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THE ROLE OF ARGININE IN POXVIRUS REPLICATION

BY

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ABSTRACT

Arginine is essential for the growth of poxviruses in a number of continuous cell lines although depletion of cells of arginine by starvation does not irreversibly affect the ability of either the cells to support virus replication or the virus to direct its replication. The yield of virus is dependent on the concentration of arginine in the growth medium. Although the major requirement for arginine in the replication cycle of vaccinia virus in HeLa cells is for incorporation into virus-specified structural protein evidence is presented to show that arginine is also involved in the synthesis of polyamines which have an essential role in the virus replication cycle. There is no indication that arginine is involved either in the formation of ATP or for the provision of other amino-acids such as proline or asparagine. Both D-arginine and L-citrulline can replace L-arginine for the growth of HeLa cells and for the

replication of vaccinia virus in these cells. However, L-citrulline can also replace L-arginine for the growth of poxviruses in both human citrullinaemia and mouse S180 cells which are unable to utilise this aminoacid and have an absolute requirement for arginine.

Evidence is presented that the utilisation of citrulline in poxvirus-infected cells involves its conversion to arginine through argininosuccinic acid by the enzymes argininosuccinate synthetase and lyase. In both HeLa and mouse L cells these enzymic activities are enhanced following virus-infection. The enhanced enzymic activity requires both transcription and translation and is controlled by the synthesis of virus-specified DNA.

It is concluded that the ability of poxvirus-infected cells to synthesise arginine from citrulline is directed by the invading viral genome and that the mechanism is either virus-induced modification of pre-existing host-coded functions or the direct expression of specific virus-coded functions.

CHAPTER 1

INTRODUCTION

This work was undertaken as an extension of observations made in this laboratory concerning the involvement of arginine in the replication of vaccinia virus. There is an essential requirement for arginine for the growth of vaccinia virus in continuous cell cultures (Wells, 1967) and there is a quantitative relationship between the availability of arginine and the yield of virus (Archard & Williamson, 1971). Arginine is required for at least two events in the growth cycle of vaccinia virus in HeLa cells. One is an early event, related to the synthesis of virus-specified DNA, which proceeds maximally in the presence of 0.03mM arginine. The second is a quantitatively greater requirement involved in the synthesis of virus-specified structural protein(s) on which the

maturation of the virus particle eventually depends. Maximal yield of virus is produced at a concentration of 0.09mM arginine (Archard & Williamson, 1971). In KB cells infected with vaccinia virus although virus-specified DNA synthesis proceeds in the absence of arginine, arginine is required for the synthesis of late mRNA (Obert, Tripier & Guir, 1971).

There have been no direct investigations to determine whether arginine is used per se in poxvirus-infected cells or whether derivatives of arginine metabolism are involved. Studies using radioactively-labelled arginine, however, have indicated that some products of arginine metabolism are incorporated into mature virus particles (Archard, 1971). In other metabolic systems arginine has been shown to be involved in the synthesis of purines and pyrimidines (Hutson & Downing, 1968) and also as a source of polyamines (Morris & Pardee, 1966; Hirschfield, Rosenfeld, Leifer & Maas, 1970) which have been shown to play an important role in nucleic acid and protein metabolism (Abraham, 1968; Inouye & Pardee, 1970). The work presented concerns the intermediate metabolism of arginine in cell cultures infected with poxviruses.

The chemical composition of a virus particle is essentially nucleic acid, DNA or RNA, associated with protein. The extracellular virion is metabolically inert: all dynamic events involving the synthesis of virus-specific macromolecules take place only when the virus infects the host-cell. Since the virus is dependent on the

host-cell for expression of its genetic material it is essential to have an understanding of cell metabolism, with respect to specific nutritional requirements and to synthetic capabilities, so that changes in metabolism in virus-infected cells can be evaluated with respect to the presence of invading viral genome.

1.1 Nutritional requirement for arginine in cell culture

When excised tissue is grown in cell culture changes occur in the nutritional requirements of the cells. In the whole animal cells become metabolically differentiated. A nutrient which may be synthesised in one type of tissue may be transported from some topographically removed site to another type of tissue which is restricted with respect to the synthesis of that nutrient. Therefore, established cells in culture may show essential requirements for nutrients that were not exhibited in the whole animal (Eagle, 1965).

There are eight amino-acids essential for nitrogen balance in man (Rose, Wixon, Lockhart & Lambert, 1955). Cells cultured in vitro require an additional five amino-acids: arginine, cyst(e)ine, glutamine, histidine and tryptophan (Eagle, 1959). Thus, although arginine is important in vitro it is not essential in vivo. There are a large number of transaminases in mammalian cells but attempts to substitute arginine by α -keto- δ -guanidoacetic acid and α -keto-carbamidovaleric acid have been unsuccessful both in vivo and in vitro in KB, HeLa, mouse fibroblasts and mouse S180 cell cultures (Eagle, 1959).

Many different cell cultures can utilize citrulline but not ornithine in place of arginine: citrulline biosynthesis cannot be induced by progressive removal of citrulline from the culture medium (Eagle, 1959). Thus, ornithine transcarbamoylase, the enzyme responsible for citrulline biosynthesis, appears to be inactive in mammalian cells in vitro. These observations indicate that either citrulline is not synthesised in these cell cultures or is synthesised in insufficient amounts to permit cell growth.

Although established cell cultures have been derived from tissues possessing diverse functions and enzymatic patterns, they have exhibited striking similarities in their nutritional requirements. The reactions involved in the Krebs-Henseleit urea cycle, namely the conversion of ornithine through citrulline to arginine, have been studied in a number of cell lines (Tytell & Neumann, 1964). Of 31 cell lines examined, none could grow on ornithine but varied in their abilities to grow with citrulline. Most established lines of malignant or "normal" origin readily utilized citrulline in place of arginine. However, transplantable rat Walker carcinoma and mouse sarcoma 180, which are stable cell lines but passaged in the host rather than in cell culture, are unable to utilize citrulline. Primary cell cultures of kidney, lung and testis of the same species can grow in medium containing citrulline.

The nature of arginine as a growth factor for mammalian cells is primarily for protein synthesis. A particular function of arginine

involves the synthesis of basic proteins, such as histones, which act as stabilisers of chromatin (Smart & Bonner, 1971) and of proteins involved in intracellular transport mechanisms (Ryser, 1968). The fact that most cells can utilize citrulline as a substitute for arginine indicates that they can convert the former through arginino-succinic acid to arginine. However, there is little evidence that other intermediates of the Krebs-Henseleit urea cycle are involved per se in protein synthesis. There are only two references to citrulline or ornithine being incorporated into protein. Citrulline has been detected in the medulla protein of the quill of the African porcupine and in the inner root sheath of the hair follicle of the guinea pig (Steinart, Harding & Rogers, 1969). Ornithine has been detected in certain $\alpha_1 - \alpha_2$ globulins. (Stetten, Aakesson & Alvsaker, 1971). Thus, although these amino-acids have important roles in intermediary metabolism, neither citrulline nor ornithine have significant roles in protein synthesis.

1.2 Arginine synthesis

Ornithine and carbamoyl phosphate are necessary for in vivo synthesis of arginine via citrulline. Ornithine is synthesised from a non-amino substrate, α -ketoglutarate, provided by the citric acid cycle. This is transaminated to form glutamate which may be 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 hydrogen ions.

Amino nitrogen is made available for arginine biosynthesis from L-glutamine by coupling with carbon dioxide in the presence of ATP. This reaction is catalysed by carbamoyl phosphate synthetase. Carbamoyl phosphate thus formed is condensed with ornithine by a transcarbamoylation reaction to form citrulline. Ornithine transcarbamoylase occurs in mammalian, but not avian, liver, in higher and lower plants and in certain microorganisms. Conversion of citrulline to arginine is accomplished in a two-step reaction. Argininosuccinate synthetase catalyses the ATP-dependent formation of an acylguanidium linkage between aspartic acid and citrulline forming argininosuccinic acid. This is cleaved in the presence of argininosuccinate lyase producing arginine and fumarate (Dagley & Nicholson, 1970) (Fig. 1.1).

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 (Shimke, 1964). Defects in the urea cycle have been demonstrated in vivo by the presence of abnormally high level of intermediates in patients urine. The accumulation of citrulline in body fluids, citrullinaemia, has been associated with mental retardation (McMurray, Mohyuddin, Bayer & Rathbun, 1964). Cells cultured in vitro from patients with citrullinaemia appear to have lost substrate specificity with respect to the enzyme argininosuccinate synthetase (Tedesco & Mellman, 1967). Cultured

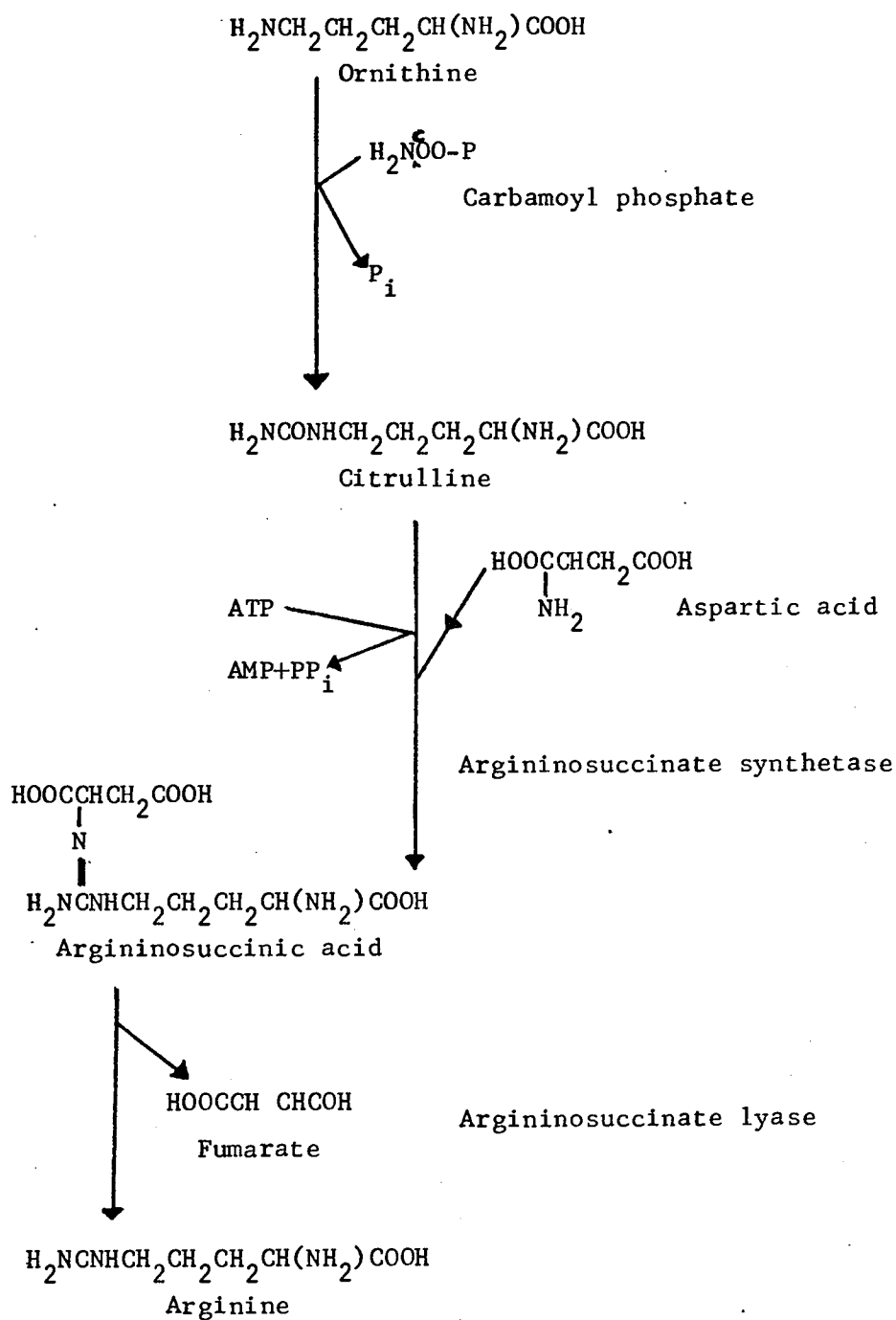


Fig. 1.1 Arginine biosynthesis

cells will grow with arginine but not with citrulline. Argininosuccinicacidaemia is a rare disorder of amino-acid metabolism in which argininosuccinic acid is excreted in high amounts (Westall, 1960). The site of the disorder is unknown but presumably is involved with inadequate activity of the enzyme argininosuccinate lyase.

1.3 Catabolism of arginine

The reactions described for the synthesis of arginine form part of the Krebs-Henseleit urea cycle (Fig. 1.2). The cycle is completed by the removal of nitrogen from arginine with the concomitant formation of urea and the regeneration of ornithine by the enzyme arginase. Exogenous arginine supplied to cell cultures can be catabolised to ornithine providing an alternative substrate for the synthesis of two other amino-acids, proline and hydroxyproline. This involves the enzyme L-ornithine-2-oxoacid amino transferase and the presence of pyridoxal phosphate. Ornithine is deaminated in the presence of a 2-oxoacid from the citric acid cycle to yield glutamate-4-semialdehyde and a second amino-acid. The product undergoes a spontaneous reaction involving the loss of water and culminating in ring closure to form L-pyrroline-5-carboxylate. Conversion to proline is effected by pyrroline-5-carboxylate reductase in the presence of NAD and hydrogen ions (Dagley & Nicholson, 1970). Further oxidation of proline appears to take place after incorporation of proline on the ribosomes (Peterforsky & Underfriend, 1963) (Fig 1.3). The ability of cells in vivo to synthesise proline from ornithine is

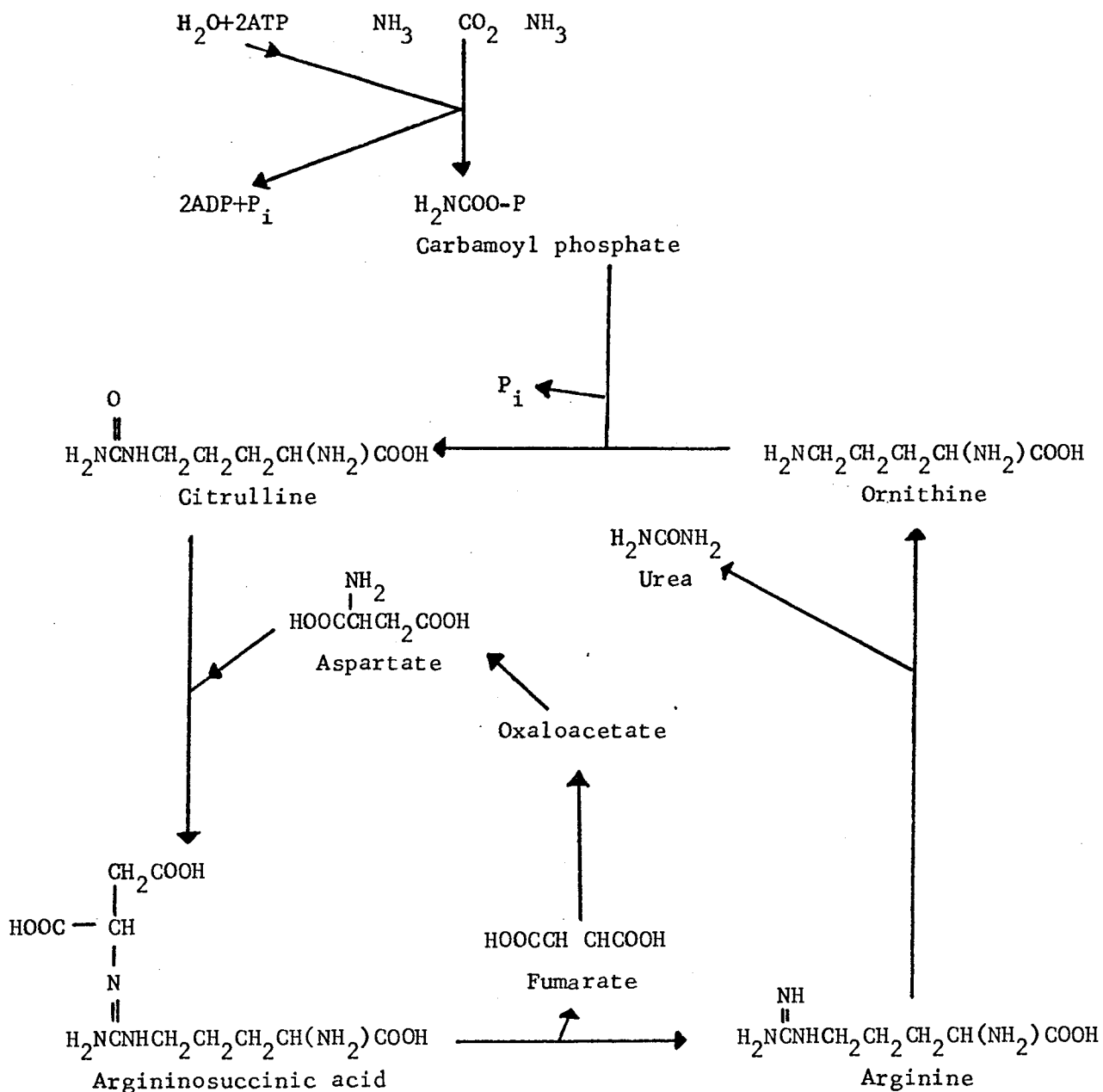


Fig. 1.2 Krebs-Henseleit urea cycle

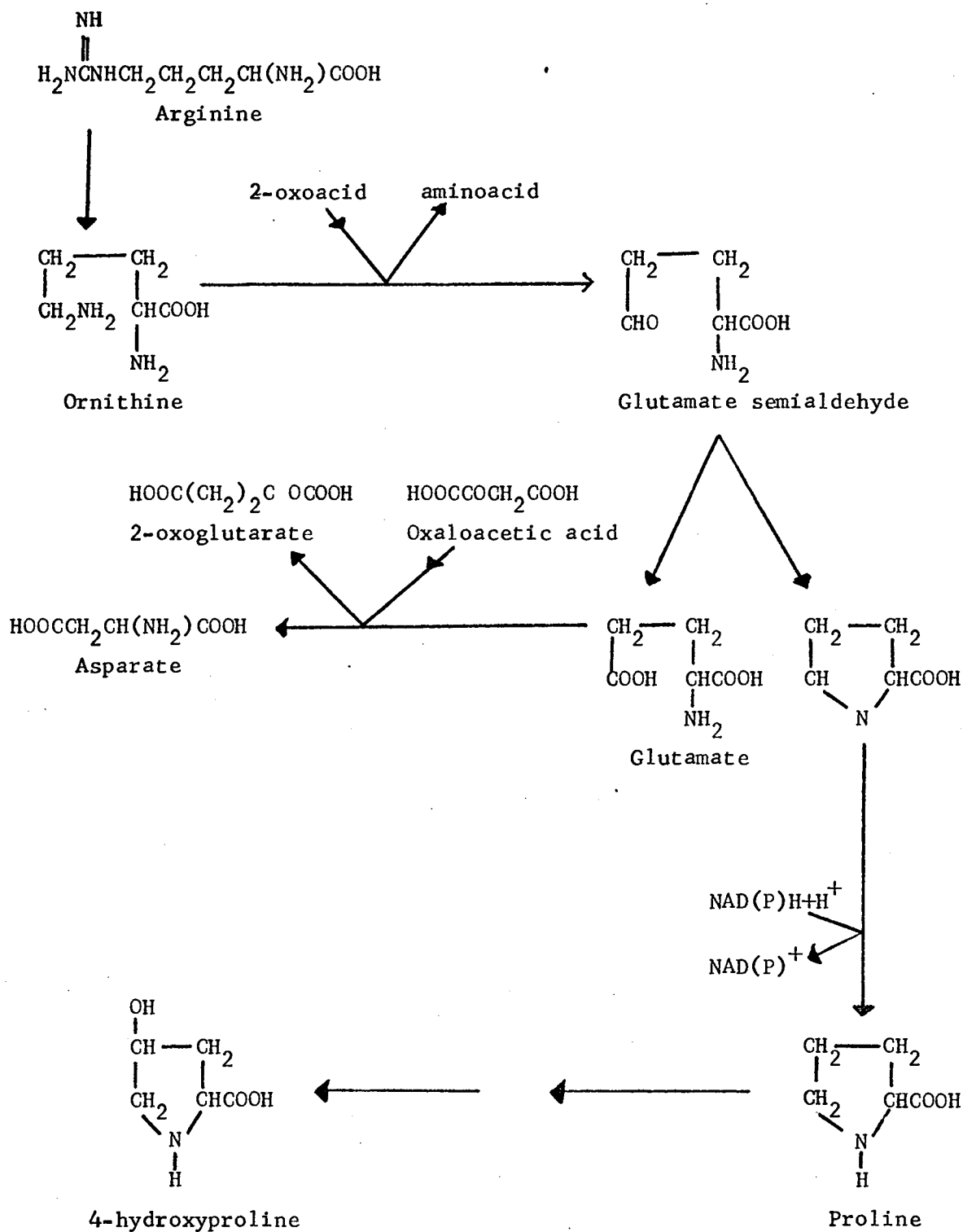


Fig. 1.3 The involvement of arginine in the biosynthesis of other aminoacids

apparently unchanged in vitro because proline is not included in cell culture medium (Eagle, 1959).

In addition to the catabolism of arginine via arginase, ornithine can be regenerated from arginine in mammals by reaction with glycine catalysed by glycine amidinotransferase. The second product, guanidoacetate is methylated in the presence of 5-adenosylmethionine and guanidoacetate transmethylase to form creatine. This is subsequently phosphorylated by creatine kinase, an ATP-dependent reaction, resulting in creatine phosphate production. In a non-enzymic reaction phosphate is released and ring closure occurs to form creatine which can be excreted (Dagley & Nicholson, 1970) (Fig 1.4).

1.4 The utilization of arginine for purine and pyrimidine biosynthesis

Preformed purines and pyrimidines are not essential in mammalian diet since the organism is able to synthesise these compounds. The synthesis of nucleotides may be coupled to arginine metabolism at several points. Both glutamate and aspartate act as functional groups in the formation of purines. The nitrogen in position 1 is derived from the amino nitrogen of aspartic acid, those in positions 3 and 9 from the amide nitrogen of glutamine. The carbon atom in position 6 is derived from carbon dioxide.

The initiation of pyrimidine biosynthesis necessitates the formation of ureido-succinic acid by the condensation of carbamoyl

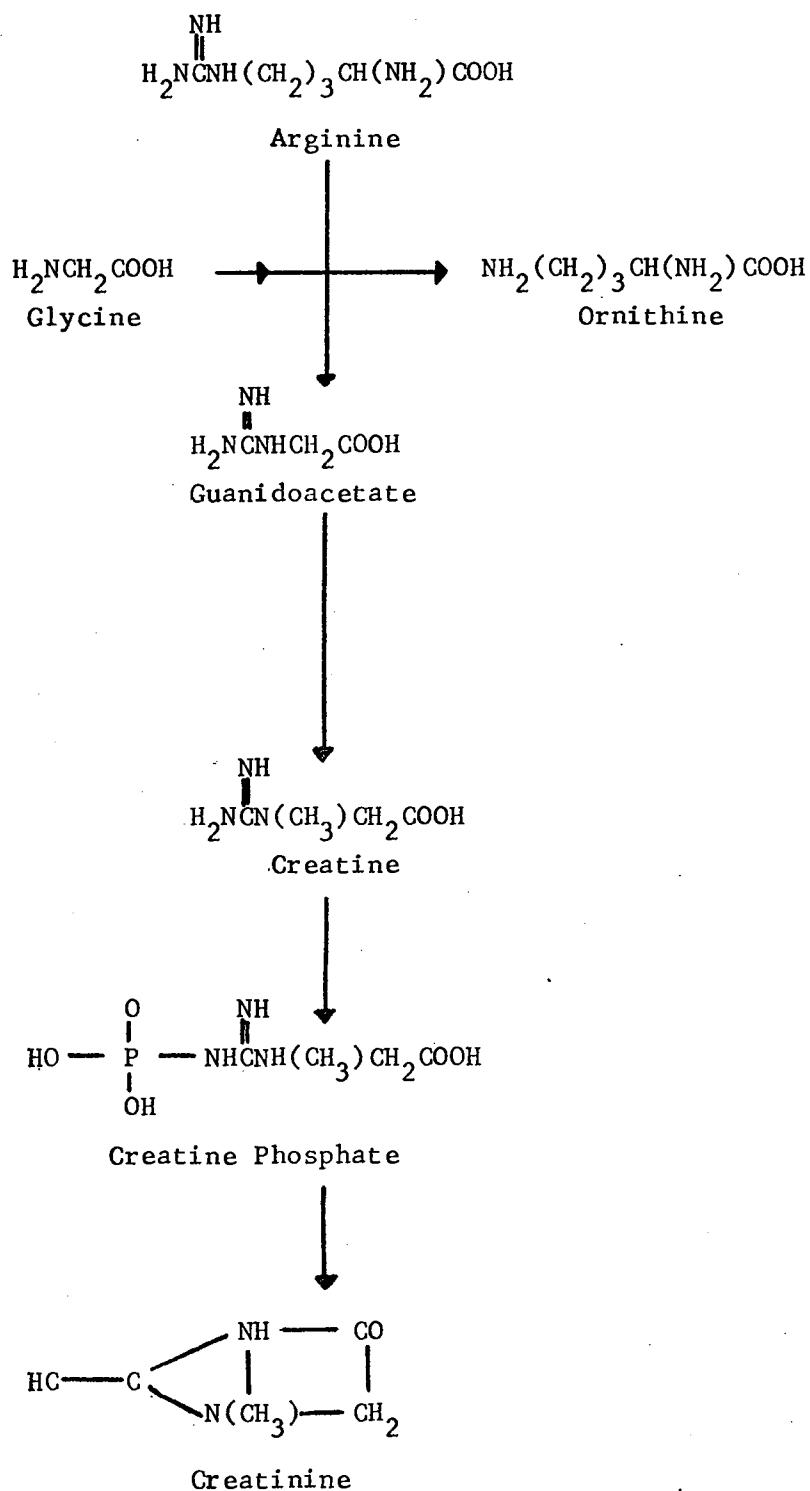


Fig. 1.4 Creatinine biosynthesis

phosphate with aspartic acid catalysed by the enzyme aspartate transcarbamoylase. This reaction is dependent on the prior formation of metabolically active carbamoyl phosphate from carbon dioxide and the amino group from glutamine (Fig 1.5).

The importance of carbon dioxide or bicarbonate anions for nucleic acid synthesis has been demonstrated by a number of authors. HeLa and human conjunctiva cells will not grow if bicarbonate is removed from the culture medium (Gayer & Change, 1958) but cell multiplication does occur if bicarbonate is restored. Swim and Parker (1958) investigated the carbon dioxide requirement for several established strains of fibroblasts of human, murine and rabbit origin. In the absence of bicarbonate, but maintaining optimal pH, multiplication of cell cultures in tightly stoppered tubes was about 50% that of controls indicating that the cells produced sufficient carbon dioxide to promote growth. Although multiplication of HeLa cells is abolished in the absence of carbon dioxide, the conversion of (^{14}C)-acetate to carbon dioxide is normal showing that general cell metabolism is still high (Gayer & Neimark, 1958). Chang, Liepins and Margolish (1961) fed (^{14}C)-labelled metabolites to cells to determine where the label accumulated. In the presence of bicarbonate most of the label was found in the acid soluble fraction, there being more label associated with purines than pyrimidines. A mixture of oxaloacetic acid and ribosides replaced the requirement for bicarbonate. Similar results have been obtained in mouse L cells

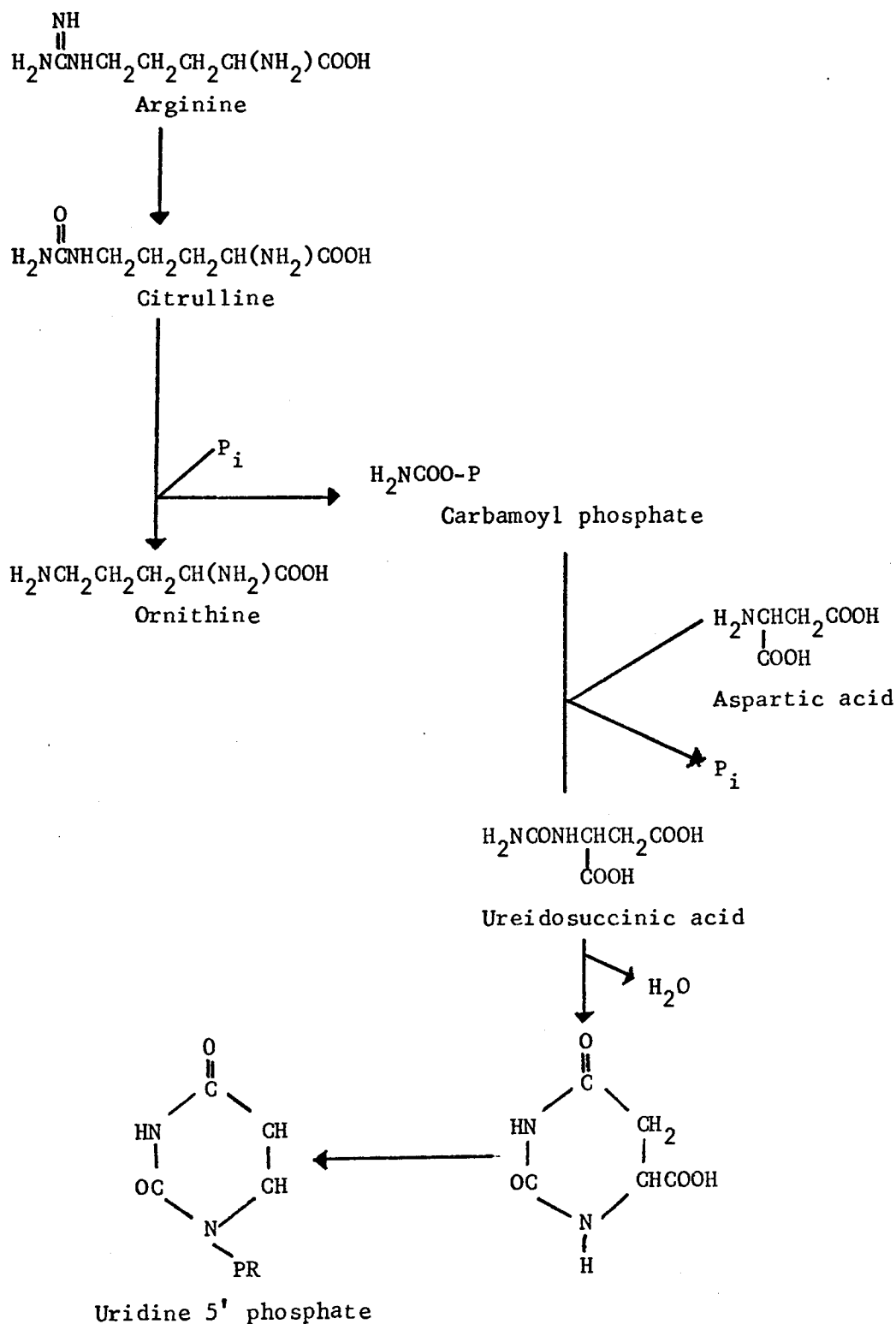


Fig. 1.5 The involvement of arginine in nucleotide biosynthesis (*Lactobacillus leichmanii*)

(Gwatkin & Siminovitch, 1960).

