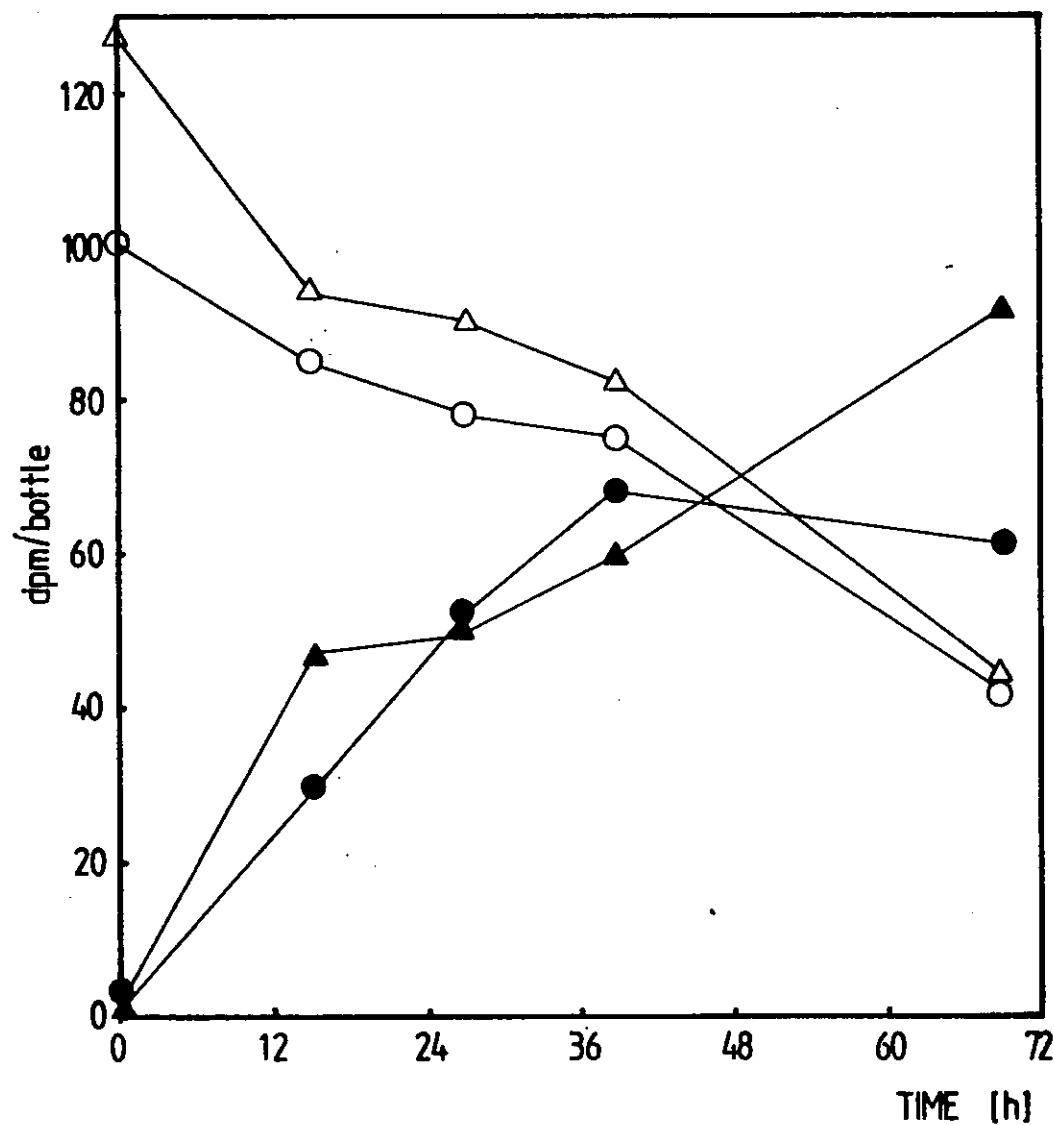
4. The effect of arginine deprivation on DNA synthesis in rabbitpox infected BHK 21 cells.

DNA synthesis in vaccinia infected HeLa cells is markedly suppressed in the absence of arginine (Archard & Williamson, 1971). Pulse labelling experiments showed that arginine is necessary for two functions in vaccinia virus replication in HeLa cells. The first is an early event

Figure 4.1. Loss of ^3H -leucine and guanido- ^{14}C -arginine from uninfected, prelabelled cell material into unlabelled medium.

$\triangle \text{---} \triangle = ^3\text{H}$ in precipitate
 $\circ \text{---} \circ = ^{14}\text{C}$ in precipitate
 $\blacktriangle \text{---} \blacktriangle = ^3\text{H}$ in medium
 $\bullet \text{---} \bullet = ^{14}\text{C}$ in medium

(a) complete medium



(b) minus arginine

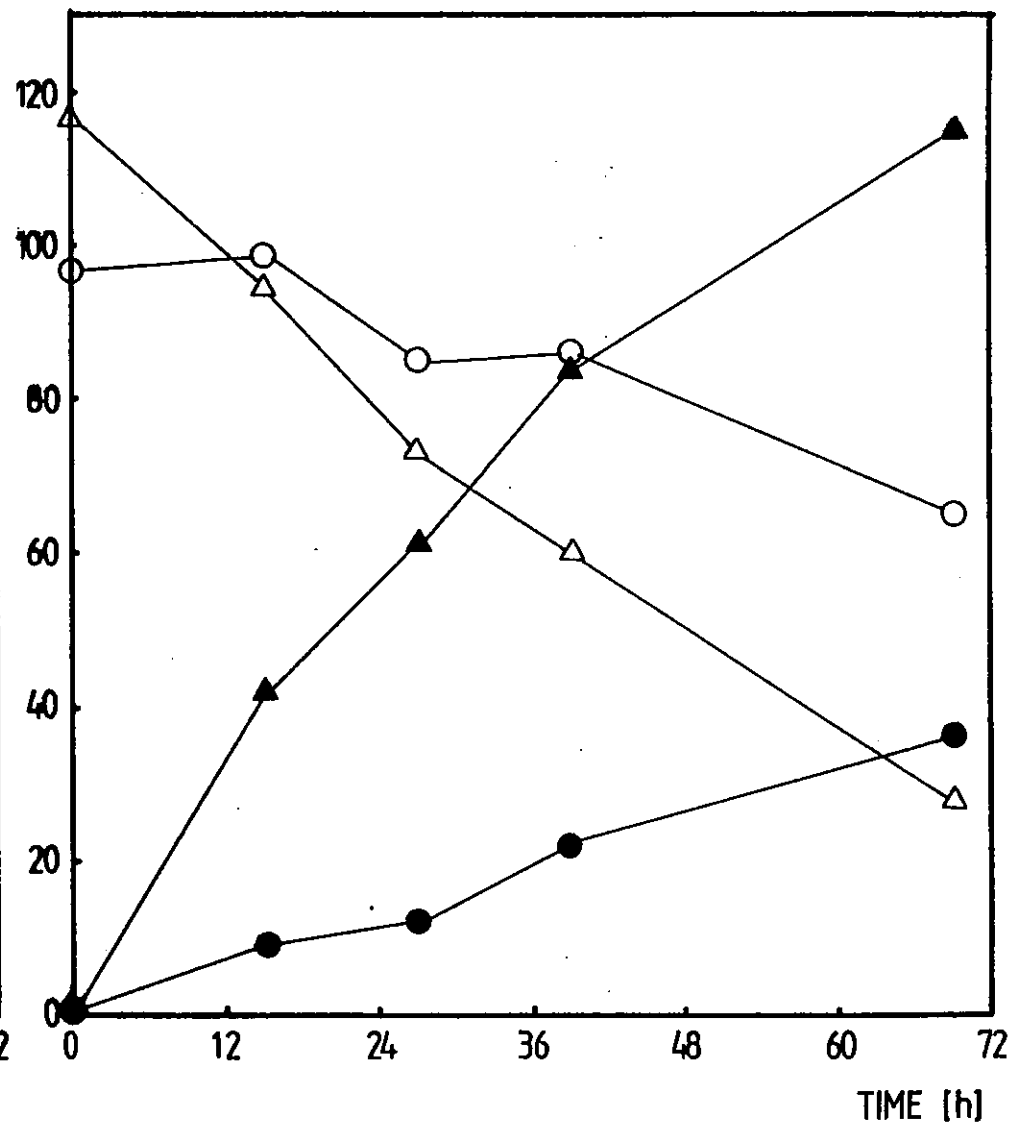
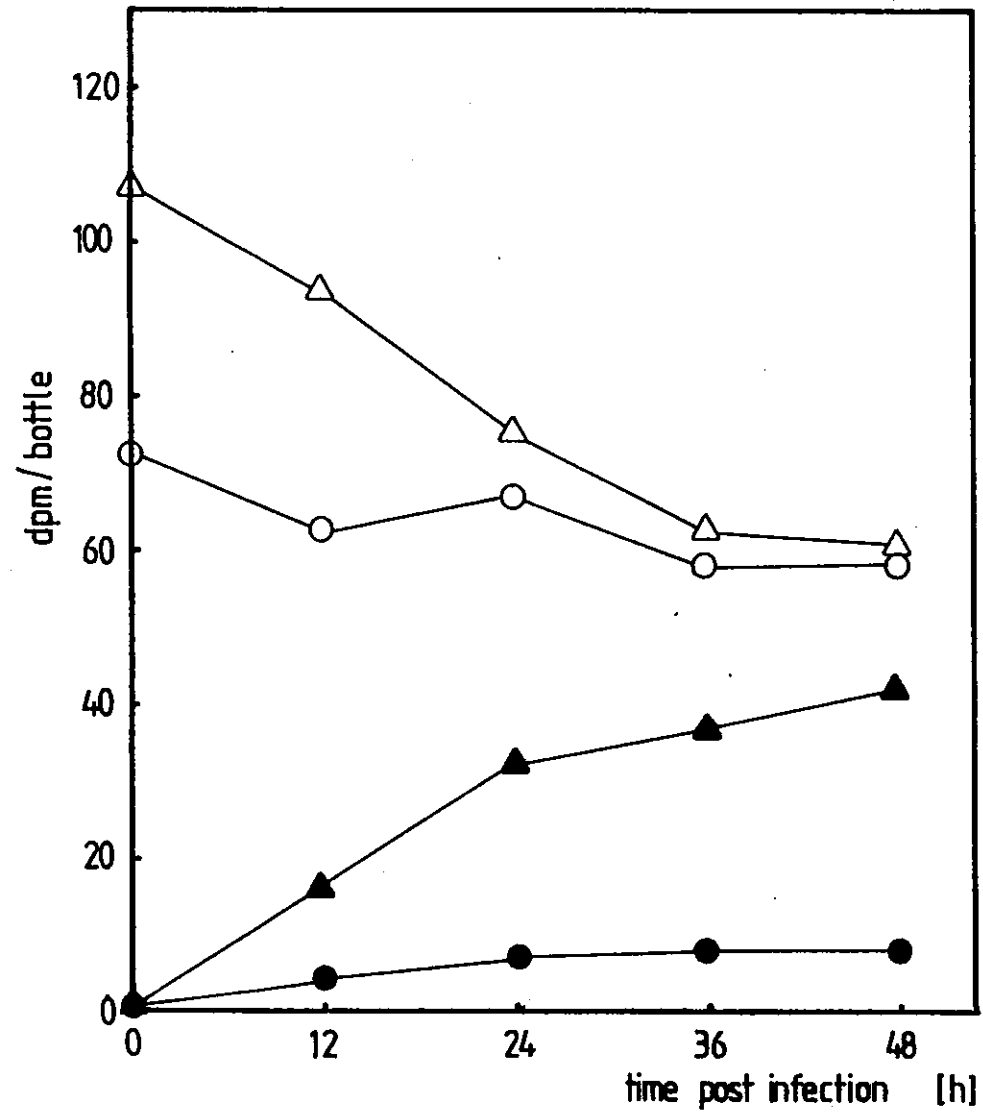


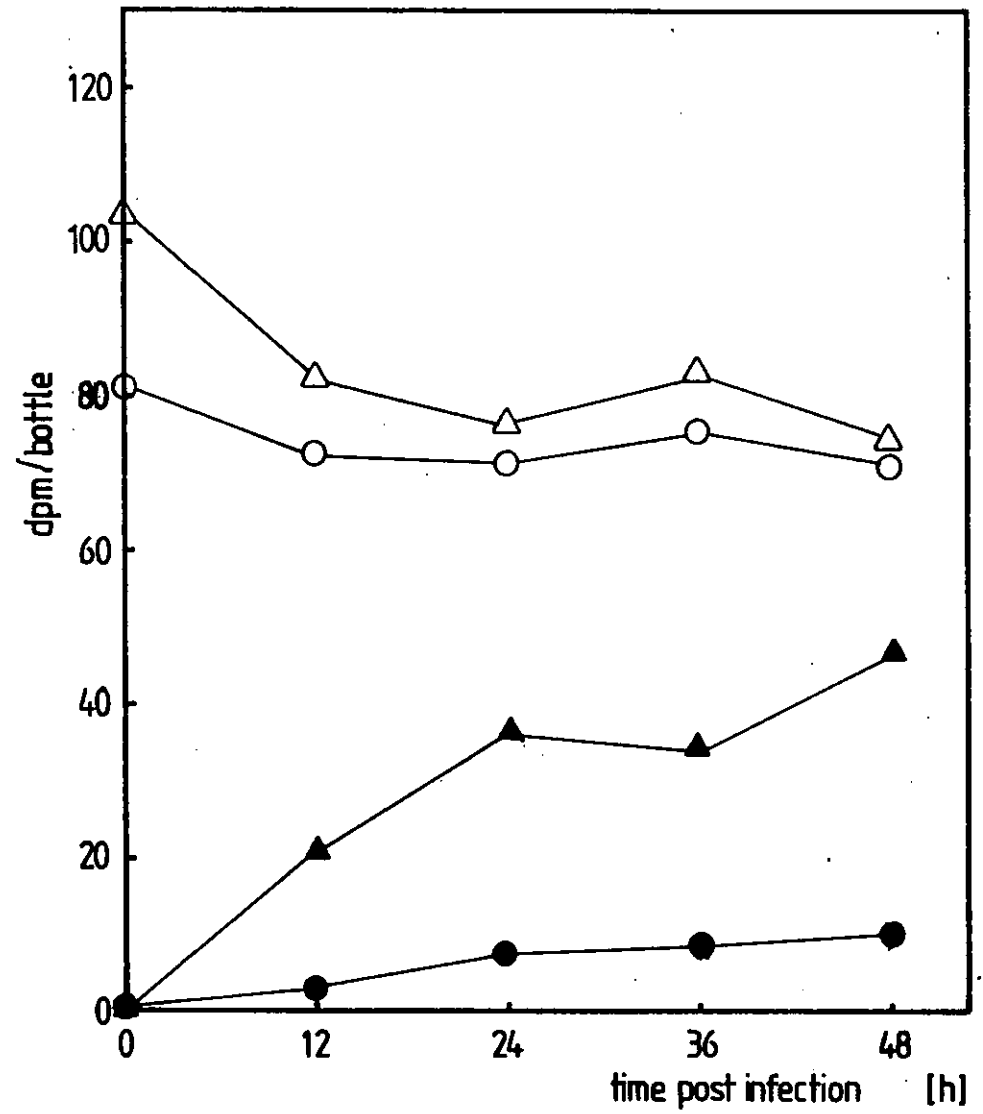
Figure 4.2. Loss of ^3H -leucine and guanido- ^{14}C -arginine from infected, prelabelled BHK cell material incubated in unlabelled PME minus arginine.

$\triangle \longrightarrow \triangle = ^3\text{H}$ in precipitate
 $\circ \longrightarrow \circ = ^{14}\text{C}$ in precipitate
 $\blacktriangle \longrightarrow \blacktriangle = ^3\text{H}$ in medium
 $\bullet \longrightarrow \bullet = ^{14}\text{C}$ in medium

(a) Rabbitpox-infected



(b) Cowpox-infected



associated with virus-specified DNA synthesis and the second, a quantitatively greater requirement involved with later events and the formation of structural proteins.

DNA synthesis in vaccinia infected KB cells in the absence of arginine was also investigated by Obert, Tripier & Guir (1971). Vaccinia infected KB cells were maintained in the absence of arginine and at 8 hours post infection, fluorouracil deoxyribose (FUdR), an inhibitor of DNA synthesis was added simultaneously with arginine. This was followed by incubation for a further 24 hours. Any further DNA synthesis was thus prevented, but the presence of arginine allowed any existing completed DNA to form mature progeny virus. DNA synthesis was found to be reduced by only 30% under these conditions in comparison with complete medium.

In the present study, the extent of DNA synthesis occurring in rabbitpox infected BHK 21 cells which could give rise to mature progeny in the absence of arginine and with citrulline replacing arginine was determined using a similar method.

a) Dose response of rabbitpox-infected BHK 21 cells to FUdR

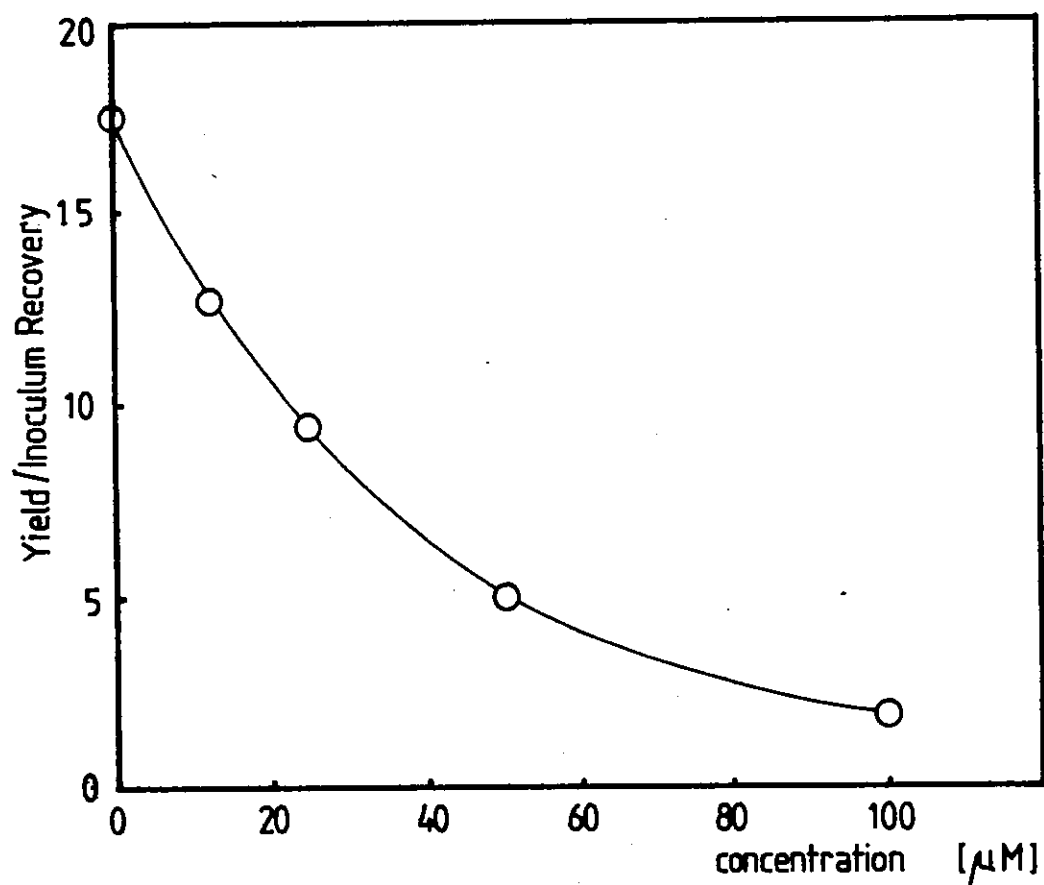
The mode of action of FUdR is to inhibit synthesis of thymidine, one of the essential substrates for DNA synthesis. Initially the dose response of rabbitpox-infected BHK 21 cells was measured with FUdR to determine the minimum concentration necessary for complete inhibition of virus replication.

Table 4.4 and figure 4.3 show the 24 hour yields of rabbitpox virus in BHK 21 cells after incubation in complete medium with various concentrations of FUdR.

TABLE 4.4. Dose response of rabbitpox-infected BHK 21 cells to FUdR.

	Titre	Yield
Inoculum Recovery	3.16×10^5	-
12.5 μ M FUdR	4.00×10^6	12.7
25 μ M FUdR	3.00×10^6	9.5
50 μ M FUdR	1.60×10^6	5.0
100 μ M FUdR	6.38×10^5	2.0
- ARGININE CONTROL	6.22×10^5	1.97
+ ARGININE CONTROL	5.57×10^6	17.63

Figure 4.3. Dose response curve of rabbitpox-infected BHK21 cells to FUDR at 24 h.p.i.