McCoy, Maxwell and Kruse (1961) demonstrated a requirement for carbon dioxide or bicarbonate for the growth of the Jenson, JA-1 and JA-2 sarcomas in vitro. When the cells were grown in the presence of (^{14}C)-labelled carbon dioxide the isotope was associated with aspartic acid, glutamic acid and proline as well as in purines and pyrimidines. Uracil partially spared the requirement for carbon dioxide but no other base was active. Thus, these cells in vitro must have the capacity to synthesize carbamoyl phosphate.

The formation of carbamoyl phosphate is essential both for the continuation of the Krebs-Henseleit urea cycle and for nucleic acid synthesis in microorganisms. Lwoff and Monod (1947) investigated the role of carbon dioxide in the microbial environment. On defined medium containing glucose the growth of E.coli was inhibited in the absence of carbon dioxide. At high population densities this inhibition was suppressed by succinate and glutarate from the citric acid cycle and by glutamate, aspartate, glutamine and asparagine. In the growth of some auxotrophic mutants of E.coli uracil can replace arginine (Yashne & Gorini, 1964). A non-specific acetyl phosphokinase from these auxotrophs can phosphorylate carbamate in vitro but the enzyme does not function in vivo. Mutants lacking carbamoyl phosphate synthetase cannot grow on medium lacking arginine or uracil (Charles & Roberts, 1968). From similar work with mutants of Neurospora, Charles and Broadbent (1964) have proposed that there are two anabolic

pathways involving carbamoyl phosphate synthetase. One pathway leads to the formation of pyrimidines and the other to arginine.

In Lactobacillus leichmanii the guanido carbon of arginine is a source of one 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 transcarbamoylase facilitates the cleavage of citrulline to ornithine with release of carbon dioxide which is fixed as carbamoyl phosphate by a reaction involving carbamoyl phosphate synthetase and inorganic phosphate. (Fig. 1.5.).

1.5 The urea cycle and carbohydrate metabolism

The formation of several precursors in the urea cycle is dependent on the functions of other metabolic pathways, principally the citric acid cycle. The involvement of aspartic acid and glutamic acid in the urea cycle is an important link directly coupling the metabolism of amino-acids and that of carbohydrates. Transamination of α -keto-glutarate to L-glutamate and a keto acid occurs in the presence of pyridoxal phosphate. Glutamate is further utilized by conversion to glutamine and is subsequently involved in carbamoyl phosphate synthesis. In addition, oxaloacetic and fumaric acids are released from the citric acid cycle and by transamination reactions provide aspartic acid as a precursor for the urea cycle.

1.6 The synthesis of polyamines

Those polyamines to which important cellular functions have been ascribed, namely, putrescine, spermidine and spermine, are derived from arginine. These substances have an important role in growing cells in which protein synthesis is paralleled by increases in the intracellular levels of spermidine (Russell & Snyder, 1968). This association may be a reflection of the requirement for spermidine in the expression of DNA-dependent RNA polymerase activity (Fox & Weiss, 1964). Polyamines are associated also with ribosomes being necessary for their structural integrity possibly by interaction with the acid moieties of the nucleic acid (Cohen & Lichtenstein, 1960). A similar function may be served by the polyamines shown to be present in T-even bacteriophage (Hershey, 1957) turnip yellow mosaic virus particles (Beer & Kosuge, 1970) and herpes simplex (Gibson & Roizman, 1971).

Putrescine biosynthesis in mammalian cells is effected by the decarboxylation of ornithine by ornithine decarboxylase. There is evidence that there are two pathways for putrescine biosynthesis in E.coli, one involving arginine and the second ornithine. In the first instance arginine is decarboxylated to agmatine by the enzyme arginine decarboxylase. This is followed by the conversion of agmatine to putrescine by agmatine ureohydrolase. The second pathway, similar to that in mammalian cells, is effected by ornithine decarboxylase (Fig. 1.6). Mutants of E.coli blocked in the capacity to synthesise putrescine from arginine because of a reduction in the enzyme, agmatine

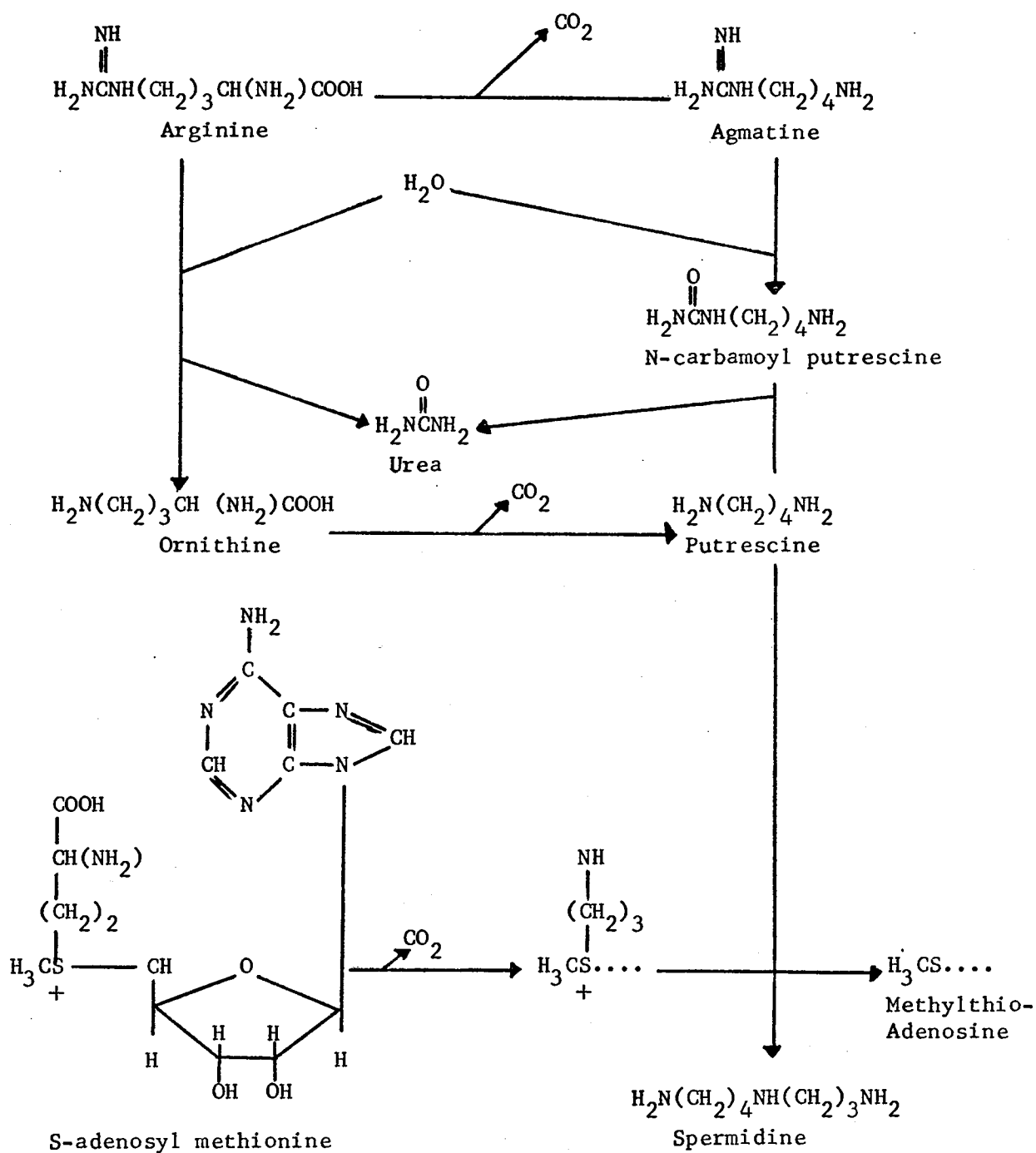


Fig. 1.6 Polyamine biosynthesis

ureohydrolase, can make putrescine in arginine-free medium by the ornithine decarboxylase pathway. However, addition of arginine to the medium suppresses the synthesis of ornithine thereby reducing the synthesis of putrescine from ornithine resulting in inhibition of cell growth (Morris & Pardee, 1966; Hirschfield et al, 1970).

In Streptococcus faecalis arginine can be catabolised through putrescine to yield carbamoyl phosphate as an energy source (Roon & Barke, 1971). N-acetyl-putrescine and putrescine have been identified as intermediates in the fermentation of agmatine (see previous section). Putrescine transcarbamoylase, an induceable enzyme, converts N-acetyl-putrescine to putrescine and carbamoyl phosphate. This enzyme can be identified in cells grown in the presence of agmatine and is distinguishable from ornithine transcarbamoylase because it is induced by agmatine but not by arginine and can be separated from ornithine transcarbamoylase by gel filtration (Roon & Barke, 1971).

1.7 The role of arginine in virus growth

a) Adenoviruses

Type 2 adenovirus replication in continuous cell cultures has an absolute requirement for arginine (Bonifas & Schlesinger, 1959). The nature of the arginine dependent step was investigated by Rouse and Schlesinger (1967) using immunofluorescence and haemagglutination techniques to detect virus-specific proteins. In the absence of arginine there was production of hexons and penton-fibre subunits but not of complete virus particles. Addition of arginine 28 - 32 hours

after infection in arginine-free medium, however, permitted immediate formation of progeny virions. Simultaneous addition of FUdR did not inhibit virus replication suggesting that viral DNA synthesis occurs in the absence of arginine. Furthermore, (^3H)-thymidine added during arginine deprivation was found in mature virions after restoration of arginine.

Bonifas (1967) has shown an increased rate of incorporation of arginine into adenovirus-infected cells immediately before the production of progeny virus. Further studies by Russell and Becker (1967) indicated that the arginine-requiring step involved a component of the "P" antigen, a complex antigen, not related to capsid antigens. Synthesis of P-antigen was implicated with the formation of an internal maturation factor associated with virus assembly. Histochemical studies of infected cells with phenanthroquinone and copper phthalocyanine-neutral red, specific stains for arginine residues, revealed characteristic structures in the form of intranuclear rings and rosettes. The same structures have been visualised by phase contrast microscopy and fluorescent antibody with adenovirus "P" antiserum. In the absence of arginine no rings or rosettes were demonstrable. These results indicated that these intranuclear structures characteristic of adenovirus-infected cells are sites of accumulation of arginine-rich, basic proteins (Russell, Brodaty & Armstrong, 1971).

Prage, Petterson and Philipson (1968) investigated directly the internal proteins of adenovirus type 2. Purified virus particles were

sequentially digested yielding an extract which in immunodiffusion tests gave a reaction of identity with an antiserum prepared against virus particles but not with antisera to hexon, penton-fibre or host-cell extracts. By dual labelling with (^3H)-arginine and (^{14}C)-threonine this core material was shown to be rich in arginine and polyacrylamide gel electrophoresis showed two protein bands. Analysis of the protein on Sephadex G150 gave a single peak corresponding to an assymetric molecule, molecular weight $2-3 \times 10^4$. Further analysis showed that these internal proteins contained about 20% arginine. Similar results were obtained by Laver (1970).

b) Herpes viruses

Tankersley (1964) examined the growth of herpes simplex virus in cultured human oesophageal epithelium cells. Eleven of the amino-acids present in Eagle's minimal essential medium, including arginine, are essential for progressive infection in minimally infected cells. In the absence of arginine there is no production of CPE or infective virus. When arginine is replaced in the medium there is a prompt and extensive infection. Similar observations have been made by Inglis (1968) using herpes simplex virus in RK_{13} cells and in HeLa, HEp2 and KB cells (Jeney, Gonczol & Vaczi, 1967).

Analysis by density gradient centrifugation of extracts from cells infected in the absence of arginine showed that no complete virus was produced but virus-specified proteins were present in the soluble fraction (Becker, Olshevsky & Levitt, 1967). In addition,

virus-specified DNA synthesis proceeded but remained sensitive to DNAase becoming resistant to digestion only after the addition of arginine to the virus-infected cells.

Courtney, McCombs and Benyesh-Melnick (1970) showed that in cells infected in the absence of arginine both immunofluorescent and complement fixing, virus-specific antigens were found primarily in the cytoplasm. Release of the arginine block at 10 hours post-infection resulted in the prompt appearance of increasing amounts of nuclear immunofluorescent and complement fixing antigens. Within 6 hr of arginine addition, the extent of intranuclear immunofluorescence and the titres of complement fixing antigen approximated those of infected cells that had been maintained in the presence of arginine throughout. Using polyacrylamide gel electrophoresis Olshevsky and Becker (1970) have also shown that virus-specified protein synthesis is not abolished in the absence of arginine. Virus-specific proteins detected included a core protein, rich in arginine, suggesting the availability of endogenous arginine from protein turnover. Similar studies have shown three virus-specified proteins found in the cytoplasm of cells devoid of arginine which migrate in electrophoresis with proteins found in the nucleus of virus-infected, control cells (Courtney et al, 1971). Mark and Kaplan (1971) using the related pseudorabies virus showed also that in the absence of arginine virus structural proteins fail to migrate from their cytoplasmic site of synthesis to their assembly site in the nucleus. The possible mechanism suggested is that absence of arginine prevents the

production of protein(s) in infected cells which may be responsible for the permeation of structural proteins into the nucleus.

In primary human, chick and monkey tissues the replication of herpes simplex virus in the absence of arginine is unimpaired (Jeney, Gonczol & Vaczi, 1967). Synthesis of DNA in uninfected primary cell cultures was not affected by the removal of arginine but in HeLa cell cultures, under similar conditions, synthesis was severely limited. The growth of herpes simplex virus in primary cell cultures but not in continuous cell cultures was attributed to the degree to which arginine deprivation affects cellular metabolism.

Arginine is essential for the growth of another herpesvirus, cytomegalovirus (Miramishima & Benyesh-Melnick, 1969). In arginine-free conditions no cytopathic effect (CPE), viral antigen or progeny virus are produced. Addition of arginine to such cultures two days post-infection results in the production of CPE and progeny virus within a further two days compared to 4 - 5 days in cultures maintained throughout in complete medium.

The replication of Epstein-Barr virus, present in lymphocytes cultured from patients with Burkitt's lymphoma, is inhibited in the presence of arginine. In complete medium mature virus particles cannot be detected within the cells (Henle & Henle, 1968). Removal of arginine from cultures impairs cellular RNA and protein synthesis (Becker & Weinberg, 1970) and arrests cell division at the G1 phase

of mitosis (Osunkaya, 1970). However, there is an increase in the number of cells synthesising Epstein-Barr virus (Becker & Weinberg, 1971). 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 the turnover of cellular protein in amounts sufficient for virus-specified events. Infection of cotton tail rabbits with Shope papilloma virus causes the induction of a virus-specified arginase which may be involved in removing cellular control so that infection can proceed (Rogers & Moore, 1963). In this instance, arginase may function to provide conditions similar to those postulated for the replication of Epstein-Barr virus.

c) Papovaviruses

Arginine is required for a late function in the replication of SV40 virus (Goldblum, Ravid & Becker, 1968). In arginine-deprived cells there is no production of CPE, virus structural antigen or progeny virus but formation of 'T' antigen is not affected. Addition of arginine to arginine-deprived cells 5 days post-infection resulted in the production within 48 hr of progeny virus to titres comparable to those of infected cells maintained in complete medium for 5 days.

Winters and Consigli (1969) demonstrated that arginine is essential for the replication of polyoma virus. In addition, in the absence of arginine virus-specified DNA is synthesised but remains

sensitive to DNAase. Protein synthesis, as shown by immunofluorescence and radiochemical techniques, is unaffected.

The requirement for arginine in adenovirus, herpesvirus and the papovavirus groups is essentially one involved in late events in the growth cycle of these viruses. The experimental evidence indicated that arginine is involved in the formation of functional or structural protein(s) essential for the production of progeny virus. All these viruses have an intranuclear stage in the replication of their genomes and in the expression of virus-specific proteins.

The poxviruses are the most complicated group of animal viruses whose genetic material is a large single molecule of double-stranded DNA, molecular weight 1.6×10^8 daltons (Joklik, 1962). On the basis of Watson's estimate of the size of bacteriophage gene as 10^6 daltons (Watson, 1965), the poxvirus genome has the coding potential for about 150 proteins. Apart from structural proteins, other proteins may have specific enzymatic functions involved with the replication of the virus (Kit, 1963). Cairns (1960) used (^3H)-thymidine and autoradiography to determine the initiation site for vaccinia virus replication. Both DNA and virus-specified protein synthesis, as judged by reaction with fluorescein-coupled antibody, take place in the cytoplasm of infected cells.

1.8 The arginine requirement of poxviruses

In human embryonic fibroblasts the requirement for bicarbonate

in variola virus replication can be partially overcome by the addition of increased concentrations of arginine (Wells, 1967). The amino-acid requirements for vaccinia virus in mouse L cells has been studied by Holterman (1969). The essential amino-acids for virus growth coincided with those found to be essential for the growth of L cells (Eagle, 1959).

Archard and Williamson (1971) investigated the role of arginine in the replication of vaccinia virus in HeLa cell cultures. A quantitative relationship was shown between exogenous arginine concentration and the yield of infective virus, maximal yield being obtained with 0.09mM arginine. In complete medium, virus-specified DNA synthesis begins at 2 - 3 hr post-infection reaching a maximum at about 5 hr post-infection. Progeny virus begins to appear at 6 hr post-infection. Pulse-labelling experiments with (^{14}C)-arginine showed that there is a marked stimulation in the rate of incorporation of arginine within 50 min of exposing cells to virus. This is followed by a rapid decrease in the rate of incorporation but there is a second peak at 3 hr. Purified vaccinia virus from cell cultures maintained in the presence of (^{14}C)-arginine gave a single peak after density gradient centrifugation that was coincidental with the 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 a sub-optimal

concentration of 0.03 mM arginine followed by simultaneous addition, at 6 hr post-infection, of FUDR and 0.06 mM arginine 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 elaboration of virus-specified, structural proteins.

Obert, Tripier and Guir (1971) have demonstrated a similar essential requirement for vaccinia virus replication in KB cells. In this virus-infected cell system, although there was no production of progeny virus in the absence of arginine, virus-specific DNA synthesis was 70% of that occurring in the presence of arginine. Simultaneous addition of FUDR and arginine at 8 hr post-infection to cell cultures previously deprived of arginine resulted in a yield of virus that was 60% of that produced by cultures infected in the presence of arginine. Further studies showed that only early virus-specified mRNA species were produced in the absence of arginine. In addition, electron microscopy studies showed the formation of virus-specific membrane structures in the cytoplasm of cells infected in the absence of arginine.

Vaccinia virus infection has been shown to result in the suppression of synthesis of macromolecules specified by the host-cell

(Joklik, 1966). The activity of cellular DNA-dependent RNA polymerase has been shown to be reduced in vaccinia virus-infected cells (Balandin, Kozlova, Lushinov, Peterson & Cherbukina, 1971). Cellular DNA is not degraded but there is an accumulation of basic protein of a histone nature.

These observations suggest a specific requirement for arginine at different stages in the replication of vaccinia virus. Assuming similar requirements in different host-cell systems, there appear to be at least four essential events. The first event involves the production of protein(s) that suppress the synthesis of host-specific macromolecules. Secondly, arginine is essential for the synthesis of virus-specific DNA. Transcription at this stage, leading to the production of late mRNA, shows a similar requirement. Finally, the availability of arginine is essential for the formation of virus structural proteins. The observed differences between HeLa and KB cell cultures may be interpreted on the basis of differences in the intracellular concentrations of arginine following starvation.

1.9 The effect of mycoplasma on cell metabolism

Mycoplasma are submicroscopic, free-living organisms which possess cellular organisation similar to bacteria but lack a rigid cell wall. They are intracellular parasites dependent on the host-cell for certain metabolic functions, notably the provision of some amino-acids for growth. Kenny and Pollock (1963) showed that cells contaminated with mycoplasma exhibit a slower growth rate. Other

effects are the production of chromosome aberrations and inhibition of mitosis (Stanbridge, 1971).

Schimke (1963) demonstrated that non-fermenting mycoplasma, such as M. orale, can utilize arginine for the production of ATP by a dihydrolase pathway. (Shimke & Barile, 1963). Arginine is deaminated in the presence of arginine deiminase to produce citrulline and ammonia. The kinetics for this reaction are similar to those in S. faecalis, where arginine is used as an energy source through the formation of putrescine. (Shimke, 1967) (see pyrimidine section). Ornithine transcarbamoylase facilitates the decarbamoylation of citrulline yielding ornithine with the concomitant synthesis of carbamoyl phosphate by a non-enzymic reaction involving the carbamido group from citrulline and inorganic phosphorus. Thus far the pathway is similar to that in L. leichmanii where arginine serves as a substitute for pyrimidine synthesis (Hutson & Downing, 1968). Carbamate phosphokinase catalyses the ADP-dependent formation of ATP from carbamoyl phosphate. Furthermore, cellular arginase has little effect on the break down of arginine by this pathway and infection with M. arginini rapidly depletes cell cultures of arginine (Stanbridge, 1971) (Fig 1.7).

Careful microscopic examination may not reveal any morphological changes following the contamination of cells with mycoplasmas. However, the yield of adenovirus and vaccinia virus is reduced in such cultures (Rouse, Bonifas & Schlesinger, 1967; Singer, Fitzgerald, Barile &

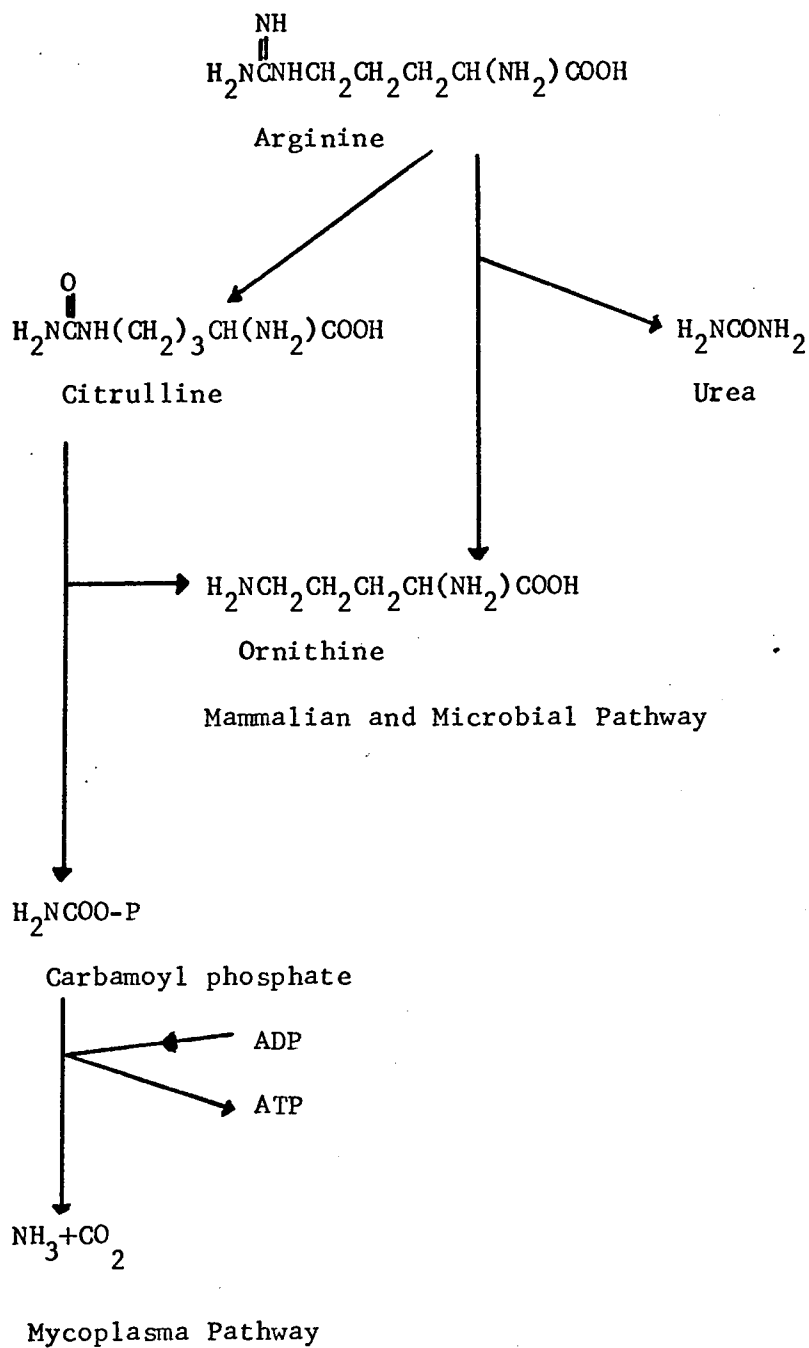


Fig. 1.7. Pathways of arginine catabolism

Kirschstein, 1970). In both cases, the maximal yields can be achieved by the addition of further arginine to the cell cultures. Hargreaves and Leach (1970) have demonstrated similar results with herpes simplex virus but an increased titre in mycoplasma-contaminated cells subsequently infected with respiratory syncytial virus.

Since contamination of cell cultures with mycoplasmas can affect both cell metabolism and virus production, interpretation of results can be hazardous and any biochemical measurements necessitate that these organisms are eradicated from the system.

The experiments presented in this thesis are a continuation of the work of Archard (1971). Attempts have been made to determine how arginine is metabolised in a number of cell lines and if infection with poxvirus alters this metabolism. Studies have been made to investigate the importance of intermediate metabolism in poxvirus replication. Biological and biochemical techniques were used to investigate the role of specific metabolites and the effect of structural analogues. From these studies further work was carried out to investigate some of the enzymes of the Krebs-Henseleit urea cycle in both uninfected and poxvirus-infected cells.

CHAPTER 2

MATERIALS AND METHODS

2.1 Cell culture

The growth of the Lister and International Health Division strain (IHD) of vaccinia virus and rabbitpox virus was studied in a number of cell lines in the presence and absence of arginine. Stock cultures of HeLa (Gey, Coffman & Kubick, 1952) mouse L (De Brys, 1949) and mouse sarcoma 180 (Foley, Drolet, McCarthy, Goulet, Dokos & Filler, 1960) were grown in Eagle's minimal essential medium (MEM, Eagle, 1959, Flow Laboratories, Irvine, Scotland) supplemented with 5% unheated calf serum (Fraburg Limited, Maidenhead, Berkshire, England) in either 12 oz prescription bottles with 35 ml of medium or 40 oz roller bottles with 150 ml of medium. All media routinely contained 200 I.U. penicillin, 200 μ g/ml streptomycin and 50 μ g/ml

nystatin. Confluent monolayers were passaged as follows. The spent medium was removed and the cells washed with phosphate buffered saline 'A' (PBSA, Dulbecco, 1954; Oxoid Limited, London, England). The cultures were treated with 0.02% w/v ethylene diamino tetracetic acid (EDTA, British Drug Houses Limited, Poole, Dorset, England), in a sufficient volume to cover the monolayer, for 5 min. Detached cells were recovered by low speed centrifugation (100g, 2 min) and resuspended in a small volume of serum-free medium. After estimation of numbers of viable cells using a Neubauer haemocytometer (Hawksley, London, England) the cells were diluted as necessary into growth medium to a final concentration of $2.5-3 \times 10^5$ cells/ml, distributed to bottles and incubated at 37°C.

Human citrullinaemia cells (Tedesco & Mellman, 1967; Bio-Cult Laboratories Limited, Glasgow, Scotland) were grown in 12 oz prescription bottles in a modified minimal essential medium (Glasgow modified, MacPherson & Stoker, 1962; Bio-Cult Laboratories Limited) supplemented with 1.25mM arginine and 10% unheated foetal calf serum (Fraburg Limited). After removal of medium confluent monolayers were washed with PBSA and incubated at 37°C for 5 min with 1 ml of 0.05% trypsin (Baird & Tatlock Limited, London, England). Detached cells were recovered as previously described and bottles were seeded with $5-6 \times 10^5$ cells/ml taking 7-8 days to become confluent.

Vero cells (Yasamova & Kanakita, 1963; Bio-Cult Laboratories Limited) were grown in 40 oz roller bottles in medium 199 (Morgan,

Morton & Parker, 1950; Flow Laboratories Limited). They were seeded at $2-3 \times 10^5$ cells/ml and produced confluent monolayers in 2-3 days. The cells were passaged by a similar method to that used for HeLa cells.

2.2 Mycoplasma testing

The importance of freedom from mycoplasma contamination has already been emphasised: HeLa, mouse L and mouse S180 cells were examined for contamination at frequent intervals during the course of the work. Samples of cell suspension were inoculated into PPLO broth (Morton & Leece, 1954; Difco Laboratories, Detroit, Michigan, U.S.A.), supplemented with 20% horse serum (Oxoid Limited). Any intact cells were disrupted by ultrasonic treatment of the broth for 1 min (Headland Ultrasonic bath,^{40Kc} Surgical Supplies Limited, London, England) before incubating for 7 days at 37° . After this period, samples were inoculated onto PPLO agar (Morton, Smith & Leberman, 1951; Difco Laboratories) supplemented as above. Plates were incubated at 37° either aerobically or anaerobically and examined microscopically for characteristic 'fried-egg' colonies at intervals between 7 and 14 days. At no time were contaminants found by this method. In addition, the same cells were frequently passaged in antibiotic-free medium to determine any undetected bacterial contamination.

2.3 Pirt-modified Eagle's medium

The culture medium (Table 2.1) used was a modification of Eagle's MEM (PME) described by Birch and Pirt (1969). From experiments

Inorganic salts (BSS.)	mg per litre	L-amino acids	mg per litre
NaCl	8,000	Histidine	100
KCl	400	Lysine	175
CaCl ₂	140	Isoleucine	180
MgSO ₄ ·7H ₂ O	100	Leucine	180
Na ₂ HPO ₄ ·12H ₂ O	60	Methionine	30
KH ₂ PO ₄	60	Phenylalanine	65
MgCl ₂ ·6H ₂ O	100	Threonine	100
NaHCO ₃	1,100	Tryptophan	20
		Valine	150
Vitamins	mg per litre	Alanine	90
		Serine	20
i-Inositol d-Biotin Choline Nicotinic acid Pantothenic acid Pyridoxal Phosphate Thiamine Riboflavin Hypoxanthine B ₁₂ Folic acid	mg per litre	Glycine	15
		Tyrosine	70
		Cystine	75
		Glutamine	200
		Na glutamate	1,530
		D-sugars	mg per litre
		Glucose	1,000

Table 2.1 Composition of Pirt's-modified Eagle's medium

with mouse LS cells these authors concluded that one of the major roles of serum for cells in culture is to provide choline and that many chemically defined media are deficient in this component. Serum also contains free amino-acids and experiments were to be conducted in which it was important to define the exact concentration of a particular amino-acid, arginine, supplied to the cells. It was decided, therefore, to omit serum from experimental media and to employ a medium rich in choline. In addition, the medium contained elevated levels of certain amino-acids shown to be essential for the maximum growth of mouse LS cells (Griffiths & Pirt, 1967). The cellular requirement for glutamine, which is particularly labile, was supplied by a mixture of glutamine and glutamic acid. The pH of the medium was maintained at 7.3 by the addition of 14mM N-2-hydroxyethyl piperazine-N'-2-ethane sulphonic acid (HEPES, Williamson & Cox, 1968; Calbiochem Limited, London, England). As an additional precaution against contamination by mycoplasma, kanamycin sulphate at 200 µg/ml (Bristol Laboratories, Feltham, England) was added to the medium of virus-infected cells.

2.4 Depletion of intracellular pools of arginine

Virus replication studies in HeLa, mouse L, mouse S180 and human citrullinaemia cells were made in cell cultures in 6 x $\frac{5}{8}$ " test-tubes.

HeLa, mouse L or mouse S180 cells suspended in growth medium were seeded into test-tubes in 1 ml aliquots at a concentration of

4×10^5 cells/ml. Confluent monolayers containing about 1×10^6 cells/ml test-tube were attained after 1 - 2 days incubation at 37° . After the removal of growth medium, HeLa and mouse L cells monolayers were maintained in PME without arginine or serum for 18 hr before infection. This starvation time was sufficient to deplete the cell pools of free arginine without causing any apparent cytological changes in the cells. Under these conditions mouse S180 cells became detached from the glass and, in consequence, they were maintained in a similar medium for a shorter period of 6 hr before infection. This proved to be sufficient time to deplete the cell pool of arginine without any effect on the microscopic appearance of the monolayer. Human citrullinaemia cells were grown in Glasgow modified MEM with 10% foetal calf serum and because of their lower growth rate were seeded at a concentration of $7-8 \times 10^5$ cells/ml. Confluent monolayers were produced after 2 days and the cells were maintained without arginine or serum for 18 hr before infection without any apparent cytological changes in the cells.

2.5 Measurement of cell growth

HeLa, mouse L or mouse S180 cells in PME without arginine, supplemented with 5% unheated calf serum were distributed at known concentrations in 4 oz prescription bottles. HeLa and mouse L cells were incubated at 37° in this medium for 18 hr but mouse S180 cells were maintained without arginine for only 6 hr. After this time cells were recovered from two bottles, resuspended in PBSA containing 0.01%

trypan blue (British Drug Houses Limited) and total and viable counts determined. This viable count provided the base line for the number of cells initially present. The media on the remaining cells was removed and experimental media supplemented with 5% calf serum were added. Incubation proceeded for 1 - 2 days at which time cells grown in the different media used were harvested in duplicate samples and total viable counts determined.

2.6 Preparation of virus stocks

Stocks of Lister vaccinia virus were prepared in confluent monolayers of HeLa cells grown in 40 oz roller bottles. The growth medium was removed and the cells infected with 1×10^8 p.f.u. of virus (0.1 - 1.0 p.f.u./cell) in 20 ml medium without serum. After adsorption of the virus for 2 hr at 37° a further 30 ml of medium without serum was added and incubation proceeded at 37° for 2 days. At this time most of the cells were detached from the glass but remaining cells were suspended by gently shaking the bottles. The cell pellet was collected by low speed centrifugation (100g, 5 min), the supernatant discarded and the cell pellet resuspended in 5 ml Hanks balanced salt solution (BSS, Hanks, 1949). After 2 cycles of freezing and thawing, disruption of cells was completed by treatment with ultrasonics for 2 min and the cell debris removed by centrifugation at 1,500g for min. The virus-containing supernatant was collected and the pellet resuspended in a further aliquot of Hanks BSS. After further treatment with ultrasonics the virus-containing supernatant was

recovered as before. The pooled supernatants were centrifuged at 15,000 rev/min for one hour using the SW50L rotor in a Spinco Model L ultracentrifuge, the supernatant was discarded and the virus pellet resuspended in 10 ml Hanks BSS. After distribution in 1 ml aliquots stock virus preparations were kept at -50° .

Lister vaccinia virus from HeLa cells was established by 3 serial passages in mouse L cells before preparation of partially-purified virus from these cells. The IHD strain of vaccinia virus and rabbitpox virus were established similarly in mouse S180 cells. Stock virus preparations were obtained as described previously. In view of the difficulty experienced in preparing sufficient quantities of human citrullinaemia cells, experiments involving this cell line were made using Lister vaccinia virus from HeLa cells.

2.7 Measurement of virus infectivity

a) Infection of cell cultures

Since the amount of virus inoculated onto cells varied according to the type of experiment, individual data will be presented in the text. For infection of cell monolayers in $6 \times \frac{5}{8}$ " test-tubes, the maintenance medium was removed and virus was adsorbed for 1 hr from 0.2 ml of medium (appropriate medium indicated in the text) without serum. Adsorption and all further procedures were carried out at 37° . The inocula were removed and the cells washed with 1 ml Hanks BSS, to remove any unadsorbed virus. Appropriate media in 1 ml aliquots were added to each tube. Duplicate samples were frozen immediately

at -50° for subsequent titration of infective virus, to determine the inoculum recovery. The remaining tubes were incubated at 37° , duplicate samples were removed as indicated in the results, and frozen at -50° until titration for infective virus.

b) Infectivity titrations

After 2 cycles of freezing and thawing cells from replicate samples were scraped into the medium using a Pasteur pipette and the resultant suspensions were pooled into 10 ml centrifuge tubes. Cell disruption was completed by treatment with ultrasonics for 1 min and serial 10-fold dilutions of the resultant cell extract were made in medium 199. Three dilutions from each sample were plated in triplicate onto confluent monolayers of Vero cells grown in test-tubes. Appropriate dilutions in 0.2 ml amounts were adsorbed for 1 hr at 37° . After removal of inocula, 1 ml of medium 199 supplemented with 2% calf serum was added and the infected cell cultures incubated at 37° for 2 days. The medium was then removed and the cells stained with carbol fuchsin (Solmedia Limited, London, England). Plaques about 1 mm in diameter were counted against a light background. The arithmetic mean of counts from dilutions containing between 30 - 300 plaques was used as an estimate of the total recoverable infective virus.

2.8 Use of radiochemicals

Experiments involving the use of radiochemicals were carried out in HeLa, mouse L or mouse S180 cell monolayers grown in test-tubes

or 4 oz prescription bottles. Protein synthesis was studied in infected and control cell cultures using uniformly labelled (^{14}C) phenylalanine (specific activity 312 mCi/mM). The incorporation of arginine into infected and control cell cultures was followed using guanido-labelled (^{14}C) arginine (specific activity 50 mCi/mM). The incorporation of citrulline into infected or control cell cultures and into a purified virus preparation was followed using carbamoyl-labelled (^{14}C) citrulline (specific activity 57 mCi/mM or 24.2 mCi/mM). All labelled compounds were supplied to cell cultures in media containing a known concentration of the unlabelled compound. Cell cultures were equilibrated to this concentration of unlabelled compound for 18 hr prior to infection.

Confluent monolayers in tubes, containing about 1×10^6 cells, and similar monolayers in 4 oz prescription bottles, containing about 1×10^7 cells, were infected with virus at a multiplicity of 5 p.f.u./cell using methods previously described. After the removal of unadsorbed virus and washing with unlabelled medium, 1 ml aliquots of experimental medium containing the appropriate labelled precursor were added to test-tube cultures; 10 ml of similar medium were added to cell cultures in 4 oz prescription bottles. At this stage similar medium was added to uninfected, control cell cultures. Cell cultures were incubated at 37° and samples consisting of 5 tubes or one 4 oz prescription bottle were frozen at -50° at appropriate times.

Pulse labelling experiments were carried out by adding radio-

actively-labelled compounds to cell cultures at various times post-infection. After 20 min further incubation, these cell cultures were rapidly frozen at -50° .

Incorporation of radioactive label was determined by submitting cells to 2 cycles of freezing and thawing. Replicates from each sample were pooled into 10 ml centrifuge tubes and cell disruption was completed by treatment with ultrasonics for 2 min. The resulting extracts were made 10% w/v with trichloroacetic acid and flocculation of the resulting precipitates were assisted by immersing the centrifuge tubes in a water bath at 56° for 5 min. The precipitates were centrifuged at 1,500 g for 5 min and the supernatant discarded. After washing 3 times with 5 ml of 5% trichloroacetic acid the precipitates were dissolved in 0.5 ml Soluene (Packard Instruments Limited, Wembley, Middlesex, England) and quantitatively transferred to scintillation vials by washing in a total volume of 10 ml Bray's scintillation liquid (Bray, 1960). The incorporated radioactivity was determined by counting in a Packard three channel liquid scintillation spectrometer, model 3310. Appropriate quench corrections were made using a channels ratio method. The radioactivity was expressed as disintegrations per minute.

2.9 Production and purification of radioactively-labelled virus

A confluent 40 oz roller bottle of HeLa cells was infected with 5 p.f.u./cell of Lister vaccinia virus in 20 ml PME without arginine or serum. After adsorption at 37° for 2 hr the inoculum was removed,

50 ml of medium containing 0.2 uCi/ml of carbamoyl-labelled citrulline was added and incubation carried out for 2 days at 37°. The cells were harvested as previously described and the virus extracted twice from the cells with 2 ml aliquots of 0.01M Tris-HCl buffer, pH 8.6. The pooled supernatants were extracted twice with equal volumes of Arcton 113 (Imperial Chemical Industries Limited, Mond Division, Runcorn, Cheshire, England) and the phases separated by centrifugation (1000 g, 5 min). The upper aqueous phase containing the virus particles was retained; cell debris and lipid collected in the Arcton layer were discarded. The virus was sedimented from the aqueous phase by centrifugation in a Spinco model L Ultracentrifuge at 15,000 g for 30 min. The virus pellet was resuspended by ultrasonic treatment in 0.25 ml 0.001M Tris-HCl, pH 8.6 and layered onto columns of 50% w/v sucrose in the same buffer. The low ionic strength of the buffer reduced aggregation of the virus particles. The virus was sedimented through the sucrose column at 130,000 g for 60 min and the procedure repeated. A non-linear sucrose density gradient in 0.001M Tris-HCl, pH 8.6 was made by layering 5 x 0.9 ml aliquots of sucrose solutions with concentrations from 50 to 70% w/v and allowing diffusion at +4° overnight. The virus pellet was resuspended in 0.2 ml 0.001M Tris-HCl and placed on the top of the gradient which was centrifuged at 130,000 g for 90 min. The virus band was recovered by puncturing the base of the tube. Examination of this band in the electron microscope revealed complete virus particles and no cellular debris.

2.10 Chromatography

Two way paper chromatography was carried out by a descending method. Whatman no. 1 chromatography paper $45 \times 45 \text{ cm}^2$ was used (W. & R. Balston Limited, England) and the procedure was carried out in a Shandon Chromo tank (Shandon Limited, London, England).

Solvent system 1: was but-1-ol-acetic acid-water, 4 : 1 : 5. After separation of the mixture into 2 phases, upper and lower phases were collected using a separating funnel. The upper phase was used as solvent and the lower water-rich phase was retained and used to saturate the atmosphere in the chromatography tank prior to the start of the experiment.

Solvent system 2: was phenol-ammonia. This was prepared by saturating 500 g of phenol with 125 ml of distilled water in a water bath at 37° . This was stored in a dark bottle to prevent oxidation of the phenol and consequent blackening. Immediately before use 2.5 ml of concentrated ammonia were added to the resulting solution. A solution of 5% phenol was used to saturate the atmosphere in the chromatography tank prior to the addition of the solvent.

Ninhydrin reagent was made by dissolving 0.625 g indane-trione hydrate in 2.5 ml glacial acetic acid and making up the volume to 250 ml with acetone.

All the reagents were supplied by British Drug Houses Limited.

Samples were spotted onto the paper in a volume of 0.035 ml.

After thorough drying chromatograms were lowered into the tank over guide rails and held in place in the troughs with tie bars. Two beakers containing the water-rich phase from the first solvent and fitted with filter papers, to enhance the equilibration of the atmosphere in the chromatography tank, were placed at the bottom of the tank which was then sealed with Apiezon grease (Edwards High Vacuum Limited, Crawley, Sussex, England). After equilibration for 1 hr, 75 ml of solvent system 1 were administered to each of the 2 troughs through valves at the top of the tank. These were resealed and the chromatograms were left for 24 hr or until the solvent front was within 1 cm of the lower edge of the paper. At this time they were removed and dried in an oven at 37°.

The chromatograms were placed in the tank as before and the atmosphere equilibrated with 5% phenol for 1 hr before introducing the second solvent. After administering the solvent the chromatograms were left for a similar length of time before being removed and dried. The dried chromatograms were sprayed with ninhydrin reagent and developed by heating at 95° for 5 min. Control chromatograms were run in parallel, with known amino-acids, each at a concentration of 5 µg/µl. After development with ninhydrin reagent, amino-acids were located as purple spots except for proline which gave a yellow spot. The R_f values of these known amino-acids were determined and compared with values given in standard tables (Biochemists Handbook, Long).

2.11 Protein Determination

Protein determination of cell extracts was carried out using a buiret-phenol method (Sutherland, Cori, Haynes & Olsen, 1949).

Stock solution 1: 2% solution of sodium bicarbonate in 0.1M sodium hydroxide.

Stock solution 2a: 1% solution of copper sulphate.

Stock solution 2b: 2% solution of sodium-potassium-tartrate.

Stock solution 3: prepared by mixing 50 ml of stock solution 1 with 0.5 ml each of stock solutions 2a and 2b. This was prepared fresh for each experiment.

Stock solution 4: Folin-Ciocalteau's reagent.

All the reagents were supplied by British Drug Houses Limited.

Samples of cell extract were dissolved in 1M sodium hydroxide and 5 ml of stock solution 3 were added to 1 ml of this alkaline solution. After standing for 10 min, 0.5 ml of Folin-Ciocalteau's reagent was added and mixed by gentle shaking. After a further 30 mins, during which time a blue chromogen develops in the presence of amino groups, the mixture was examined in a EEL colorimeter (Evans Electroselenium Limited, Halstead, Essex, England) with a red filter, 607. The colorimeter was standardised against a blank which contained 1 ml of 1M sodium hydroxide but no protein and had been treated in the same way as the test material.

A standard curve was prepared using bovine serum albumen (British Drug Houses Limited) by diluting a stock solution in 1M sodium hydroxide to cover a range of concentrations between 30 - 300 ug/ml.

There was a linear relationship between the concentration of protein in the sample and the optical density value obtained from the EEL colorimeter (Fig. 2.1). The concentration of protein in experimental samples was determined using this standard curve.

2.12 Enzyme assays

An in vitro enzyme assay system was used to determine the levels of certain enzymes in the Krebs-Henseleit urea cycle in infected and control cells. The assays were dependent on the ultimate release of $^{14}\text{CO}_2$ from guanido-labelled (^{14}C) arginine or carbamoyl-labelled (^{14}C) citrulline. The method was based on that employed by Carulli, Kaihata and Wagner (1968) who developed a closed system suitable for assaying arginase in serum, plasma and tissue homogenate. The apparatus, developed for work presented in the text, consisted of two liquid scintillation vials joined at the neck by a rubber collar. A canula was inserted through the collar so that liquid could be admitted to the lower vial (Plate 2.1). The canula was connected externally to 0.5 mm i.d. plastic tubing that could be sealed using a screwclip. The upper vial was lined with a cylinder 4 cm x 8 cm of Whatman no. 1 filter paper which was soaked with 0.4 ml of 0.1N potassium hydroxide (British Drug Houses Limited) which served to trap evolved carbon dioxide as potassium hydrogen carbonate. The apparatus was maintained in an upright position in a metal rack. All reactions were carried out at 37° in a water bath and racks were gently shaken. The recovered $^{14}\text{CO}_2$ was estimated by separating the vials at the end of an experiment, drying those containing filter papers over phosphorus pentoxide (British Drug Houses Limited)

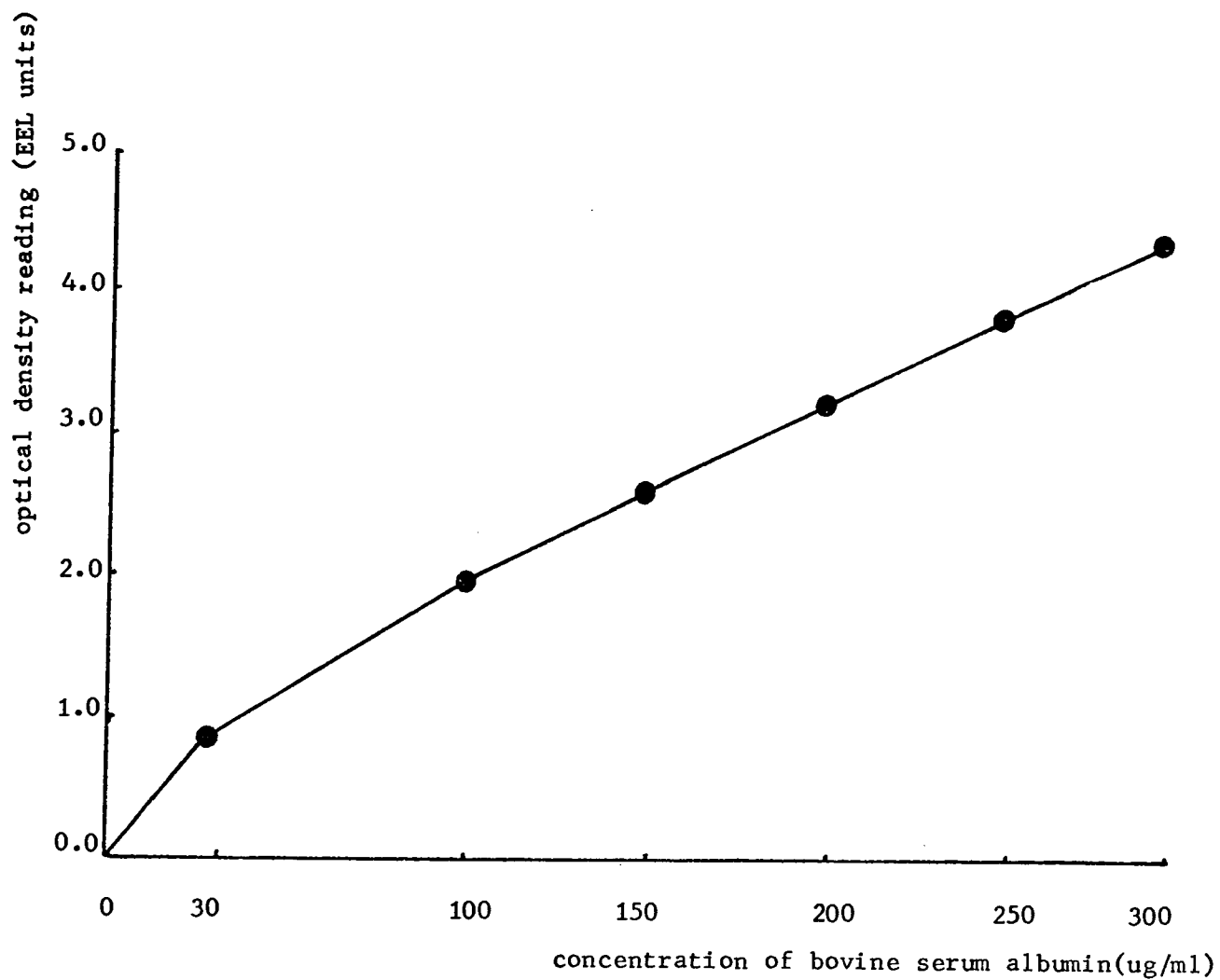


Fig. 2.1 Standard curve for protein estimation



- A) Filter paper soaked with KOH.
- B) Plastic tubing for addition of reagents.
- C) Reaction mixture.

Plate 2.1 Apparatus for enzyme assays.

in a vacuum desiccator overnight and adding 20 ml of toluene liquid scintillant (4g 2-5-diphenyloxazole, PPO; 50mg 1-4-bis (2(4methyl-5-phenyloxazolyl))-benzene, POPOP; Packard Instruments Limited in 1000 ml toluene) to each vial. The recovered radioactivity was determined by counting in a Packard spectrometer. Assessment of the quantitative recovery of carbon dioxide in this system was made using (^{14}C)-labelled sodium bicarbonate or (^{14}C)-labelled urea.

a) Quantitative recovery of $^{14}\text{CO}_2$

The quantitative efficiency of this system was determined by recovering $^{14}\text{CO}_2$ from (^{14}C)-labelled sodium bicarbonate following the addition of acid. Volumes of 0.5ml (^{14}C)- NaHCO_3 were added to the lower vial of the absorption apparatus, 1.0 ml 1N hydrochloric acid were added through the canula and the apparatus sealed. The upper vials were removed at intervals of from 1 to 5 hr after the addition of acid and processed for assessment of recovered radioactivity as described. Quantitative recoveries were determined by comparison with control vials in which the same volume of (^{14}C)- NaHCO_3 was applied directly to the filter paper cylinder after soaking with potassium hydroxide. The results shown in Table 2.2 indicate that the recovery of $^{14}\text{CO}_2$ was greater than 95% and that this maximal recovery was achieved after absorption for one hour. Correlation between replicate samples was also greater than 95%.

Further experiments were made to determine the quantitative recovery of $^{14}\text{CO}_2$ and the efficiency of determination of radioactivity

Table 2.2. Determination of absorption time for quantitative recovery of $^{14}\text{CO}_2$

Time (hr)	dpm/sample
1	94,714 : 93925
2	93,550 : 90,867
5	93,029 : 92,151
Control : 0.5ml ^{14}C - HCO_3 applied directly to filter paper	95,103 : 95,246

in this system. Volumes of 0.5 ml (^{14}C)-urea containing 10 to 0.05 μmoles urea were added to the lower vials, 0.05 μmoles urea were added to the lower vials, 0.5 ml urease solution added and the apparatus closed immediately. The urea and urease solutions were made in 0.01 M phosphate buffer pH 7.0 and the amount of urease was sufficient to hydrolyse completely the urea to ammonia and $^{14}\text{CO}_2$. After incubation at 37° for 20 min, 1.0 ml 1N hydrochloric acid was added and the reaction mixtures incubated for a further hour. After this time the upper vials were recovered and processed for measurement of radio-activity as described. The quantitative recovery was determined by applying 0.5 ml of a solution containing 10 μmoles (^{14}C)-urea directly onto the filter paper. The efficiency of counting was determined by adding 0.1 ml of the same preparation to 10 ml scintillation liquid containing 30% ethyl alcohol.