The FUdR was found to be only partially effective in preventing virus replication as even the highest concentration permitted a low yield. This was taken into consideration in further experiments.

b) Effect of adding FUdR and arginine at 24 hours post infection

After depletion of the intracellular pools of arginine, confluent monolayers of BHK 21 cells were infected with rabbitpox virus at 5 p.f.u. per cell and incubated in PME minus arginine or with citrulline or arginine at 0.5 mM concentration. At 24 hours post infection medium containing 0.5 mM arginine and 100 μ M FUdR was added to the infected cultures and the subsequent virus yields were measured after a further 24 hours. The yields of virus were also measured at 24 and 48 hours post infection under all conditions without any inhibitor added. The results are shown in table 4.5. Without FUdR present there were similar low yields in the absence of arginine and with citrulline at 24 and 48 hours post infection. However 24 hours after FUdR and arginine were added a slightly higher yield was obtained from infected cells maintained with citrulline than in the absence of arginine. This suggests that rabbitpox is able to undergo a small amount of DNA synthesis if citrulline replaces arginine. But the yield was only 20% of that in the presence of arginine.

The virus yield in the presence of complete medium was lower in those infected cultures to which FUdR and arginine were added at 24 hours post infection compared with cultures maintained in the initial medium throughout. This observation suggests that FUdR may be associated with some mechanism of inactivation of virus particles.

TABLE 4.5. Yields of rabbitpox virus in BHK 21 cells when FUdR plus arginine is added to the medium at 24 hours post infection in the presence and absence of arginine and when citrulline replaces arginine.

	Titre	Yield
Inoculum Recovery	6.20×10^5	-
24 h.p.i.		
- Arginine	8.68×10^5	1.4
+ Citrulline	1.05×10^6	1.7
+ Arginine	2.14×10^7	34
48 h.p.i.		
- Arginine	8.12×10^5	1.31
+ Citrulline	7.24×10^5	1.17
+ Arginine	1.63×10^7	26.3
48 h.p.i. after addition of FUdR + arginine at 24 h.p.i.		
- Arginine	1.12×10^6	1.8
+ Citrulline	2.16×10^6	3.5
+ Arginine	1.12×10^7	18

5. Discussion

BHK 21 cells were found to be unable to utilise citrulline in place of arginine. Increasing the concentration did not result in greater incorporation into cell protein, showing that this was not due to a substrate affinity change of the enzymes.

The yields of eight orthopoxviruses were measured in BHK 21 cells in the presence and absence of arginine and the ability to utilise citrulline was also determined. The results obtained fell into two categories. The first group consisted of rabbitpox and vaccinia which produced low but significant yields of virus in the presence of citrulline but also gave similar yields of virus in the absence of arginine. All the other viruses tested; cowpox, buffalopox, elephantpox, anteaterpox, cheetahpox and camelpox fell into the second category and were unable to replicate in BHK 21 cells unless exogenous arginine was present.

Since similar yields of rabbitpox and vaccinia were obtained both in the presence of citrulline and in the absence of arginine, this suggests that these viruses do not obtain their arginine from utilisation of citrulline, but rather a pool of arginine becomes available from breakdown of cell protein which is either induced by the virus or else occurs as a result of autolysis. The former suggestion is more likely since the "cowpox-like" viruses are unable to replicate under the same conditions. However the ability to replicate may depend on the quantitative requirements of each of the viruses for arginine, although these are thought to be similar judging from their dose response to arginine

in other cell systems (see chapter 6).

Further experiments were carried out to study the effects of arginine deprivation on infected and sham-infected BHK 21 cells. With sham infected cells, prelabelled with ^{14}C -arginine and ^3H -leucine and incubated in unlabelled complete medium, protein turnover resulted in both ^{14}C and ^3H radioactivity appearing in the medium at a steady rate. Under arginine deprived conditions, however ^{14}C appeared at a much slower rate than ^3H , the latter being similar to that in complete medium. This shows that the arginine is preferentially conserved for reuse in protein synthesis under arginine deprived conditions.

With cowpox and rabbitpox virus infected BHK 21 cells similarly labelled, both amino acids appeared in the medium at a slower rate than in sham infected cells showing inhibition of host-specific protein turnover following virus infection. Again the arginine was conserved more than leucine in the absence of exogenous arginine.

Arginine is involved with at least two stages in the replication cycle of vaccinia virus in HeLa cells, an early event associated with virus-specific DNA synthesis and a quantitatively greater amount is required in a later event involved with virus assembly. Rabbitpox-infected BHK 21 cells were investigated to determine whether sufficient arginine could be made available to allow complete DNA synthesis under arginine deprived conditions or with citrulline replacing arginine.

The extent of rabbitpox DNA replication occurring in BHK 21 cells under arginine deprived conditions was investigated by adding FUDR and arginine at 24 hours post infection

which prevented further DNA synthesis but allowed any existing DNA, capable of forming infective progeny to complete the replication cycle.

The presence of FUdR in the medium at the end of the replication cycle under one-step growth conditions tended to reduce the virus yields compared with appropriate controls, suggesting that exposure of infected cell cultures to the inhibitor for several hours results in some inactivation of the virus particles. However, although the yields of rabbitpox in the absence of arginine and with citrulline were similar under normal conditions, further replication in the presence of FUdR and arginine resulted in a subsequent virus yield which was slightly higher with infected cultures maintained previously in the presence of citrulline. This suggests that citrulline may be utilised by rabbitpox-infected BHK 21 cells to produce arginine which allows further DNA synthesis to occur.

The BHK 21 cell system was found to be more useful than the CAM cell system for comparison of the orthopoxviruses. The virus yields were much higher and the results much more consistent. The orthopoxviruses could be classified into those "rabbitpox-like" viruses which were able to replicate to a limited extent in BHK 21 cells in the absence of arginine with perhaps some utilisation of citrulline and the "cowpox-like" viruses which could only replicate in the presence of exogenous arginine.

However, the ability of rabbitpox-infected BHK 21 cells to produce infective progeny in the absence of arginine masks any ability to utilise citrulline and so the search continued for other cell lines with a similar metabolic

but which do not allow the replication of orthopoxviruses under arginine deprived conditions, and also give high yields of infective progeny in complete medium.

CHAPTER 5 REPLICATION OF ORTHOPOXVIRUSES IN S180 CELLS

Mouse sarcoma 180 (S180) cells were reported by Tytell & Neuman (1960) to be unable to grow if citrulline replaced arginine in the culture medium. The replication of rabbitpox in S180 cells was studied by Cooke & Williamson (1973). It was found that although cell multiplication and protein synthesis was suppressed in the presence of 0.5 mM citrulline, a yield of infective virus was obtained from rabbitpox-infected S180 cells, which was only slightly less than that in the presence of 0.1mM arginine.

Chesters (1976) seeking to compare the abilities of other orthopoxviruses to utilise citrulline found that in complete medium S180 cells did not support the growth of any of the other viruses investigated except cowpox, and this gave a very low yield. However, cowpox was found to be unable to produce infective progeny when citrulline replaced arginine.

Further experiments with S180 cells are now presented which study the replication of some other orthopoxviruses not investigated in the earlier studies, together with some investigations of the extent of DNA synthesis in S180 cells infected with rabbitpox and other viruses and maintained in the presence and absence of arginine or with citrulline.

1. Characterisation of the Cell System

Initially, experiments were carried out to confirm the nature of the metabolic lesion. The ability of the S180 cells to utilise arginine and citrulline was determined by the incorporation of guanido- ^{14}C -arginine and carbamyl- ^{14}C -citrulline into cell protein. Overall protein synthesis was measured by the incorporation of ^3H -leucine into cell protein. Confluent monolayers of S180 cells were prepared and after depletion of the intracellular pools of arginine the cells were incubated in PME minus arginine, containing ^3H -leucine at $0.5\mu\text{Ci/ml}$ and with citrulline or arginine at 0.5 mM concentrations with the respective radiolabel at $0.1\mu\text{Ci/ml}$. No serum was used in the culture media. After 24 hours, the total amounts of radioactivity incorporated into TCA precipitable material were determined.

The results in table 5.1 show that if citrulline replaced arginine, the incorporation of ^{14}C -label was reduced by 96%, thus showing the inability of the cells to utilise citrulline. Overall protein synthesis was reduced by about 70% in the absence of arginine or if citrulline replaced arginine. When argininosuccinate replaced arginine, there was also a reduction of about 70% in protein synthesis suggesting that S180 cells are unable to use this intermediate.

In earlier experiments (Cooke, 1973) S180 cells were shown to be able to grow to a limited extent when the concentration of citrulline was increased. In the presence of 5 mM citrulline after 3 days the number of cells had increased fourfold compared with an eightfold increase in the presence of 0.5 mM arginine: in the absence of arginine

TABLE 5.1. Incorporation of ^3H -leucine with guanido- ^{14}C -arginine, carbamyl- ^{14}C -citrulline or unlabelled arginino-succinate into high molecular weight material of S180 cells.

	^{14}C -dpm	^3H -dpm
- ARGININE	-	604,127
+ CITRULLINE (0.5 mM)	21,256	552,305
+ ARGININO- SUCCINATE (0.5 mM)	-	486,858
+ ARGININE (0.5 mM)	510,732	1,787,997

or citrulline, no increase in cell numbers occurred. This experiment was repeated with the SL80 cells presently used to determine their growth characteristics under similar conditions.

SL80 cells were seeded at 10^6 cells/bottle into 4 oz medical flats in FME with concentrations of 0, 0.5, 1, 2, 3, 4 and 5 mM concentrations of citrulline and 0.5 mM arginine. All media were supplemented with 5% foetal calf serum. After 3 days the cells were harvested and counted in trypan blue. The viability in all cases was almost 100% and table 5.2 shows the cell counts obtained. In the presence of arginine the cell numbers increased about 3-fold. However in the absence of arginine and with all concentrations of citrulline, no increase was observed, unlike previous reports. Since the cells were seeded at quite a high concentration, the experiment was repeated in tubes with a lower seeding concentration of cells. After 3 days the controls containing arginine grew confluent but under all other conditions there was no discernable growth. Thus the SL80 cells used in these experiments appear to have different characteristics compared with previous studies.

2. Replication of orthopoxviruses in SL80 cells

a) Infective Centre Titration

An infective centre titration for rabbitpox in SL80 cells was carried out as previously described. The results are shown in figure 5.1. The multiplicity of infection required to infect 100% of the SL80 monolayer was found to be 2; infectivity titrations were made in Vero cells.

TABLE 5.2. Cell counts of S180 cells after 3 days' incubation in PME - arginine supplemented with different concentrations of citrulline. All media contained 5% calf serum.

CITRULLINE CONCENTRATION (mM)	SAMPLE A (cells/bottle)	SAMPLE B (cells/bottle)
0	1.10×10^6	1.05×10^6
0.5	1.50×10^6	9.50×10^5
1.0	1.15×10^6	1.14×10^6
2.0	1.03×10^6	8.90×10^5
3.0	1.01×10^6	1.09×10^6
4.0	9.50×10^5	1.02×10^6
5.0	1.09×10^6	1.04×10^6
CONTROL (0.5 mM ARG)	2.35×10^6	3.55×10^6

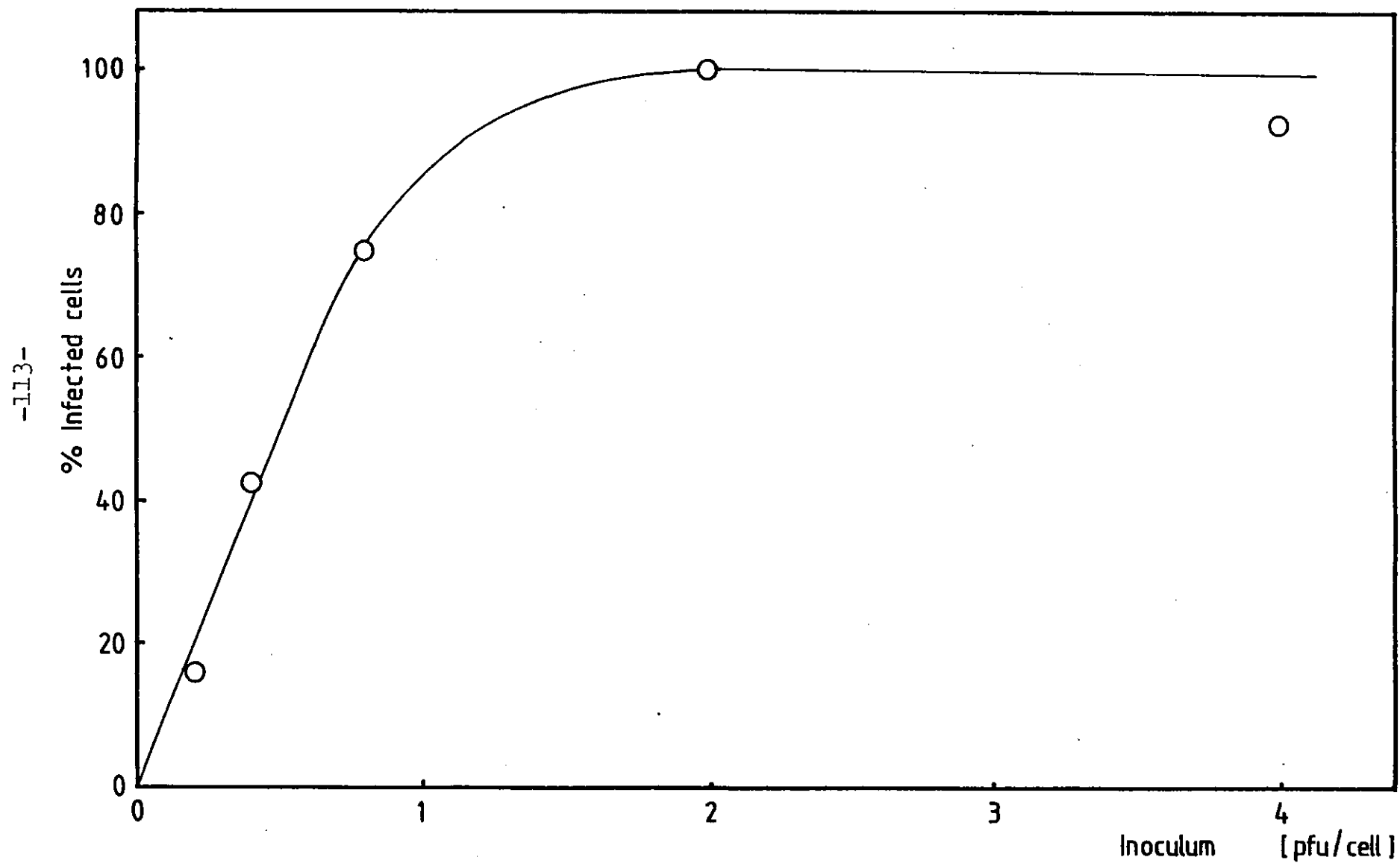


Figure 5.1. Infective centre titration for rabbitpox in S180 cells.

b) Dose response of rabbitpox-infected S180 cells to arginine and citrulline

In order to determine the minimum concentrations of arginine and citrulline required to give optimum virus growth in S180 cells, the virus yields were measured at various concentrations of the amino acids. Confluent monolayers of S180 cells were prepared and the intracellular pools of arginine depleted. After infection with rabbitpox virus under one-step growth conditions, cultures were maintained in PME minus arginine without serum, or supplemented with varying concentrations of arginine and citrulline up to 1 mM. Virus yields at 24 hours post infection were titrated in Vero cells. This experiment was repeated with a much lower inoculum, allowing multiple cycles of infection to take place and, consequently, magnifying any differences. These latter results can be compared with those presented previously by Cooke (1973).

The results in table 5.3 show that in both experiments the yield of rabbitpox is much lower in the presence of citrulline than was previously reported, being at most only 5% of that with an equimolar concentration of arginine. Thus the cells appear to be showing altered characteristics. This could be due to a change in the cells, a change in the virus or a change in the growth conditions. A number of modifications were attempted to improve the yield of rabbitpox in the presence of citrulline, in order to ascertain the nature of the apparent restriction on virus growth.

TABLE 5.3. Dose response of rabbitpox-infected SI80 cells to varying concentrations of arginine and citrulline at 24 hours post infection.

a) With one-step growth conditions (Inoculum Recovery = 6.2×10^5)

ARGININE (mM)	YIELD	CITRULLINE (mM)	YIELD
0.00	1	0.00	1
0.005	1	0.005	1
0.01	3.6	0.01	1
0.05	36	0.05	2
0.10	42	0.10	2.2
0.50	61	0.50	3
1.0	63	1.0	3.2

b) With a low inoculum (Inoculum Recovery = 5.6×10^4)

ARGININE (mM)	YIELD	CITRULLINE (mM)	YIELD
0.00	1 (1)	0.00	1 (1)
0.005	1 (1)	0.005	1 (1)
0.01	1.6 (6)	0.01	1.7 (1)
0.05	51.1 (45)	0.05	1.3 (1.3)
0.10	139.0 (230)	0.10	1.7 (20)
0.50	188.0 (269)	0.50	8.1 (200)
1.0	319.0 (316)	1.0	19.0 (220)

(Nos. in brackets from Barbara Cooke, 1973)

i) Stocks of Sl80 cells

The Sl80 cells used in the experiments described were examined for their cytological appearance by fluorescent staining to investigate mycoplasma contamination which might be responsible for inhibiting virus growth in the presence of citrulline. Such examination did not reveal any cytoplasmic DNA but many very large vacuoles were seen which are more characteristic of senescent cells. Since the Sl80 cell line has been known to have changed its characteristics once before in its history, a new stock of Sl80 cells was obtained from Flow Laboratories, with a lower passage number (94) for further experiments. These cells were stained and did not appear to contain large vacuoles. However with this stock of Sl80 cells, no improvement in the virus yield in the presence of citrulline was achieved.

ii) Stocks of rabbitpox virus

Virus stocks, which had been used in the original experiments, were retrieved from liquid nitrogen storage and passaged in Sl80 cells. When this stock was used to infect Sl80 cells, again there was no increase in the virus yield obtained in the presence of citrulline.

iii) Nutritional Conditions

Since neither the use of original virus stocks nor a low passage stock of Sl80 cells resulted in an improved virus yield when citrulline replaced arginine, it is possible that the divergence from the original results is due to some change in the nutritional requirements of the system.

Initially, medium was made up using fresh stocks of amino acids and vitamins, but this again had no effect on the virus yields. One particular vitamin, pyridoxal, was considered, since vitamin B6 is known to participate in arginine metabolism. This essential factor is available as three derivatives; pyridoxal, pyridoxal phosphate and pyridoxine. The virus yields were measured using all three alternatives in case there might be differences in the ability of the cells to take up the vitamin in different forms. However similar results were obtained in all cases.

Changes in the serum used to grow the SL80 cells were considered as a possible reason for the change in cell behaviour, since serum contains many growth factors, some of which have been shown to be essential for the replication of poxviruses in other cell systems. However serum used in the original experiments was not available. Neither the use of newborn calf serum nor foetal calf serum in the growth medium of uninfected cells or in the maintenance medium of virus infected cells had any effect on the virus yield in the presence of citrulline.

iv) The use of galactose as a carbon source

The use of galactose as a carbon source instead of glucose has been shown to increase the efficiency of carbohydrate metabolism in some human cell lines (Eagle et al, 1959). Since arginine metabolism is closely linked with the tricarboxylic acid cycle, such carbohydrate substitution might increase the ability of rabbitpox-infected cells to utilise citrulline. In preliminary experiments, cells were seeded in tubes in PME made up with galactose instead

of glucose as the carbon source, with 5% calf serum. The cultures were examined microscopically over the following 3 days, but no growth was observed although similar cultures became confluent after incubation in the same medium but containing glucose. These results indicate that galactose cannot be metabolised by S180 cells, and no further experiments were carried out.

v) The effect of a low concentration of trypsin

It has been reported that in the presence of low concentrations of proteolytic enzymes, certain cells have a faster growth rate, suggesting that the cells become more metabolically active (Brown & Kiehn, 1977; Carney & Cunningham, 1977). The mode of action is not clear, but it could possibly be due to the enzymes breaking down cell proteins and overstimulating the repair mechanisms.

The effect of the presence of a low concentration of trypsin on the growth of S180 cells and the replication of rabbitpoxvirus was investigated.

Cells were seeded in 4 oz medical flats at 10^6 cells per bottle and incubated in PME containing trypsin at $10\mu\text{g/ml}$ with and without arginine and with varying concentrations of citrulline. Control cultures were set up without trypsin. All media contained 5% calf serum. After incubation for 48 hours the cells were harvested, counted in trypan blue and a protein assay carried out. In the presence of trypsin 95% of the cells in all samples were found to be viable. Table 5.4 shows that in the absence of trypsin under all conditions of arginine deprivation no cell multiplication took place and no increase in cell protein occurred. In the

TABLE 5.4. The effect of a low concentration of trypsin on the growth of S180 cells.