The results presented in Fig. 2.2 show that there was a linear, quantitative recovery over the range 10 to 0.05 μmoles (^{14}C)-urea and the total recovery was greater than 95%. The efficiency of counting was 60%.

b) Conversion of citrulline to arginine

The conversion of citrulline to arginine is effected by two enzymes, arginosuccinate synthetase and arginosuccinate lyase. The assay was designed to follow the ultimate production of arginine from citrulline and no attempts to differentiate the two steps in this conversion have been made. The reaction mixture for each sample

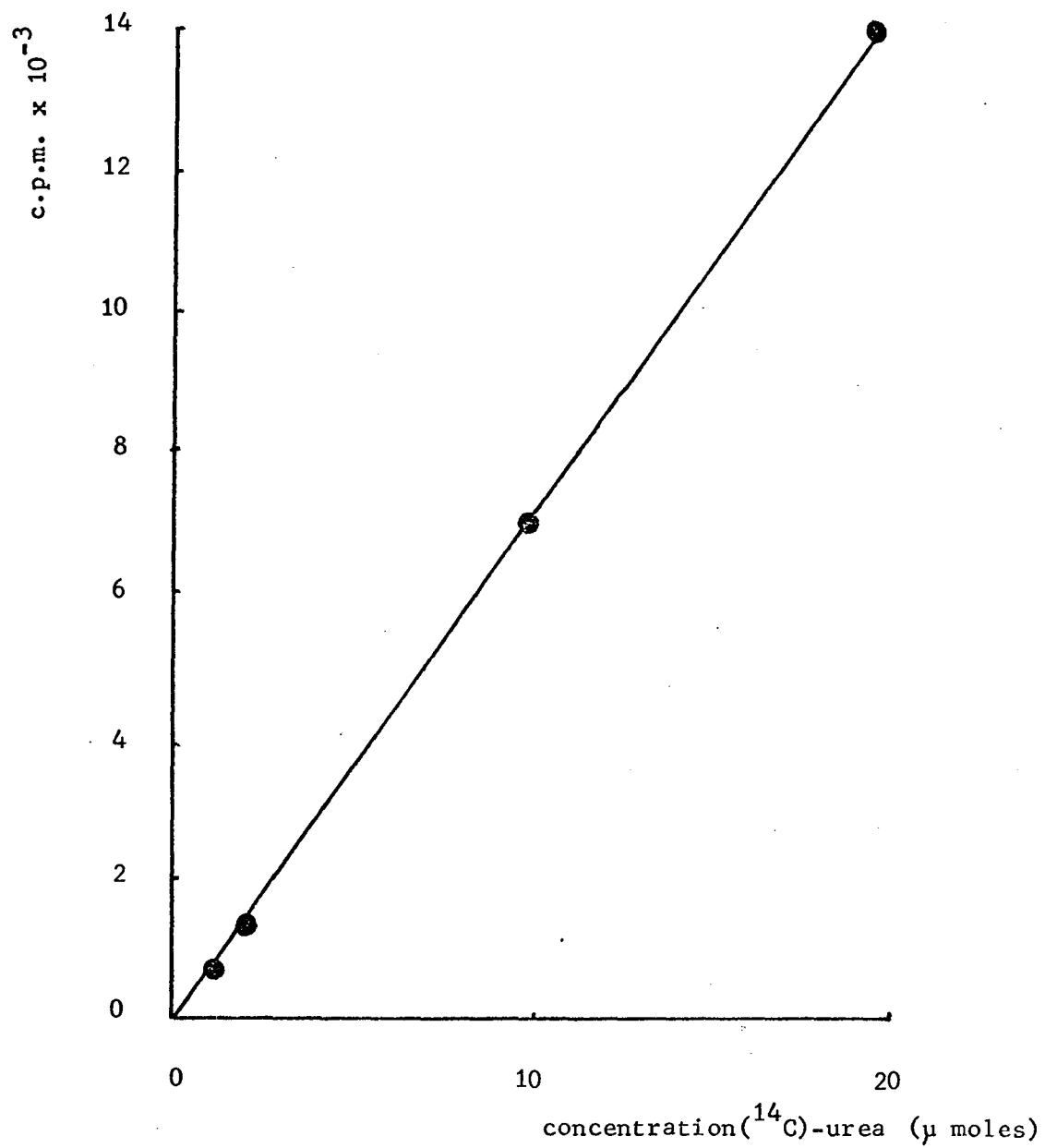


Fig. 2.2 Quantitative recovery of $^{14}\text{CO}_2$ from (^{14}C) -urea

consisted of : -

2.5 μ moles aspartic acid	250 units arginase
1.25 μ moles adenosine triphosphate, ATP	25 μ moles citrulline
20 μ g pyruvate kinase	1 μ mole (14 C) carbamoyl-labelled citrulline
5 μ g potassium chloride	(specific activity 0.1mCi/mM, Radiochemical Centre)

All chemicals were obtained from Sigma Chemical Company Limited, London, England, except potassium chloride (British Drug Houses Limited).

c) Purification of arginase

The purification of arginase was based on a method by Bach, Hawkins and Swaine (1968). Partially purified arginase (specific activity 22 or 35 units/ng) in 100 mg aliquots was dissolved with stirring for 80 mins in 10 ml manganese-maleate buffer, pH 6.8. The bulky precipitate that remained was removed by centrifugation at 1,500 g for 5 min and the supernatant dialysed at $+4^{\circ}$ for 20 hr, with one change, against one litre volumes of the same buffer. The pH of the dialysate was raised to 7.3 with 0.1N sodium hydroxide (British Drug Houses Limited) and warmed to 30° in a water bath, before immersing in a second water bath of 65° for 3 min. The precipitate that formed was removed by centrifugation (1,500g, 5 min) and the arginase-containing supernatant was collected. The remaining activity of the arginase and that of contaminating enzymes responsible for the conversion of citrulline to arginine was determined. This was carried out by the method described below using guanido-(14 C)-arginine and carbamoyl-(14 C)-citrulline

respectively as substrates. This method of purification gave a ten-fold reduction in the background count from contaminating enzymes without significantly affecting the arginase activity.

d) Assay procedure

Confluent cell monolayers in 40 oz roller bottles were maintained in suitable medium for 18 hr prior to infection. The cells were infected with 5 p.f.u./cell of virus by methods previously described. Control cultures were mock infected in the same way. All cell cultures were subsequently incubated at 37° for 18 hr in 50 ml of experimental medium without serum. The cells were harvested and resuspended in 4 - 5 ml Tris-HCl buffer, pH 8.0. After two cycles of freezing and thawing, cell disruption was completed by ultrasonic treatment for 2 min. Aliquots containing 1.0 ml of these extracts were taken for enzyme assay and further samples were used for protein determination. The reaction mixture was added to scintillation vials in a total volume of 1.0 ml, and equal volume of cell extract added and the system closed. After 1 hr incubation with shaking at 37°, the apparatus was removed, immersed in a second water bath at 70° for 20 min and 0.5 ml 1N hydrochloric acid was added to each sample. One ml of 1.6N sodium hydrogen phosphate (British Drug Houses Limited) warmed to 37° was added to each sample to raise the pH to 7.0, 0.5 ml urease (Sigma Chemical Company Limited) in a solution at 10 units/ml in phosphate buffer pH 7.0 added and the system closed immediately. After shaking at 37° for 20 min, 1.0 ml 1N hydrochloric acid was added to each sample

to stop any further reaction and enhance the release of carbon dioxide. After a further hour the vials were separated and processed as previously described.

Controls were carried out for each experiment to determine the background level of enzyme activity from the commercial arginase preparation by omitting cell extracts from the reaction mixtures and making up the volume with 1.0 ml Tris-HCl buffer, pH 8.0. The radioactive counts from the controls were subtracted from those in experimental samples to determine the activity of enzymes in the cell extracts. The results were calculated from the disintegrations/min, knowing the total concentration of citrulline in the reaction mixture, as μ moles of citrulline converted to arginine/hour/mg cell protein.

e) Arginase assay

Confluent monolayers of cells in 12 oz prescription bottles were maintained in suitable experimental media for 18 hr prior to infection. At this time cells were infected with 5 p.f.u./cell of virus by methods previously described. After adsorption at 37° for 1 hour the cells were washed with Hanks BSS and incubated for 18 hr in 25 ml of the medium supplemented with 0.1 μ C/ml (¹⁴C)-guanido-labelled arginine. Control cell cultures were mock infected and treated in the same way. After incubation, the cells were harvested and used for protein determination. Aliquots of 0.1 ml of labelled medium taken before and after incubation were put into 10 ml Bray's liquid scintillant and the radioactivity counted to determine the total activity

remaining in the medium after incubation.

Aliquots of media were used to determine the arginase activity in the cells. Aliquots of 0.5 ml were placed into the lower vials and the apparatus assembled. Urease in 0.01 M phosphate buffer pH 7.0 was added in 0.5 ml amounts and the system closed immediately. All further procedures were identical to those described for the determination of the conversion of citrulline to arginine. Samples were examined in duplicate and the results from the same sample agreed to within 97%.

CHAPTER 3

THE EFFECT OF POTENTIAL PRECURSORS AND ANALOGUES ON THE REPLICATION OF VACCINIA VIRUS IN HELA CELLS

3.1 Introduction

On the basis of available data, arginine is required in vaccinia virus-infected HeLa cells for events leading to the production of virus-specified DNA: a further quantitatively greater amount of arginine is required for the production of mature virus particles (Archard & Williamson, 1971). Synthesis of virus-specified DNA proceed maximally in the presence of 0.03mM arginine but maximal yield of infectious virus requires a final concentration of 0.1mM arginine. Arginine per se is incorporated into virus particles so that at least part of the arginine requirement of pox-virus infected cells is involved in the synthesis of structural components of the virus. The synthesis of these structural components

probably takes place after the synthesis of virus-specified DNA.

Since there is a dose-response relationship between the availability of arginine and the production of vaccinia virus between 0.03 and 0.1mM arginine, it would appear that at least 60-70% of the total arginine requirement is for the synthesis of virus-specified structural protein. If arginine is metabolised for the production of factors essential for intermediate stages in the replication cycle this will be a quantitatively lower requirement than for arginine itself.

With regard to other arginine-dependent events there is no direct evidence that arginine per se is involved. Products of arginine metabolism are known to play important roles in both prokaryotic and eukaryotic cells. If products of arginine metabolism are important in vaccinia virus replication, supplementation of media available to vaccinia virus-infected cells with these products should have a sparing effect on the requirement for arginine. Experiments were carried out to investigate such effects. Since production of infectious virus was used as a criterion for substitution, experiments could only be carried out in the presence of arginine. However, if there is any substitution effected by these added chemicals, then arginine normally directed into metabolic pathways would become available for incorporation into virus-specified proteins. This could be experimentally detected as a sparing effect on the utilization of arginine making more available for the production of progeny virus. Experiments carried out in the complete absence of arginine would be futile unless the chemical showed a total

substitution, because any early events which might proceed in the presence of that chemical would be masked by the absence of arginine required for later events and maturation. Consequently by the criterion of infectivity, no virus growth would be detected. However, total substitution could be detected in this system if the material substituted acted as a precursor of arginine biosynthesis. Therefore supplementation of medium containing a suboptimal concentration of arginine with possible products of arginine metabolism would reveal any sparing effect on arginine utilization by increasing the yield of infectious virus.

Initially, a dose response curve of the growth of vaccinia virus in HeLa cells supplied with PE medium containing different known concentrations of arginine was prepared. Cells were maintained for 18 hr in PE medium without arginine and subsequently infected in similar medium with 10^4 p.f.u./test-tube of Lister vaccinia virus (See Materials and Methods). After infection cell cultures were maintained for 48 hr in PE medium containing different concentrations of arginine. A low inoculum was used so that any effect produced would be magnified at the end of the 48 hr incubation period as a result of multiple cycles of infection. The results obtained (Fig. 3.1) are in agreement with those of Archard & Williamson (1971). Starvation of cells did not irreversibly block their capacity to facilitate virus growth when arginine was subsequently added to the medium. There was no significant production of virus at a concentration of 0.01mM arginine or below. Maximum yield was obtained at 0.1mM and further addition of arginine above this concentration did not enhance the yield of virus. At a concentration of 0.05mM there

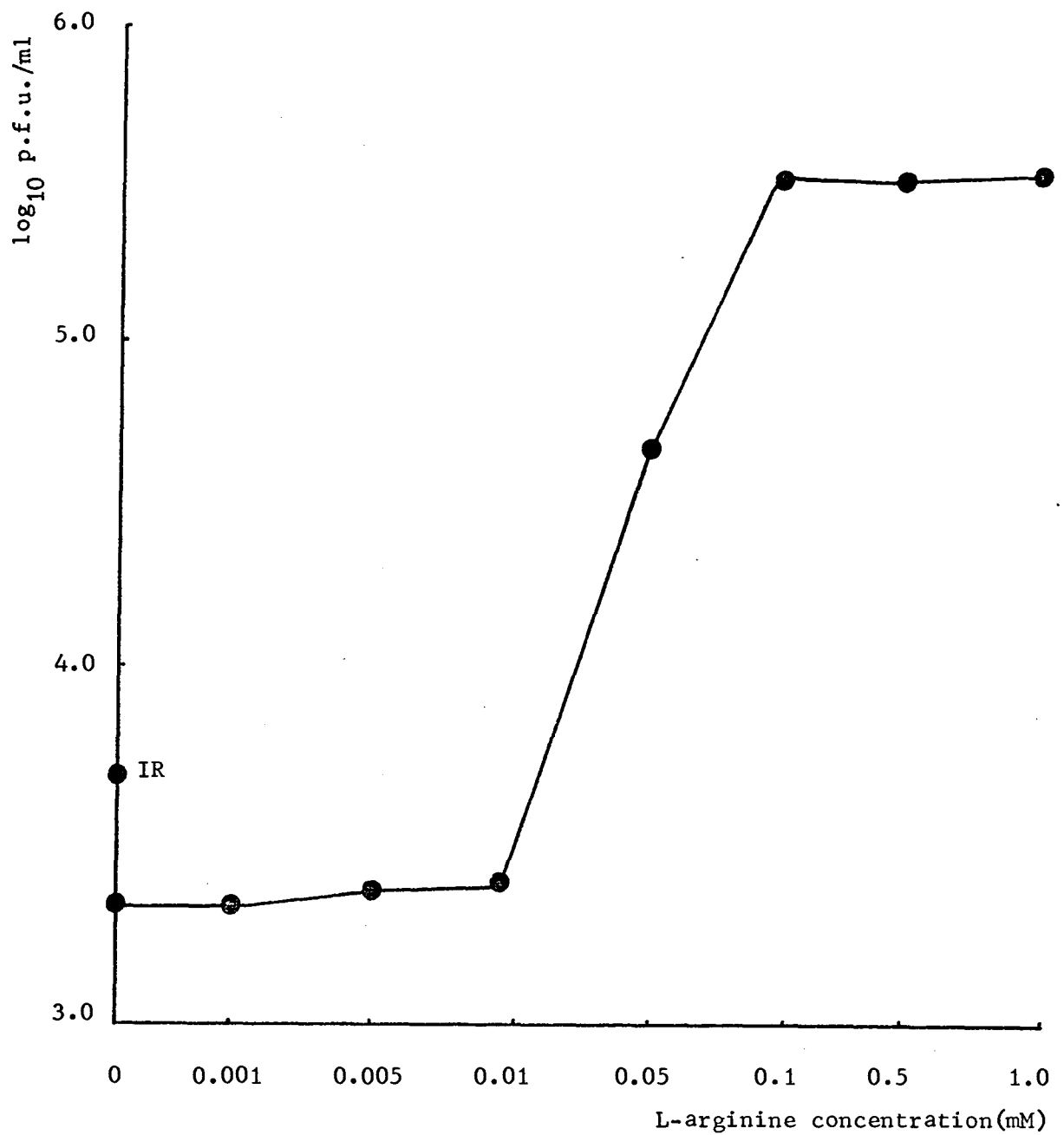


Fig. 3.1 The growth of vaccinia virus in HeLa cells maintained in PE medium containing L-arginine at different concentrations

IR inoculum recovery

was a yield of virus about 60% of the maximal value. Since virus growth was suboptimal at 0.05mM arginine, this concentration was used in subsequent experiments in which sparing effects of different chemicals were investigated. Any sparing effect would be significant up to a value of 40% increase in titre above that detected in the presence of that concentration of arginine alone.

In all experiments to demonstrate a sparing effect, cultures of HeLa cells in test-tubes were maintained for 18 hr in PE medium without arginine or serum before infection and subsequently infected with 10^4 p.f.u./test-tube of Lister vaccinia virus. After adsorption, media containing 0.05mM L-arginine and concentrations between 0.001 and 1.0mM of the test chemical were supplied to the cell cultures. After incubation at 37° for 48 hr yields of infective virus were determined (See Materials and Methods).

3.2 Substitution experiments

a) L-proline

L-proline can be synthesised from arginine through its catabolism to ornithine. Since proline is not an essential requirement for cell growth in vitro (Eagle, 1959) it is assumed that cells in vitro retain their capacity to synthesise this amino-acid. The dose-response relationship between the concentration of arginine and the yield of infective virus was identical in the absence or presence of added proline. The results (Fig. 3.2) indicate that proline does not enhance the yield of vaccinia virus in HeLa cells in the presence of a suboptimal

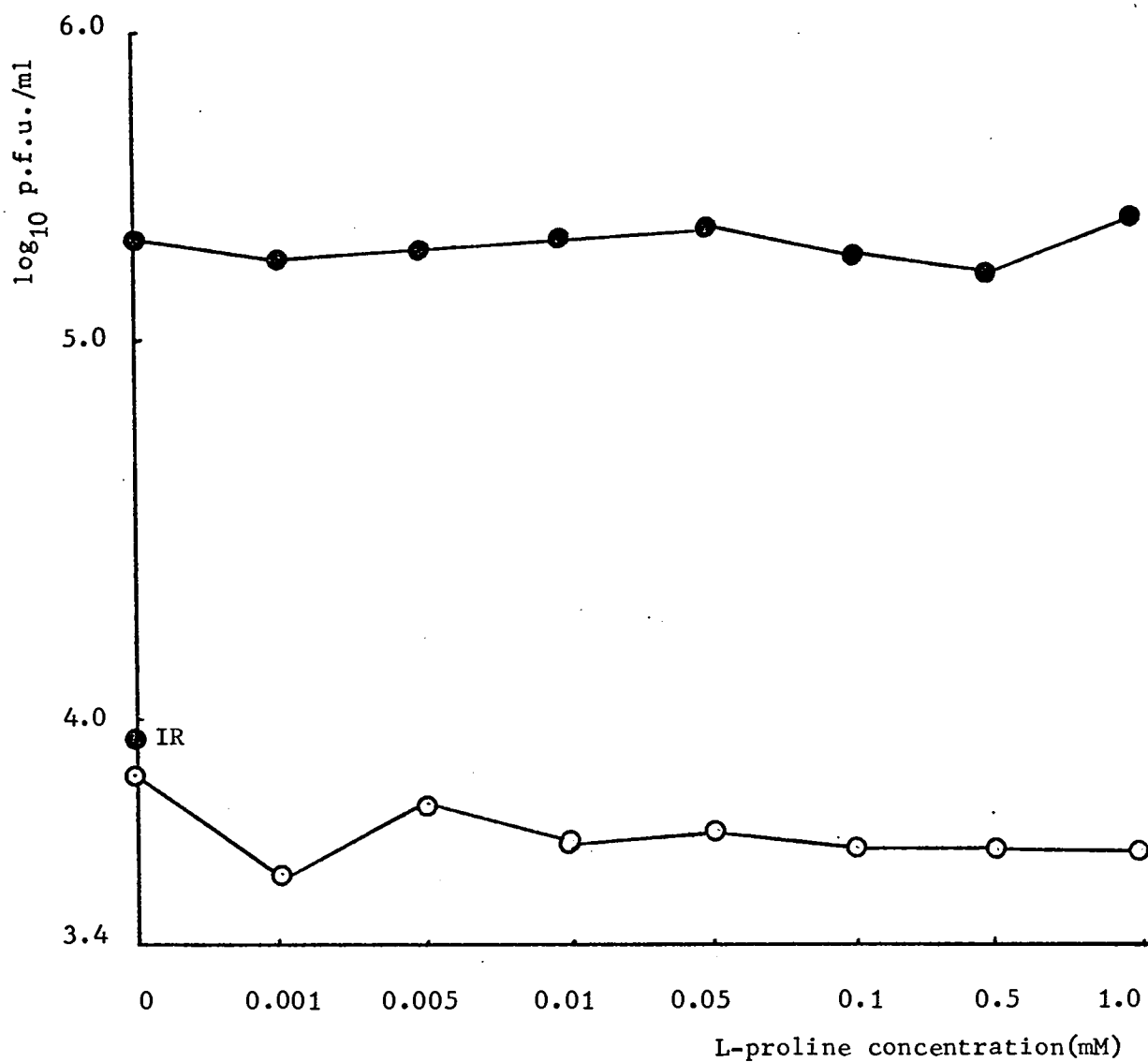


Fig. 3.2 The growth of vaccinia virus in HeLa cells maintained in PE medium containing L-proline at different concentrations in the absence and in the presence of 0.05mM L-arginine.

○—○, L-proline;

●—●, L-proline with 0.05mM L-arginine

concentration of arginine.

b) L-asparagine

L-asparagine could be synthesised from arginine by a reversal of the reactions of the Krebs-Henseleit urea cycle by conversion of arginine through citrulline to ornithine with concomitant production of aspartic acid. Asparagine is formed from aspartic acid by a transamination reaction. The results were almost identical to those found for proline. Asparagine does not enhance the production of progeny virus in cultures maintained in the presence of a sub-optimal concentration of arginine.

c) D-arginine

Eagle (1959) demonstrated that mammalian cells in culture have an essential requirement for the L-isomers of certain amino-acids: only D-cyst(e)ine was shown to be an exception. Experiments were designed firstly to establish whether HeLa cells would grow in PE medium in which D-arginine replaced the L-isomer. Estimation of cell growth was determined by viable cell counts of cells in PE medium with either 0.6mM L- or D-arginine. The results (Table 3.1) indicate that HeLa cells can grow to the same extent in the presence of L- or D-isomer at this concentration and infer that the cells must convert the D-isomer to its L-form. This may involve the action of a specific racemase. Alternatively, a pathway initiated by D-amino acid oxidase may be involved to remove the α -amino grouping which would yield a racemic mixture of L- and D-arginine after transamination (Diagram 3.1). Addition of

Table 3.1 The growth of HeLa cells in the presence of L-arginine or D-arginine

Medium	Viable cell count at 48hr ($\times 10^{-6}$)
0.6mM L-arginine	2.89
0.6mM D-arginine	2.84
arginine free	0.31

Viable cell count at time 0hr

0.4×10^6

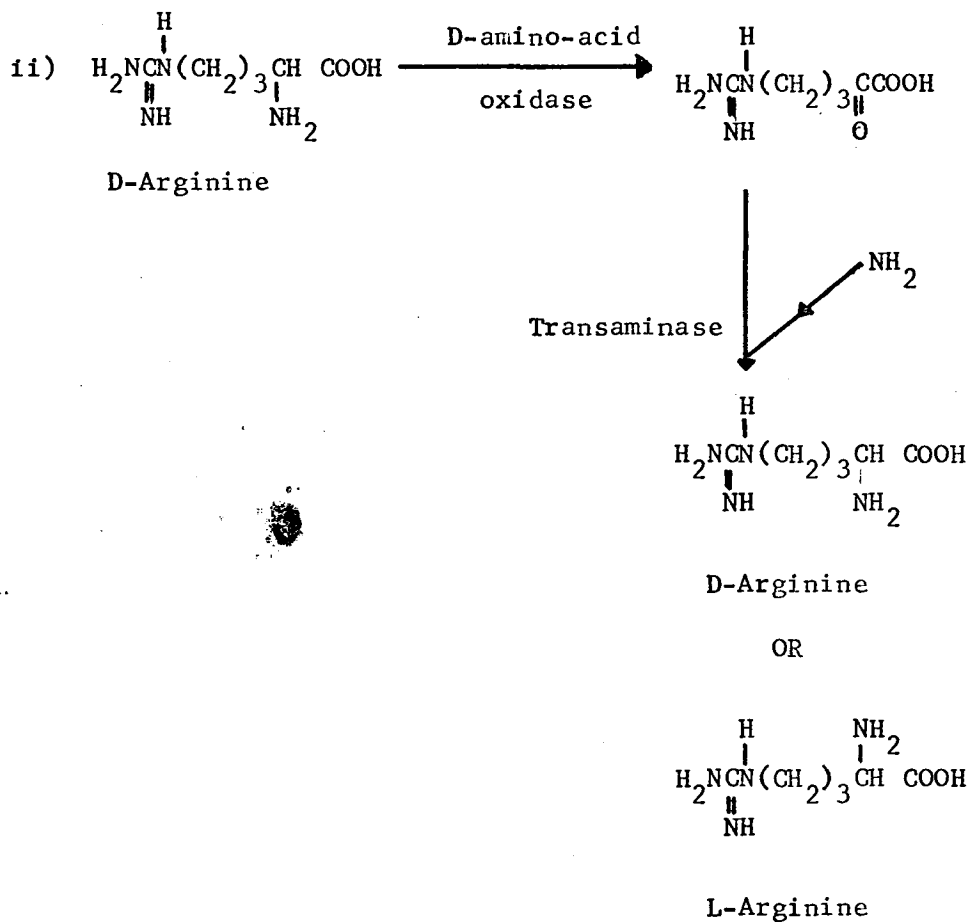
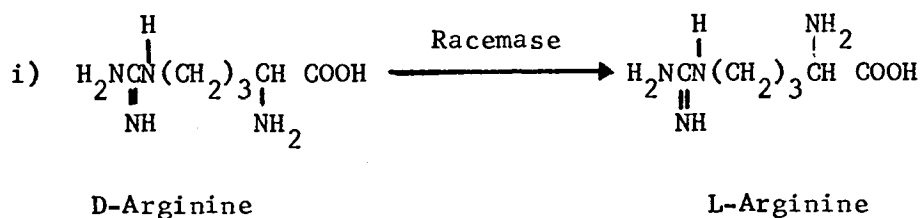


Diagram 3.1. Possible mechanisms for the conversion of D-arginine to L-arginine

different concentrations of D-arginine to cell cultures infected in the presence of the suboptimal concentration of L-arginine results in an elevated production of infective virus at each concentration up to 0.5mM of the D-isomer (Fig. 3.3). Further experiments to determine the growth of vaccinia virus in HeLa cells in the presence of D-arginine alone show that the D-isomer can effectively substitute for L-arginine but the maximal yield is not obtained until a concentration of 0.5mM D-arginine. This demonstrates that the D-isomer is not utilized as effectively as the L-isomer and supports the suggestion that a racemic mixture may be produced within the cell.

d) Galactose

Mammalian cells growing in medium containing different hexoses at a concentration of 20mM utilize 69 μ M per mg of cell protein synthesised in the case of glucose compared to 19 μ M for galactose. At this concentration of substrate cells grown with glucose produce 81 μ M of lactic acid compared to 4.5 μ M of lactic acid from similar cultures grown with galactose (Eagle, Barton, Levy & Schulze, 1959). Polakis and Bartley (1965) showed that Saccharomyces cerevisiae cells grown on galactose possess mitochondria whereas similar cells grown on glucose do not and that the activities of respiratory enzymes in cells cultured on galactose are higher than those grown on glucose.

Galactose is converted to glucose before further catabolism. This involves a reaction mediated by uridine triphosphate (UTP). After phosphorylation, galactose-1-phosphate is converted to UDP-galactose

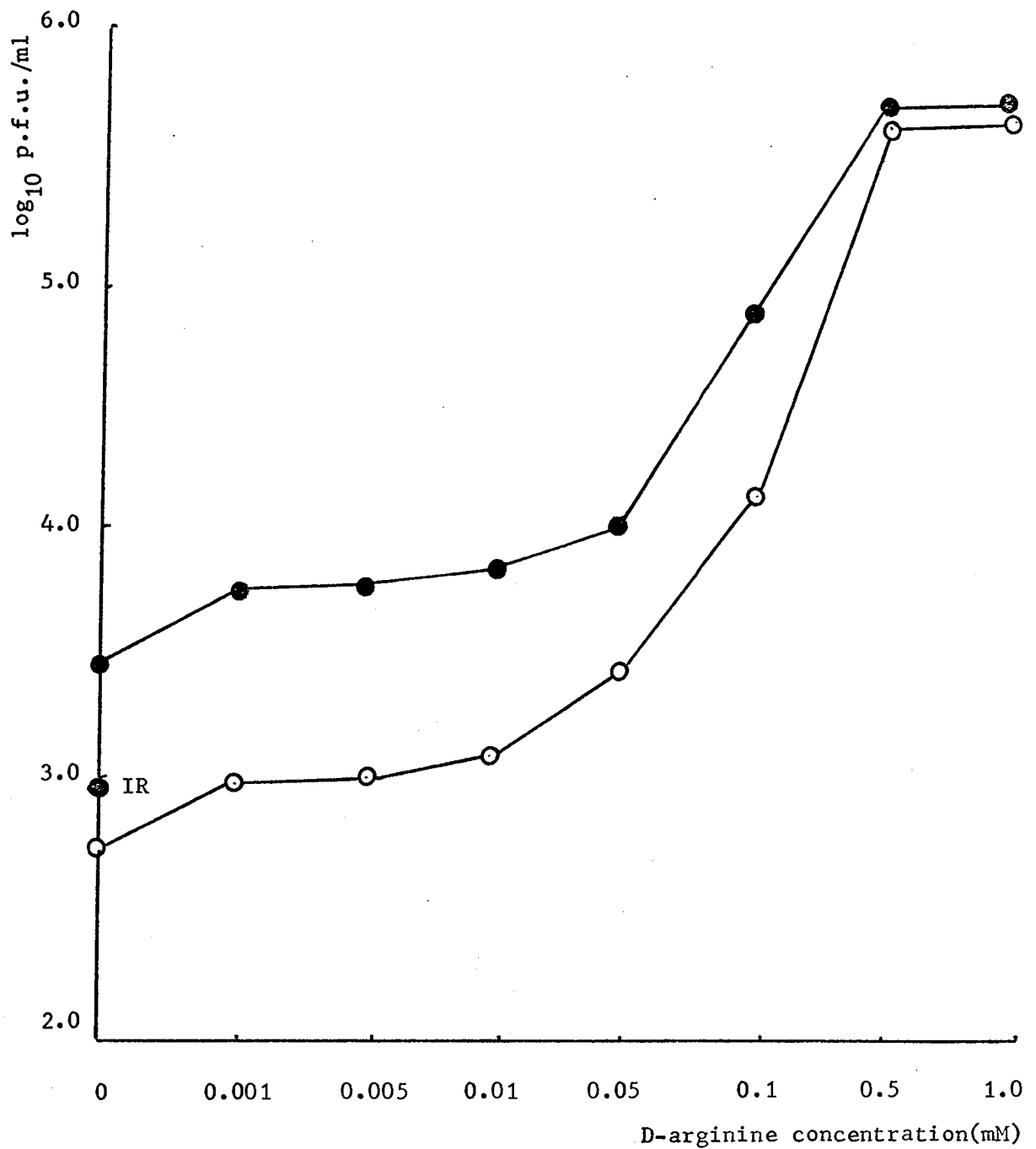


Fig. 3.3 The growth of vaccinia virus in HeLa cells maintained in PE medium containing D-arginine at different concentrations in the absence and in the presence of 0.05mM L-arginine.

○—○, D-arginine;

●—●, D-arginine with 0.05mM L-arginine

with the concomitant release of pyro phosphate by the enzyme UDP-galactose-pyrophosphorylase. UDP-glucose-epimerase, an NAD dependent enzyme, converts this to UDP-glucose which is subsequently catabolised to glucose-1-phosphate by hexose-1-phosphate-uridyltransferase. The production of energy by anaerobic pathways alone is insufficient for cell growth in the presence of galactose and in consequence other supplementary pathways which metabolize hexose by aerobic pathways are induced (Polakis & Bartley, 1966). Thus, in these conditions the citric acid cycle becomes fully primed. Since this cycle is intimately associated with the Krebs-Henseleit urea cycle for the provision of intermediates, any diversion of arginine metabolism with regard to the synthesis of these intermediates in poxvirus-infected cells should be arrested by providing galactose in place of glucose in the growth medium. Also, if arginine were involved as a source of energy in virus-infected cells, as has been indicated in mycoplasma-infected cells (Schimke, 1967), then the provision of galactose should increase the availability of ATP and exert a sparing effect on the utilization of arginine for this purpose.

HeLa cells were serially passaged three times in media containing 1% glucose or 1% galactose. After this time monolayer cultures of the same cells were prepared in test-tubes. Dose-response curves with L-arginine were prepared by methods previously described to determine whether virus production was enhanced in cell cultures maintained with galactose in place of glucose. There was no difference between yields of virus in the presence of either hexose at any concentration of L-arginine.

3.3 Polyamines

Since arginine is incorporated as a structural component of vaccinia virus particles and since the requirement for arginine in later events of the replication cycle determines quantitatively the amount of virus produced, it seems likely that arginine is incorporated directly into the virus. If vaccinia virus-infected cells are supplied with media containing (^{14}C)-guanido or (^{14}C)-uniformly-labelled arginine the ratio of the labelled compounds detected in purified virus particles should be the same as that in the medium supplied for virus growth if none of the arginine is metabolised in the cells. Archard (1971) demonstrated that this is not so and concluded that there is some metabolism of arginine in vaccinia virus-infected cells.

In microbial cells, in which there is a ready availability of arginine, polyamines are derived from ornithine produced by arginine metabolism, whereas in lower concentrations of arginine ornithine is synthesised directly from glutamate and subsequently decarboxylated (Morris & Pardee, 1966). Similarly, in mammalian cells ornithine can be produced from arginine as a result of arginase activity.

Experiments were carried out to determine whether polyamines could enhance the yield of vaccinia virus in HeLa cells maintained in the presence of a suboptimal concentration of arginine. If polyamines are necessary for the stabilization of virus-directed RNA polymerase during the production of early mRNA species, then by providing arginine at a growth limiting concentration and adding known amounts of a

particular polyamine an enhanced yield of virus may be expected from cultures supplemented with exogenous polyamine.

a) Agmatine

Agmatine is produced by the decarboxylation of arginine. The pathway involved is essentially microbial and has not been demonstrated in mammalian cells. Agmatine is an intermediate in the synthesis of spermine. Agmatine ureohydrolase converts agmatine to putrescine which is converted to spermine in the presence of S-adenosyl-methionine. Agmatine was found to be toxic to cells at a concentration of 0.1mM. There was no sparing effect on the utilization of arginine below this concentration and no attempts were made to quantitate any effect at and above 0.1mM agmatine.

b) Spermine

In mammalian cells the action of arginase provides ornithine from arginine as the substrate for spermine biosynthesis. Ornithine decarboxylase facilitates the production of putrescine from ornithine. The synthesis of spermine is completed with S-adenosyl-methionine similar to the microbial system. Addition of spermine at concentrations between 0.001 and 1.0mM, to cell cultures maintained in the presence of a sub-optimal concentration of arginine did not enhance the yield of vaccinia virus above that found in samples maintained with arginine alone.

c) Cadaverine

Cadaverine is derived from lysine. Its effects were

examined additionally to compare with results from cell cultures supplied with polyamines derived from arginine. Cadaverine did not effect the yield of vaccinia virus in HeLa cells maintained with a suboptimal concentration of arginine.

3.4 Precursors

Various authors (Tytell & Neumann, 1960; Schimke, 1964) have established that many established cell cultures can utilize citrulline, but not ornithine, as a replacement for arginine for cell growth. Results were obtained in HeLa cells in agreement with this (Table 3.2). Failure to grow in the presence of ornithine alone has been attributed to a decrease in the activity of the enzyme ornithine transcarbamoylase in cell cultures in vitro (Eagle, 1959).

a) L-ornithine

Vaccinia virus-infected cell cultures were maintained in media containing ornithine at concentrations between 0.001 and 1.0mM. Further cell cultures were maintained in the presence of a suboptimal concentration of arginine supplemented with ornithine at the same concentrations. The results indicate that ornithine cannot substitute per se for arginine for the growth of vaccinia virus in HeLa cells, nor was there any detectable increase in virus production from cell cultures maintained with ornithine and a suboptimal concentration of arginine. The results were similar to those for L-proline (Fig 3.2). Since mammalian cells synthesise polyamines from ornithine it may have been

Table 3.2 The growth of HeLa cells in the presence of L-ornithine, L-citrulline or L-arginine

Medium	Viable cell count at 24hr ($\times 10^{-6}$)
2.59mM L-ornithine	1.16
2.59mM L-citrulline	2.24
2.59mM L-arginine	2.48
arginine free	1.44

Viable cell count at time 0hr 1.22×10^6

expected that the presence of ornithine would enhance virus production in the presence of a suboptimal concentration of arginine if polyamines are involved in the expression of the vaccinia virus genome. The results suggest that if there is utilization of ornithine in some early virus-specified event, this is a minor component and that the method employed here is not sensitive enough to detect any effect.

b) L-citrulline

L-citrulline can effectively replace arginine for the growth of vaccinia virus in HeLa cells (Fig. 3.4). There was an increase in infective virus above the virus inoculum at a concentration of 0.05mM and maximal yield was obtained with 0.1mM L-citrulline. There was a dose-response relationship between the yield of virus and the concentration of this compound: yields of virus in the presence of citrulline at concentrations of 0.05mM and above were greater at each concentration than those from cell cultures maintained with an equimolar concentration of arginine. These results have been observed repeatedly by other workers in this laboratory.

3.5 Analogues

HeLa cell cultures were maintained in PE medium without arginine or serum for 18 hr prior to infection, subsequently infected with 1-2 p.f.u./cell vaccinia virus and maintained for 24 or 48 hr in experimental media containing L-arginine at a concentration sufficient to permit maximal yield of virus. Analogues of arginine and ornithine at concentrations between 0.001 and 1.00mM were added to such cultures to

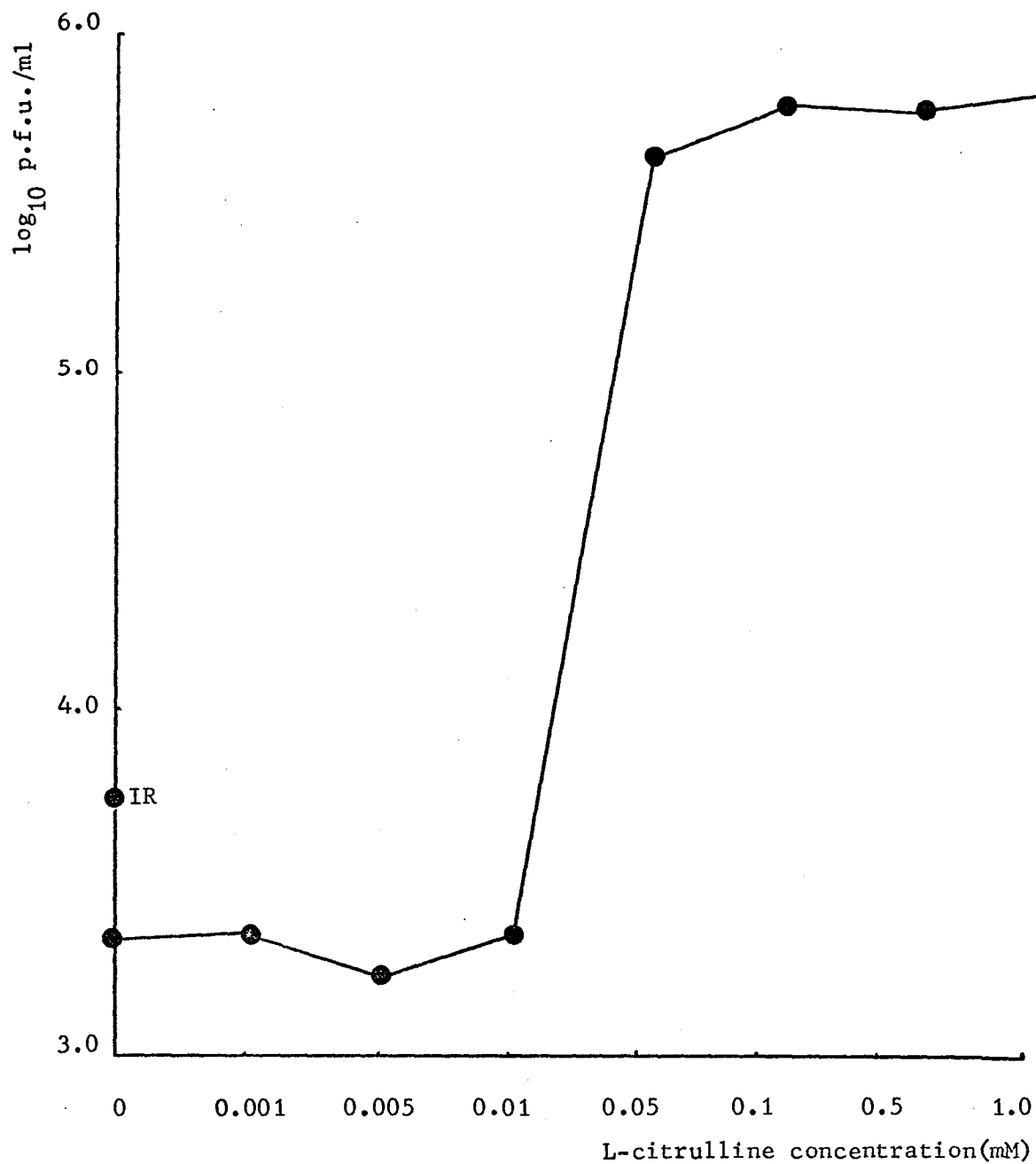


Fig. 3.4 The growth of vaccinia virus in HeLa cells maintained in PE medium containing different concentrations of L-citrulline in the absence of L-arginine

determine their effect on the production of infective virus. In addition, the effect of β -methyl-aspartic acid, at concentrations between 0.5 and 5.0mM, was examined to determine if it would reduce the yield of vaccinia virus in cell cultures maintained with an optimal concentration of L-citrulline.

a) Canavanine

Canavanine is a structural analogue of arginine. Hare (1969) demonstrated that canavanine inhibits cellular DNA synthesis by the production of abnormal canavanyl proteins rather than by a general non-specific inhibition of either RNA or protein synthesis.

Canavanine inhibits the synthesis of Semliki Forest virus RNA if added to cell cultures within 2 hr post-infection. At 3 hr or later the growth cycle is no longer arrested by the addition of the compound. Furthermore, inhibition can be reversed by the addition of arginine to the medium. The site of inhibition appears to be at the active configuration of virus-specified RNA polymerase (Ranki & Kaariainen, 1970).

In adenovirus-infected cells virus-specified DNA replication, the synthesis of capsid components and maturation of virus particles are all inhibited if canavanine is added to cell cultures immediately after infection. The synthesis of the tumour "T" antigen is not affected. Virus-specified DNA synthesis and the synthesis of capsid components is only partially inhibited when canavanine is added at a later time, but assembly is completely blocked. In the absence of arginine both

viral DNA and capsid components are synthesised in reduced amounts but maturation is inhibited (Rouse & Schlesinger, 1967). This block has been attributed to an arginine-rich internal component (Prage, Petterson & Philipson, 1968) and since canavanine binds to arginyl-tRNA it competitively inhibits incorporation of arginine into proteins containing arginine (Neurath, Krzyzek & Arfman, 1970). This may explain why maturation is inhibited in adenovirus-infected cells.

Experiments were carried out in HeLa cells infected with vaccinia virus to determine the effect of L-canavanine on the production of infective virus in the presence of 0.1mM L-arginine. Inhibition was greater than 99% at a concentration of 0.05mM L-canavanine (Fig. 3.5). Since arginine is incorporated into vaccinia virus specified proteins (Archard & Williamson, 1971) and since canavanine binds to arginyl-tRNA (Neurath et al, 1970), inhibition of virus production must occur at the level of protein synthesis on the ribosomes by competition between arginine and canavanine for the sites of incorporation.

A similar experiment was carried out to determine whether the production of vaccinia virus would be inhibited by canavanine in HeLa cells maintained with 0.1mM L-citrulline. There was no inhibition of virus production at a concentration of 0.05mM canavanine but inhibition was greater than 90% at 0.1mM and complete above this concentration (Fig. 3.6). The production of arginine from citrulline through arginosuccinic acid is reversible; in vivo and in cell culture, intracellular conditions favour the reaction towards arginine production.

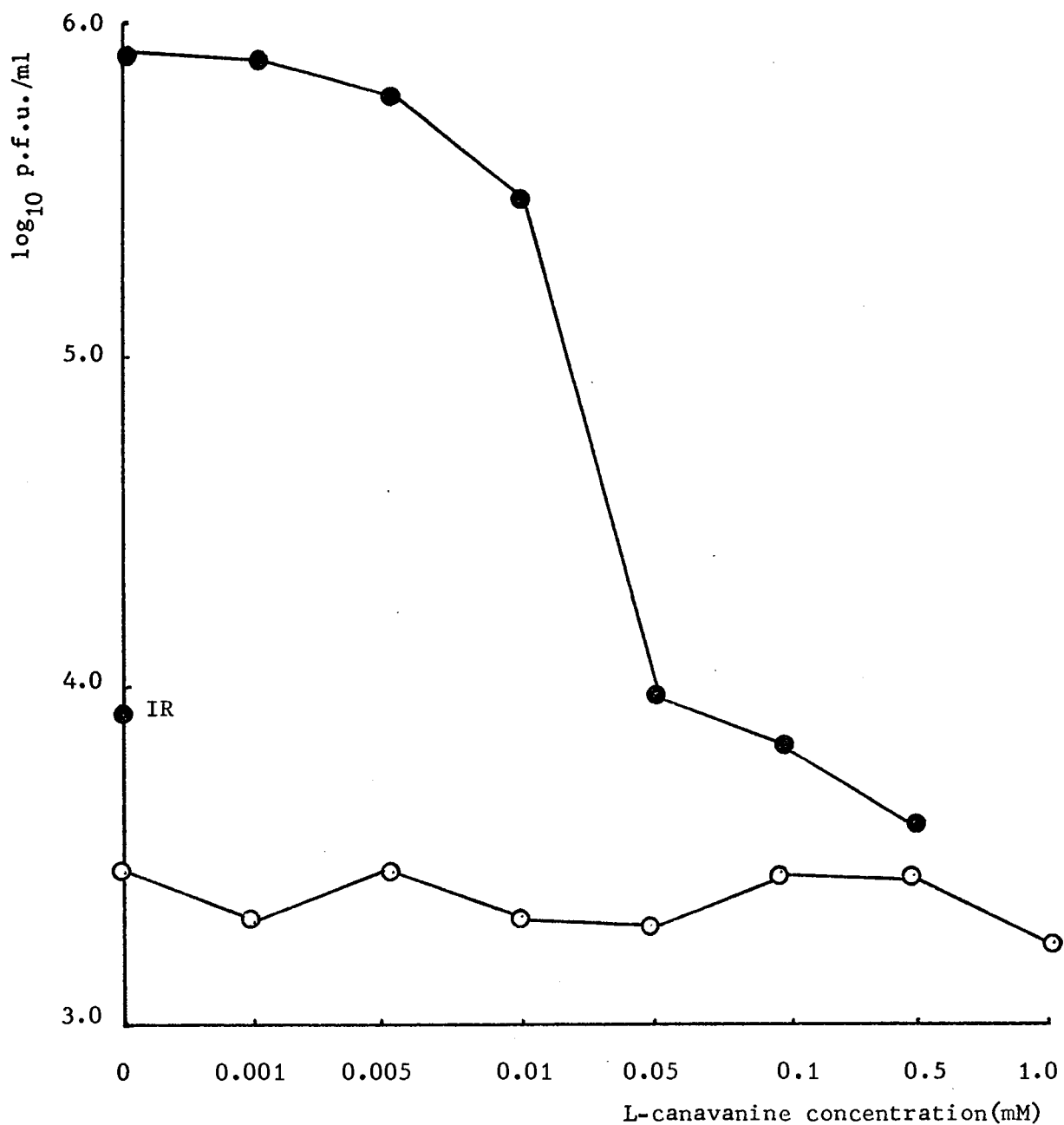


Fig. 3.5 The effect of L-canavanine on the growth of vaccinia virus in HeLa cells maintained in PE medium in the absence and in the presence of 0.1mM L-arginine

○—○, L-canavanine;

●—●, L-canavanine with 0.1mM L-arginine

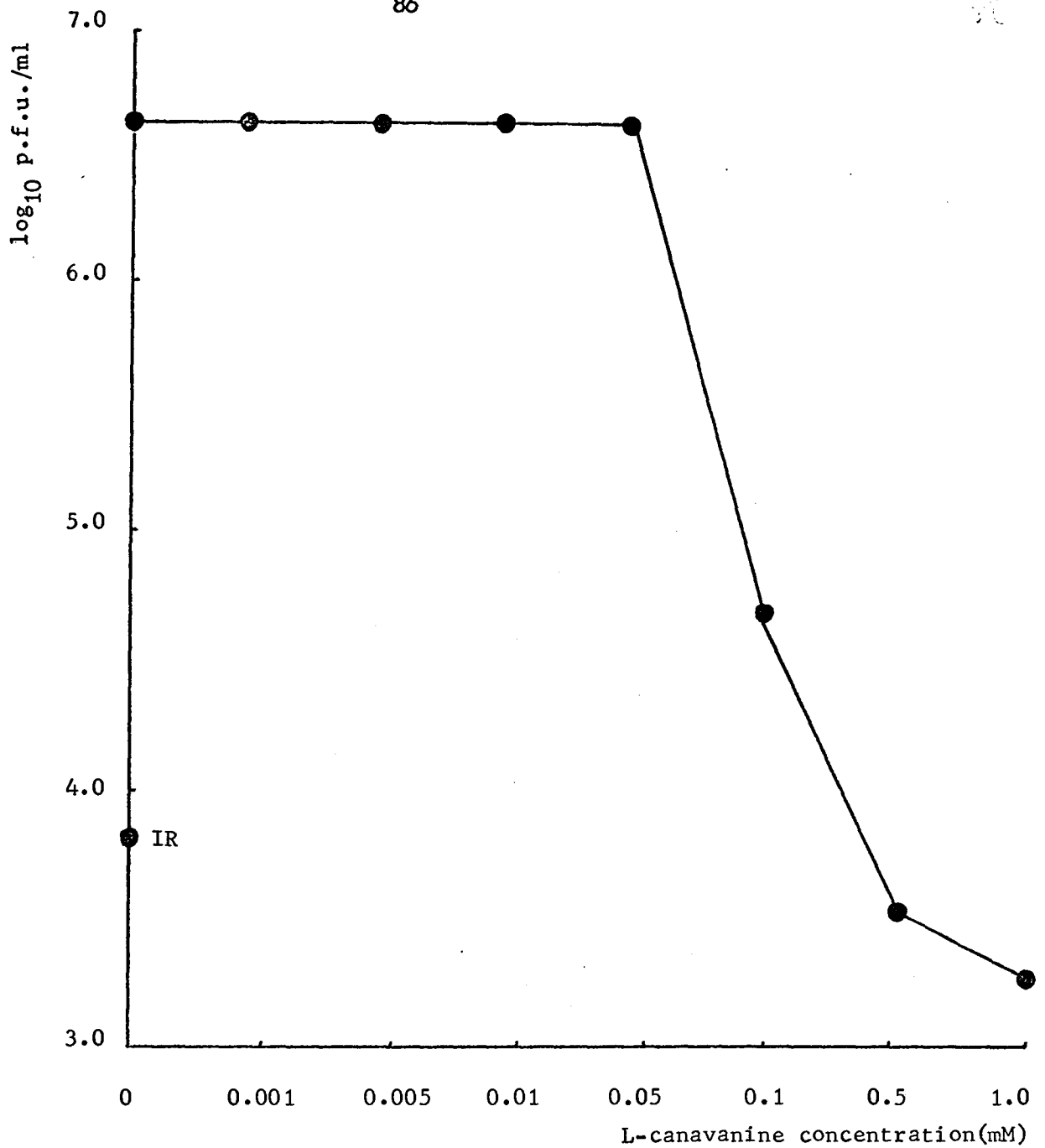


Fig. 3.6 The effect of L-canavanine on the growth of vaccinia virus in HeLa cells maintained in PE-A* medium containing 0.1mM L-citrulline

*PE-A, PE medium without arginine

Using a cell-free system Walker (1953) demonstrated that canavanine competitively inhibits argininosuccinate lyase activity by reacting with fumarate to form canavanosuccinic acid at a molar ratio of canavanine to arginine greater than unity. In the cell this would block the production of arginine from citrulline by the competitive inhibition of argininosuccinate lyase. Thus, in this instance, inhibition is primarily at the level of conversion of citrulline to arginine. In HeLa cells infected with vaccinia virus there is a marked stimulation in the activity of enzymes converting citrulline to arginine (see Chapter 5). Since inhibition is not complete until a molar ratio canavanine : citrulline greater than unity is reached, the analogue is only weakly inhibitory to these enzymes (Walker, 1953). In view of the elevated levels of enzyme activity in infected cultures maintained with citrulline it would be expected that a higher concentration of canavanine would be needed to inhibit these enzymes than in similar cultures maintained with arginine. Canavanine, in such cells, would be converted to canavanosuccinic acid which would not inhibit the incorporation of arginine at the ribosomal level. Hence, in infected cell cultures maintained with citrulline, any arginine that is synthesised would be available for incorporation into functional protein. In similar cultures maintained with arginine protein synthesis would be high because of the availability of the substrate and the canavanine block would be expressed in the synthesis of defective proteins. This could explain why inhibition of vaccinia virus growth requires a higher concentration of canavanine in cell cultures maintained with citrulline than with arginine.

A further experiment was carried out to determine whether the inhibition by canavanine of vaccinia virus growth in HeLa cells reflects any differences in the amounts of (^{14}C)-guanido-labelled arginine or (^{14}C)-carbamoyl-labelled citrulline incorporated into high molecular weight material in these cells. Cell cultures were maintained in media containing 0.1mM L-citrulline or L-arginine for 18 hr prior to infection, subsequently infected with 5 p.f.u./cell of vaccinia virus and after adsorption were maintained in similar media containing 0.1 $\mu\text{Ci/ml}$ labelled compound and 0.5mM L-canavanine. Additional infected cell cultures were maintained in similar media without canavanine. Uninfected cell cultures were treated similarly. After incubation for 20 hr at 37° the amounts of radioactivity incorporated into each sample were calculated by methods previously described (Table 3.3). The results indicate that at a concentration of canavanine inhibiting virus replication the uptake of citrulline into vaccinia virus-infected cells is reduced by 72% compared to control infected cells, whereas that of arginine is reduced by only 17%. In uninfected cell cultures there is a 67% reduction in the amount of citrulline incorporated into cells in the presence of canavanine compared to a 34% decrease in cultures maintained with arginine.

These results suggest that canavanine has a more pronounced effect on protein synthesis in cell cultures, both infected and uninfected, maintained with citrulline than with arginine, but since data is based on the amounts of radioactivity incorporated into high molecular weight

Table 3.3 The effect of 0.5 mM L-canavanine on the incorporation of (^{14}C)-guanido-labelled arginine and (^{14}C)-carbamoyl-labelled citrulline into uninfected and vaccinia virus infected HeLa cell cultures

Medium	Incorporated radioactivity dpm/mg protein	
	Control cells	Infected cells
0.1mM L-arginine	24,000	18,776
0.1mM L-arginine + L-canavanine	15,864	15,689
0.1mM L-citrulline	19,416	17,874
0.1mM L-citrulline + L-canavanine	6,472	5,068

material this may be an over-simplification. Canavanine inhibits the conversion of citrulline to arginine by conversion of canavanine to canavanosuccinic acid. This inhibits argininosuccinate lyase activity. In order for the radioactivity associated with labelled citrulline to be incorporated into protein citrulline must be converted to arginine. Thus, the radioactivity associated with TCA precipitable material from uninfected and infected cells exposed to labelled citrulline in the presence of canavanine must reflect the incorporation of arginine into protein. In view of the enhanced argininosuccinate synthetase and lyase activity of cells maintained in the presence of citrulline (See Chapter 5) these enzyme activities will be greater than in cells maintained in the presence of arginine. Therefore, a greater proportion of canavanine will be converted to canavanosuccinic acid. The incorporation of labelled arginine into TCA precipitable material in the presence of canavanine does not necessarily reflect the synthesis of functional protein. Since canavanyl residues will be incorporated in addition to the arginyl residues the resulting protein will lack biological activity. Canavanine is a weak inhibitor of argininosuccinate lyase activity whereas canavanine and arginine bound to RNA probably compete equally for incorporation into protein. It is concluded that the low level of competitive inhibition by canavanine of the argininosuccinate synthetase and lyase activity allows some conversion of citrulline to arginine. In addition, conversion of canavanine in the same system to canavanosuccinic acid reduces the degree of inhibition at the level of protein synthesis allowing some synthesis of functional protein. Inhibition by canavanine of the

utilization of arginine occurs exclusively at the level of protein synthesis. The observed differences in the degree of inhibition of virus replication by canavanine in the presence of equimolar concentrations of citrulline and arginine reflect differences in the efficiency of this inhibitor on the enzymatic conversion of citrulline to arginine and the incorporation of arginine into protein.

b) Canaline

Canaline is a structural analogue of ornithine. The possible role of polyamines in vaccinia virus-infected cells has already been mentioned. Since ornithine decarboxylase initiates the synthesis of polyamines from ornithine, supplying canaline to vaccinia virus-infected cells may determine whether there is such a requirement. A dose-response curve was prepared similar to that described for canavanine. Cell cultures were maintained for 24 hr after infection with 0.5mM L-arginine in PE medium supplemented with 0.001 to 1.0mM canaline. There was almost complete inhibition of virus growth at a concentration of 0.01mM canaline (Fig. 3.7).