	- TRYPSIN		+ TRYPSIN (10 μ g/ml)	
	Cells/ Bottle	Protein Bottle(mg)	Cells/ Bottle	Protein/ Bottle(mg)
- ARGININE	1.27 x 10 ⁶	100	1.70 x 10 ⁶	140
+ 0.5 mM CITRULLINE	1.21 x 10 ⁶	97	1.98 x 10 ⁶	125
+ 2 mM CITRULLINE	1.13 x 10 ⁶	110	2.12 x 10 ⁶	125
+ 5 mM CITRULLINE	1.17 x 10 ⁶	102	1.91 x 10 ⁶	120
+ 0.5 mM ARGININE	2.45 x 10 ⁶	160	2.36 x 10 ⁶	150

presence of arginine the cell numbers doubled and cell protein increased by about 60%. Similar increases were observed in cell numbers following the addition of trypsin to complete medium. However in all other conditions either in the absence of arginine or if citrulline replaced arginine, the cell numbers increased by 60% and the cell protein increased by about 25%. Thus in the presence of trypsin some growth of the S180 cells was allowed in the absence of arginine, although this appeared to be independent of the presence of citrulline. Since serum was used in this experiment, it is possible that the trypsin was breaking down the serum proteins, thus creating a pool of arginine and allowing multiplication to take place.

Further experiments were carried out to measure the growth of S180 cells in the absence of arginine in media containing various concentrations of calf serum, with and without trypsin. S180 cells were seeded at 4.5×10^5 cells per bottle in PME minus arginine containing varying concentrations of serum from 0 to 10% with and without trypsin at $10 \mu\text{g/ml}$. Control cultures were set up with PME plus arginine with 5% serum. After 3 days the cells were harvested and viable counts made in trypan blue and the results obtained are shown in table 5.5. In the absence of trypsin there was no cell growth with any concentration of serum unless arginine was also present; such conditions resulted in a 5-fold increase in cell numbers. In the presence of trypsin however, cell growth in the absence of arginine was, again, observed but the extent to which this occurred was found to be related to the serum concentration. Similar cell growth with 10% serum in the absence of arginine if trypsin was

TABLE 5.5. 48 hour viable counts of SL80 cells grown with varying concentrations of serum in PME minus arginine, with and without trypsin at $10\mu\text{g/ml}$.

	- TRYPSIN	+ TRYPSIN
- ARGININE - SERUM	CELLS DIED	CELLS DIED
- ARGININE + 0.5% SERUM	6.5×10^4	1.65×10^5
- ARGININE + 2% SERUM	1.1×10^5	3.7×10^5
- ARGININE + 5% SERUM	1.4×10^5	5.0×10^5
- ARGININE + 10% SERUM	1.2×10^5	6.3×10^5
+ ARGININE + 5% SERUM	6.1×10^5	6.5×10^5

present and with control cultures without trypsin maintained with 0.5 mM arginine and 5% serum. These results suggest that there is an interaction between the trypsin and the serum resulting in the formation of a pool of exogenous arginine which is utilised for cell growth.

In order to investigate the effect of trypsin on the metabolic activity of S180 cells, an experiment was carried out to measure the incorporation of carbamyl- ^{14}C -citrulline, guanido- ^{14}C -arginine and ^3H -leucine into cell protein, with and without trypsin present in the medium.

Confluent monolayers of S180 cells were prepared and the intracellular pools depleted of arginine. These cultures were maintained subsequently in PME minus arginine containing ^3H -leucine at $0.5\mu\text{Ci/ml}$ with either carbamyl- ^{14}C -citrulline or guanido- ^{14}C -arginine, both amino acids at 0.5 mM concentration and specific activity of $0.1\mu\text{Ci/ml}$. Cultures were set up with and without trypsin in the medium at $10\mu\text{g/ml}$. After 24 hours incubation the cells were harvested and the radioactivity associated with the TCA precipitable material determined. This experiment was carried out in the absence of serum. As a result, in all samples with trypsin present, the cells had fallen off the glass into the medium but when stained with trypan blue were shown to be 95% viable. As can be seen in table 5.6, the presence of trypsin appeared to inhibit the utilisation of citrulline for protein synthesis, as this was reduced by 75% of that incorporated in the absence of the enzyme. The incorporation of arginine was also reduced in medium containing trypsin but only by 29%. The incorporation of ^3H -leucine was also reduced by the presence of trypsin.

TABLE 5.6. The effect of trypsin at $10\mu\text{g/ml}$ on the incorporation of carbamyl- ^{14}C -citrulline, guanido- ^{14}C -arginine and ^3H -leucine into S180 cells.

	- TRYPSIN		+ TRYPSIN	
	^3H -dpm/ bottle	^{14}C -dpm/ bottle	^3H -dpm/ bottle	^{14}C -dpm/ bottle
- ARGININE	329,661	-	280,341	-
+ CITRULLINE (0.5 mM)	669,083	26,717	635,221	6,926
+ ARGININE (0.5 mM)	-	2,946,284	-	2,091,972

This experiment was carried out using dual isotopes and with the samples maintained in the presence of arginine both with and without trypsin, a large amount of ^{14}C -radioactivity was found in the tritium channel such that the tritium d.p.m.s could not be determined and also the ^{14}C counts are likely to be inaccurate. This may explain why the proportion of citrulline to arginine incorporated under the control conditions shown in table 5.6 disagrees with the results shown in table 5.1. However the other results in the table are significant since the samples contained much lower quantities of ^{14}C radioactivity.

vi) Effect of a low concentration of trypsin on the replication of rabbitpox virus in S180 cells

Experiments were carried out to determine whether the presence of trypsin in the medium would affect the ability of S180 cells to support the replication of rabbitpox virus in the presence of citrulline. Cultures of S180 cells were prepared in tubes and the intracellular pools depleted of arginine. The cells were infected with rabbitpox at a low inoculum to permit multiple cycles of virus replication. The infected cells were maintained in PME minus arginine containing trypsin at $10\mu\text{g/ml}$ and with citrulline or arginine at 0.5 mM concentration. These experiments were carried out in serum-free medium or with serum at 2%. Control cultures were also infected and maintained without trypsin or serum. At 24 hours post infection the cell cultures were examined for any cytopathic effect (c.p.e.) before harvesting the progeny virus for titration. With medium containing trypsin but lacking serum, all the cells had again fallen

off the glass into the medium. The monolayers maintained with serum were still intact. Infected cells incubated in the absence of arginine appeared rounded up if trypsin was present compared with little c.p.e. without trypsin. In the presence of citrulline with trypsin there was extensive c.p.e. with syncytia, similar to normal c.p.e. in complete medium. Without trypsin, limited c.p.e. was visible. With arginine, complete c.p.e. had taken place in medium with and without trypsin.

Table 5.7 shows the results of the titrations of the virus progeny. In the absence of trypsin there was no yield in arginine minus conditions and a low yield if citrulline replaced arginine. The yield in complete medium was similar whether trypsin was present or not. However the yield of rabbitpox when citrulline replaced arginine was greatly increased if trypsin was present but this was accompanied by an increase in the yield in the absence of arginine. The increases in the presence of trypsin were greater in the absence of serum even though the cells were detached from the glass. This suggests that the trypsin is more effective in its mode of action when serum is not present and that when the cells fall off the glass into the medium they do not lose the ability to support rabbitpox virus replication.

TABLE 5.7. The effect of trypsin at $10\mu\text{g/ml}$ on the yields of rabbitpox virus in S180 cells in the presence and absence of arginine and with citrulline replacing arginine, with and without serum.

24 hour virus yields			
	- TRYPSIN No Serum	+ TRYPSIN No Serum	+ TRYPSIN 2% Serum
- ARGININE	1.57	45	25
+ CITRULLINE (0.5 mM)	7.35	98	39
+ ARGININE (0.5 mM)	230	221	226

(Inoculum Recovery = 4.3×10^4)

vii) Sub-optimal concentrations of arginine supplementing
citrulline

It is possible that low concentrations of arginine might be needed to synthesise the proteins which can in turn utilise citrulline more effectively. Consequently, sub-optimal concentrations of arginine were supplemented to citrulline to determine whether any enhancement of virus yield could be obtained.

Monolayers of SL80 cells were prepared in tubes and the intracellular pools of arginine depleted. The starved cells were infected with rabbitpox virus and maintained in PME containing 0.5 mM citrulline supplemented with three sub-optimal concentrations of arginine. Infected controls were also incubated in PME with the sub-optimal arginine concentrations alone and also with arginine or citrulline at 0.5 mM and without arginine. The 24 hour yields of virus were harvested and titrated. The results in table 5.8 show no increase in the yield of rabbitpox virus in the presence of citrulline after supplementation with sub-optimal concentrations of arginine.

viii) Spread of virus from cell to cell

The cytopathic effect produced by rabbitpox in SL80 cells in the presence of citrulline appears to be completely different from that in the presence of arginine. Syncytia are observed in complete medium whereas only rounding up of the infected cells occurs with citrulline. Thus, the virus may not be able to spread from cell to cell so quickly in the presence of citrulline. To test this hypothesis, the

TABLE 5.8. Yields of rabbitpox virus in SL80 cells with and without citrulline, supplemented with sub-optimal concentrations of arginine.

Inoculum Recovery = 3.48×10^4

Arginine Supplement (mM)	Yield - Arginine	Yield + Citrulline
0	1.34	1.30
0.005	2.00	1.38
0.01	5.50	8.90
0.05	71	50.3
0.50	143	-

Sl80 cells were infected with rabbitpox virus at different multiplicities of infection and the subsequent virus yields determined from infected cultures maintained with either arginine or citrulline.

After depletion of arginine pools, Sl80 monolayers were infected with rabbitpox using 10^4 , 10^5 and 10^6 p.f.u. per cell culture. Infected cells were maintained in PME minus arginine, supplemented with 0.5 mM citrulline or 0.5 mM arginine. No serum was used. The 24 hour yields of progeny virus were harvested and titrated..

The results in table 5.9 show that the highest inoculum used produced a yield of rabbitpox with citrulline which was about 10% of that with arginine. With both the lower inocula, where multiple cycles of infection were able to occur, the yield in the presence of citrulline was 3% of that with arginine. Since both the lower inocula gave almost identical yields, it suggests that a similar number of replication cycles had taken place and that infection of the whole monolayer was not complete at 24 hours post infection. With the lower multiplicity, the reduction in virus yield in the presence of citrulline relative to virus yields obtained with arginine would be expected since, if the first cycle of replication gave 10% of the yield with arginine, there would be a reduction following the second cycle of infection. The yields in the presence of citrulline were higher with a lower multiplicity of infection than the highest, indicating that some limited spread of virus had occurred.

TABLE 5.9. 24 hour yields of rabbitpox virus in S180 cells plus and minus arginine and with citrulline replacing arginine, with varying input inocula.

Inoculum Recovery	8.5×10^4	8.8×10^3	1.1×10^3
- Arginine	1.6	2.1	2.5
+ Citrulline (0.5 mM)	18.0	27.0	28.0
+ Arginine (0.5 mM)	193	975	947

c) Comparison of orthopoxvirus yields in S180 cells

Since many modifications of the S180 cell system had been investigated unsuccessfully to try to enhance the yield of rabbitpox in the presence of citrulline, further experiments proceeded to determine the abilities of other orthopoxviruses to replicate in S180 cells under similar conditions. S180 monolayers in which the intracellular pools had been depleted were infected with 10 different orthopoxviruses and incubated in PME with and without arginine and with citrulline replacing arginine. No serum was used. The yields of progeny virus were measured at 24 hours post infection.

The results in table 5.10 show that of the 10 orthopoxviruses tested, 8 produced progeny virus in complete medium with yields ranging from 3.5 with camelpox to over 200 with ectromelia.

The viruses fell into four main categories; (1) Rabbitpox produces no yield in the absence of arginine, a low yield with citrulline which is 10% of the yield with arginine. (2) Vaccinia (IHD) and ectromelia produce significant yields in the absence of arginine, slightly higher yields with citrulline and high yields with arginine. Ectromelia produced the higher yield perhaps since its natural host is the mouse and the virus is replicating in a cell system derived from mouse tissue. (3) Cowpox, anteaterpox, cheetahpox, elephantpox and camelpox, all of which will only replicate in S180 cells in complete medium. The yields obtained from this group are lower than the others but are significant. (4) Both vaccinia (Lister) and buffalopox did not produce any progeny virus in S180 cells in complete medium.

TABLE 5.10. Yields of different orthopoxviruses in SL80 cells in the presence and absence of arginine and when citrulline replaces arginine.

POXVIRUS (Inoculum Recovery)	- ARGININE	+ CITRULLINE	+ ARGININE
RABBITPOX (2.0×10^5)	1.1	10.3	100
VACCINIA (IHD) (1.4×10^4)	10.0	13.5	166
ECTROMELIA (1.0×10^4)	73	102	237
COWPOX (1.2×10^5)	1	1	21.6
ANTEATERPOX (1.0×10^5)	1	1	33
CHEETAHPOX (1.0×10^5)	1	1	10
ELEPHANTPOX (2.0×10^5)	1	1	6.7
CAMELPOX (1.0×10^5)	1	1	3.5
BUFFALOPOX (1.0×10^5)	1	1	1
VACCINIA (Lister) (1.0×10^5)	1	1	1

3. Studies of DNA synthesis in orthopoxvirus-infected S180 cells

As mentioned previously, arginine may be required for at least two events during vaccinia virus replication in HeLa cells, the first being an early event on which virus-specific DNA synthesis depends and the second, a quantitatively greater requirement, is for a later event associated with virus maturation.

The requirement for arginine during virus-specific DNA synthesis in various poxvirus-infected S180 cells was investigated by using two inhibitors of DNA synthesis and by measuring the incorporation of ^3H -thymidine into cytoplasmic, perinuclear and nuclear fractions of orthopoxvirus-infected S180 cells.

a) Use of Inhibitors of DNA Synthesis.

Fluorouracil deoxyribose (FUdR) inhibits thymidine synthesis and another DNA inhibitor, cytosine arabinoside (ara C) blocks DNA polymerase, preventing chain elongation. Both inhibitors were used in similar experiments to those described previously with poxvirus-infected BHK cells (Chapter 4) to determine the extent of DNA synthesis, which could give rise subsequently to mature virus progeny under arginine deprived conditions or with citrulline replacing arginine.

Initially the dose response of rabbitpox virus infected S180 cells was measured with the two inhibitors of DNA synthesis, to determine the minimum concentration resulting in complete inhibition of virus replication. Table 5.11. shows the 24 hour yields of rabbitpox virus in S180 cells

TABLE 5.11. Dose response of rabbitpox-infected SI80 cells to inhibitors FUDR and Ara C.

	TITRE	YIELD
Inoculum Recovery	3.16×10^5	-
12.5 μ M FUDR	6.35×10^5	2.01
25 μ M FUDR	3.76×10^5	1.19
50 μ M FUDR	3.38×10^5	1.07
100 μ M FUDR	3.25×10^5	1.03
62.5 μ M Ara C	4.80×10^5	1.53
125 μ M Ara C	5.15×10^5	1.63
250 μ M Ara C	3.34×10^5	1.06
500 μ M Ara C	3.09×10^5	0.98
- ARGININE control	5.97×10^5	1.89
+ ARGININE control	5.09×10^6	16.1

in complete medium containing various concentrations of FUdR and ara C. The concentrations of the inhibitors found to be effective were $50\mu\text{M}$ FUdR and $125\mu\text{M}$ ara C. These concentrations were used in all further experiments.

In order to determine the extent of virus-specific DNA synthesis in orthopoxvirus-infected cells under arginine deprived conditions which was able to subsequently give rise to infective progeny, infected cells were incubated for 24 hours in the limiting medium before the addition of arginine together with an inhibitor of DNA synthesis. This prevented further DNA synthesis but the presence of arginine allowed any existing DNA to be utilised in the formation of mature progeny virus. Confluent monolayers of SL80 cells with intracellular pools depleted of arginine were infected with different orthopoxviruses; rabbitpox, cowpox, anteatpox vaccinia (IHD) and ectromelia. The infected cells were maintained in PME with and without arginine or with citrulline. At 24 hours post infection, arginine together with one of the inhibitors was added to the maintenance medium to final concentrations of 0.5 mM arginine with $50\mu\text{M}$ FUdR or $125\mu\text{M}$ ara C and infected cultures incubated for a further 24 hours. Control cultures were incubated in complete medium with the inhibitors present from the time of infection and others were maintained for 48 hours under the three conditions but without the inhibitors. The results are shown in table 5.12. Both FUdR and ara C appear to have completely inhibited the replication of rabbitpox, cowpox and anteatpox. Ectromelia was completely inhibited by ara C but a low yield was obtained in complete medium containing FUdR. Vaccinia (IHD) was only partially inhibited

TABLE 5.12. Biological measurement of DNA synthesis.

RP = rabbitpox (1.8×10^4), CP = cowpox (2.8×10^4),
 AP = anteatpox (10^5), E = ectromelia (7.8×10^3)
 and V = vaccinia (IHD) (2.0×10^3).

Yield at 48 h.p.i.	RP	CP	AP	E	V
- Arginine	1.87	1.0	0.8	15.5	150
+ Citrulline	7.4	1.1	0.54	28.2	224
+ Arginine	154	9.2	33	592	4000
+ Arginine + 50μ M FUdR	1.2	0.77	0.8	5.9	400
+ Arginine + 125μ M Ara C	1.5	0.85	0.5	0.2	300
48 h.p.i. after inhibitor and arginine added at 24 h.p.i.					
- Arginine (FUdR)	1.4	1.6	1.6	33	800
+ Citrulline (FUdR)	1.4	2.9	2.1	33	1000
+ Arginine (FUdR)	8.5	10.7	35	85	1200
- Arginine (Ara C)	0.9	1.1	0.75	27	300
+ Citrulline (Ara C)	0.9	0.65	0.82	31	600
+ Arginine (Ara C)	4.2	2.76	13.5	31	1000

by FUDR and ara C at the concentrations used. The high yields obtained with vaccinia(IHD) in this experiment were probably a result of the low inoculum. With rabbitpox, cowpox and anteatpox viruses no increase in the yield was observed following addition of arginine and inhibitor. In fact the yields were lower than the appropriate controls suggesting that addition of the inhibitors caused inactivation of extant infective virus particles, or inhibited further cycles of replication. The yields of ectromelia and vaccinia (IHD) incubated in the absence of arginine or with citrulline increased compared with the appropriate controls on addition of arginine and inhibitor at 24 h.p.i. but, again, reduced virus yields were obtained at 48 hours compared with the controls.

b) Incorporation of ^3H -thymidine into poxvirus-infected SL80 cells

In order to determine directly the extent of virus-specific DNA synthesis the incorporation of ^3H -thymidine into high molecular weight material present in the cytoplasmic fraction of virus infected cells was measured under different conditions of arginine deprivation.

Since poxviruses replicate in the cytoplasm, virus specific DNA can be separated from host cell nuclear DNA by cell fractionation. This can be achieved by detergent treatment of infected cells as described fully in Materials and Methods. Initially the cells are treated with Nonidet P40 (NP40) to rupture the cytoplasmic membrane, leaving the nucleus in tact. The crude nuclei are pelleted to leave the first cytoplasmic fraction in the supernatant. The nuclei are resuspended for a short time in a mixed detergent solution

of NP40 and sodium deoxycholate. The latter slowly attacks the nuclear membrane removing any remaining cytoplasmic material. After repelleting the nuclei, the second, perinuclear cytoplasmic fraction is recovered. The host cell DNA can be prelabelled with ^{14}C -thymidine in order to indicate whether the nuclear membrane has been ruptured by the second detergent treatment. This also gives a reference value for the amount of cell material present in each sample.

Confluent monolayers of Sl80 cells were prepared in 4 oz. medical flat bottles and exposed to ^{14}C -thymidine at $0.1\mu\text{Ci/ml}$ to prelabel the host cell DNA. However, even after 24 hours, the levels of radioactivity associated with TCA precipitable material were very low suggesting that unlike other cells, Sl80 cells do not scavenge free nucleotides from the surrounding medium. This hypothesis was tested by maintaining cells in medium containing ^3H -thymidine with two different concentrations of FUDR which inhibits host cell thymidine synthesis. (The ^3H -thymidine used in this laboratory has a 200 times greater specific activity than the ^{14}C)

As can be seen from table 5.13, the presence of FUDR caused the incorporation of ^3H -thymidine to increase over the control incubated in its absence, thus supporting the hypothesis.

Experiments to measure the extent of virus specific DNA synthesis in poxvirus infected Sl80 cells by incorporation of ^3H -thymidine were carried out without prelabelling host cell DNA since the uninfected cells appeared to be unable to incorporate ^{14}C thymidine on their own. Use of FUDR to facilitate incorporation would be most unsuitable since DNA synthesis is being investigated and the presence of a

TABLE 5.13. The effect of FUdR on the incorporation of ^3H -thymidine into S180 cells.