Although canaline is a structural analogue of ornithine there have been no reports that it causes inhibition by direct competition with ornithine. It has been shown that canaline is a potent inhibitor of pyridoxal-dependent enzymes strongly inhibiting ornithine decarboxylase, liver ornithine keto-aminotransferase and tyrosine aminotransferase in vitro. The inhibition is due to the strong binding of canaline to pyridoxal phosphate. In reactions involving ornithine,

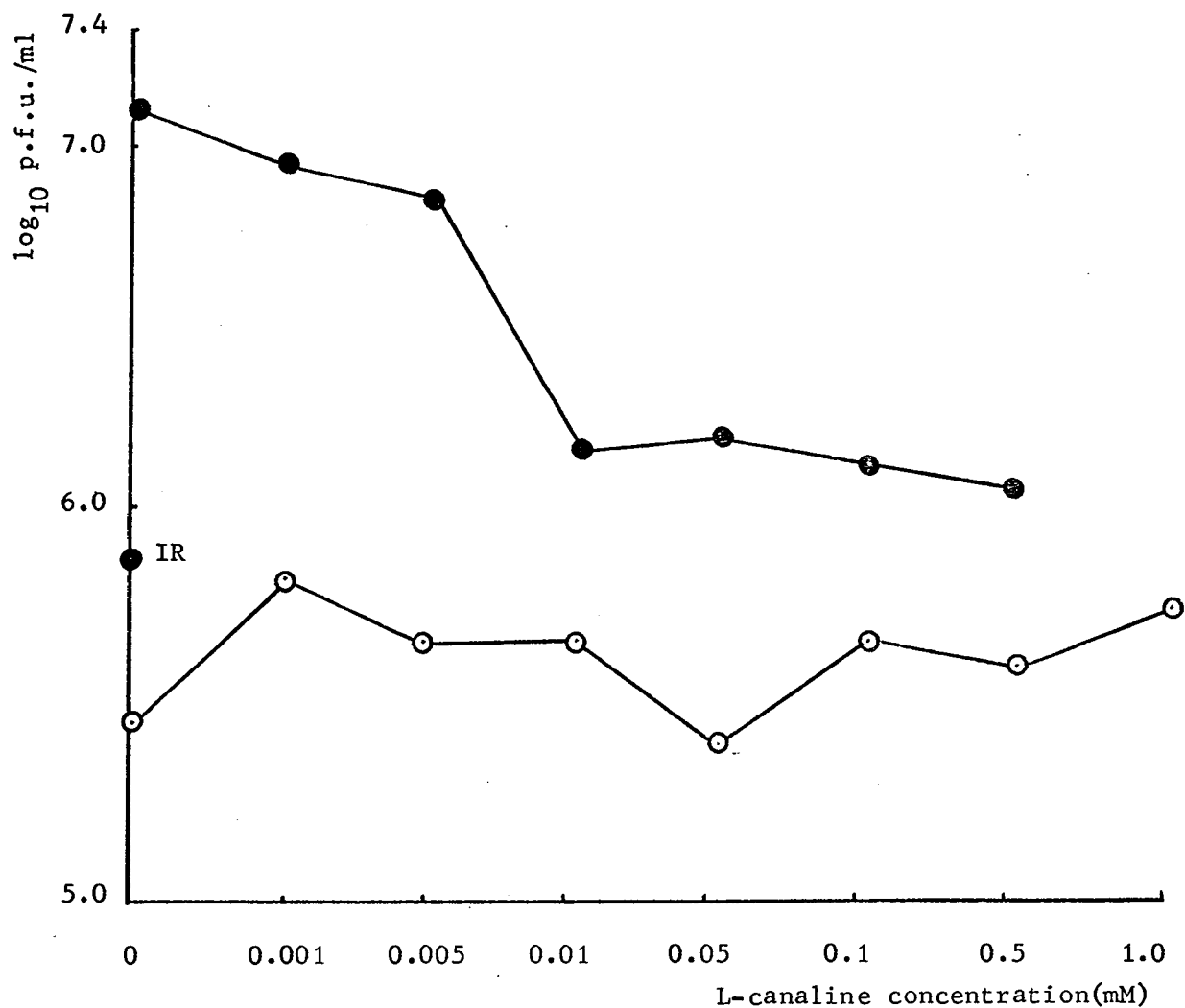


Fig. 3.7 The effect of L-canaline on the growth of vaccinia virus in HeLa cells maintained in PE medium containing 0.5mM L-arginine

○—○, L-canaline;

●—●, L-canaline with 0.5mM L-arginine

inhibition cannot be reversed by the addition of ornithine. (Kekomaki, Janna, Rahiala, Raina & Raiha, 1969). In view of this it is only possible to conclude that canaline inhibits the growth of vaccinia virus in these cells at a ratio one mole : 50 moles arginine. The mechanism of inhibition cannot be inferred from the data presented since canaline may affect other pathways besides those originated from the Krebs-Henseleit urea cycle. Further investigations are required to elucidate the mechanism of this effect.

c) β -methyl-DL-aspartic acid

β -methyl-DL-aspartic acid is an analogue of aspartic acid. Bayer and McMurray (1969) investigated the nature of inhibition produced by this analogue concluding that it inhibits carbamoyl phosphate synthetase and arginosuccinate synthetase in vitro. Results by other workers (Rowe & Miller, 1971) have been inconclusive about the mechanism of inhibition and observations made in vivo have not been confirmed in vitro. Since citrulline must be converted, in part, to arginine for incorporation into mature vaccinia virus particles, experiments were carried out to determine whether virus production would be inhibited in vaccinia virus-infected HeLa cell cultures maintained with 0.1mM L-citrulline and concentrations of β -methylasspartic acid between 0.5 and 5.0mM for 24 hr after infection. Aspartic acid is a non-essential amino-acid for cell growth in culture and it is involved in a number of biosynthetic pathways other than the Krebs-Henseleit urea cycle. Since its availability in the cell will not be limited, compared to essential

amino-acids higher concentrations of β -methylaspartic acid were used than in other experiments of this type. There was a reduction in virus yield of 25% at the highest concentration of the analogue (Table 3.4). This suggests that either a higher concentration of analogue is required to effect total inhibition of virus growth or that the intracellular pool of aspartic acid is such that there is a permeation barrier which restricts the amount of β -methylaspartic acid that can enter the cell.

d) Octopine

Octopine is a structural analogue of arginine. Its properties have not been investigated thoroughly. Preliminary experiments (Table 3.5) demonstrated that, at a concentration of 0.1mM L-arginine, the growth of vaccinia virus in HeLa cells was completely inhibited with 1.0mM octopine, and partially inhibited, by about 50%, at an equimolar concentration, suggesting that there may be direct competition between octopine and arginine for incorporation into virus-specified proteins. The high concentration of octopine required to inhibit virus growth completely may be a reflection of susceptibility of octopine to cellular amino-acid oxidases which can effectively convert octopine to arginine.

3.6 Discussion

On the basis of the results of experiments designed to investigate any sparing effect on the utilization of arginine by supplementation of media supplied to infected cells with possible metabolic derivatives,

Table 3.4 The effect of β -methyl aspartic acid on the growth of vaccinia virus in HeLa cells maintained in PE-A medium containing 0.1mM L-citrulline

Concentration of β -methyl aspartic acid	$\log_{10}$ p.f.u./ml at 24hr
0.0	6.82
0.5	6.77
1.0	6.56
2.0	6.50
5.0	6.44

Inoculum recovery at 0hr

5.133 $\log_{10}$ p.f.u./ml

Infectivity titre in citrulline
-free medium at 24 hr

5.34 $\log_{10}$ p.f.u./ml.

Table 3.5 The effect of L-octopine on the growth of vaccinia virus in HeLa cells maintained in PE medium containing 0.1mM L-arginine.

Concentration of L-octopine (mM)	Log ₁₀ p.f.u./ml at 48hr
0.0	5.36
0.1	4.61
1.0	3.80

Inoculum recovery at 0hr	3.61 log ₁₀ p.f.u./ml
Infectivity titre in arginine-free medium at 48hr	3.57 log ₁₀ p.f.u./ml

arginine does not appear to be a quantitatively significant source of formation of the metabolites tested. Interpretation of these results, however, can be made only within the limits of the tests applied. The data of Archard and Williamson (1971) indicate that the prime requirement for arginine in vaccinia virus-infected cells is in the synthesis of structural protein. Biochemical techniques directed towards the analysis of other virus-specified macromolecules should offer a more sensitive system. It may be concluded that arginine does not serve significantly as a source of non-essential amino-acids, for example, proline, nor as a source of intermediates in the citric acid cycle. Additionally, experiments with vaccinia virus-infected cells using galactose as a source of carbohydrate indicate that arginine does not function as an energy source.

Since the growth of vaccinia virus in HeLa cells is impaired in media containing ornithine but is not impaired in media containing citrulline or D-arginine, it appears that pathways for the synthesis of L-arginine in uninfected cells are also operative in virus-infected cells. This is demonstrated again in experiments using analogues of the Krebs-Henseleit urea cycle. Canavanine can completely inhibit the growth of vaccinia virus in HeLa cells in media containing an optimal concentration of citrulline and β -methylaspartic acid is also partially inhibitory under the same conditions. Since citrulline must be converted to arginine before incorporation into virus-specified proteins, these data infer that argininosuccinic acid is the intermediate because both

analogues can inhibit the synthesis of this compound. In addition, canavanine inhibits the growth of vaccinia virus in HeLa cells in media containing an optimal concentration of arginine by competing with arginine at the ribosomal level. That octopine is a less effective inhibitor than canavanine suggests that octopine may be metabolised in virus-infected cells. This suggests that amino-acid oxidases may be operative in the system such that a significant amount of octopine is converted to arginine and consequently a higher concentration of this analogue would be required to effect inhibition.

Although no requirements for polyamines for the production of vaccinia virus in HeLa cells could be demonstrated in supplementation experiments, it has been demonstrated that in infected KB cells addition of spermine or spermidine to arginine-deprived KB cell cultures infected with vaccinia virus leads to the appearance of late virus-specified mRNA (Obert, personal communication). Furthermore, although polyamines do not exert a sparing effect on the utilization of arginine in cells infected with herpes simplex virus, it has been shown that exogenous ornithine is incorporated into polyamines if it is added to cultures prior to infection (Gibson & Roizman, 1971).

Canaline inhibited the growth of vaccinia virus in HeLa cells by more than 90% at a molar ratio canaline : arginine, 1 : 50. Although canaline is a structural analogue of ornithine it is unlikely that inhibition of virus growth is a result of competition between ornithine and canaline for incorporation into protein because ornithine is not

normally involved in protein synthesis. Kekomaki et al (1969) demonstrated that canaline binds to pyridoxal phosphate and consequently inhibits the activity of pyridoxal-dependent enzymes. The enzymes involved are transaminases and decarboxylases such as ornithine decarboxylase which initiates polyamine synthesis. On the basis of Obert's results it appears that polyamines have a significant role in vaccinia virus-infected cells. Furthermore, the available data indicate that most of the other decarboxylases and transaminases which function in vivo would be repressed in vitro because their products are supplied in the growth medium or are not associated with this type of cell. In view of this, if the observed inhibition of vaccinia virus growth by canaline is due to the antagonism of the synthesis of some component mediated by a pyridoxal-dependent enzyme, this is likely to be the synthesis of polyamines from ornithine decarboxylase.

CHAPTER 4

THE REPLICATION OF POXVIRUSES IN MOUSE S180 AND HUMAN CITRULLINAEMIA CELLS

4.1 Introduction

The results in Chapter 3 show that citrulline can completely replace arginine for the growth of vaccinia virus in HeLa cells. Since arginine is incorporated per se into mature virus particles, citrulline must either be converted to arginine in vaccinia virus-infected cells or must replace arginine for involvement in virus-specified events.

Two lines of cells, mouse S180 and human citrullinaemia have been shown to have a metabolic defect such that these cells have an absolute requirement for arginine for growth but neither will grow in medium containing citrulline as a substitute for arginine (Tytell & Neuman, 1960; Tedesco & Mellman, 1967). The human citrullinaemia

cell line was derived from the skin of a child who was excreting abnormally high levels of citrulline in the urine. Tedesco & Mellman (1967) investigated the site of the metabolic lesion in these cells and showed that the enzyme argininosuccinate synthetase had markedly reduced substrate specificity. The Michaelis constant (K_m) for the reaction was $10-100 \times 10^{-3} M$ compared to $0.4 \times 10^{-3} M$ for "normal" fibroblasts. Experiments were carried out to determine whether pox-viruses are able to replicate in these two cell lines in media containing citrulline as a substitute for arginine.

4.2 Growth of mouse S180 cells

The site of the metabolic lesion in mouse S180 cells has not been investigated previously. The growth of these cells in media containing 0.1mM L-arginine was compared, by methods previously described, with that of similar cell cultures in media containing the same concentration of L-citrulline. Mouse L cells were grown in media containing 0.1mM L-arginine or L-citrulline as a control cell line. The results show that citrulline cannot replace arginine for the growth of mouse S180 cells (Table 4.1), confirming the results of Tytell and Neuman (1960). In addition, these cells did not grow with 0.1mM argininosuccinic acid suggesting that there is at least some defect in the enzyme argininosuccinate lyase. Citrulline replaced arginine for the growth of mouse L cells, but no growth occurred in the absence of either of these amino-acids (Table 4.2).

Determinations of protein synthesis in mouse S180 cells were

Table 4.1 The growth of mouse S180 cells in the presence of L-arginine, L-citrulline or arginosuccinic acid

Medium	Viable cell count at 48hr (x 10 ⁻⁶)
0.1mM L-arginine	4.04
0.1mM L-citrulline	1.83
0.1mM arginosuccinic acid	1.80
arginine free	1.30

Viable cell count at time 0hr

1.60 x 10⁶

Table 4.2 The growth of mouse L cells in the presence of L-arginine or L-citrulline

Medium	Viable cell count at 48hr ($\times 10^{-6}$)
0.1mM L-arginine	4.66
0.1mM L-citrulline	4.84
arginine free	0.98

Viable cell count at time 0hr

1.0×10^6

made by measuring the amounts of radioactively-labelled amino-acids taken up by these cells over a period of 20 hr. Cell cultures in 4 oz prescription bottles were maintained in PE medium containing 0.5mM L-arginine or L-citrulline for 6 hr. At this time the media was changed for similar media containing 0.2 $\mu\text{Ci/ml}$ (^{14}C)-guanido-labelled arginine or (^{14}C)-carbamoyl-labelled citrulline. Additional cultures were maintained in media containing 0.2 $\mu\text{Ci/ml}$ (^{14}C)-uniformly-labelled phenylalanine. Cell cultures were incubated at 37° for 20 hr and the total amounts of radioactivity incorporated into high molecular weight material determined by methods previously described.

Similar experiments were carried out with HeLa and mouse L cells which had been maintained for 18 hr in PE medium containing 0.1mM L-arginine or L-citrulline and were subsequently given similar media containing 0.1 $\mu\text{Ci/ml}$ (^{14}C)-carbamoyl-labelled citrulline or (^{14}C)-guanido-labelled arginine. Cultures of HeLa cells were also incubated in similar media containing 0.1 $\mu\text{Ci/ml}$ (^{14}C)-uniformly-labelled phenylalanine. Incubation and all subsequent procedures were similar to those for mouse S180 cells.

The results show that the level of incorporation of citrulline into high molecular weight material in mouse S180 cells maintained in media containing 0.5mM L-citrulline was about 4% of that into similar cell cultures maintained in the presence of arginine at a similar concentration. Under these conditions overall protein synthesis, as assessed by the incorporation of (^{14}C)-phenylalanine, was reduced by

about 75% (Table 4.3a and b). At a 5-fold lower concentration of citrulline or arginine the uptake of citrulline into HeLa mouse L cell cultures was about 45% and 72% respectively, compared to that of similar cultures maintained with arginine (Table 4.4a and 4.5). Furthermore, in HeLa cells this level of incorporation of citrulline was sufficient to maintain protein synthesis at a rate similar to that of cell cultures in the presence of arginine (Fig. 4.4b).

4.3 Poxvirus growth in mouse S180 cells

Preliminary experiments were carried out to determine whether these cells are sensitive to poxvirus infection. Of the viruses tested both IHD vaccinia virus and rabbitpox virus grew well in media containing arginine, but the Lister strain of vaccinia virus gave poor results and was not used for subsequent work.

Mouse S180 cells are very sensitive to arginine deprivation. In cell cultures maintained without arginine or serum for 18 - 20 hr the cells become refractile and detach from the glass. Autolysis of cellular material must occur because if such cells are infected with rabbitpox virus or IHD vaccinia virus and maintained in the absence of arginine there is some virus growth after about 40 hr. Further investigations indicated that starvation for a period of 6 hr was sufficient to deplete the intracellular pool of arginine without causing any apparent cytological damage to cell monolayers. Thus, in all subsequent experiments where it was necessary to deplete these cells of arginine, cell cultures were maintained for 6 hr in PE medium,

Table 4.3.a Incorporation of (^{14}C)-guanido-labelled arginine and (^{14}C)-carbamoyl-labelled citrulline into TCA-precipitable material in mouse S180 cells

Medium	Total incorporation ($\mu\text{m}/\text{mg}$ cell protein)
0.5mM L-arginine	0.0445
0.5mM L-citrulline	0.0019

Table 4.3.b Incorporation of (^{14}C)-uniformly-labelled phenylalanine into TCA-precipitable material in mouse S180 cells in the presence of 0.5mM L-arginine or L-citrulline

Medium	Total incorporation ($\mu\text{m}/\text{mg}$ cell protein)
0.5mM L-arginine	0.0414
0.5mM L-citrulline	0.012

Table 4.4.a Incorporation of (^{14}C)-guanido-labelled arginine and (^{14}C)-carbamoyl-labelled citrulline into TCA-precipitable material in HeLa cells

Medium	Total incorporation ($\mu\text{m}/\text{mg}$ cell protein)
0.1mM L-arginine	0.0359
0.1mM L-citrulline	0.0151

Table 4.4.b Incorporation of (^{14}C)-uniformly-labelled phenylalanine into TCA-precipitable material in HeLa cells in the presence of L-arginine or L-citrulline

Medium	Total incorporation ($\mu\text{m}/\text{mg}$ cell protein)
0.1mM L-arginine	0.081
0.1mM L-citrulline	0.077

Table 4.5 Incorporation of (^{14}C)-guanido-labelled arginine and (^{14}C)-carbamoyl-labelled citrulline into TCA-precipitable material in mouse L cells.

Medium	Total incorporation ($\mu\text{m}/\text{mg}$ cell protein)
0.1mM L-arginine	0.059
0.1mM L-citrulline	0.046

without arginine or serum, prior to infection.

The growth of IHD vaccinia virus and rabbitpox virus and rabbitpox virus was examined in mouse S180 cells in PE medium containing 1.0mM L-arginine or L-citrulline. Cell cultures were maintained in PE medium without arginine or serum for 6 hr before infection and subsequently infected with 1 - 2 p.f.u./cell of virus, in similar medium, by methods previously described. After adsorption cell cultures were incubated at 37° in experimental media and samples were examined for the production of infective virus at intervals up to 72 hr post-infection (p.i.). Both IHD vaccinia virus and rabbitpox virus grew equally well in media containing arginine or citrulline (Table 4.6a and b).

Further experiments were carried out to determine whether the growth of rabbitpox virus in mouse S180 cells in the presence of arginine or citrulline showed a dose response relationship similar to the growth of vaccinia virus in HeLa cells under similar conditions. Cell cultures were starved as before and subsequently infected with 10^4 p.f.u./test-tube of rabbitpox virus in similar medium. After adsorption cell cultures were supplied with media containing either L-arginine or L-citrulline at concentrations between 0.001mM and 1.0mM and incubated at 37° for 48 hr.

In mouse S180 cells maximum yield of rabbitpox virus was reached at a concentration of 0.1mM arginine, at 0.05mM the yield of virus was

Table 4.6.a Growth of rabbitpox virus in mouse S180 cells
in the presence of L-citrulline and L-arginine

Medium	Log ₁₀ p.f.u./ml		
	24hr	48hr	72hr
1.0mM L-arginine	7.15	7.38	7.90
1.0mM L-citrulline	7.14	7.37	7.86

Inoculum recovery at 0hr 4.83

Table 4.6.b Growth of IHD vaccinia virus in mouse S180 cells
in the presence of L-citrulline and L-arginine

Medium	Log ₁₀ p.f.u./ml		
	24hr	48hr	72hr
1.0mM L-arginine	7.15	7.36	7.87
1.0mM L-citrulline	7.11	7.40	7.84

Inoculum recovery at 0hr 4.92

about 90% of the maximum value (Fig. 4.1). In cell cultures maintained with citrulline there was no appreciable yield of virus below a concentration of 0.1mM but at this concentration the yield was about 50% of the maximum value. Maximum yield was reached at 0.5mM citrulline and further addition of citrulline above this concentration did not enhance the yield of virus. Thus, although mouse S180 cells failed to grow at a concentration of 0.1mM citrulline and host-cell directed protein synthesis is reduced by 75% at 0.5mM citrulline, intracellular conditions in virus-infected cells are such that the growth of rabbitpox virus is permitted. Furthermore, virus growth in the presence of citrulline is a real effect and not produced by the autolysis of cellular material, releasing arginine, because no virus production was detected below a concentration of 0.1mM citrulline or in the complete absence of citrulline or arginine.

In cell cultures maintained with arginine, growth of rabbitpox virus was detected at lower concentrations of arginine than in the vaccinia virus - HeLa cell system. This may indicate differences in permeation of arginine into the two types of cell or specific nutritional requirements for arginine by different viruses.

4.4 Incorporation of citrulline into rabbitpox-infected mouse S180 cells

Labelling experiments were undertaken to investigate the effect of virus infection on the uptake of (^{14}C)-labelled amino-acids into high molecular weight material in HeLa and mouse S180 cells. Confluent

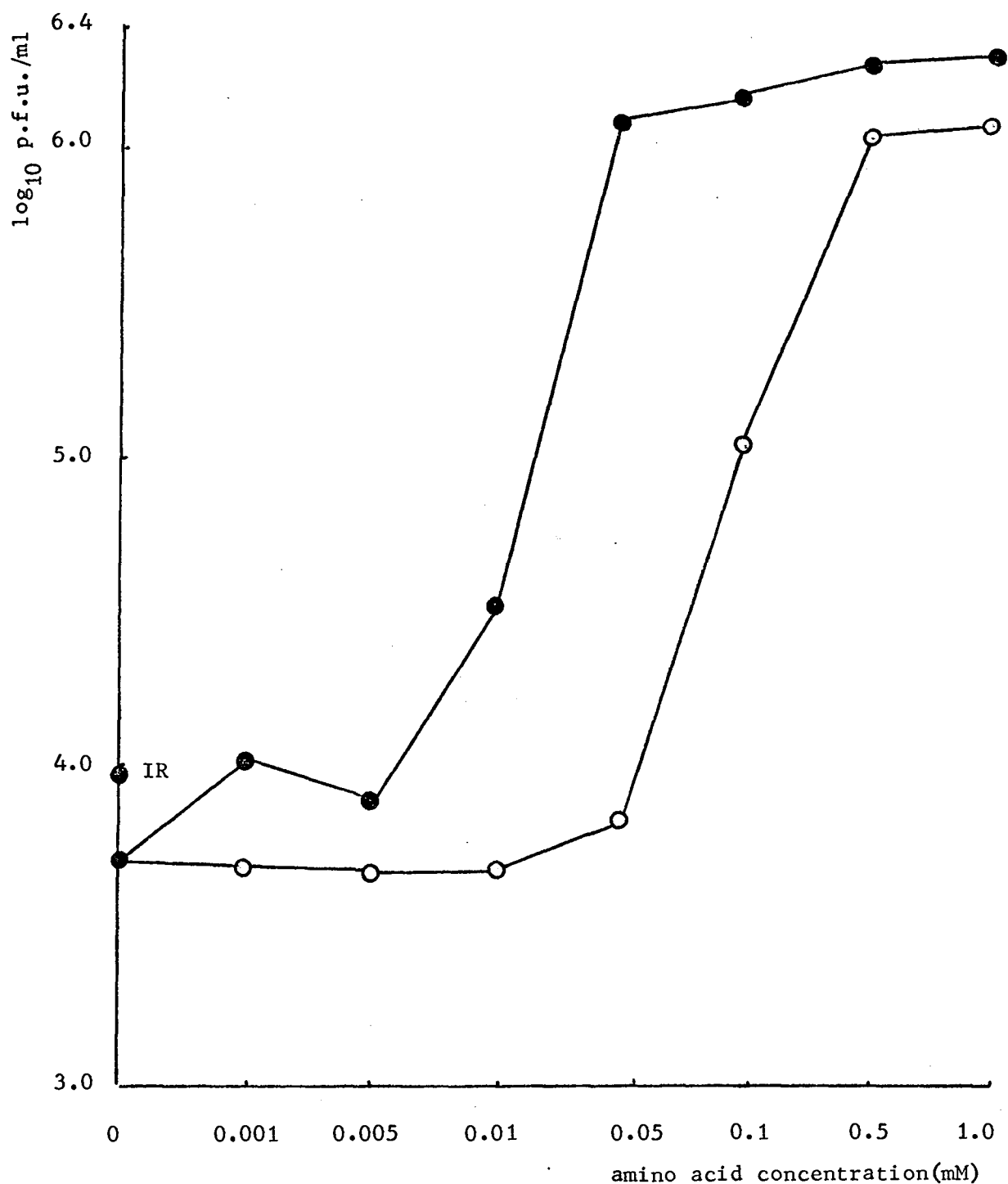


Fig. 4.1 The growth of rabbitpox virus in mouse S180 cells in the presence of different concentrations of L-arginine or L-citrulline

○—○ L-citrulline ●—● L-arginine

monolayers of cells in 4 oz prescription bottles were maintained and infected in PE medium containing 0.1mM L-citrulline, by methods previously described. Mouse S180 cells were infected with rabbitpox virus and HeLa cells with vaccinia virus at a multiplicity of 5 p.f.u./cell. After adsorption cell cultures, together with uninfected, control cultures, were incubated in similar medium containing 0.2 μ Ci/ml (14 C)-carbamoyl-labelled citrulline at 37° for 20 hr. At this time the amounts of radioactivity incorporated into high molecular weight material were determined by methods previously described. In both cell systems there was a stimulation of incorporation of (14 C)-labelled citrulline into virus-infected cells (Table 4.7).

Archard and Williamson (1971) used a pulse-labelling technique to follow the time course of incorporation of (14 C)-labelled arginine into vaccinia virus-infected HeLa cells. There is a stimulation of the rate of incorporation of arginine immediately after infection followed by a rapid decline. At 3 hr p.i. there is a slight but reproducible stimulation in the rate of incorporation and a further stimulation of incorporation at 5 hr p.i. In uninfected cell cultures the rate of incorporation of arginine remains constant. Mouse S180 cells were maintained and subsequently infected in PE medium containing 0.1mM L-arginine or L-citrulline with 5 p.f.u./cell of rabbitpox virus. After adsorption cell cultures were incubated at 37° in similar media for up to 7 hr. Samples were taken at the times indicated in the results and the media changed for similar media containing 0.1 μ Ci/ml (14 C)-guanido-

Table 4.7 Incorporation of (^{14}C)-carbamoyl-labelled citrulline into HeLa and mouse S180 cells infected with vaccinia virus and rabbitpox virus respectively

Cell line	Total incorporation ($\mu\text{m}/\text{mg}$ cell protein)
HeLa uninfected	0.0151
HeLa + vaccinia virus	0.0248
Mouse S180 uninfected	0.0029
Mouse S180 + rabbitpox virus	0.0064

labelled arginine or 0.2 $\mu\text{Ci/ml}$ (^{14}C)-carbamoyl-labelled citrulline. After incubation in this media for 20 min further incorporation was inhibited and the amounts of radioactivity incorporated into each sample determined by methods previously described. Similar experiments were undertaken with HeLa cells infected with vaccinia virus in medium containing 0.1mM L-citrulline and pulse-labelled with the same medium containing 0.1 $\mu\text{Ci/ml}$ (^{14}C)-carbamoyl-labelled citrulline.

In mouse S180 cells infected with rabbitpox virus in medium containing arginine the results were similar to the pattern of incorporation of arginine into high molecular weight material in HeLa cells infected with vaccinia virus, but the third peak reached a maximum at 6 hr p.i. (Fig. 4.2). The rate of incorporation of (^{14}C)-carbamoyl-labelled citrulline into HeLa cells infected with vaccinia virus was similar to that for (^{14}C)-labelled arginine in this system (Archard & Williamson, 1971; Fig. 4.3). The level of incorporation of labelled amino-acids in uninfected mouse S180 and HeLa cells remained constant throughout the length of the experiments and after the first hour p.i. was higher in uninfected cell cultures than in virus-infected cell cultures.

In mouse S180 cells maintained with citrulline the rate of incorporation of labelled precursor was about 200-fold lower than in similar cultures maintained with arginine (Fig. 4.4). There was no stimulation of the rate of incorporation of (^{14}C)-carbamoyl-labelled citrulline immediately after infection but there were two distinct peaks

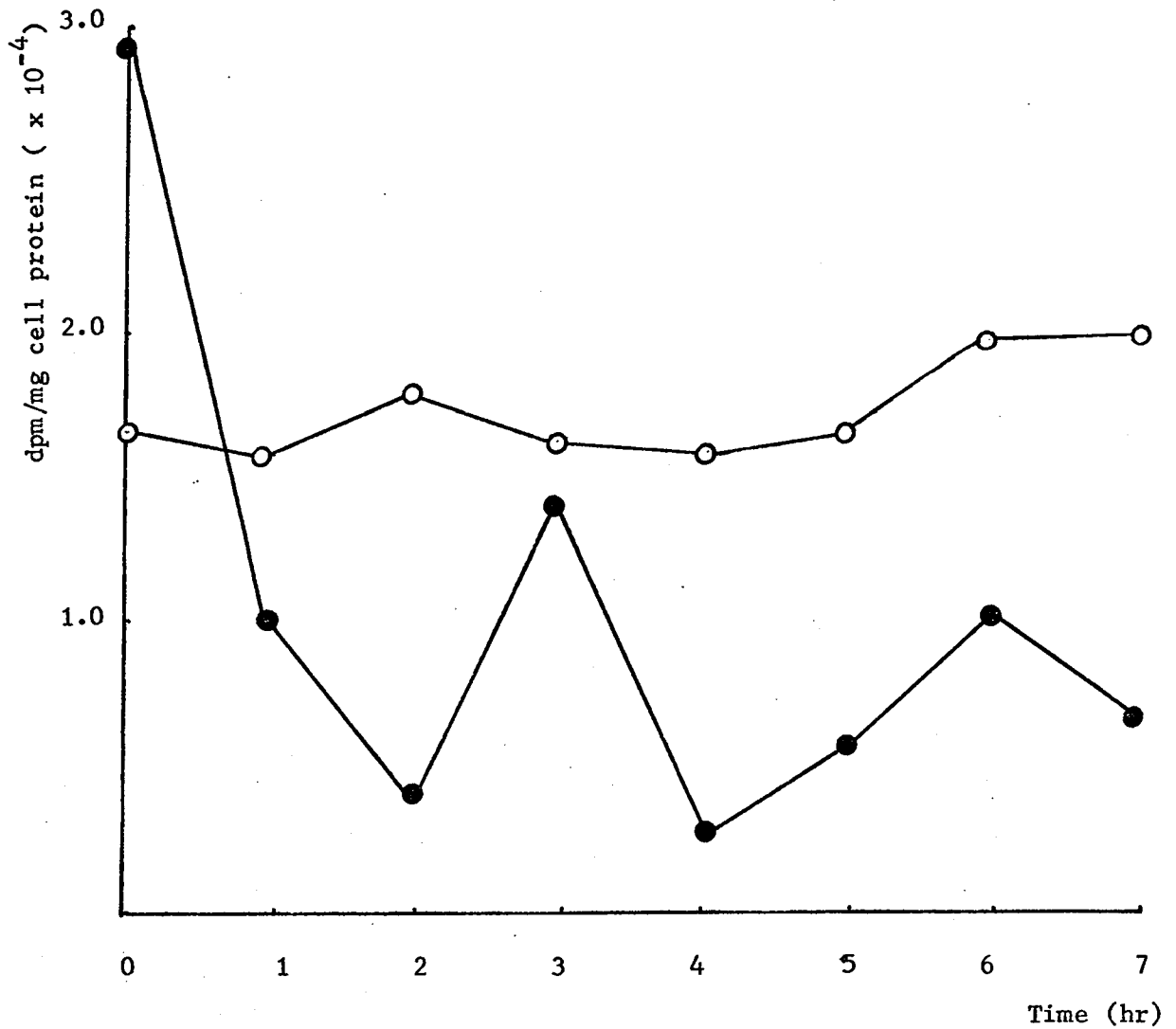


Fig. 4.2 Rates of incorporation of (¹⁴C)-guanido-labelled arginine into rabbitpox virus-infected mouse S180 cells



, Control cell cultures;



, Infected cell cultures

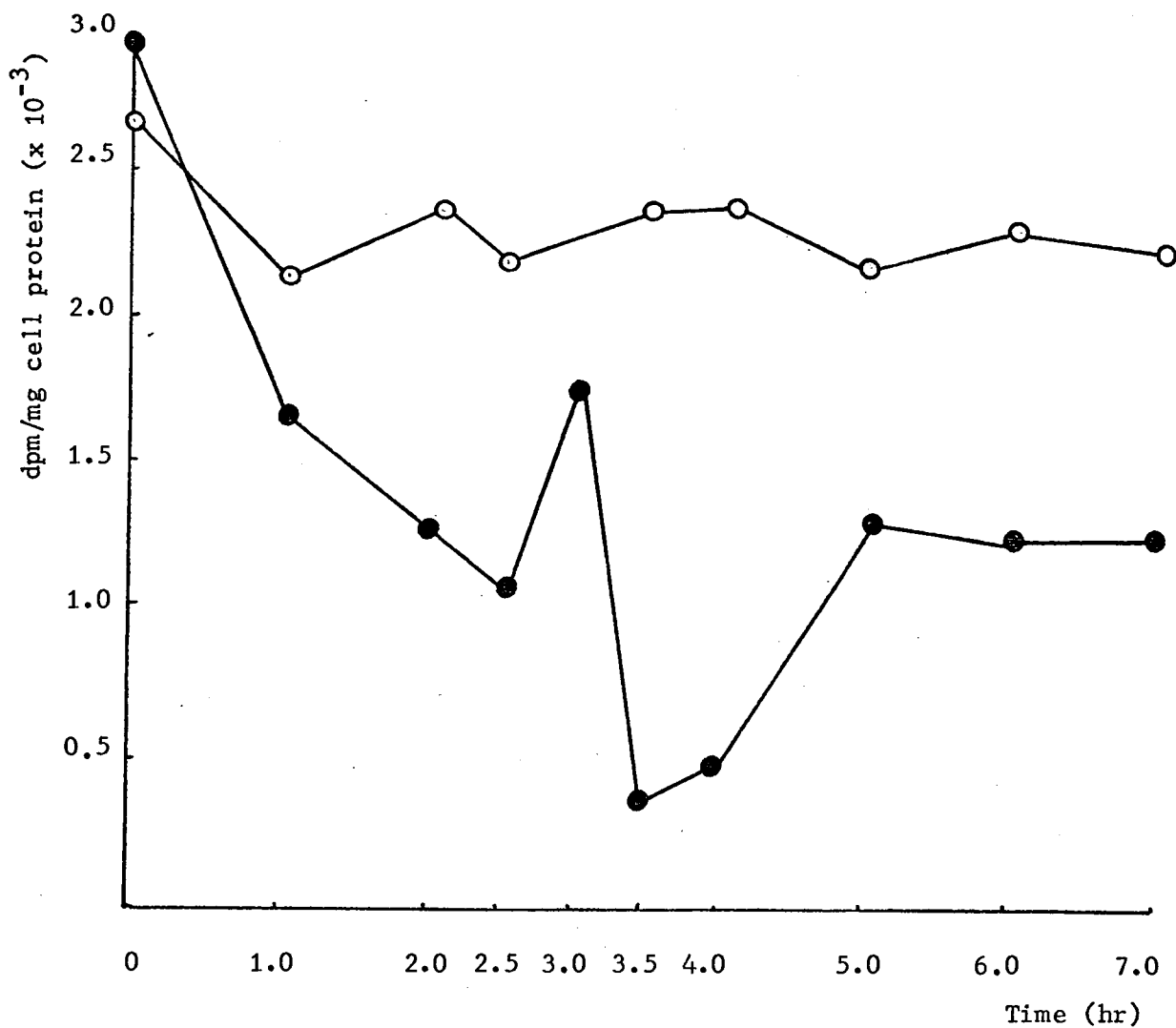


Fig. 4.3 Rates of incorporation of (^{14}C)-carbamoyl-labelled citrulline into vaccinia virus-infected HeLa cells

○—○ , Control cell cultures;

●—● , Infected cell cultures

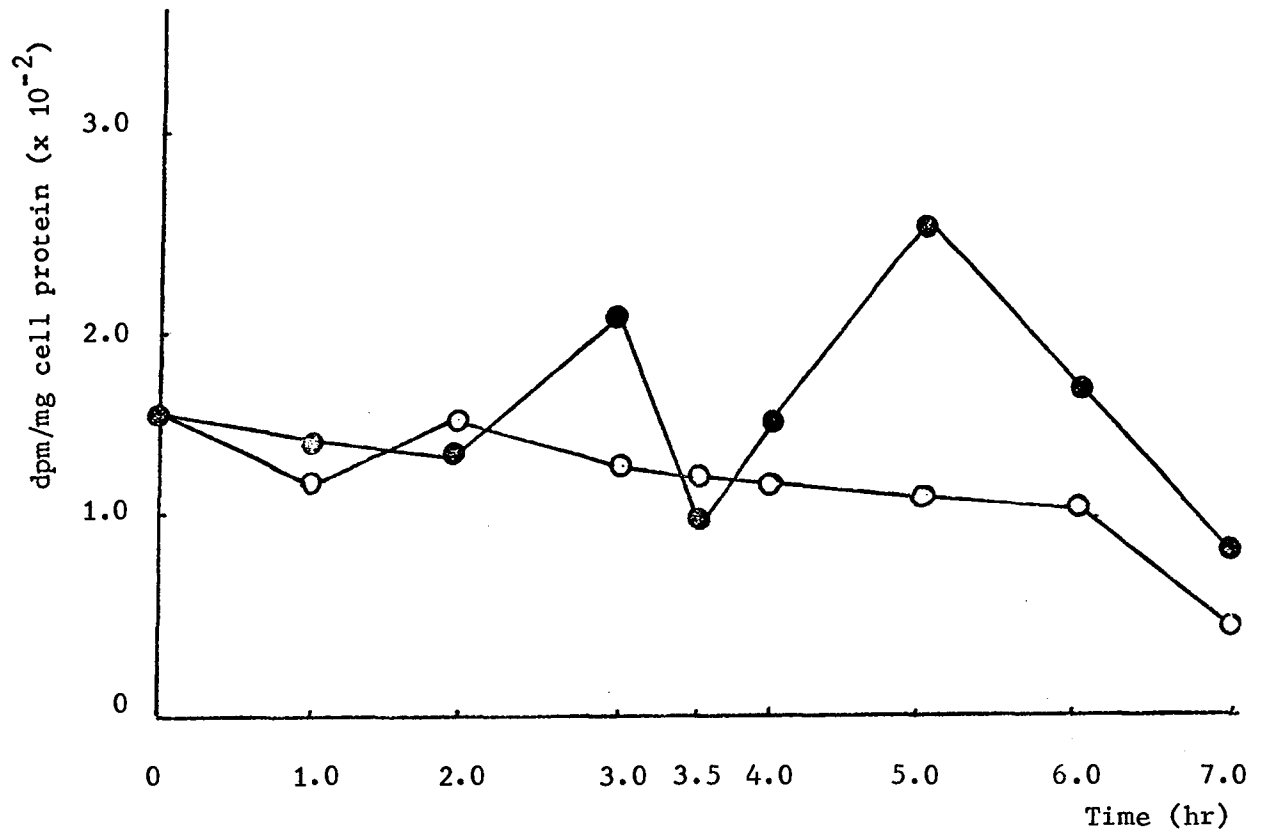


Fig. 4.4 Rates of incorporation of (^{14}C) -carbamoyl-labelled citrulline into rabbitpox virus-infected mouse S180 cells.

○—○ , Control cell cultures;

●—● , Infected cell cultures

at 3 hr and 5 hr p.i. Furthermore, at these times rates of incorporation were markedly greater in infected cell cultures than in uninfected cell cultures, which remained constant in their rate of uptake of citrulline throughout the experiment.

Since the level of incorporation of citrulline is low in mouse S180 cells further investigations of this type were carried out with HeLa cells infected with vaccinia virus. Experiments were made to determine whether the observed stimulations in the rate of incorporation of citrulline into poxvirus-infected cells are sensitive to the inhibition of transcription. Experimental procedures were as before. Vaccinia virus-infected cell cultures were maintained in PE medium containing 0.1mM L-citrulline and 0.5 μ g/ml actinomycin D (Sigma Chemical Company Limited). Control infected cell cultures were maintained in similar medium without the inhibitor. Cell cultures were pulse-labelled for 20 min at the times indicated in the results with the same media containing 0.1 μ Ci/ml (14 C)-carbamoyl-labelled citrulline. The results obtained show that actinomycin D reduced the early incorporation of citrulline and inhibits the peaks of incorporation of citrulline at both 3 hr and 5 hr p.i. in vaccinia virus-infected HeLa cells (Fig. 4.5).

4.5 Growth curve of rabbitpox virus in mouse S180 cells

The yield of rabbitpox virus from mouse S180 cells maintained with 0.5mM citrulline was similar to that from cultures maintained with the same concentration of arginine, but the level of incorporation of citrulline compared to arginine was reduced 20-fold in such cells.

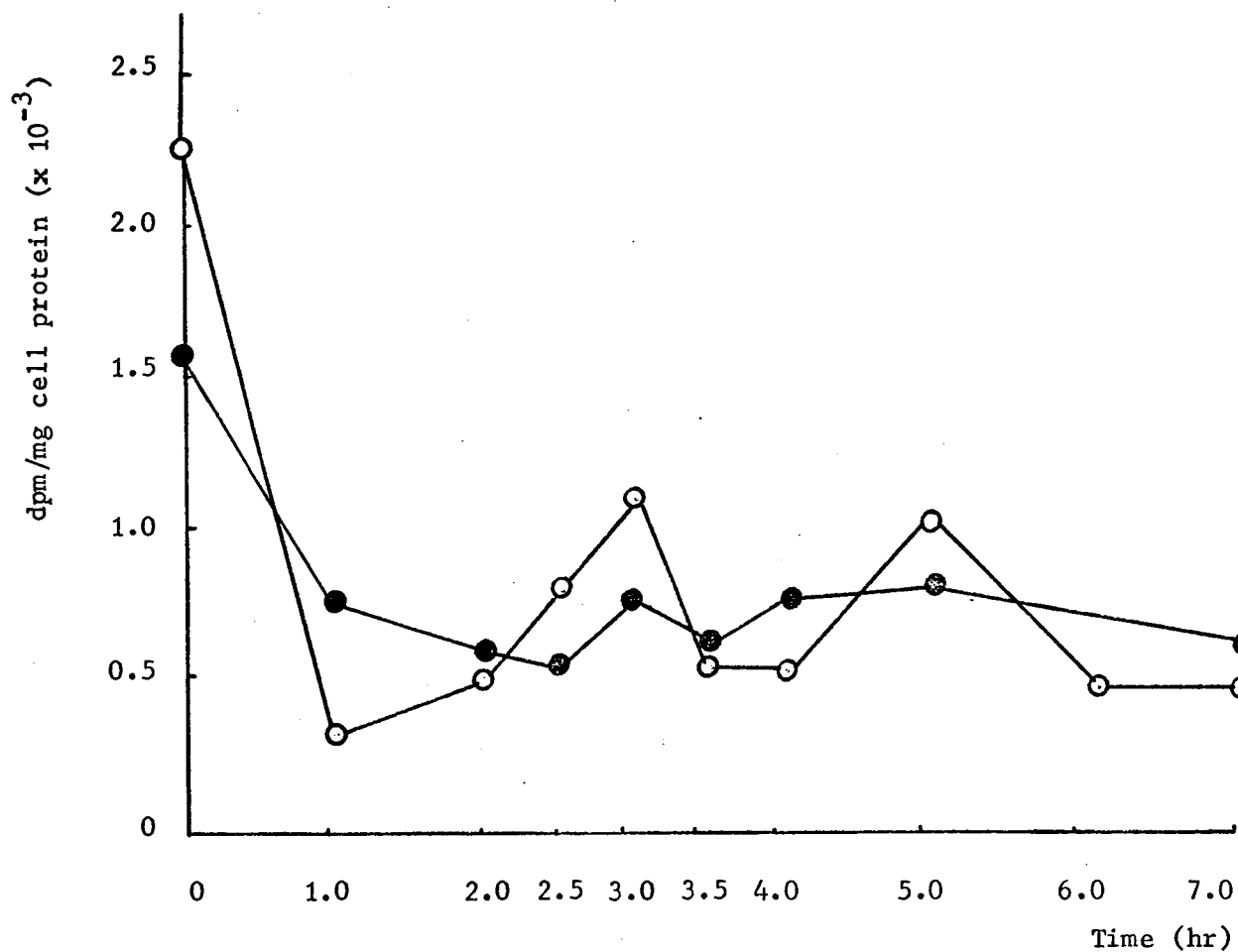


Fig. 4.5 Rates of incorporation of (^{14}C) -carbamoyl-labelled citrulline into vaccinia virus-infected HeLa cells in the presence of $0.5 \mu\text{g/ml}$ actinomycin D

●—● , Infected cells + ;
actinomycin D

○—○ , Control infected
cells

Growth curves were prepared, therefore, to determine whether there are differences in the time course of appearance of progeny virus from mouse S180 cells maintained in medium containing 1.0mM L-citrulline compared to 1.0mM L-arginine. Cell cultures were maintained in PE medium without arginine or serum as before, subsequently infected with 1 - 2 p.f.u./cell of rabbitpox virus in similar medium and after adsorption were maintained in media containing 1.0mM L-arginine or L-citrulline. Samples were examined for the production of infective virus at the times indicated in the results up to 22 hr p.i. In media containing citrulline or arginine virus production was detected between 6 - 8 hr p.i. and the rate of appearance of progeny virus was similar in both types of medium (Fig. 4.6).

4.6 The growth of Lister vaccinia virus in human citrullinaemia cells

Dose response curves were prepared with human citrullinaemia cells infected with Lister vaccinia virus. Cell cultures were maintained without arginine or serum for 18 hr before infection, subsequently infected with 10^4 p.f.u./test-tube of vaccinia virus and maintained in media containing L-citrulline at concentrations between 0.01 and 1.0mM. Further cell cultures were maintained in medium containing 1.0mM L-arginine. Tedesco and Mellman (1967) demonstrated that these cells cannot convert citrulline to arginine efficiently below a concentration of 10mM citrulline yet cells infected with vaccinia virus gave a maximum yield of virus at a concentration of 0.5mM citrulline and virus production could not be enhanced by increasing the concentration of citrulline

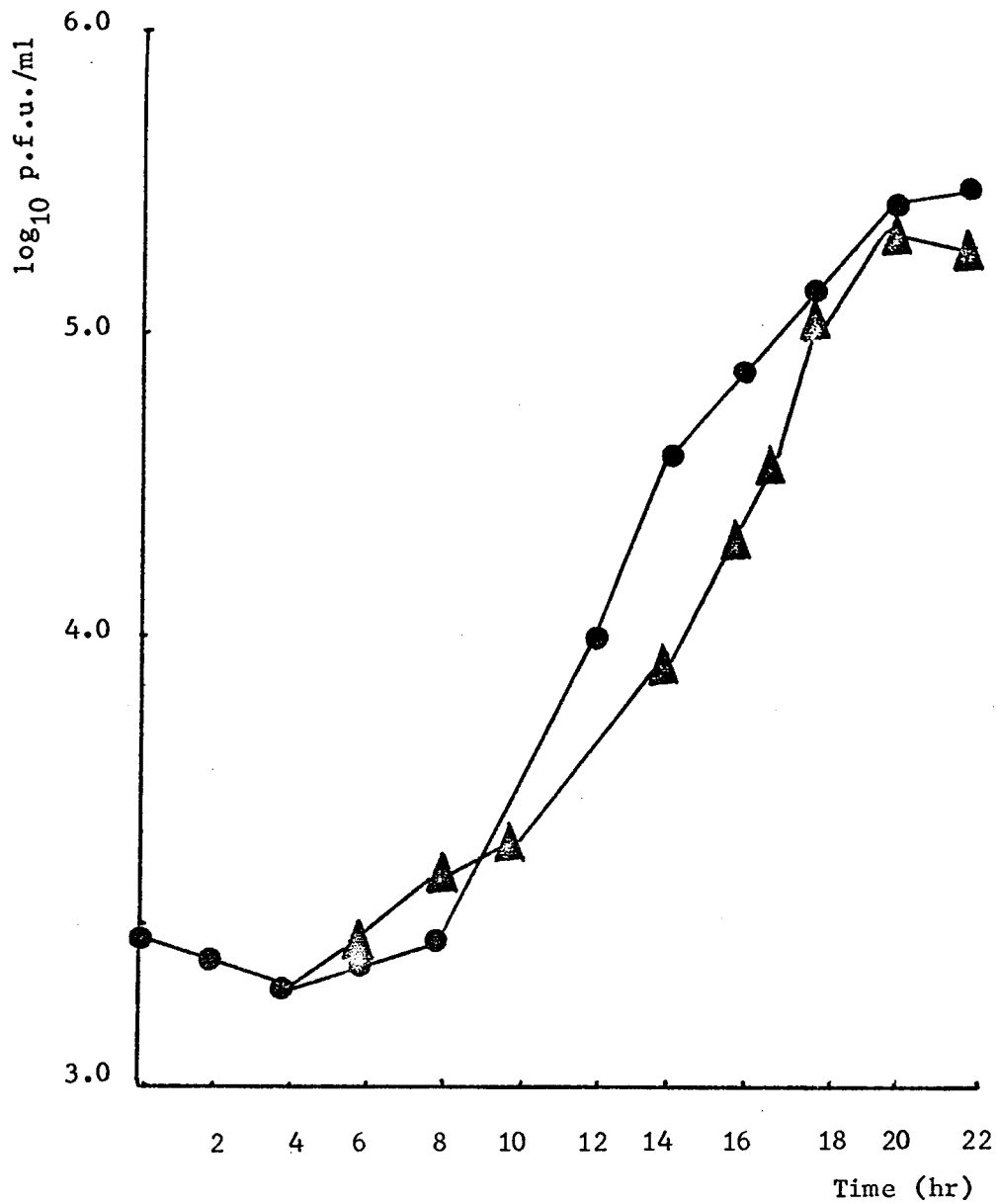


Fig. 4.6 Growth of rabbitpox virus in mouse S180 cells in the presence of L-arginine or L-citrulline.

▲ — ▲, 1.0mM L-arginine; ● — ●, 1.0mM L-citrulline

(Fig. 4.7). Furthermore the maximum yield of virus in these conditions was similar to that from cell cultures maintained with an optimal concentration of arginine.

4.7 Utilization of citrulline in the synthesis of arginine-containing virus structural protein

Although both mouse S180 and human citrullinaemia cells have metabolic lesions which inhibit cell growth in medium containing citrulline, this does not inhibit the growth of poxviruses in these cells in similar medium. Since arginine is required per se for the growth of these viruses, experiments were carried out to determine whether (^{14}C)-carbamoyl-labelled citrulline supplied to virus-infected cells could be detected in mature virus particles as (^{14}C)-guanido-labelled arginine or whether under these conditions citrulline can substitute per se for arginine. Experiments involved the use of two-way paper chromatography and in view of the quantities of labelled virus required, HeLa cells infected with vaccinia virus were used. The level of incorporation of citrulline is high in such infected cell cultures and thus this system offers greater facility.

Confluent monolayers of HeLa cells in 40 oz roller bottles were infected with 5 p.f.u./cell of Lister vaccinia virus and after adsorption were maintained at 37° for 48 hr in PE medium containing 0.1 mM L-citrulline and $0.2\ \mu\text{Ci/ml}$ (^{14}C)-carbamoyl-labelled citrulline. The virus was subsequently harvested and purified by methods previously described. The purified preparation was dialysed for 48 hr against distilled water to remove sucrose. After this time the virus pellet

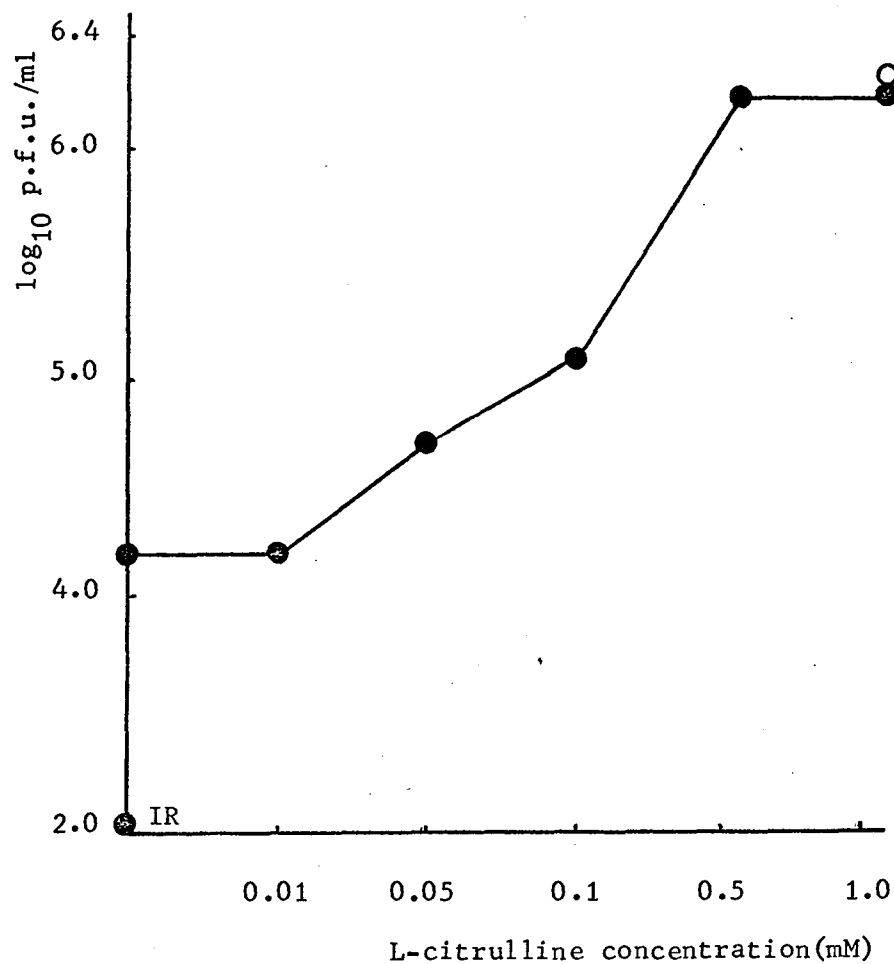


Fig. 4.7 The growth of vaccinia virus in human citrullaemia cells in the presence of different concentrations of L-citrulline

O, 1.0mM L-arginine

●—●, L-citrulline

was resuspended in 1 ml of 6M hydrochloric acid and heated in a sealed ampoule in a water bath at 100° for a further 48 hr to break-down the virus particles to constituent amino-acids. After evaporating off the acid in vacuo over potassium hydroxide the pellet was resuspended in a small volume (0.07 ml) of a mixture of unlabelled amino-acids in distilled water. Samples of this mixture were spotted onto chromatography paper. A control, consisting of unlabelled amino-acids alone was included to locate each of the amino-acids and to determine their Rf values. Two-dimensional paper chromatography was employed with but-1-ol-acetic acid as the first solvent and phenol-ammonia as the second by methods previously described. After drying the chromatograms the control was stained with ninhydrin reagent, the constituents of the amino-acid mixture located and Rf values for the amino-acids in each solvent calculated. The separated amino-acids were identified by comparison of Rf values determined experimentally with values given in standard tables (Long, King & Sperry, Biochemists Handbook).

One of the experimental chromatograms was cut into strips 4 cm wide and scanned for radioactivity in a Panax System D.0212/XGD-.01 (Panax Equipment Limited, Redhill, Surrey). A single peak was detected and its Rf value calculated from its location on the chromatography paper. The peak corresponded to arginine on the control. Since radioactivity can be estimated with greater sensitivity by counting in a liquid scintillation spectrometer, a second experimental chromatogram

was cut into small squares at the site corresponding to arginine from the calculated Rf values. In addition, other samples were taken from positions on the chromatogram corresponding to arginine precursors and derivatives, viz. citrulline, ornithine, proline and aspartic acid. The site corresponding to threonine was also examined as a control for the level of radioactivity in the background. Samples were counted in 10 ml Bray's liquid scintillant in a Packard 3 channel spectrometer, model 3310. The results confirmed the finding from the scanning experiment. No additional radioactivity, above the low background count from threonine, could be detected from samples containing citrulline, ornithine, proline or aspartic acid. However, there was a significant and measurable count corresponding to arginine (Table 4.8).

Thus, in poxvirus-infected cells citrulline is converted to arginine for incorporation into mature virus particles and citrulline does not substitute per se for incorporation into virus specified structural protein(s). Since the amino-acid preparation used in these experiments was labelled in the carbamoyl group, if proline or ornithine are involved in the synthesis of virus-specified proteins this would not be detected in this system because the carbamoyl group, after conversion to the guanido group of arginine, is lost as urea by the action of cellular arginase. Thus, if arginine is in part catabolised to either of these amino-acids for incorporation into mature virions, similar experiments using a uniformly labelled arginine preparation

Table 4.8 Radioactivity associated with amino acids from purified vaccinia-virus grown in HeLa cells supplied with 0.2 μ Ci/ml (14 C)-carbamoyl-labelled citrulline

Amino acid	dpm
Arginine	295
Citrulline	37
Ornithine	37
Aspartic Acid	45
Proline	60
Threonine	38

may demonstrate this.

4.8 Discussion

There are marked increases in macromolecular synthesis in mouse S180 cells and human citrullinaemia cells maintained in the presence of citrulline following poxvirus infection. At a concentration of 0.5mM citrulline there was a 20-fold reduction in the amount of radioactive label incorporated into high molecular weight material in uninfected cells maintained in medium containing (^{14}C)-carbamoyl-labelled citrulline compared to that of (^{14}C)-guanido-labelled arginine at the same concentration. This was reflected in a marked reduction in protein synthesis in cells maintained with citrulline, as indicated by the incorporation of (^{14}C)-uniformly-labelled phenylalanine. However, at this same concentration of citrulline the yield of rabbitpox virus was maximal in these cells. Even at a concentration of 0.1mM citrulline, a level at which there was no detectable growth of mouse S180 cells, the yield of rabbitpox virus was about 50% of the maximum value. These results offer two possible explanations. Either the levels of enzymes which convert citrulline to arginine in mouse S180 cells are insufficient for cell growth in media containing citrulline but sufficient arginine is synthesised for the growth of rabbitpox virus, or in virus-infected cells the enzymes effecting arginine biosynthesis are virus-directed.

An enhanced level of citrulline incorporation into high molecular weight material was noted in rabbitpox virus-infected mouse

S180 cells and the pattern of incorporation of citrulline in such cell cultures follows closely that of arginine in vaccinia virus-infected HeLa cells (Archard & Williamson, 1971). Both these results indicate virus-directed functions because the changes in the rate of incorporation of citrulline in HeLa cells were not observed in cell cultures treated with actinomycin D. Thus, transcription is necessary for these events to take place. In HeLa cells the mRNA species for the synthesis of argininosuccinate synthetase and lyase have a long half-life (See chapter 5). Hence, these results suggest the need for new mRNA species to be synthesised.

The growth curves of rabbitpox virus in mouse S180 cells indicate that events taking place in the presence of citrulline are occurring at the same time as in similar cultures maintained in media containing arginine. Furthermore, they occur at the same time as events in vaccinia virus-infected HeLa cells (Archard, 1971) in which there are host-coded functions for arginine biosynthesis. In view of this any alteration of metabolism in mouse S180 cells as a result of virus infection must be an "early" virus-directed event.