	^3H -thymidine incorporated (dpm)
Minus FUdR	462,133
25 μM FUdR	2,167,406
50 μM FUdR	3,065,255

DNA inhibitor before infection could seriously affect subsequent virus replication.

Sl80 cells were prepared in 4 oz. medical flat bottles, were depleted of arginine and infected with either rabbitpox or cowpox viruses. After infection, cultures were maintained in PME containing ^3H -thymidine at $0.5\mu\text{Ci/ml}$ with unlabelled thymidine added to a final concentration of $20\mu\text{M}$. Maintenance media were with or without arginine or with citrulline replacing arginine. Sham infected cells were also incubated in similar media containing arginine. At 24 hours post infection the cells were harvested, fractionated as described and the radioactivity associated with TCA precipitable material from each fraction determined. The results are shown in table 5.14. In the first two fractions the levels of incorporation of ^3H -thymidine into infected cells were much greater than in sham infected cells. This is indicative of poxvirus specific DNA synthesis. The low levels of radioactivity in the cytoplasmic and perinuclear fractions of sham infected cells are probably due to host DNA of mitochondria. The incorporation of radioactivity into the nuclear fractions tended to be lower in infected cells. This suggests that host DNA synthesis has been inhibited by virus infection.

With rabbitpox-infected cells maintained in the absence of arginine, the incorporation of ^3H -thymidine into both cytoplasmic and perinuclear fractions was 19% of that in the presence of arginine, but if citrulline replaced arginine incorporation increased to 45%. This indicates an increase in the amount of DNA synthesis in the presence of citrulline. There were also increases following a similar trend in the nuclear fraction. With cowpox-infected cells incubated in

TABLE 5.14. Biochemical measurement of DNA synthesis in rabbitpox and cowpox infected SL80 cells.

	CYTOPLASMIC (³ H-dpm)	PERINUCLEAR (³ H-dpm)	NUCLEAR (³ H-dpm)
Sham-infected + Arginine	25,939	14,749	40,870
Rabbitpox - Arginine	49,088	34,688	6,194
Rabbitpox + Citrulline	116,336	80,975	9,665
Rabbitpox + Arginine	256,434	166,941	41,740
Cowpox - Arginine	40,650	31,486	5,850
Cowpox + Citrulline	39,954	34,382	4,526
Cowpox + Arginine	171,208	105,765	11,808

the absence of arginine incorporation of ^3H -thymidine was found to be 23% in the cytoplasmic fraction and 30% in the perinuclear fraction of that in the respective arginine controls. However if citrulline replaced arginine there was no increase in the incorporation suggesting that, unlike rabbitpox, cowpox is unable to utilise this amino acid.

Similar experiments were made with vaccinia (IHD) which is able to produce progeny virus in the absence of arginine and buffalopox which is unable to grow in SL80 cells. The results are shown in table 5.15. Again in the first two fractions with infected cells there were much higher levels of ^3H -thymidine incorporated than in the sham infected controls. However in this experiment the nuclear fractions contained more radioactivity than the perinuclear fractions, suggesting that the second detergent treatment had not been as effective as in the previous experiment. Thus the nuclear fractions may have contained virus-specific DNA which would have been recovered in perinuclear fractions after longer detergent treatment.

In the cytoplasmic and perinuclear fractions of vaccinia (IHD) infected cells maintained in the absence of arginine, the levels of incorporation were about 65% of those with arginine and no increase was observed when citrulline replaced arginine. The nuclear fractions all contained about the same amounts of radioactivity.

Although buffalopox does not produce any infective progeny virus in SL80 cells, some virus specific DNA synthesis occurred. In the absence of arginine or in the presence of citrulline, the levels of incorporation of ^3H -thymidine in the cytoplasmic and perinuclear fractions were similar being

TABLE 5.15. Biochemical measurement of DNA synthesis in vaccinia (IHD) and buffalopox infected SI80 cells.

	CYTOPLASMIC (³ H-dpm)	PERINUCLEAR (³ H-dpm)	NUCLEAR (³ H-dpm)
Sham-infected - Arginine	7,238	1,673	89,960
Sham-infected + Arginine	9,580	1,920	117,991
Vaccinia (IHD) - Arginine	10,284	3,193	270,877
Vaccinia (IHD) + Citrulline	10,476	3,736	226,106
Vaccinia (IHD) + Arginine	16,078	4,818	224,412
Buffalopox - Arginine	13,766	3,345	265,399
Buffalopox + Citrulline	9,201	3,293	253,009
Buffalopox + Arginine	23,258	6,846	399,201

about 50% of those with arginine and the nuclear fractions contained 65% of the control. Thus the presence of citrulline did not appear to make any difference to the amount of virus-specific DNA synthesised in vaccinia (IHD) or buffalopox infected SL80 cells.

c) Acridine Orange Staining

The extent of DNA synthesis occurring in poxvirus-infected SL80 cells was also studied qualitatively using acridine orange staining at various times post infection as described in Materials and Methods. During the poxvirus replication cycle, large discrete cytoplasmic inclusions, which are 'factories' of DNA synthesis, can be observed from 4 hours post infection. At later times they become diffuse as mature progeny virus are formed.

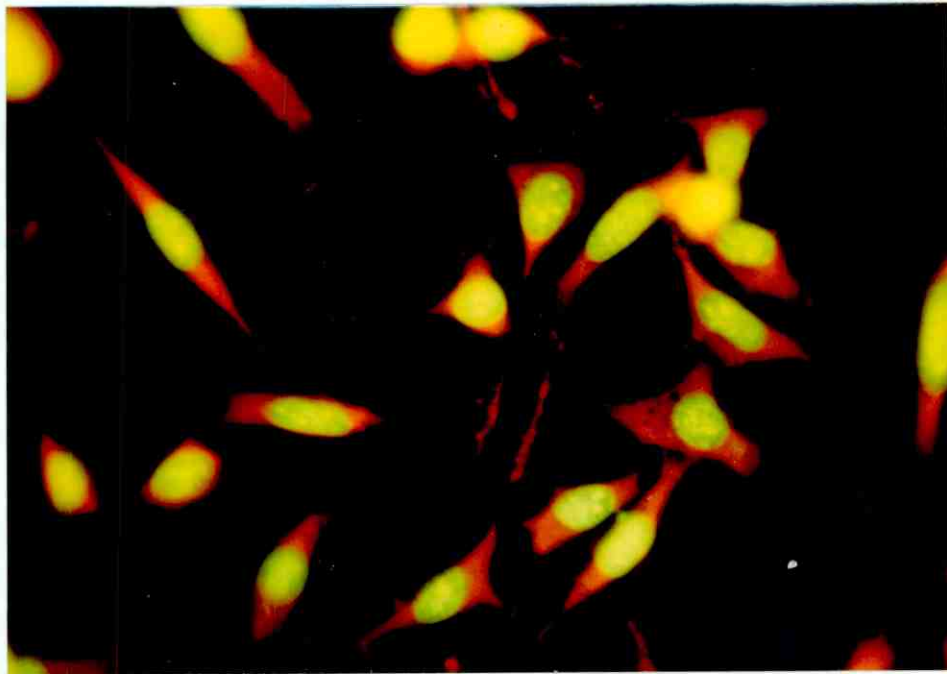
SL80 cells were seeded at low population density on to circular coverslips and intracellular pools depleted immediately by incubation in arginine deprived medium. After infection with rabbitpox or cowpox viruses under one step growth conditions infected cells were incubated in PME with and without arginine or with citrulline. At 7 and 24 hours post infection the cells were fixed and stained with acridine orange before mounting on microscope slides.

At 7 hours post infection, cytoplasmic inclusions were clearly visible with both cowpox and rabbitpox infected cells under all conditions. In rabbitpox infected cells at 24 hours post infection, as expected, the cytoplasmic inclusions had become diffuse and barely visible. The infected cells had rounded up and some syncytia had formed from adjacent cells. This cytopathic effect had occurred under all conditions.

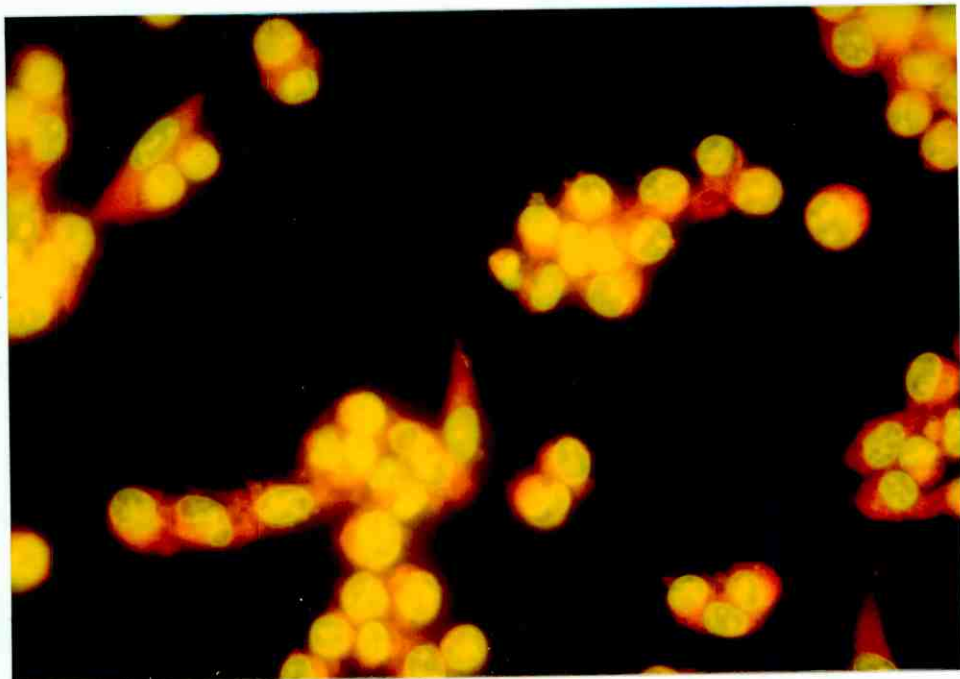
In cowpox-infected S180 cells in complete medium at 24 hours post infection, again the normal cytopathic effect was present with diffuse cytoplasmic inclusions. However in the absence of arginine or when citrulline replaced arginine, discrete cytoplasmic inclusions were still clearly visible at 24 hours post infection.

Photomicrographs of rabbitpox and cowpox infected S180 cells stained with acridine orange at 24 hours post infection are shown in figures 5.3 and 5.4.

Figure 5.3. Photomicrographs of rabbitpox-infected SI80 cells stained with acridine orange.

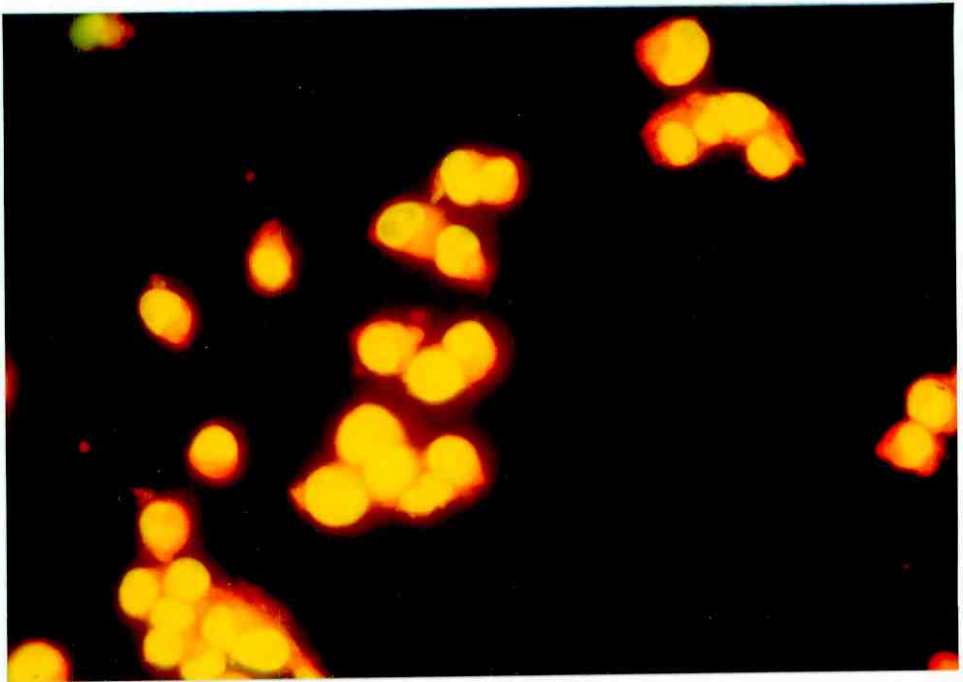


(a) Sham-infected cells

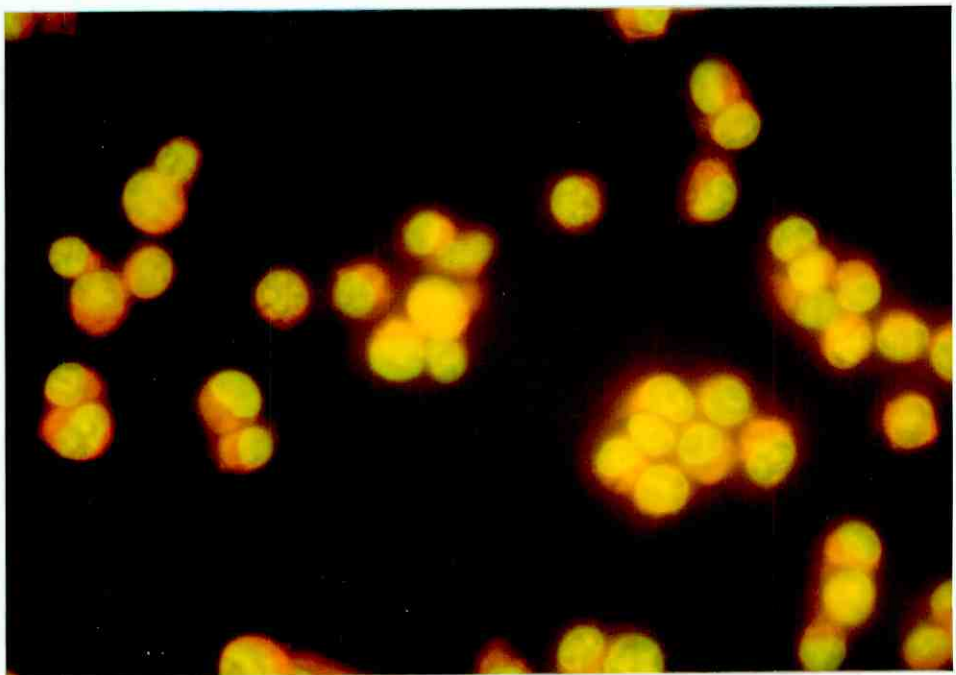


(b) Rabbitpox-infected cells maintained in the absence of arginine at 24 h.p.i.

Figure 5.3 continued.

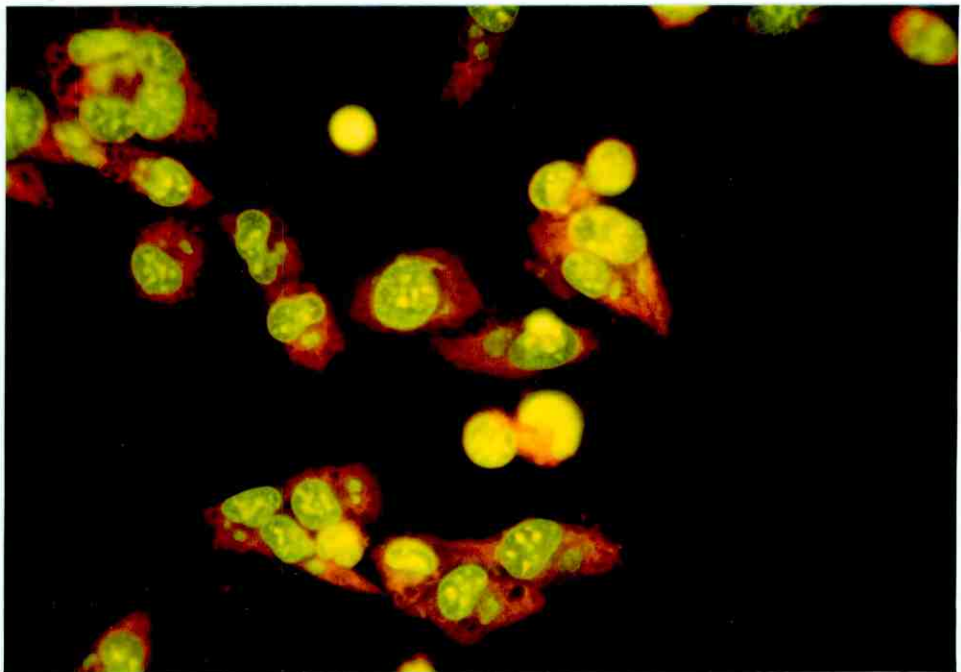


(c) Rabbitpox-infected cells maintained
with citrulline at 24 h.p.i.



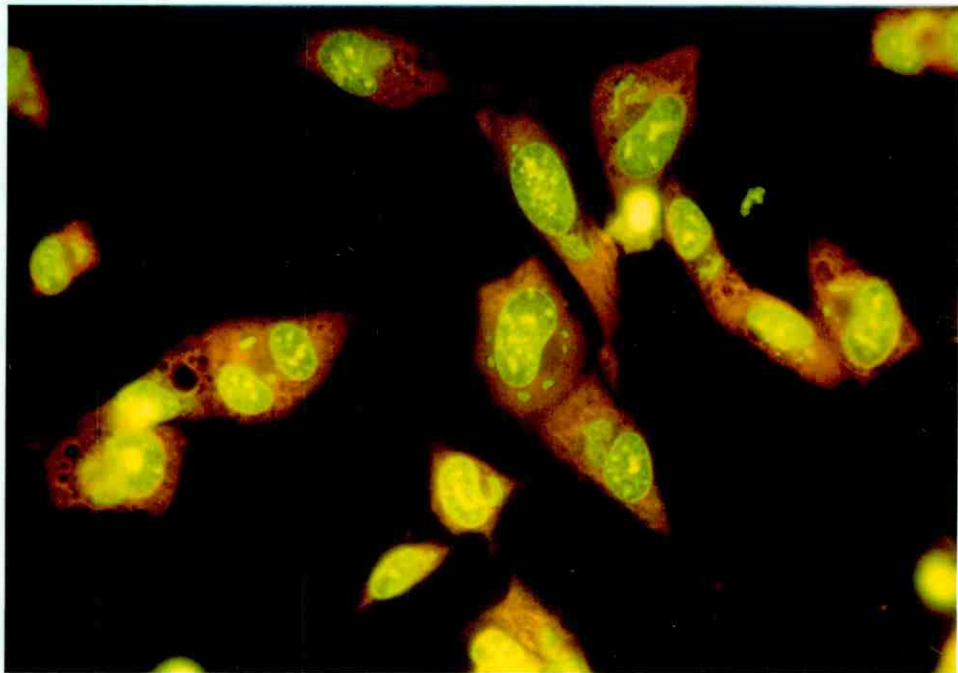
(d) Rabbitpox-infected cells maintained
with arginine at 24 h.p.i.

Figure 5.4. Photomicrographs of cowpox-infected SL80 cells stained with acridine orange.

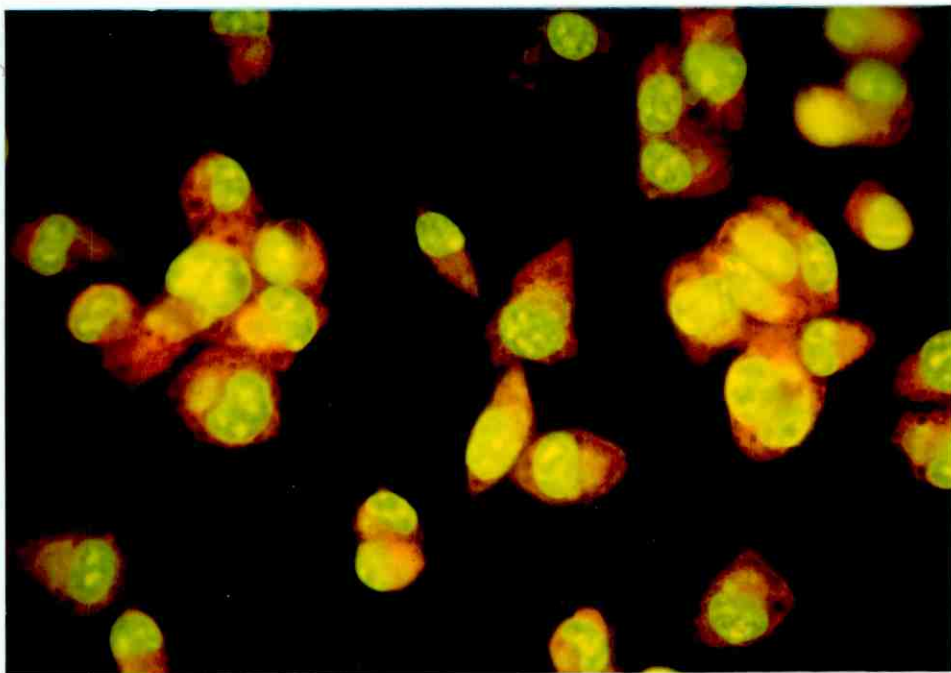


(a) Cowpox-infected cells maintained in the absence of arginine at 24 h.p.i.

Figure 5.4. continued.



(b) Cowpox-infected cells maintained with citrulline at 24 h.p.i.



(c) Cowpox-infected cells maintained with arginine at 24 h.p.i.

4. Discussion

Mouse Sarcoma 180 cells used in this study were unable to utilise citrulline but, in other characteristics, the cells had altered compared with earlier studies. Increasing the concentration of citrulline did not improve the growth of cells or increase the amount of carbamyl-¹⁴C-citrulline incorporated, whereas previously S180 cells had resumed growth when the concentration of citrulline was increased from 0.5 to 5 mM.

Following infection with rabbitpox virus, the yield in the presence of citrulline was much lower than previously reported, being at most only 10% of that in complete medium. These experiments indicated again that the S180 cells used in this study had altered characteristics. This must be as a result of changes in either the cells, the virus, or a change in the growth conditions. A number of modifications of the cell system were attempted to ascertain the reason for the altered results.

The cells were examined microscopically for their cytological appearance by fluorescent staining to show any mycoplasmal contamination. No cytoplasmic DNA was revealed but the cells were shown to contain many large vacuoles, characteristic of senescent cells. Since the S180 cell line is known to have changed its characteristics once before in its history, a new stock was obtained with a lower passage number and was shown not to contain large vacuoles. However with these cells there was no improvement in the yield of rabbitpox with citrulline. The stock of rabbitpox virus was found not to be implicated since virus from the original stock gave very limited yields in the presence of citrulline.

The most likely cause for the change in characteristics is the nutritional conditions, especially the serum since fresh stocks of amino acids and vitamins made no difference. Unfortunately, serum used in the original experiments was not available. Neither the use of newborn or foetal calf serum had any effect on the virus yield, and the yields were similar from infected cells maintained with or without serum, although the cells required serum to grow confluent.

Attempts were made to stimulate the growth rate of SL80 cells since it was noticed that this was slower than with previous experiments. Galactose was used as an alternative carbon source which has been shown to increase the efficiency of carbohydrate metabolism but preliminary experiments showed that galactose cannot be metabolised by SL80 cells.

The effect of a low concentration of trypsin on the growth of SL80 cells was investigated since this has been shown to increase the growth rate in certain cells. The presence of trypsin was found to have no effect on the growth rate of SL80 cells but under arginine deprived conditions the growth rate was greater and cell protein increased although this appeared to be independent of the presence of citrulline. However serum was used in these experiments and further experiments showed that the extent of cell growth occurring in the absence of arginine stimulated by trypsin was dependent on the concentration of serum. This suggests that the trypsin may be breaking down the serum and producing free arginine allowing cell growth to resume. Alternatively the serum may contain essential growth factors for SL80 cells and the trypsin may not be able to stimulate cell growth without these factors.

The levels of radioactivity incorporated with all the amino acids used were reduced when trypsin was present, particularly with citrulline which was reduced by 75% in the presence of the enzyme. Without serum present trypsin caused the cells to round up and detach from the glass. This may have caused a reduction in the capacity of the cells to take up the amino acids since their spherical shape would cause them to have a smaller surface area. The uptake mechanism may have been impaired by the trypsin, since the enzyme is known to attack membrane proteins. This may also have resulted in increased protein turnover, creating a pool of arginine and thus reducing the requirement for exogenous arginine. If a pool of arginine were formed, this would markedly reduce any utilisation of citrulline since the enzymes required for conversion to arginine would be repressed by feedback inhibition. This may explain why the reduction in incorporation of ^{14}C -citrulline was more marked than with the other amino acids.

Since trypsin did appear to stimulate the growth rate of S180 cells, its effect on rabbitpox replication was investigated. The yield in complete medium was similar whether trypsin was present or not. However the yield from rabbitpox-infected S180 cells when citrulline replaced arginine was greater with trypsin present but this was accompanied by an increased virus yield in the absence of arginine. The yields of virus were greater when trypsin was used in the absence of serum, even though this resulted in the cells becoming detached from the glass. This indicates that trypsin is more effective in its mode of action when serum is not present and that the resuspended cells do not lose their

to support rabbitpox replication. Thus under arginine deprived conditions, although the increased metabolic activity of S180 cells in the presence of trypsin is dependent on serum, the increase in replication of rabbitpox in S180 cells with trypsin is actually less when serum is present. Serum may interact with trypsin and reduce its activity.

The ability to utilise citrulline appears not to be affected in the presence of trypsin since increases in the yields with citrulline are always accompanied by increased virus yield in the absence of arginine. This suggests that a pool of arginine may be formed by interaction between the trypsin and cell proteins which may result from stimulation of protein turnover. Since the presence of trypsin did not increase the capability of rabbitpox-infected S180 cells to utilise citrulline, no further experiments were made with other poxviruses, but more modifications of the cell system were attempted.

The yield of rabbitpox was measured in the presence and absence of citrulline with different sub-optimal concentrations of arginine. However no increase in the yields with citrulline was observed over the controls. This may have been unsuccessful due to repression of any mechanism to synthesise the enzymes due to feedback inhibition by the arginine.

It was observed that the cytopathic effect produced by rabbitpox-infected S180 cells maintained with citrulline appeared to be completely different from that in the presence of arginine. Syncytia were observed in complete medium, whereas only rounding up of the cells occurred when citrulline replaced arginine. This may result in the virus

being unable to spread from cell to cell so easily and may account for the lower yields with citrulline. The yield of rabbitpox in SI80 cells with citrulline was never more than 10% of that with arginine. Thus either all the rabbitpox-infected cells in the presence of citrulline produce only 10% of the yield of infective progeny with arginine, or alternatively only 10% of the infected cells are able to produce infective progeny with citrulline. In either case it is probable that with multiple cycles of replication the virus spreads much more slowly in the presence of citrulline compared with arginine.

Thus many modifications of the SI80 cell system were investigated to try to enhance the yield of rabbitpox in the presence of citrulline. But low passage SI80 cells infected with the original stock of rabbitpox virus maintained in fresh medium still gave low yields compared with previous results. The only determinant which could have caused this is the serum used originally in the growth medium of the cells. None of this was available during the present experiments. Since serum contains many undefined growth factors there is a strong possibility that the serum used in later experiments lacked an essential growth factor involved with induction of argininosuccinate synthetase/lyase activity in SI80 cells.

Further experiments proceeded to determine the abilities of other poxviruses to replicate in SI80 cells when citrulline replaced arginine. Ten poxviruses were compared and these fell into four categories. The first contains rabbitpox, which gave no yield in the absence of arginine and a low yield with citrulline which was about 10% of that with arginine. The next category contains vaccinia (IHD) and

ectromelia which produced significant yields in the absence of arginine, slightly higher yields with citrulline and high yields with arginine. Ectromelia was found to be the most virulent of the poxviruses tested. This is interesting since its natural host is the mouse and it is replicating in a cell system derived from mouse tissue. The third category contains cowpox, anteatpox, cheetahpox, elephantpox and camelpox, all of which will only replicate in SL80 cells when exogenous arginine is present. Finally vaccinia (Lister) and buffalopox are unable to produce any infective progeny virus in SL80 cells even in complete medium.

The extent of virus specific DNA which was able to give rise to infective progeny was determined using two inhibitors of DNA synthesis, FUdR and Ara C, in similar experiments to those with BHK 21 cells. The poxviruses studied appeared to have different sensitivities to the inhibitors. The more virulent poxviruses, vaccinia (IHD) and ectromelia were much less sensitive to the inhibitors than other poxviruses. Perhaps some virus specific DNase activity was acting on host specific DNA resulting in the formation of a pool of nucleotides, thus by-passing the inhibition by FUdR. In experiments where infected cells were maintained for 24 hours before addition of arginine and an inhibitor of DNA synthesis, no increase in yields were observed when citrulline replaced arginine with any of the viruses tested. The presence of the inhibitors caused reduction in the titres compared with the controls, suggesting that this caused inactivation of extant infective virus particles, or inhibited further cycles of replication.

The extent of virus specific DNA synthesis occurring under arginine deprived conditions was measured directly by the incorporation of ^3H -thymidine into high molecular weight

material present in the cytoplasmic fractions of poxvirus-infected SL80 cells. This gave no indication of the extent of functional DNA synthesis, only a quantitative measurement of virus specific DNA synthesised.

With rabbitpox infected cells, DNA synthesis was found to occur to a greater extent when citrulline replaced arginine than in medium lacking arginine. With cowpox infected cells levels of DNA synthesis in the absence of arginine were similar to those with rabbitpox under similar conditions but there was no increase in the incorporation of ^3H -thymidine with citrulline. This supports the view that unlike rabbitpox, cowpox is unable to utilise this amino acid. Since vaccinia (IHD) is able to replicate in SL80 cells in the absence of arginine, this masked any ability to utilise citrulline. The levels of DNA synthesis were similar in the absence of arginine. Although buffalopox did not produce infective progeny virus in SL80 cells, some virus specific DNA synthesis was detected. This suggests that in complete medium, DNA synthesis may take place but subsequent events in the replication cycle are impaired.

The extent of DNA synthesis occurring in cowpox and rabbitpox infected SL80 cells was also studied qualitatively by acridine orange staining of infected cells. At 7 hours post infection cytoplasmic inclusions were clearly visible with both rabbitpox and cowpox infected cells under all conditions. In rabbitpox infected cells at 24 hours post infection as expected, the cytoplasmic inclusions had become diffuse and barely visible under all conditions. However with cowpox infected cells incubated in the absence of arginine or with citrulline, discrete cytoplasmic inclusions were still clearly

visible at 24 hours. This suggests that unless exogenous arginine is present, the replication of cowpox is arrested after virus specific DNA synthesis has occurred.

Although the S180 cells had altered their characteristics from earlier experiments, several poxviruses were compared successfully and some interesting information was gained using this cell system.

CHAPTER 6 THE REPLICATION OF POXVIRUSES IN CEF3

Chick embryo fibroblasts (CEFs) were not investigated by Tytell and Neuman (1960) but primary cultures of chick embryo lung, heart and chorioallantoic membrane were reported to be unable to grow if citrulline replaced arginine. Chick embryo fibroblasts, prepared in this laboratory were also found to have an absolute requirement for arginine and only incorporated very low levels of radioactivity into cell protein from medium containing carbamyl- ^{14}C -citrulline. Since CEFs are known to support the replication of most orthopoxviruses this provided yet another cell system for comparison of the abilities of different orthopoxvirus-infected cells to induce argininosuccinate synthetase/lyase activity, particularly between rabbitpox and cowpox viruses.

1. Characterisation of the Cell System

The metabolic deficiency of the CEFs was demonstrated by measuring the incorporation of carbamyl- ^{14}C -citrulline compared with guanido- ^{14}C -arginine into cell protein. Overall protein synthesis was measured under both conditions and in arginine free medium by the concomitant incorporation of ^3H -leucine.

Confluent monolayers of CEFs were prepared in 4oz. bottles. After depletion of the intracellular pools of arginine the cells were incubated in PME minus arginine containing ^3H -leucine at $0.5\mu\text{Ci/ml}$ with and without guanido- ^{14}C -arginine or with carbamyl- ^{14}C -citrulline replacing arginine, both amino acids at 0.5 mM concentration and $0.1\mu\text{Ci/ml}$. Serum was present at

2%. After 24 hours, the cells were harvested, washed three times in unlabelled PME and the radioactivity associated with the TCA precipitable and soluble fractions was determined.

Table 6.1 shows that the incorporation of ^{14}C radioactivity was reduced by 99% in the presence of citrulline compared with cultures incubated in an equimolar concentration of arginine. Overall protein synthesis was reduced by 75% under arginine free conditions and by 72% when citrulline replaced arginine. The levels of amino acids in the intracellular pools as determined by the radioactivity associated with the TCA soluble fractions were found to be similar, showing that both citrulline and arginine are taken up in the same amounts. The uptake of leucine was also found to be similar in all three conditions.

2. Effect of arginine deprivation on the intracellular pools of CEFs.

Wigand and Kümel (1978) found that if HeLa cells were deprived of arginine, the intracellular levels of the amino acid fell to a minimum within 30 minutes. In this study the effect of withdrawal of exogenous arginine from CEF cultures was measured by similar techniques. CEFs were seeded into 4 oz bottles, maintained in medium containing guanido- ^{14}C -arginine at $0.1\mu\text{Ci/ml}$ for 24 hours and the labelled cells were washed twice before transferring to prewarmed PME minus arginine. At various time intervals the medium was removed, the cultures chilled on ice and washed once with PME minus arginine at 4°C . The cells were resuspended in 3 ml versene, frozen and thawed twice and the radioactivity

TABLE 6.1. Incorporation of ^3H -leucine, guanido- ^{14}C -arginine and carbamyl- ^{14}C -citrulline into TCA precipitable and soluble fractions of CEFs.

	TCA PRECIPITABLE		TCA SOLUBLE	
	^{14}C -dpm /bottle	^3H -dpm /bottle	^{14}C -dpm /bottle	^3H -dpm /bottle
- ARGININE	-	61,955	-	7,604
+ CITRULLINE (0.5 mM)	1,575	70,723	1,973	8,301
+ ARGININE (0.5 mM)	158,925	250,393	1,923	10,429

associated with the TCA soluble fraction determined. The TCA precipitable material from each sample was dissolved in 2N NaOH and retained for protein assays. Figure 6.1 shows the percentage of radioactivity remaining in the soluble fractions after standardising against the protein concentration. A similar result to that of Wigand and Kümel in HeLa cells was observed with CEFs. The level of arginine in the intracellular pools dropped to a minimum within half an hour of arginine deprivation. The arginine concentration however had only been reduced to about 50% of the original level, thus leaving a certain amount available for protein synthesis.

3. Replication of orthopoxviruses in CEFs

a) Infective Centre Titration

In order to determine the appropriate multiplicity of infection required for one step growth conditions in CEFs, an infective centre titration was carried out, as previously described. Figure 6.2 shows the percentage of infected cells with varying multiplicities of infection for rabbitpox and cowpox viruses. It appears that 5 p.f.u./cell is the optimum multiplicity of infection for both viruses since the number of infective centres at 10 p.f.u./cell does not increase further. But under these conditions, only 35% of the monolayer is infected with rabbitpox and 54% with cowpox. However CEFs were very difficult to resuspend to a monocellular suspension, even though they were treated with a trypsin/versene mixture for 30 minutes. This may account for the reduction in the yield of infective centres. Alternatively the cells may have formed multiple layers, thus blocking some from exposure to

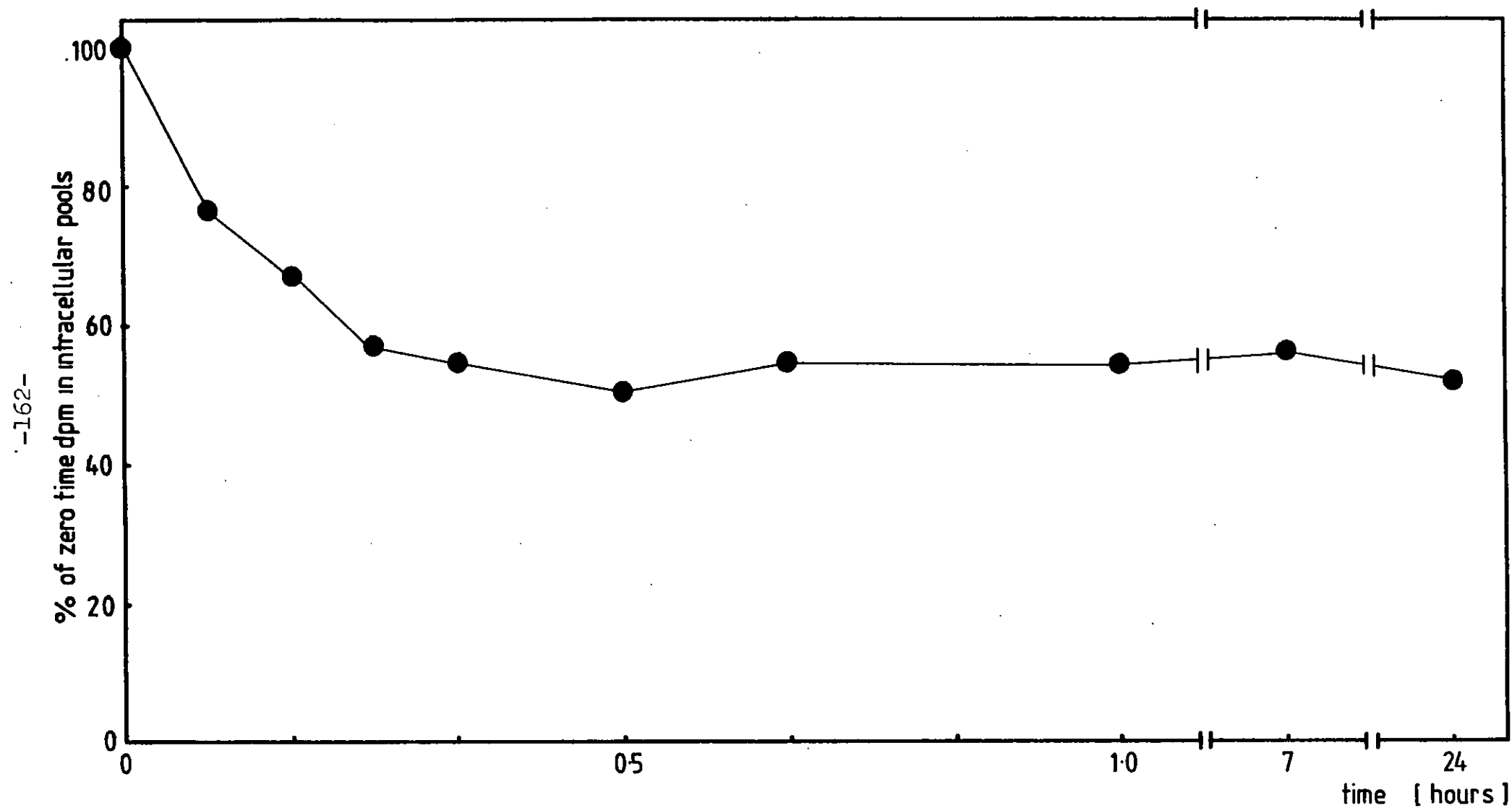
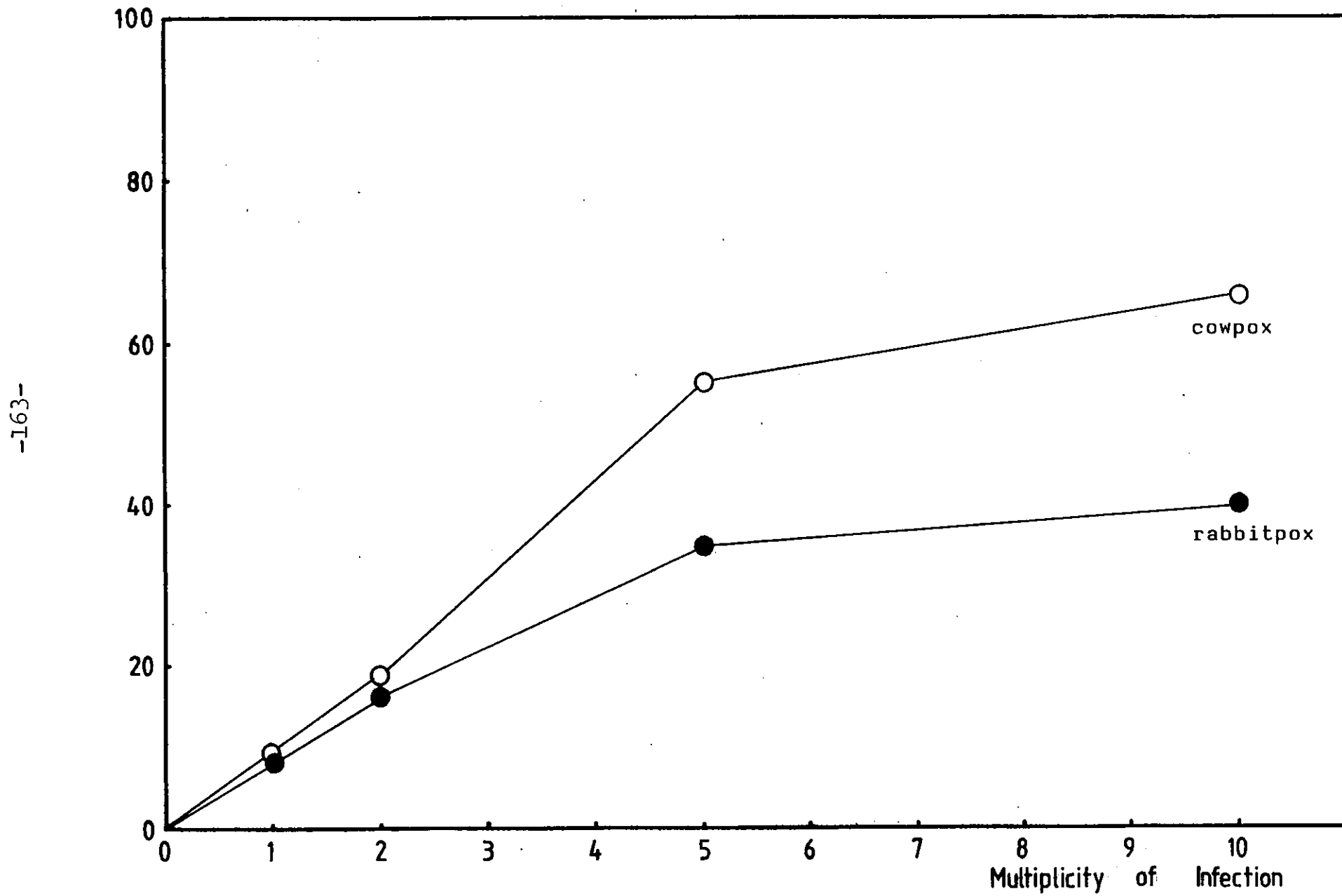


Figure 6.1. Effect of arginine deprivation on the intracellular pools of CEFs.

Figure 6.2. Infective centre titration for rabbitpox and cowpox in CEFs.



virus. This would also reduce the number of infective centres.

b) Dose response of rabbitpox and cowpox infected CEFs to arginine

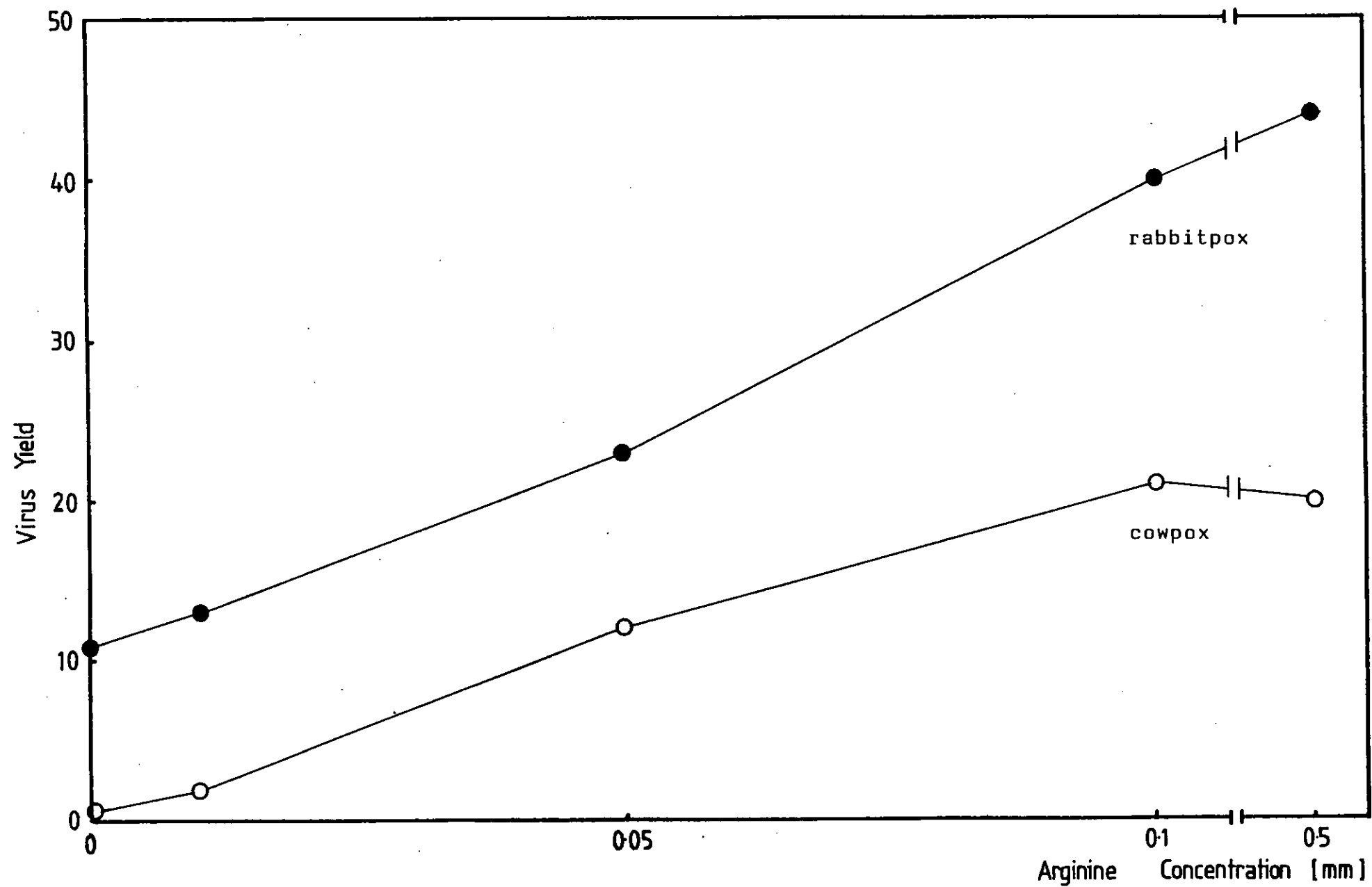
In order to determine the optimum arginine concentration which gives rise to the maximum yield of virus, the dose response of rabbitpox and cowpox infected CEFs to different concentrations of arginine was measured. Confluent monolayers of CEFs were prepared in tubes and were infected with cowpox and rabbitpox viruses. The infected cells were maintained in PME minus arginine supplemented with; 0, 0.01, 0.05, 0.1 and 0.5 mM concentrations of arginine. The yields of virus were measured at 24 hours post infection. The results are shown in figure 6.3. Unlike cowpox, rabbitpox infected CEFs gave a yield of virus in the absence of arginine. The yields of both viruses increased linearly with arginine concentration up to 0.1 mM without further increases at higher concentrations. Thus, 0.1 mM arginine is the minimum concentration required to give an optimum yield and the concentration of 0.5 mM used in all experiments thus ensures that arginine is present in excess.

c) Growth curves of rabbitpox and cowpox in CEFs

The growth characteristics of rabbitpox and cowpox viruses in CEFs were studied by following the replication cycle for 36 hours under one step growth conditions.

Cultures of CEFs in tubes were infected with cowpox or rabbitpox at 5 p.f.u./cell and the virus inocula used were titrated immediately. After adsorption infected cells were maintained in PME with or without arginine and with citrulline

Figure 6.3. Dose response of rabbitpox and cowpox infected CEFs to arginine.



replacing arginine. Both amino acids were at 0.5 mM concentration. The yields of rabbitpox virus in complete medium and with citrulline in place of arginine were measured from samples of infected cultures, recovered every 2 hours for 28 hours post infection and subsequently every 4 hours until 36 hours post infection. Virus yields from infected cells maintained in the absence of arginine were determined from cultures recovered every 6 hours. The yields from cowpox infected CEFs were measured every 2 hours for 28 hours and then at 32 and 36 hours post infection in complete medium. Yields from cowpox-infected cells maintained without arginine or with citrulline were measured at 12, 24 and 36 hours post infection.

The resulting growth curves for rabbitpox and cowpox are shown in figures 6.4 and 6.5 respectively. Both viruses followed a similar growth pattern in complete medium with an eclipse period of about 4 hours followed by production of infectious progeny virus to a maximum virus yield at 24 hours post infection. After this time the titres began to decline, indicating that some thermal inactivation was taking place.

With cowpox infected CEFs there was no yield of virus unless arginine was present. In rabbitpox infected cells a low background yield in the absence of arginine was observed. In the presence of citrulline, the rabbitpox growth curve initially followed the same kinetics as in complete medium, but subsequently formed a plateau at 12 hours at a slightly higher titre than that in the absence of arginine.

Figure 6.4. Growth curve for rabbitpox in CEFs plus and minus arginine and with citrulline.

- 0.5mM arginine.
- ▲—▲ 0.5mM citrulline.
- minus arginine.

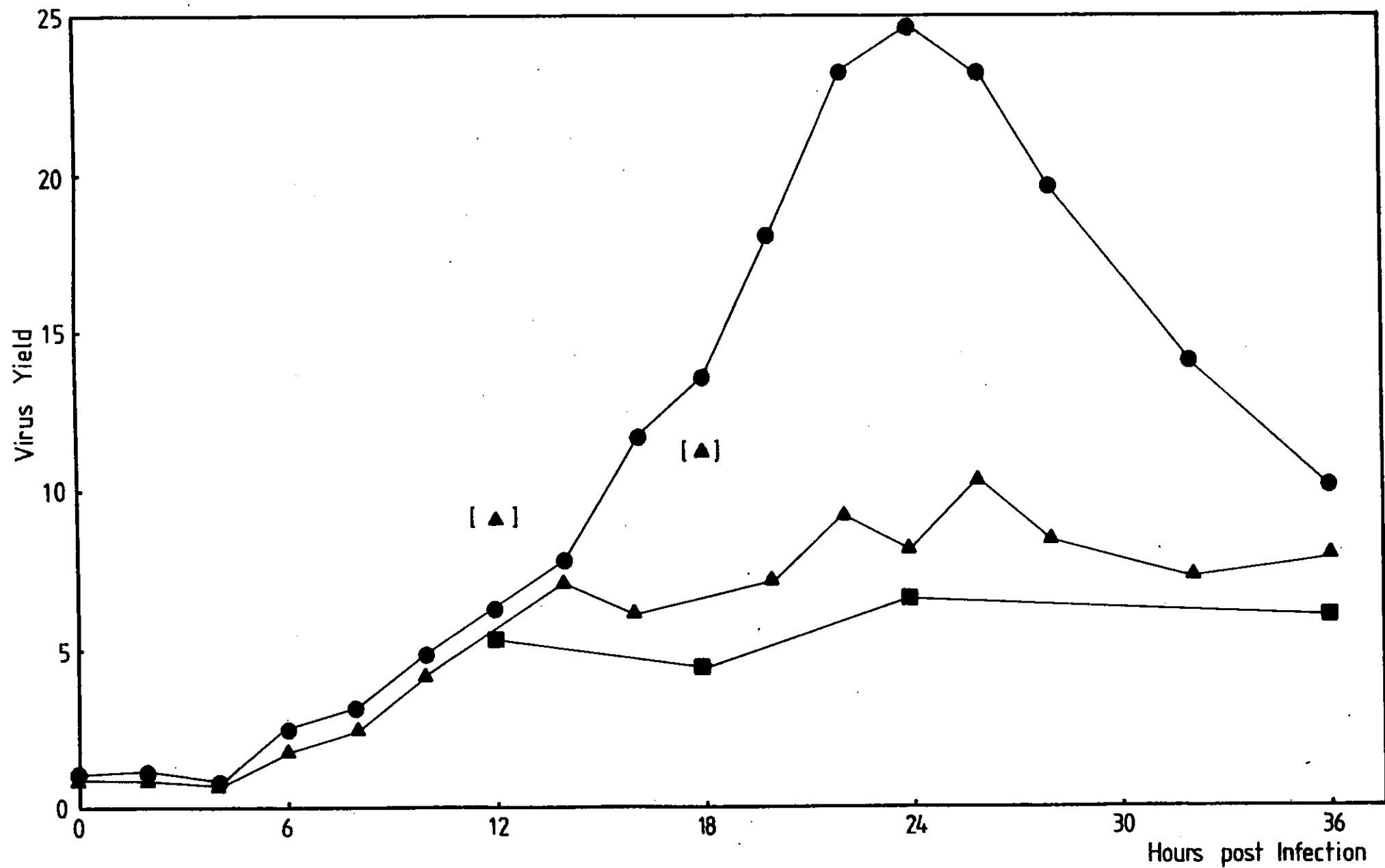
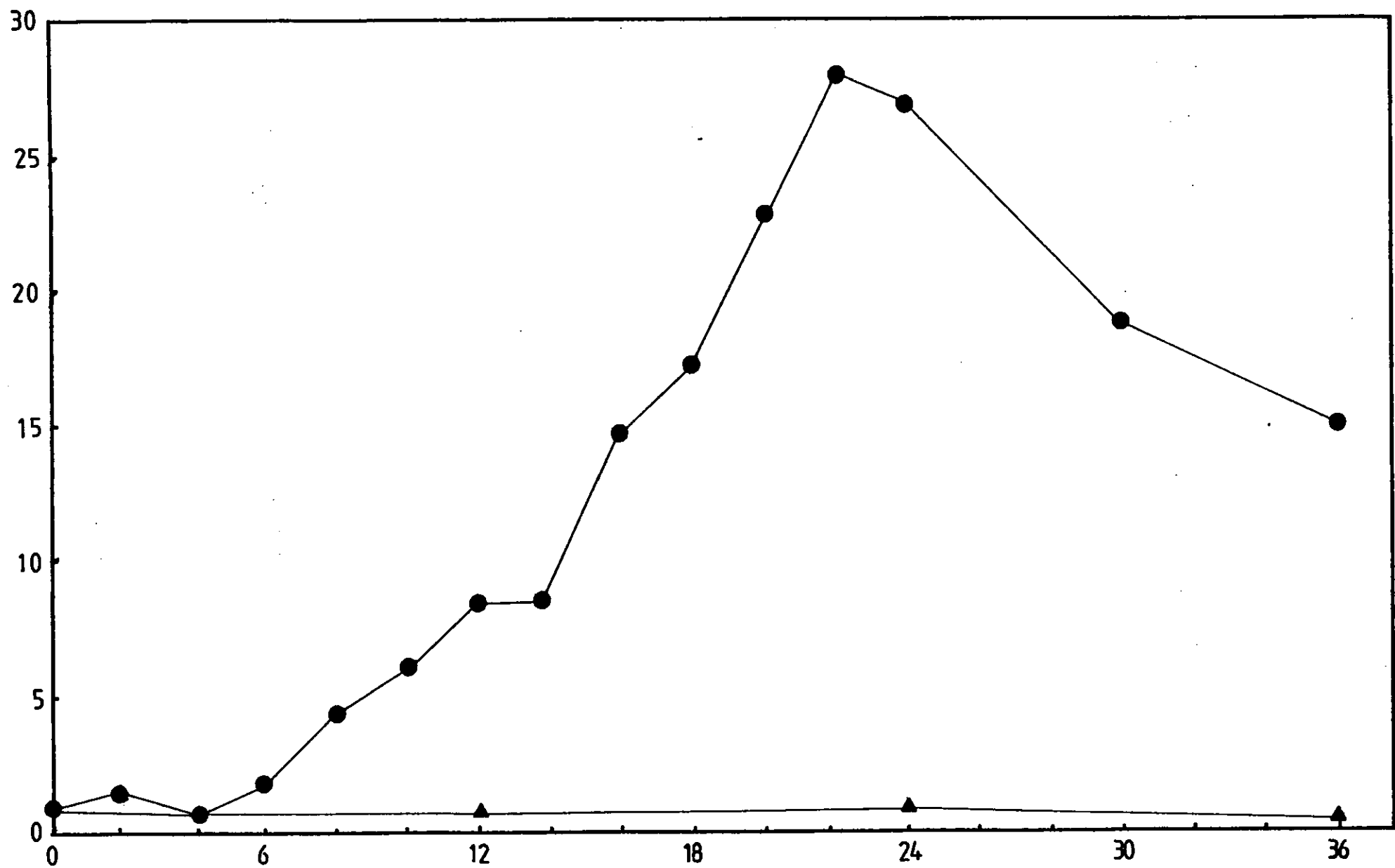


Figure 6.5. Growth curve of cowpox in CEFs
in PME plus and minus arginine
and with citrulline.

●—● 0.5mM arginine.
▲—▲ 0.5mM citrulline.
and minus arginine.



d) Comparison of poxvirus yields in CEFs

The yields of 8 orthopoxviruses were compared in CEFs in the presence and absence of arginine and if citrulline replaced arginine.

Confluent monolayers of CEFs were prepared in tubes and infected with different poxviruses at approximately 10^5 p.f.u./cell and incubated in PME with and without arginine or with citrulline, both amino acids being at 0.5 mM concentration. The virus yields were obtained at 24 hours post infection and are shown in figure 6.6. The actual virus yields in the absence of arginine, with citrulline and with arginine are shown in histograms a, b and c and yields without arginine and with citrulline are expressed as a percentage of the yield with arginine in histograms d and e. The results are taken from the averages of a series of experiments. The yields in complete medium varied from camelpox and monkeypox which were about 500 down to ectromelia with a yield of about 30. Although the yields of rabbitpox and ectromelia were relatively low in complete medium, these viruses were able to produce significant yields under arginine deprived conditions being about 13% and 18% of their respective yields with arginine. The other poxviruses gave much lower yields in arginine free medium compared with those in complete medium. The ability to utilise citrulline was found not to be an "all or nothing" effect, but this varied from rabbitpox which produced a yield with citrulline which was 20% of that with arginine to cowpox with 1% of the yield in complete medium. All the poxviruses gave slightly higher yields with citrulline than in the absence of arginine apart from cowpox and monkeypox.

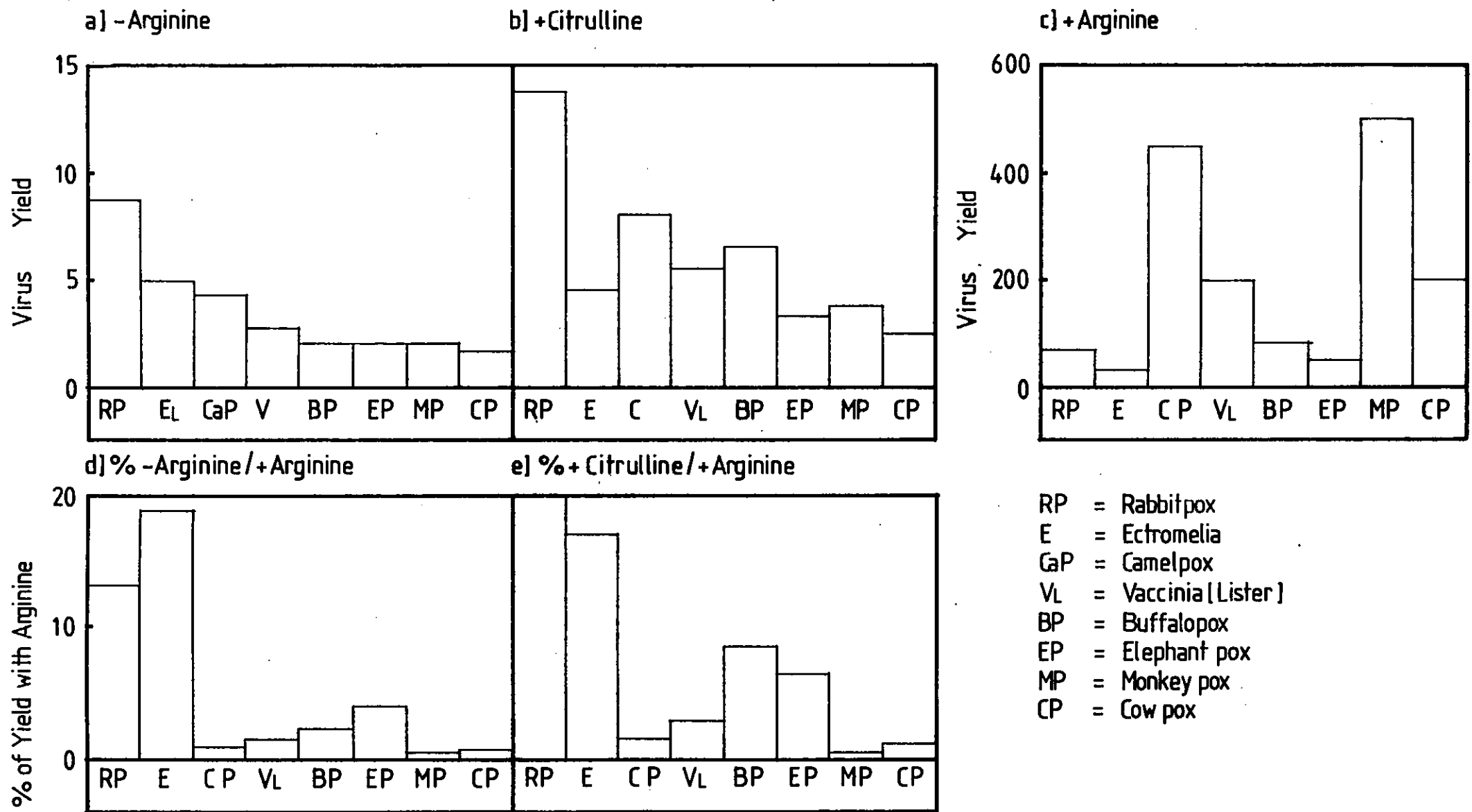


Figure 6.6. Comparison of poxvirus yields in CEFs plus and minus arginine and with citrulline.

e) Virus utilisation of host arginyl residues.

Since rabbitpox is able to produce a yield of progeny virus in the absence of any exogenous arginine, it is possible that the arginine essential for replication is derived from breakdown of host cell protein. To investigate this possibility an experiment was carried out to try to detect radioactive virus specific protein under arginine deprived conditions from infected cells which had been labelled before infection with ^{14}C -arginine. However it was necessary to use a method of purification which eliminates as much host cell material as possible.

The technique attempted used an immunoabsorbent consisting of antivaccinia serum bound to microcrystalline cellulose, activated with cyanogen bromide. Mixing the immobilised, virus specific antibodies with extracts from infected cells binds virus specific antigen and thorough washing of the complex eliminates host-specific cell material.

The immunoabsorbent was prepared as described in Materials and Methods. CEF cultures were prepared in 16 oz medical flat bottles and labelled with guanido- ^{14}C -arginine. After infection with rabbitpox virus under one step growth conditions, one series of infected cultures were maintained for 48 hours in PME without arginine. A second series was sham infected and maintained under similar conditions. A third series of rabbitpox infected cultures was maintained in complete medium containing guanido- ^{14}C -arginine at $0.5\mu\text{Ci/ml}$. Cells from infected and sham infected cultures were recovered into 2 ml PBS 'A' and uninfected cells labelled with ^3H -leucine added to infected cells to act as an internal control to show the presence of host cell material. After two cycles of freezing

and thawing the cell suspensions were treated with ultrasonics and the crude cell debris pelleted by low speed centrifugation. The supernatant extracts were mixed with approximately 100 mg immunoabsorbent in a Petri dish in 8 ml coupling buffer and kept for 12 hours at 4°C. After washing several times with coupling buffer until there was no radioactivity present in the washings, the immunoabsorbent suspension was dissolved in 1 ml solubene and the total amount of radioactivity present determined. The results from the first experiment are shown in table 6.2 i). Sample A is the test material, extract from rabbitpox infected cells, labelled before infection with ^{14}C -arginine and maintained subsequently in arginine free medium. Sample B from rabbitpox infected cultures maintained in the presence of ^{14}C -arginine would be expected to contain ^{14}C radioactivity in virus specific antigen. Samples A and B were mixed with uninfected cells labelled with ^3H -leucine and the presence of ^3H radioactivity indicates non-specific binding of host cell material, which may be caused by insufficient washing. This is indicated by the third sample C, an extract of uninfected CEFs labelled with ^{14}C -arginine. Since there is no virus-specific antigenic material in this sample, radioactivity associated with the immunoabsorbent would not be expected unless non-specific binding was taking place.

The experiment was repeated and only a fraction of the immunoabsorbent was analysed for radioactivity. The results again showed substantial amounts of ^3H radioactivity present with both infected cell extracts. The remainder of the immunoabsorbent was washed further with 1 M NaCl for 3 hours at 4°C. Although this did not result in elimination of

Table 6.2. Radioactivity associated with immunoadsorbent.

Sample A - extract from rabbitpox-infected CEFs, prelabelled before infection with guanido- ^{14}C -arginine and maintained in the absence of arginine for 48 hours + uninfected CEFs labelled with ^3H -leucine.

Sample B - extract from rabbitpox infected CEFs maintained with guanido- ^{14}C -arginine for 48h + uninfected CEFs labelled with ^3H -leucine.

Sample C - extract from sham-infected CEFs labelled with guanido- ^{14}C -arginine before infection and maintained in the absence of arginine for 48 hours.

i) First Experiment

	^{14}C -dpm	^3H -dpm	$^{14}\text{C}/^3\text{H}$
A	5,231	13,712	0.38
B	24,525	3,099	7.90
C	60,047	-	-

ii) Second Experiment

A	1,232	3,320	0.37
B	1,266	422	3.0
C	6,880	-	-

1st Salt Wash

A	1,013	1,782	0.56
B	1,741	442	4.0
C	3,770	-	-

2nd Salt Wash

A	3,096	5,512	0.7
B	5,669	1,766	3.2
C	2,662	-	-

^3H radioactivity there was a slight increase in the ratio of ^{14}C to ^3H in the test and positive control samples. After a second wash with salt for 12 hours at 4°C the ratio increased further. This indicates that although non-specific binding could not be eliminated, ^{14}C radioactivity tended to be retained compared with ^3H activity. This suggests that the virus specific antigen from rabbitpox infected CEFs labelled before infection with ^{14}C -arginine and maintained subsequently under arginine free conditions may contain ^{14}C -arginyl residues derived from host cell protein.

4. DNA Synthesis in rabbitpox and cowpox infected CEFs.

The extent of DNA synthesis occurring in rabbitpox and cowpox infected CEFs was investigated under conditions of arginine deprivation in a similar way to that previously described with the SI80 cell system in Chapter 5. The amounts of virus specific DNA synthesised were determined by the incorporation of ^3H -thymidine into TCA precipitable material of cytoplasmic and perinuclear fractions of virus infected cells at 24 hours post infection. The cells were prelabelled with ^{14}C -thymidine to act as a reference for the amount of cell material present in each sample and to ensure that the cytoplasmic fractions were not contaminated with nuclear material.

Monolayers of CEFs in 4 oz medical flat bottles were prelabelled for 24 hours with complete medium (MEM) containing ^{14}C -thymidine at $0.1\mu\text{Ci/ml}$. These cultures were infected or sham infected with rabbitpox or cowpox under one-step growth conditions and incubated in PME containing ^3H -thymidine

at $0.5 \mu\text{Ci/ml}$ to a final concentration of $20 \mu\text{M}$. This labelled medium was used with and without arginine and with citrulline replacing arginine. At 24 hours post infection the cells were fractionated by detergent treatments as previously described and the radioactivity associated with the TCA precipitable material from each fraction determined.

The CEF cells were found to take up the ^{14}C -thymidine much more readily than the SI80 cells. Table 6.3 shows the incorporation of ^3H -thymidine after normalising against the ^{14}C -radioactivity present in each sample. In the sham infected cultures there is a low level of ^3H -radioactivity in the cytoplasmic and perinuclear fractions probably associated with host cell mitochondrial DNA. In the virus infected cells the levels of ^3H radioactivity are much higher in these fractions

In rabbitpox infected cells, the amount of DNA synthesis occurring in the absence of arginine in all three fractions is about 45% of that in complete medium. When citrulline replaces arginine this increases to about 75% showing that the presence of citrulline allows further DNA synthesis to take place. In cowpox infected cells in the absence of arginine the synthesis of DNA is 43% in the cytoplasmic and 56% in the perinuclear fractions of that in the presence of arginine. Unlike rabbitpox infected cells, in the presence of citrulline there is no further increase in the amount of ^3H -thymidine incorporated, showing again that cowpox is unable to utilise this amino acid. The incorporation of ^3H -thymidine into nuclear fractions of infected cultures was similar under all conditions.

Table 6.3. Biochemical measurement of DNA synthesis in rabbitpox and cowpox infected CEFs.

	CYTOPLASMIC (³ H-dpm)	PERINUCLEAR (³ H-dpm)	NUCLEAR (³ H-dpm)
Sham-infected	58,900	63,692	127,098
- Arginine	41,700	85,354	116,385
Sham-infected	71,465	74,132	192,758
+ Arginine	88,406	79,253	240,703
Rabbitpox	135,248	97,766	400,547
- Arginine	130,597	76,672	314,529
Rabbitpox	217,643	143,299	592,908
+ Citrulline	191,764	167,830	648,702
Rabbitpox	261,718	179,598	779,331
+ Arginine	291,673	215,475	790,840
Cowpox	185,289	142,321	751,793
- Arginine	196,002	144,165	716,531
Cowpox	177,643	143,796	533,237
+ Citrulline	166,558	141,088	590,142
Cowpox	424,754	218,933	762,438
+ Arginine	444,713	239,588	613,214

5. Discussion

Chick embryo fibroblasts were found to be unable to grow when citrulline replaced arginine and incorporated very low levels of citrulline into cell protein compared with arginine, even though both amino acids are taken up in similar amounts. This indicated that CEFs are unable to express argininosuccinate synthetase/lyase activity. Since CEFs are known to support the replication of many poxviruses, this provided yet another cell system with which to compare the abilities of different poxvirus-infected cells to induce argininosuccinate synthetase/lyase activity.

The effect of arginine deprivation on the intracellular pools of CEFs was investigated. Wigand and Kümel (1978) found that when HeLa cells were placed on an arginine free medium, the level of the amino acid fell to a minimum within 30 minutes. This was also found to be true with CEFs although the arginine level was only reduced by 50%, even after 24 hours. This suggests that depletion of intracellular pools as described in Materials and Methods is not really necessary before infecting cells since the inoculum is always adsorbed for one hour from a balanced salt solution lacking arginine, and arginine requiring events are known not to take place during the first 30 minutes of poxvirus replication (Archard & Williamson, 1971). However, since only 50% of the intracellular arginine is removed, this low level present must be taken into account in interpreting results.

An infective centre titration revealed that 5 p.f.u./cell is the optimum multiplicity of infection for both rabbitpox and cowpox viruses in CEFs although it appeared that 100%

of the monolayer was not infected even at high multiplicities of infection. The reduction in infective centres may have been due to difficulty in producing a monocellular suspension from infected CEF monolayers, even though they were exposed to enzyme treatment to facilitate resuspension. Alternatively the cells may have formed multiple layers thus blocking some from exposure to the virus. Since the CEFs are a primary culture, there may have been a mixed population of cells resulting in only some being susceptible to infection. This is less likely than with CAM cells since CEFs are all similar fibroblastic type cells.

The dose response of cowpox and rabbitpox viruses to arginine was investigated and the minimum concentration which resulted in the optimum yield was found to be 0.1 mM for both viruses. This is a similar concentration to that required for poxvirus replication in other cell systems such as SL80 (Cooke, 1973) and HeLa (Archard, 1971). This showed that the concentration of 0.5 mM used in all following experiments ensured that excess arginine was present.

The replication of rabbitpox and cowpox viruses in CEFs was followed over a 36 hour period in the presence and absence of arginine and with citrulline in place of arginine. In complete medium, both viruses underwent an eclipse phase of about 4 hours followed by increases in infective virus titre with maximum levels at 24 hours post infection. After this the titres of both viruses began to fall, indicating that some thermal inactivation had taken place. Under arginine deprived conditions or when citrulline replaced arginine, there was no increase in the yield of cowpox. But with rabbitpox a low yield was obtained in the absence

of arginine and there was a slightly higher yield with citrulline. Replication of rabbitpox in the presence of citrulline appeared to follow similar kinetics to that in complete medium for the first 12 hours, but the subsequent virus titre did not increase further. This suggests that in rabbitpox virus infected CEFs, when no exogenous arginine is present, a pool of arginine becomes available inside the cell which permits a limited amount of virus replication and in the presence of citrulline, further virus replication is effected by the limited provision of arginine from citrulline indicating the presence of some argininosuccinate synthetase/lyase activity. Since neither process occurs with cowpox infected CEFs, these events must both result from rabbitpox virus directed mechanisms.

The abilities of 8 orthopoxviruses; rabbitpox, ectromelia, camelpox, vaccinia (Lister), buffalopox, elephantpox, monkeypox and cowpox to replicate in CEFs under arginine limiting conditions were compared. Rabbitpox and ectromelia were found to give relatively high yields in the absence of arginine compared with other viruses. The ability to utilise citrulline was not an "all or nothing" effect, but varied from rabbitpox, which gave 20% of the yield with arginine, to cowpox, which gave 1% of the yield in complete medium. All the poxviruses gave slightly higher yields in CEF cultures maintained with citrulline than in the absence of arginine indicating some argininosuccinate synthetase/lyase activity, albeit at very low levels, especially cowpox infected cultures.

Since rabbitpox and ectromelia are able to produce infective progeny in the absence of exogenous arginine, the arginine

essential for replication must be derived either from de novo synthesis or from breakdown of host cell protein. As de novo synthesis cannot occur in uninfected cells and has not been found to occur in any other type of cell infected with rabbitpox, this is unlikely to be the reason. The possibility of utilisation of host arginyl residues was investigated by the demonstration of virus-specific antigens containing ^{14}C -radioactivity from rabbitpox infected CEFs labelled before infection with ^{14}C -arginine and maintained after infection in the absence of arginine. Extracts from such cultures combined with extracts from uninfected ^3H -labelled cells, were mixed and allowed to react with an immunoabsorbent consisting of an antivaccinia serum bound to microcrystalline cellulose. By thorough washing of the complex, it was hoped to be able to detect virus antigen labelled with ^{14}C -radioactivity derived from arginyl residues in host cell protein. However despite thorough washing, much nonspecific binding to the immunoabsorbent occurred. But analysis of samples after consecutive washings revealed that ^{14}C radioactivity tended to be retained rather than ^3H , suggesting that the virus antigen which was also more likely to be retained by the immunoabsorbent did contain arginyl residues derived from host cell protein. If arginine was derived from this source, the breakdown of cell protein could either be spontaneous due to degeneration of the primary cell culture or alternatively it may be a virus directed mechanism. If the former were true, other poxviruses such as cowpox would be able to capitalise on the formation of the arginine pool. But since this does not occur and cowpox does not have a greater requirement for arginine, it is more

likely that rabbitpox and ectromelia have some virus mediated lytic ability which cowpox lacks.

The extent of DNA synthesis occurring in rabbitpox and cowpox infected CEFs with and without arginine and with citrulline was investigated by the concomitant incorporation of ^3H -thymidine into TCA precipitable material from cytoplasmic fractions of infected cells. Unlike SI80 cells, CEFs readily assimilated ^{14}C -thymidine into their DNA. Thus ^{14}C -labelled host cell nuclei could be used as a reference for the amount of cell material present and would ensure that nuclei had not ruptured during the detergent treatments.

In rabbitpox infected CEFs maintained in arginine free medium, DNA synthesis was reduced to about 45% of that in complete medium. When citrulline replaced arginine, the level of DNA synthesis increased to 75%. In cowpox infected cells a reduced level of DNA synthesis occurred in the absence of arginine but no further increase was observed in medium containing citrulline. These results again indicate that rabbitpox infected CEFs are able to utilise citrulline whereas cowpox infected cells are not.

Arginine deprivation has been shown to markedly suppress DNA synthesis in vaccinia infected HeLa cells (Archard & Williamson, 1971) but to a lesser degree in vaccinia infected KB cells (Obert, Tripier & Guir, 1971). Thus the extent of DNA synthesis occurring in the absence of arginine depends on the host cell system used.

Comparing the extents of DNA replication with the growth curves for rabbitpox and cowpox under each condition, it appears that the viruses undergo similar levels of DNA synthesis under arginine free conditions, but that only

rabbitpox is capable of producing a subsequent yield of infective progeny virus.

In the presence of citrulline, the extent of DNA synthesis increases and the resulting yield of rabbitpox is also higher than in the absence of arginine. Although cowpox undergoes a similar level of DNA synthesis under arginine deprivation, no infective progeny is produced. It is possible that cowpox replication under these conditions leads to the formation of defective particles. However, if citrulline replaces arginine there is no increase in DNA synthesis or virus yield in cowpox-infected cells.

The extent of DNA synthesis of both cowpox and rabbitpox viruses in CEFs in medium lacking arginine may be affected by the residual amount of arginine in the intracellular pools following withdrawal of exogenous arginine. This low level may be sufficient to permit limited DNA synthesis with both viruses, since Archard & Williamson (1971) showed that the level of arginine necessary for DNA synthesis in vaccinia infected HeLa cells was quantitatively lower than that for later events in the replication cycle. However rabbitpox appears to be able to obtain additional arginine for its replication, when the medium lacks arginine, possibly by utilisation of host arginyl residues. This virus also shows an ability to utilise citrulline to a limited extent which indicates some induction of argininosuccinate synthetase/lyase activity. Cowpox appears to have neither of these characteristics. Other poxviruses studied appeared to fall somewhere between these two, not exhibiting an "all or nothing" effect.

CHAPTER 7

GENERAL DISCUSSION

The objective of the work presented in this thesis was to make comparisons between different orthopoxviruses in their ability to replicate following the substitution of citrulline for arginine supplied to cell systems which cannot utilise citrulline, and thus indicate the presence of virus induced argininosuccinate synthetase/lyase activity. The results obtained have shown differences in the ability to utilise citrulline, but also, with some viruses, in their facility for replication in the absence of arginine or citrulline. Since arginine has been shown to be essential for vaccinia virus replication in HeLa cells (Archard & Williamson, 1971) this was an unexpected observation and with many viruses studied, it masked the result of replication with citrulline.

The viruses studied were; vaccinia (Lister and IHD strains), rabbitpox, cowpox, anteatpox, cheetahpox, buffalopox, camelpox, elephantpox, monkeypox and ectromelia (mousepox).

In the CAM cell system the virus yields were very low and no distinctive comparisons could be made. Rabbitpox however was able to replicate to the same degree with or without arginine. The growth of rabbitpox virus in CAM fragments showed similar results but again, very low yields were obtained with other poxviruses in the absence of arginine. In the preparation of rabbitpox virus stocks from infected CAMs a low inoculum must be used to prevent chick embryo mortality. This high virulence and the replication of rabbitpox virus in cell cultures of chick embryonic tissue in the absence of exogenous arginine may be related.

In the BHK cell system eight poxviruses were compared. The results obtained fell into two groups, those viruses unable to replicate unless arginine was present; cowpox, buffalopox, elephantpox, anteater pox, cheetahpox and camelpox; and the remaining rabbitpox and vaccinia (Lister) which could replicate to a limited extent in the absence of arginine and gave slightly higher yields with citrulline, suggesting some utilisation of citrulline.

In the S180 cell system, ten poxviruses were compared and these fell into four groups. The first contained only rabbitpox, which gave no yield in the absence of arginine and a yield with citrulline which was 10% of the yield with arginine. In the second group, vaccinia (IHD) and ectromelia gave yields in the absence of arginine and with citrulline. Ectromelia gave very high yields in S180 cells and this could again be due to the "virulence" of the virus in these cells since they are derived from mouse tissue and ectromelia's natural host is the mouse. ("Virulence" used in this context and elsewhere in this thesis refers to the ill-defined virus-host cell interactions which lead to greater or lesser yields of progeny virus.) In the third group, cowpox, anteaterpox, cheetahpox, elephantpox and camelpox gave no yield unless arginine was present. Finally, the fourth group contained vaccinia (Lister) and buffalopox which are unable to produce any infective progeny (i.e. non-permissive) in S180 cells in complete medium, although adsorption, penetration and DNA synthesis were shown to take place.

In the CEF system, the results obtained were similar to those in CAM cells but there was better resolution of the results and the virus yields obtained were higher. Again,

rabbitpox and ectromelia were found to produce significant yields under arginine deprived conditions.

The results obtained clearly depended upon the host cell system used and the differences in the yields obtained with the same virus in different systems are probably a reflection of the virulence of the virus for the particular type of cell.

However, replication of the different poxviruses in the four cell types did fall into similar categories in each cell system, indicating that the ability to utilise citrulline or to produce infective progeny in the absence of arginine is a virus-specific phenomenon rather than due to the phenotypic character of the cells.

Overall comparisons showed that the orthopoxviruses investigated fell into two main groups. Rabbitpox, ectromelia, vaccinia (IHD) and vaccinia (Lister) all appeared to behave in a similar way in the cell systems studied, and could be called "rabbitpox-like" viruses. They all seem to be able to undergo limited amounts of replication in the absence of exogenous arginine, rabbitpox and vaccinia (Lister) in BHK 21 cells, vaccinia (IHD) and ectromelia in SL80 cells and rabbitpox also in CAM cells. They also show a limited ability to utilise citrulline. Vaccinia (Lister) can be distinguished from the others by its inability to grow in SL80 cells in complete medium. Ectromelia gave very low yields in CAM and CEF cells and rabbitpox differs from ectromelia and vaccinia (IHD) in that it does not produce infective progeny in SL80 cells in the absence of arginine only with citrulline or arginine.

The second group, the "cowpox-like" viruses, contains

the remaining poxviruses examined; cowpox, elephantpox, anteaterpox, cheetahpox, camelpox, monkeypox and buffalopox. These viruses all behave in a similar way and differ from the "rabbitpox-like" viruses. In SI80 cells and BHK 21 cells they all have an absolute requirement for arginine and show a much lower tendency to be able to replicate in CEF and CAM cells in the absence of arginine. They all showed little, if any, ability to utilise citrulline. Buffalopox may be an exception in this group and differs from the other viruses in that it cannot grow in SI80 cells in complete medium. Also, buffalopox gives significantly higher yields in the presence of citrulline than in arginine deprived conditions in both CEF and CAM cells. Although no utilisation of citrulline appears to occur in BHK 21 cells, thus grouping it with cowpox, buffalopox appears to be the nearest of the group to the "rabbitpox-like" viruses.

This categorisation agrees with similar comparisons made by other methods that have been reported. Vaccinia and rabbitpox have been shown to be very closely related and have similar restriction enzyme patterns with *Hin* dIII and *Sst* I (Wittek et al, 1977). Also carnivore (anteater) pox has been grouped with cowpox using polypeptide analysis (Harper et al, 1979). Poxviruses isolated from captive elephants were found to be much more like cowpox than vaccinia by using biological methods (Baxby & Ghaboosi, 1977).

Buffalopox has been shown to have some similarities to vaccinia (Baxby, 1975). Its pox morphology on the CAM is more like vaccinia than cowpox, it does not contain 'A' type inclusions and does not produce diffusible 'LS' antigen in the CAM, both of which are characteristic of vaccinia and

not of cowpox. Gispen and Brand-Saathof (1974) also reported that serologically, buffalopox was more like vaccinia and rabbitpox than cowpox.

Thus the criteria for comparison used in this study appear to have some significance, since the results obtained suggest similar groupings to those previously reported using completely different methods of comparison.

Molecular basis for arginine metabolism in
orthopoxvirus replication.

Arginine has been shown to be essential for vaccinia virus replication in HeLa cells (Archard & Williamson, 1971) and in KB cells (Obert, Tripier & Guir, 1971). In the absence of exogenous arginine no virus yield was obtained. Citrulline could be used as a source of arginine for the growth of these cultures since the host cells can express argininosuccinate synthetase/lyase activity.

The results presented here show that vaccinia and other "rabbitpox-like" viruses are able to utilise citrulline in cells which do not express argininosuccinate synthetase/lyase activity. These viruses also appear to obtain a source of arginine for replication in the absence of exogenous arginine or citrulline.

The virus yields obtained in the presence of citrulline were very low compared with the yields in complete medium. Rabbitpox was the only virus which, when grown in SL80 cells, gave a yield with citrulline and no yield in the absence of arginine. All other virus/cell systems able to utilise citrulline gave low background yields in the absence of either amino acid, which masked the results with citrulline.

In previous work (Cooke & Williamson, 1973) rabbitpox gave much higher yields in SL80 cells when citrulline replaced arginine, about 90% of that in complete medium. The low yield obtained with citrulline in this study was probably due to unresolved changes in the nutritional conditions. An important growth factor from the serum used in the original experiments may have been necessary for the

induction of the enzyme activities.

When neither citrulline nor arginine was present in the medium, a limited amount of infective progeny was produced with the "rabbitpox-like" viruses in some cells. The source of arginine necessary for this to occur could either be due to de novo synthesis of arginine or alternatively to utilisation of host arginyl residues. De novo synthesis is unlikely to occur since this has not been reported in any other poxvirus-infected cell system. Arginine is only known to be synthesised via the Krebs-Henseleit urea cycle in mammalian cells in vivo. No mammalian cells in vitro are able to undergo de novo synthesis of arginine since the initial step involving the conversion of ornithine to citrulline occurs only with liver mitochondria. Also two of the cell systems used in this study were derived from avian tissue and the urea cycle does not occur in birds since a different method of excretion of nitrogen is in operation involving the formation of uric acid.

Utilisation of host arginyl residues is much more likely to have provided the source of arginine necessary for virus replication. Since only a small amount of replication occurred, this shows that a limited amount of arginine was available.

The formation of a pool of arginyl residues may result from 'autolysis' of the cells. This may occur particularly in CAM and CEF cells since they are primary cell cultures and the monolayers would tend to degenerate after a limited period of incubation. This may account for the yields obtained by nearly all the poxviruses in the absence of exogenous arginine in these cells. A similar phenomenon

was observed by Gonzol, Jeney & Vaczi (1967) when studying the effects of arginine deprivation in herpes simplex viruses. In continuous cell lines, HeLa, Hep 2 and KB cells there was no yield in the absence of arginine but in primary cultures of monkey kidney and human embryo fibroblasts, there was a small amount of replication in the absence of arginine. This was attributed to the degree to which arginine deprivation affects cell metabolism.

With the continuous cell lines SI80 and BHK 21 cells, only the "rabbitpox-like" viruses were able to replicate in the absence of arginine. These viruses would appear to obtain their arginine by another means, since autolysis is less likely to occur in these cells.

The ability of a particular poxvirus to replicate in the absence of arginine may depend on the amount of proteolytic activity present in the virus infected cells. The time taken before host protein synthesis is switched off following virus infection may be important. This is caused by a component of the virus particle (Shatkin & Moss, 1968) and different poxviruses may differ in the potency of expression of this function. The degradation, which balances synthesis/turnover of protein, may continue after protein synthesis is switched off and result in the formation of a pool of arginyl residues. However, if host protein synthesis continues after infection, a pool of arginine is less likely to form. These virus-mediated factors may determine "virulence" in a particular cell type.

Conversely, virus infection may accelerate host protein breakdown by producing additional lytic enzymes. An alkaline protease activity has been detected in vaccinia virus

(Arzoglou, Drillien & Kirn, 1979). This enzyme appears during late events associated with the virus assembly process and envelopment. The structural proteins have been shown to be synthesised as large polymer precursors which are then cleaved by this enzyme to form the structural units. Such proteolytic activity might result in the formation of a pool of utilisable arginyl residues from host cell protein, coinciding with the requirement for arginine at particular stages of the virus replication cycle and allow a limited yield of progeny to be formed. Although an earlier requirement for arginine has been described in vaccinia-infected HeLa cells (Archard & Williamson, 1971) associated with DNA synthesis, it was not reported to be necessary for vaccinia DNA synthesis in KB cells (Obert, Tripier & Guir, 1971).

Virus specific DNA replication was studied in S180 cells and CEFs infected with rabbitpox or cowpox viruses under arginine deprived conditions and with citrulline replacing arginine to compare one virus from each of the two groups of poxviruses defined in this study. Under arginine deprived conditions, both viruses underwent similar levels of virus-specific DNA synthesis in each cell system, reduced to 45% in CEFs and 20% in S180 cells compared with complete medium. Thus an early requirement for arginine at during DNA synthesis was sustained to the same extent for both viruses. However in CEFs, only rabbitpox was able to produce any infective progeny virus from the DNA synthesised in the absence of arginine, again showing that during late events in the replication cycle, rabbitpox was able to obtain a source of arginine unaccessable to cowpox.

In the presence of citrulline there was no increase in the amount of virus-specific DNA synthesised in cowpox-infected CEFs or SL80 cells. In cowpox-infected SL80 cells incubated either without arginine or with citrulline, discrete cytoplasmic inclusions were clearly visible as late as 24 hours post infection. Such inclusions are normally present between 4 - 8 hours post infection in complete medium, after which time they gradually disappear and become diffuse as replication progresses. This indicates that in the absence of arginine the replication of cowpox in SL80 cells is arrested between 4 - 8 hours post infection. In rabbitpox-infected SL80 cells maintained in arginine deprived conditions cytoplasmic DNA-containing inclusions become diffuse indicating normal progression of the infection.

In the presence of citrulline, rabbitpox virus DNA synthesis increased to 75% in CEFs and 45% in SL80 cells compared with complete medium. This again indicates that argininosuccinate synthetase/lyase activity is present and that it is induced during early events in the replication cycle since further DNA synthesis occurs if citrulline is present. However the enzyme activity is not sufficient to cater for the much greater requirement during later events when newly synthesised virus DNA undergoes encapsidation since the yield of infective progeny virus from rabbitpox-infected SL80 or CEF cultures maintained with citrulline is only 10% of the yield obtained in the presence of arginine.

A further point which may be considered is whether the DNA synthesised under arginine deprivation results in the formation of a reduced number of complete DNA molecules or whether synthesis of a large number of non-functional,

partial genomes occurs. In arginine deprived herpes simplex-infected BSC1 cells, virus-specific DNA synthesis is not reduced but no virus progeny is formed (Becker et al, 1978). Closer examination of the structure and size of the DNA synthesised revealed molecules of different lengths, some circular, some linear and some with branches, the latter not previously reported.

Virus-specific DNA replication in rabbitpox-infected CEFs was reduced to 45% in the absence of arginine. Of that 45% only a quarter may have been complete molecules, the remainder being partial genomes. This would result in the 10% of the total DNA forming infective progeny, irrespective of the concentration of arginine during later maturation events. This could account for no increase in virus yields in the biological experiments with DNA inhibitors, adding FUdR and arginine after 12 hours incubation without arginine and allowing any complete DNA molecules to mature in the presence of excess arginine, but preventing further DNA synthesis.

Even though cowpox DNA replication was only reduced to 50% in CEFs in the absence of arginine, no progeny virus resulted. This could be due to no complete DNA molecules having been synthesised. With cowpox-infected S180 cells, as with rabbitpox, DNA replication was reduced to 20% but, again, no progeny virus was formed, compared with a 10% yield with rabbitpox.

The "rabbitpox-like" poxviruses have been shown to induce limited amounts of argininosuccinate synthetase/lyase activity and also to undergo a small amount of replication in the absence of arginine or citrulline. The

evidence strongly suggests that this is due to utilisation of host arginyl residues. A pool of arginyl residues may result from 'autolysis' with the "cowpox-like" viruses in CAM and CEF cultures, the "rabbitpox-like" viruses perhaps also having some proteolytic activity, allowing replication in susceptible continuous cell lines under arginine deprived conditions where 'autolysis' is less likely to occur. This proteolytic activity may be associated with the "virulence" of the viruses for particular cell types and this would account for the inability of the "rabbitpox-like" poxviruses to replicate in all cell types in the absence of arginine.

Arginine Requirement of Other DNA Viruses

Arginine requirements have been reported for the replication of other DNA containing viruses. Rouse & Schlesinger (1967) showed that arginine plays a vital role in adenovirus type 2 assembly. In cells infected in the absence of arginine, viral and capsid antigens are made in reduced yield, but no infectious progeny is produced. If arginine is subsequently added to these cells there is an immediate production of infective virus shown to result from the assembly of particles from components already present in the infected cells. Bonifas (1967) showed an increase in the incorporation of arginine into adenovirus-infected cells immediately before the production of virus progeny. Further work by Sindquist & Philipson (1971), Raska et al (1972), Rouse & Schlesinger (1972) and Horowitz et al (1973) showed that under arginine deprived conditions all major structural components were synthesised, even those rich in arginine. The arginine requiring step in adenovirus type 2 replication was in the synthesis of a maturation factor, required for the assembly of all virus components into complete virus particles.

Wigand & Kümel (1978) studied the amino acid requirements of adenovirus types 1 and 9-15 for replication in HeLa cells. Like vaccinia and adenovirus type 2, the absence of any of the 13 essential amino acids for cell growth had an inhibiting effect on the virus yield, and again arginine deficiency resulted in the inhibition of an essential protein very late in the replication cycle. Hosokawa & Sing (1976) isolated an extremely basic protein from adenovirus type 5 containing

54% arginine.

The arginine requirement for herpes viruses has also been studied. Tankersley (1964) first reported that arginine was necessary for replication of herpes simplex virus in human cells. Infected cells deprived of arginine supported neither replication nor c.p.e., but when arginine was replaced, a prompt and extensive infection followed. Adsorption penetration and eclipse were found to be unaffected by arginine deprivation in herpes simplex infected RK 13 cells (Inglis, 1963). Roizman et al (1967) reported that certain complement fixing antigens did not appear in the absence of arginine. The proteins necessary for encapsidation were also found not to be present when arginine was lacking. Olshevsky & Becker (1976) showed that arginine deprivation affected synthesis and transport of herpes simplex virus proteins in BSC1 cells. In the absence of arginine, synthesis of viral peptides in the cytoplasm is reduced and these are transported very slowly to the nucleus. The peptides gradually disappear from the cytoplasm suggesting that degradation occurs before they reach the nucleus. These proteins may contain essential maturation factors.

The arginine requirement of pseudorabies, a herpes related virus was investigated by Mark & Kaplan (1971). With arginine deprived conditions, the virus yield was reduced by 95%, protein synthesis by 50% and DNA synthesis by 67%. The major structural proteins were found to be present, but they did not migrate to the cytoplasmic site of assembly from the nucleus as they did when the infected cells were supplied with arginine. Chantler & Stevely (1976) studied the synthesis of three arginine rich proteins which

were preferentially inhibited in the absence of arginine. These were found to be core proteins which are involved in the assembly process.

The replication of human cytomegalovirus, another herpes related virus was also found to require arginine. Under arginine free conditions no c.p.e., viral antigen or progeny virus are produced (Minamishima & Benyesh-Melnick, 1969).

Frog virus 3, another DNA containing virus which replicates in the cytoplasm was found to require arginine for replication in BHK 21 cells (Aubertin, 1975). The total amount of virus-specific DNA synthesised in the absence of arginine was unaffected, but its distribution in the cellular fractions had changed. The total amount of DNA synthesised in the absence of arginine remained associated with the nucleus, whereas in the presence of arginine virus-specific DNA was also found in the cytoplasm. When arginine was added to the deprived cultures virus DNA was chased from the nucleus into the cytoplasm.

The papovaviruses also show an essential requirement for arginine. In arginine deprived monkey kidney cells infected with SV40, both viral DNA and structural proteins are synthesised, but no infectious progeny is produced (Tan, 1977). The arginine requirement of polyoma virus in mouse embryo cells was investigated by Winters & Consigli (1969 & 1971). Arginine deprivation resulted in inhibition of DNA synthesis by 60% and plaque-forming activity by 90%.

Arginine appears to play a more complicated role in the replication of viruses capable of transformation of cells. The presence or absence of arginine may determine

whether the infected cell becomes transformed or whether it undergoes the lytic cycle.

The replication of Epstein Barr virus, present in lymphocytes cultured from patients with Burkitt's Lymphoma is inhibited in the presence of arginine (Henle & Henle, 1968). Removal of arginine from the cell cultures impairs cellular RNA and protein synthesis (Becker & Weinberg, 1970) and arrests cell division at the G1 phase of mitosis (Osunkaya, 1970). Virus specified DNA and capsid proteins have been detected in such cells, suggesting that the absence of arginine releases cellular control mechanisms and arginine becomes available from turnover of cell protein in sufficient amounts for virus specified events (Becker & Weinberg, 1971).

In the absence of arginine, or if arginine is competitively inhibited by canavanine, the replication of adenovirus type 5 (Russel & Becker, 1968) and type 7 (Neurath et al, 1970) is affected. The synthesis of virus specified DNA and viral components are inhibited, but virus specified tumour antigens are synthesised. Goldblum et al (1968) also investigated the effect of withdrawal of arginine and other amino acids on the synthesis of tumour and viral antigens by SV40 in BSC1 cells. In the absence of arginine there was neither c.p.e. nor increase in virus titre compared with the controls but again synthesis of T-antigen was unaffected. Control of arginine concentrations may constitute the mechanism by which the expression of a late gene function is regulated and whether a cell is transformed or goes through the lytic cycle.

Shope papilloma virus has been shown to induce a virus specific arginase in infected cells, which depletes the

cellular arginine and inhibits nuclear histone. This results in abnormally rapid growth forming the papilloma. By supplying the cells with large amounts of arginine which bypasses the enzyme, the papillomas were found to be greatly reduced (Rogers & Moore, 1963).

The involvement of arginine in determining whether cells are transformed or not by oncogenic viruses appears to be a result of the events during virus replication affected by arginine deprivation, and are similar for all DNA containing viruses. In the absence of arginine virus specific DNA synthesis may be reduced but with nearly all viruses maturation of virus progeny is severely inhibited. In such virus infected cells where transformation is possible, synthesis of T-antigens, which is an early event after infection, tends to be unaffected since they do not require arginine, and so transformation is more likely to occur under arginine deprived conditions.

Arginine is a cationic amino acid and proteins rich in arginine are intimately associated with ionic DNA molecules. Thus in all DNA viruses, such basic proteins may be involved initially in DNA replication when the DNA molecule unfolds and again to a much greater extent late in the replication cycle in the refolding of newly synthesised progeny DNA molecules which precedes encapsidation by the structural proteins. Thus the common effect with arginine deprivation in all DNA viruses is the lack of these 'maturation' factors, which are probably arginine rich proteins associated with encapsidation of virus DNA molecules.

Vaccinia DNA has been shown to be intimately associated with an arginine enriched protein and analysis by SDS-PAGE revealed a single component which shared the properties of

protamines and histones associated with eukaryotic DNA. This protein was found to be present in the cytoplasmic factories of DNA synthesis and later in the cores of mature virus particles. (Pogo, Katz & Dales, 1975) It was suggested that this molecule modulates gene activity and controls packing of DNA into the cores of mature virus particles.

Roizman & Spear, (1969) suggested that under arginine deprived conditions translation of mRNA stops at codons specifying arginine. Thus in the absence of arginine, the probability that a given peptide is synthesised completely is inversely proportional to its length and the number of arginyl residues present in the peptide. Thus the maturation factors may be very long peptides rich in arginine and these are preferentially inhibited under arginine deprivation, compared with other, structural components which, although arginine rich are shorter in length.

Alternatively, the synthesis of maturation factors could be a very late function. Prior synthesis of other arginine rich structural components could exhaust the limited cellular pool of arginyl residues in the absence of exogenous arginine.

Although arginine is used directly to synthesise proteins, it may be used also as a substrate for arginase, producing ornithine, which could be utilised further in the formation of other important metabolites. Vaccinia virus has been shown to contain polyamines, spermine and spermidine (Lanzer & Holowzak, 1975). After infection with vaccinia virus, while the synthesis of host cell protein is being shut off rapidly, synthesis of polyamines continues albeit at a lower level (Hodgson & Williamson, 1975). Polyamines might also serve

with the basic polypeptides in neutralising the charges of the DNA and facilitate the folding and packaging of DNA within the cores of the virus particles. Thus the essential 'maturation' factors may contain polyamines which would have limited availability in the absence of arginine.

Criteria for Comparison of the Orthopoxvirus Group

In comparing the orthopoxvirus group, the methods used in this study have produced a broad categorisation. An absolute effect was not observed and with many virus/cell systems only low yields were obtained. The determinations of utilisation of citrulline were complicated by background yields in the absence of arginine.

If a cell system could be found which could not utilise citrulline but would support the replication of poxviruses in complete medium and in addition give no yield in the absence of arginine, then this would be much more suitable for detecting the induction of virus specified argininosuccinate synthetase/lyase activity. If such a system could be realised then the presence of the enzymes could be quantified using a simple method of enzyme assay developed in this laboratory. Carbamyl- ^{14}C -citrulline would be used as a substrate for the enzymes, the resulting guanido- ^{14}C -arginine being converted to ^{14}C -urea by arginase and then to $^{14}\text{CO}_2$ and ammonia by urease. The $^{14}\text{CO}_2$ recovered is a measure of the argininosuccinate synthetase/lyase activity.

The utilisation of citrulline in the cell systems described indicates the phenotypic expression of a virus coded mechanism. If some poxviruses have this ability and others do not, this indicates a difference in the genotype between the viruses and is thus a good criterion for comparative studies. It is clearly better than some of the biological methods used to distinguish between different poxviruses which involve the response of a secondary host to infection, particularly since genetic differences between the hosts may affect the

response obtained to otherwise similar conditions. Serological techniques have their limitations since the orthopoxviruses have so many common antigens. The use of restriction endonucleases has been shown to be extremely useful in the comparison of the orthopoxvirus genomes. However a change in only one base sequence may result in a different fragmentation pattern. Although this is a very sensitive method for comparison, viruses which are very closely related will still show different fragmentation patterns, and thus the method is almost too sensitive. Also no information is gained as to the phenotypic behaviour of different poxviruses in infected cells. But the use of restriction enzymes is clearly the best current method for determining the relationships between the orthopoxviruses. Hybridisation techniques can also allow fragments obtained from restriction enzyme digestions to be mapped, allowing still finer comparisons.

In order to complement the studies of the poxvirus genome it is important to investigate the phenotypic characteristics resulting from the gene products. The analysis of virus induced polypeptides by polyacrylamide gel electrophoresis has been shown to be valuable in comparing the poxviruses, although many structural proteins are similar. However, non-structural proteins give a wider spectrum for comparison but the function of these different proteins is not known.

The method of comparison in this study attempts to show the presence or absence of virus induced proteins and also identifies the function of these proteins.

All the comparisons which have been made have yielded valuable information as to the dangers of the possibility of the existence of an animal reservoir of smallpox virus.

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