In human citrullinaemia cells the enzyme argininosuccinate synthetase has markedly decreased substrate specificity; at concentrations of citrulline up to 1mM this enzyme has zero velocity and a concentration of 10mM citrulline is required before the conversion of citrulline to arginine is comparable with that in "normal" fibroblasts (Tedesco & Mellman, 1967). However, a dose response curve was obtained

with these cells infected with vaccinia virus in media containing citrulline and the yield of virus was maximal at a concentration of 0.5mM citrulline. Since arginine is incorporated per se into mature virus particles and (^{14}C)-carbamoyl-labelled citrulline is detected as arginine in purified virus preparations, these results with human citrullinaemia cells suggest that there is an enhanced capacity for arginine biosynthesis in poxvirus-infected cells.

Since mouse S180 cells did not grow in media containing argininosuccinic acid there is at least some defect in the enzyme argininosuccinate lyase. In HeLa cells the growth of vaccinia virus in media containing citrulline is inhibited by both canavanine and β -methyl aspartic acid (Chapter 3) indicating that argininosuccinic acid is an intermediate of arginine metabolism in poxvirus-infected cells. Furthermore, since both mouse S180 and human citrullinaemia cells have metabolic lesions which inhibit cell growth in medium containing citrulline but virus growth is permitted in similar conditions, it appears that poxvirus-infected cells are able to overcome these metabolic defects and have an enhanced ability to convert citrulline to arginine.

Although transcription is necessary for the stimulation of incorporation of citrulline in high molecular weight material in virus-infected cell cultures, it seems unlikely that this would involve derepression of host cell iso-enzymes because this would offer no selective advantage to uninfected cells, particularly mouse S180 or

human citrullinaemia. There may be some expression of pre-existing host cell enzymes so that their activity is sufficient to permit virus growth or the invading viral genome may be responsible for the synthesis of specific enzymes which can convert citrulline to arginine from its own genetic complement.

CHAPTER 5

ARGININOSUCCINATE SYNTHETASE-LYASE AND ARGINASE ACTIVITY IN UNINFECTED AND VACCINIA VIRUS-INFECTED HELA AND MOUSE L CELLS

5.1 Introduction

The results presented previously indicate that citrulline can replace arginine per se for the growth of poxviruses in continuous cell lines. These lines include mouse S180 and human citrullinaemia cell cultures, both of which have metabolic lesions in the enzymes converting citrulline to arginine. These lesions are reflected in the inability of these two cell lines to grow in otherwise complete medium when arginine is replaced by citrulline. However, poxvirus replication proceeds in both mouse S180 and human citrullinaemia cells when arginine is replaced by citrulline. It has been shown by radioactive labelling experiments that the citrulline available to vaccinia virus-infected HeLa cells is

incorporated into virus particles as arginine. Additionally, in vaccinia virus-infected HeLa cells in the presence of citrulline, L-canavanine and β -methyiaspartic acid inhibit virus growth. These analogues are known inhibitors of the enzymes argininosuccinate synthetase and lyase, the enzymes effecting the conversion of citrulline to arginine. The activity of these enzymes is essential, therefore, for the replication of poxviruses in the presence of citrulline. These results indicate that similar enzymatic mechanisms may be operative in virus-infected mouse S180 and human citrullinaemia cells. Since these functions are not expressed under the experimental conditions used in uninfected cells, this suggests that in infected cells expression of these enzymes may be determined by the virus genome.

Poxviruses have the largest genomes of the mammalian viruses with a coding potential on the basis of the molecular weight of the virus DNA of 1.8×10^7 daltons (Joklik, 1962), of about 150 proteins. Holowzak and Joklik (1967) demonstrated that there are at least 17 polypeptides associated with structural components of vaccinia virus. Serological investigations indicated 20 soluble virus-specific components in extracts of virus-infected cells capable of reacting with antiviral serum in gel diffusion tests, which indicate that some proteins do not have a structural function (Appleyard, Hume and Westwood, 1965). Investigations have shown changes in the activities of enzymes concerned with nucleic acid biosynthesis in poxvirus-infected cells. Green and Pina (1962) demonstrated increased incorporation of thymidine into cell cultures following infection with vaccinia

virus. This increase occurred at the same time as virus-specified DNA synthesis. Magee (1962) showed that the levels of activity of thymidylate kinase and DNA polymerase increased early after infection. Vaccinia virus infection also results in increased thymidine kinase activity (Kit, Dubbs & Piekarski, 1963a). This enzyme activity was also demonstrated in thymidine kinase-deficient mutants of mouse L cells following their infection with vaccinia virus (Kit, Piekarski & Dubbs, 1963b).

Jungwirth and Joklik (1965) showed that DNA polymerase, DNAase and thymidine kinase activity is associated with the cytoplasmic fraction of vaccinia virus-infected cells. These enzyme activities are "switched off" at 4 hr p.i. but continue for at least 5 hr in infected cells treated with actinomycin D indicating that the relevant early mRNA species have long half lives. The DNA polymerase from virus-infected cells has a higher saturation level for primer than that from uninfected cells and although both polymerases have a pH optimum at 7.8 the virus induced polymerase is more active than that from the host cell at pH 9.0. Protein synthesis is required for the synthesis of DNA polymerase, DNAase and thymidine kinase in virus-infected cells and "switch off" mechanisms fail if DNA synthesis is inhibited. The synthesis of DNA polymerase and DNAase is inhibited in the presence of UV-irradiated virus but that of thymidine kinase is induced.

There are at least two DNAases in poxvirus-infected cells (Eron & McAuslan, 1966). One is an acid DNAase, with a pH optimum at 5.0,

which is synthesised under the direction of the progeny genomes. During the course of infection the enzyme undergoes a transition in vivo from a soluble to an insoluble form, but it is synthesised continuously for about 18 hr p.i. (McAuslan & Kates, 1967). Pogo and Dales (1969) demonstrated that this enzyme is an exonuclease whereas the second DNAase from infected cells, which has a pH optimum at 7.8, is an endonuclease. The two activities are localised in the core of the virus particle and sequential removal of envelopes and lateral bodies permits activation of both enzymes. It has been suggested that a specific inhibitor may be present in the lateral bodies since both DNAases are expressed as late functions in vivo but are apparently regulated by early virus-coded functions.

Vaccinia virus also contains a nucleotide phosphohydrolase enzyme with affinities for nucleotide triphosphates. The enzyme function has been located in the core of the virus particle (Pogo & Dales, 1968).

Further enzymic functions concerning RNA synthesis have been shown to be associated with poxvirus particles. Purified rabbitpox virus cores can mediate the synthesis of virus-specific RNA in vitro without the addition of exogenous DNA primer (Kates & McAuslan, 1967). Similar observations have been made in vitro using purified vaccinia virus particles (Munyon, Paoletti & Grace, 1967). Further investigations by Pitkanen, McAuslan, Hedgpeth and Woodson (1968) have demonstrated that there are two RNA polymerases which are induced in

cells infected with vaccinia virus. Both their properties and the time course of appearance are different. One catalyses the dAT (copolymer of deoxyadenylate and deoxythymidylate) - primed conversions of ATP and UTP into an acid soluble pool. This enzyme is detectable only if DNA synthesis is inhibited. In contrast, the accumulation of progeny genomes is a necessary criterion for the induction of the second enzyme. The latter activity, which is unmasked by detergent treatment, is found exclusively in maturing virus particles.

Those enzyme activities shown to be associated with poxvirus particles may probably be regarded as virus-specified functions. The other quantitative changes in enzyme activity in poxvirus-infected cells may be either virus-specified or host-specified functions. However, in other virus-infected systems metabolic pathways unique to virus-infection have been demonstrated indicating, in the absence of other criteria, their virus-specific nature. Such systems have not been demonstrated following infection with animal viruses, but have been shown in bacteria infected with bacteriophage. In Escherichia coli infected with "T"-even bacteriophage 5'-hydroxymethylcytosine replaces cytosine in bacteriophage DNA (Flaks & Cohen, 1957). In addition, glucosylating enzymes have been isolated from the same system, which transfer glucose from UDP-glucose to 5'-hydroxymethylcytosine residues in DNA (Kornberg, Zimmerman & Kornberg, 1961).

In the present study the replication of rabbitpox virus in mouse

S180 cells and of vaccinia virus in human citrullinaemia cells in the presence of citrulline suggested a virus determined expression of argininosuccinate synthetase and lyase activity. Experimental techniques to assay these activities have been developed and applied to uninfected and poxvirus-infected cell cultures.

5.2 Development of enzyme assay

The formation of arginine from citrulline in uninfected and vaccinia virus-infected cells has been quantitatively determined by measuring the conversion of (^{14}C)-carbamoyl-labelled citrulline to (^{14}C)-guanido-labelled arginine. This has been measured directly as $^{14}\text{CO}_2$ released from the (^{14}C)-guanido-labelled arginine formed following further treatment with arginase and urease. Fig. 5.1 shows that each molecule of (^{14}C)-carbamoyl-labelled citrulline converted to (^{14}C)-guanido-labelled arginine results, under the conditions used, in the release of one molecule of $^{14}\text{CO}_2$. This assay system measures the activities of both argininosuccinate synthetase and lyase. Separate measurement of these two enzyme activities was not attempted due to the unavailability of appropriately labelled argininosuccinic acid.

Preliminary investigations were carried out to establish the factors which would effect the efficiency of the assay system. The pH optimum for arginase activity was established by preparing a series of solutions, each containing 0.227 M arginine in tris-HCl buffers, covering a range of pH from 6.45 to 9.5. Volumes of 0.5 ml of each arginine solution were added to 0.5 ml of arginase in the same buffer

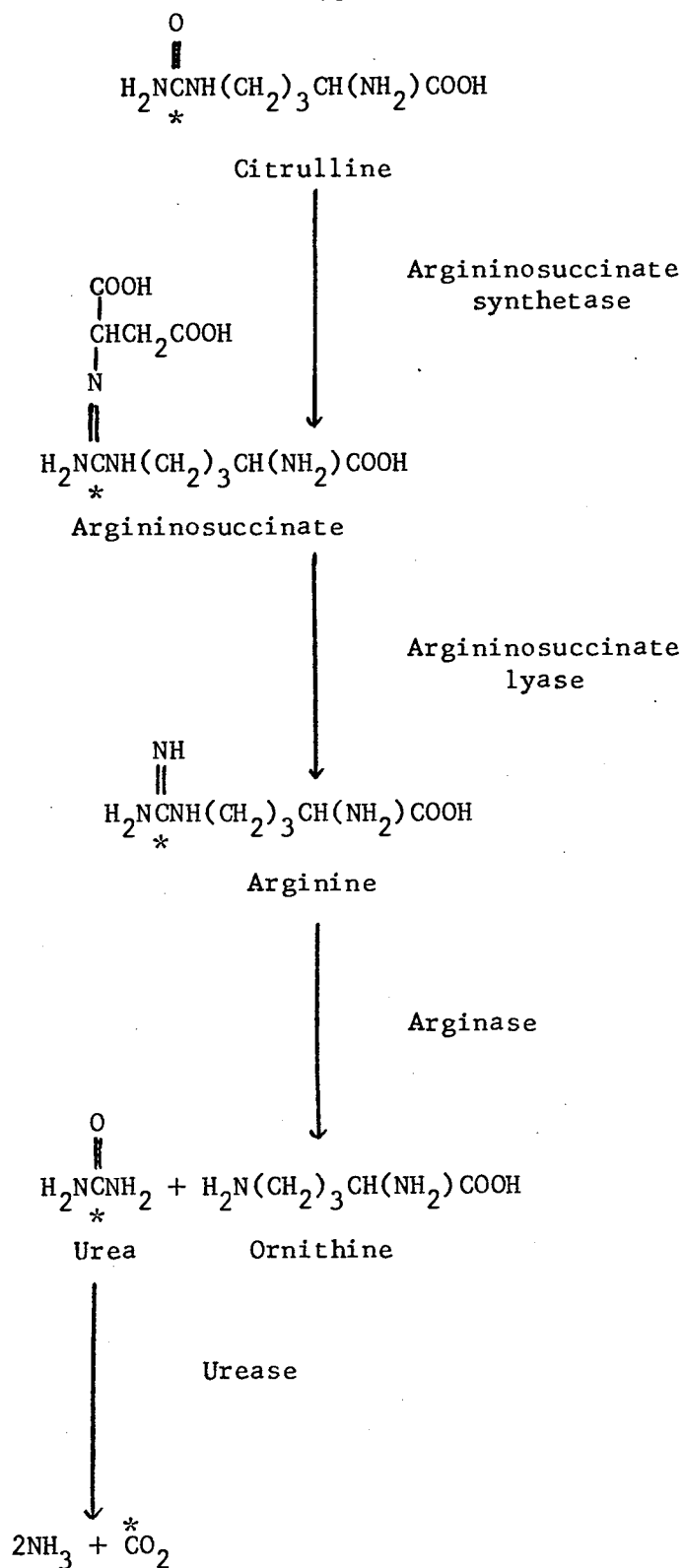


Fig. 5.1 The catabolism of (^{14}C)-carbamoyl-labelled citrulline to $^{14}\text{CO}_2$

together with 0.1 μCi (^{14}C)-guanido-labelled arginine. The mixture was allowed to react at 37° for 5 min at which time 0.5 ml 1 N HCl was added to each sample, followed by 1.6 N sodium hydrogen phosphate to adjust the pH to 7.0. Warmed urease solution (10 units/ml in 0.1 M phosphate buffer, pH 7.0) was added to each sample in 0.5 ml aliquots, the system closed and allowed to react at 37° for 20 min; 1.0 ml 1N HCl was added to inhibit further urease activity and to assist the release of $^{14}\text{CO}_2$. The evolved $^{14}\text{CO}_2$ was collected and processed by methods previously described (See Materials and Methods). The mean recovered radioactivity recorded in dpm from replicate samples at each pH was compared with the pH of that sample (Fig. 5.2). Maximum catabolism of arginine occurred at pH 8.0 and stock solutions were prepared at this pH for all subsequent experiments.

The effect of ornithine on the efficiency of the system was examined to determine whether arginase activity would be inhibited by high concentrations of this end product. Ornithine, in amounts between 0-25 μmoles , was added to the complete reaction mixture containing 25 μmoles (^{14}C)-guanido-labelled arginine as substrate. Urease was added to each sample and further procedures carried out as indicated previously. The efficiency of the system was unaffected by amounts of ornithine up to 25 μmoles .

Samples of uninfected HeLa cells in 1.0 ml aliquots were used to investigate the effect of omitting different components from the complete assay mixture. The substrate was 25 μmoles (^{14}C)-carbamoyl-

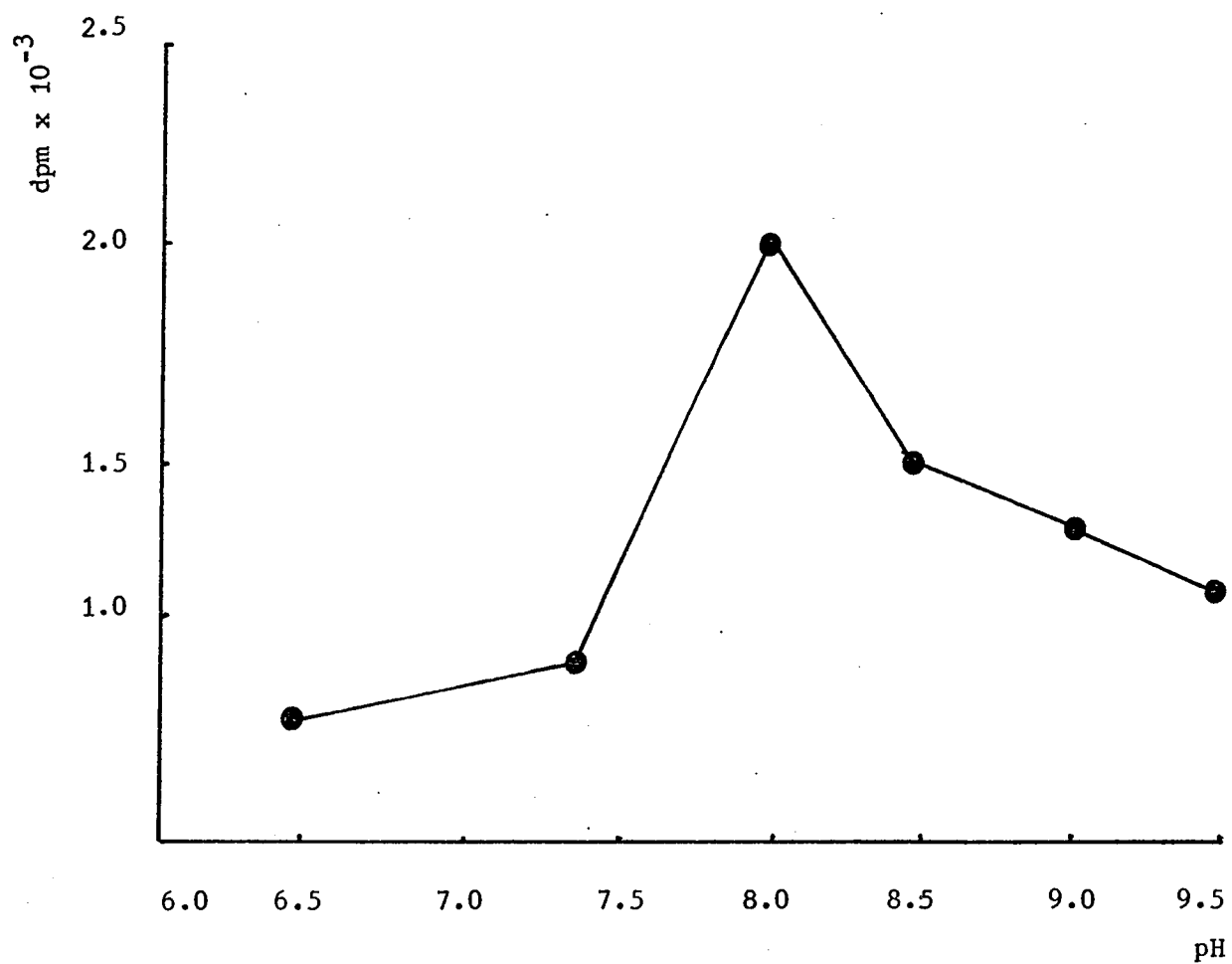


Fig. 5.2 The effect of pH on arginase activity

labelled citrulline. Samples were treated as indicated in Materials and Methods. The amount of $^{14}\text{CO}_2$ recovered from samples containing the complete assay mixture was compared to that from samples from which arginase, urease or both had been omitted. In the absence of both urease and arginase the amount of $^{14}\text{CO}_2$ recovered was reduced by more than 90% and similar reductions occurred in the absence of urease alone. Removal of arginase resulted in a 75% reduction in recovery compared with the complete assay mixture. Thus, these results show that for maximum efficiency addition of both arginase and urease is essential and also show the specificity of the system.

The addition of 0.5 ml 1N HCl alone was not sufficient to completely inhibit the activity of the arginase preparation at the end of the initial one hr incubation period. Complete inhibition of arginase activity was achieved by heating the system at 70° for 20 min prior to the addition of urease and this procedure was used in all subsequent experiments.

Although the arginase used was a partially purified commercial preparation (Sigma Chemical Company Limited) it was found to be contaminated with the enzymes argininosuccinate synthetase and lyase. After further purification by methods described by Bach, Hawkins and Swaine (1963), this background activity was reduced 10-fold but not completely abolished. Thus, in all experiments the rate of conversion of citrulline to arginine from a given sample of cell extract had to be measured above the level of the background activity. The level of contamination in the

arginase preparation made experiments with mouse S180 cells difficult. Preliminary experiments did not show any significant activity and consistent quantitative data could not be obtained. In further experiments carried out with mouse S180 cells infected with rabbitpox virus there appeared to be some stimulation in the rate of conversion of citrulline to arginine but, again, the background activity was too high for significant quantitative data to be obtained. Since human citrullinaemia cells have markedly reduced substrate specificity with respect to the enzyme argininosuccinate synthetase (Tedesco & Mellman, 1967) low levels of enzyme activity would be expected under the experimental conditions used. Furthermore, since these cells proved difficult to culture in vitro it was not possible to provide sufficient numbers of cells to attempt any enzymatic measurements. Hence, no attempts were made to investigate enzyme levels in these cells.

Both HeLa and mouse L cells can utilize citrulline in place of arginine for cell growth. Thus, the enzymes argininosuccinate synthetase and lyase must be functional in these cells. Furthermore, since the two lines of cells are from different species they may be expected to show differences in the properties of the enzymes assayed. Preliminary experiments with uninfected cells showed that the level of conversion of citrulline to arginine in both these cell lines was sufficient to measure quantitatively above the background from the arginase preparation.

5.3 Argininosuccinate synthetase-lyase activity in vaccinia virus-infected HeLa cells

Schimke (1964) investigated the concentrations of arginine and citrulline required for the maximum rate of conversion of citrulline to arginine in a number of mammalian cell lines. In HeLa cells 0.2mM L-citrulline or 0.6mM L-arginine are optimal. These concentrations of amino-acids were used for all subsequent experiments. The growth medium from confluent monolayers of HeLa cells in 40 oz roller bottles was removed and the cells maintained for 18 hr in PE medium containing the above concentrations of citrulline or arginine. Cell cultures were infected with 5 p.f.u./cell vaccinia virus in the same media and after adsorption were maintained in similar media. After this time the infected cells were recovered and disrupted together with uninfected, control cells. The rates of conversion of citrulline to arginine by these extracts were measured by methods previously described. Samples of the same cell extracts were used to determine the amounts of cell protein present by the biuret-phenol method. Results are expressed as μ moles of citrulline converted to arginine per mg cell protein per hour.

The enzyme activity of vaccinia virus-infected cells maintained in the presence of arginine was 10-fold higher than uninfected control cultures. Similar results were obtained with infected cells maintained in the presence of citrulline: enzyme activity increased 3-fold compared with control cultures. As shown by Schimke (1964), the level of enzyme activity in uninfected cells maintained in the presence of citrulline was greater than that in similar cultures maintained in the

presence of arginine (Table 5.1). These latter results indicate the operation in uninfected cells of an induction-repression mechanism of argininosuccinate synthetase and lyase activity determined by the availability of citrulline and arginine respectively. Similar results were obtained with vaccinia virus-infected cells but the level of enzyme activity in infected cell cultures in the presence of arginine was higher than that of uninfected cells in the presence of citrulline. This indicates that the mechanisms effecting changes in the levels of enzyme activity following virus infection differ from those controlling the induction-repression mechanism in uninfected cells.

5.4 Argininosuccinate synthetase-lyase activity in vaccinia virus-infected mouse L cells

Similar experiments to those described in the previous section were carried out with mouse L cells. There was a 3-fold increase in enzyme activity following virus infection in cells maintained in the presence of arginine. With cell cultures maintained in the presence of citrulline there was a 2-fold increase in enzyme activity following virus infection. Again, the enzyme activity in uninfected cells maintained in the presence of citrulline was greater than that in uninfected cells maintained in the presence of arginine but lower than that of vaccinia virus-infected cells maintained in the presence of arginine.

Thus, in two cell lines from different species marked increases in argininosuccinate synthetase-lyase activity occur following infection with vaccinia virus (Table 5.1).

Table 5.1 Argininosuccinate synthetase-lyase activity from uninfected and vaccinia virus-infected HeLa and mouse L cells

Cell line	Medium	Enzyme activity*	
		Control cell cultures	Infected cell cultures
HeLa	0.6mM L-arginine	0.0115	0.115
	0.2mM L-citrulline	0.079	0.262
Mouse L	0.6mM L-arginine	0.0325	0.09
	0.2mM L-citrulline	0.07	0.179

* μ M arginine synthesised/mg cell protein/hr

5.5 Effect of metabolic inhibitors on argininosuccinate synthetase-lyase activity in vaccinia virus-infected HeLa cells

Since the degree of stimulation of enzyme activity in vaccinia virus-infected HeLa cells maintained with arginine was 10-fold greater than that in similar uninfected cell cultures, further experiments were carried out in this system. Experiments were made to determine the role of DNA synthesis, transcription and translation in the changes in enzyme activity. Confluent monolayers of HeLa cells in 40 oz roller bottles were maintained in PE medium containing 0.6mM L-arginine and subsequently infected as before. After adsorption cell cultures were maintained in similar medium containing either 10 μ M FUDR, 0.5 μ g/ml actinomycin D or 1.5 μ g/ml puromycin (Sigma Chemical Company Limited). Uninfected cultures were treated similarly. Additional cell cultures, both uninfected and infected, were maintained in media without inhibitors.

FUDR did not inhibit the enhanced enzyme activity in HeLa cells infected with vaccinia virus. Since this inhibitor blocks the formation of thymidylate, DNA synthesis is inhibited (Cohen, Flaks, Barner, Loeb and Lichtenstein, 1960). Thus, the replication of vaccinia virus DNA is also inhibited (Salzman, 1960). The results indicate that the increase in enzyme activity in vaccinia virus-infected cells is dependent on the invading viral genomes and not upon progeny species (Table 5.2).

The enzyme activity of virus-infected cells did not increase in

Table 5.2 Argininosuccinate synthetase-lyase activity from uninfected and vaccinia virus-infected HeLa cells after treatment with inhibitors of DNA synthesis, transcription and translation

Concentration of inhibitor	Enzyme activity*	
	Control cell cultures	Infected cell cultures
10 μ M FUDR	0.021	0.24
0.5 μ g/ml actinomycin D	0.011	0.012
1.5 μ g/ml puromycin	0.0016	>0.00001
No inhibitor	0.012	0.12

* μ M arginine synthesised/mg cell protein/hr

the presence of actinomycin D but was similar to that of uninfected cells maintained with this inhibitor and also to that of uninfected cells maintained in the absence of inhibitor (Table 5.2). Since actinomycin D inhibits all DNA-directed RNA synthesis (Reich, Franklin, Shatkin and Tatum, 1961) the results indicate that transcription is necessary for the increased enzyme activity in vaccinia virus-infected cells. Also, since the level of enzyme activity in uninfected cells maintained with actinomycin D was similar to that from uninfected cells maintained in its absence, it appears that the mRNA species for the enzymes argininosuccinate synthetase and lyase have long half lives. The enzyme activity in uninfected cells in the presence of actinomycin D was investigated further. Cell cultures were maintained in PE medium without arginine for 18 hr before changing to complete medium containing 0.5 $\mu\text{g/ml}$ actinomycin D for a further 20 hr. Additional cell cultures which had been treated in the same way were maintained in complete medium without the inhibitor. The activity of enzymes from cells which had been maintained in the presence of actinomycin D was almost 80% of that from similar cultures which had been maintained in its absence. These results indicate that after inhibition of protein synthesis following withdrawal of arginine from cell cultures (Archard, 1971), enzyme activity was maintained when protein synthesis was initiated but formation of mRNA was inhibited. This indicates that the mRNA species coding for the expression of argininosuccinate synthetase and lyase have long half lives in excess of 24 hr (Table 5.3).

Table 5.3 Argininosuccinate synthetase-lyase activity from uninfected HeLa cells after starvation in arginine-free PE medium and subsequent treatment with actinomycin D

Medium	Enzyme activity*
	Uninfected cell cultures
0.6mM L-arginine	0.011
0.6mM L-arginine + 0.5 µg/ml actinomycin D	0.08

* µM arginine synthesised/mg cell protein/hr

The enzyme activity in virus-infected cells was barely detectable when infected cells were maintained in the presence of puromycin. The enzyme activity from similarly treated, uninfected cells, although reduced by about 90% compared to the level of activity in similar cultures maintained without the inhibitor, was at least 16-fold higher (Table 5.2). Thus, protein synthesis is also essential for the increased enzyme activity observed in vaccinia virus-infected cells.

5.6 Michaelis constants for argininosuccinate synthetase and lyase in uninfected and vaccinia virus-infected HeLa and mouse L cells

Cell extracts were prepared from uninfected and vaccinia virus-infected HeLa and mouse L cells which had been maintained in PE medium containing 0.6mM L-arginine. These extracts were examined for the rate of conversion of citrulline to arginine at different substrate concentrations. Preliminary experiments were carried out with uninfected and vaccinia virus-infected cells to determine the concentration of citrulline to obtain maximum reaction velocities.

a) Uninfected and vaccinia virus-infected HeLa cells

In order to facilitate interpretation of results, constant proportionality was maintained between (^{14}C)-carbamoyl-labelled and unlabelled citrulline. Assays were made with citrulline concentrations from 0.03 to 5mM with the same final volume in each assay series. The assay procedures used were as described previously (See Materials and

Methods). Results were expressed graphically as the velocity of the reaction (V) against the concentration of substrate (S) in the reaction mixture (Figs. 5.3 and 5.4). Michaelis constants were calculated from Lineweaver-Burke plots of ($1/V$) against ($1/S$).

The Michaelis constant for argininosuccinate synthetase-lyase activity in uninfected HeLa cells was $0.105 \times 10^{-3} M$ and in vaccinia virus infected HeLa cells was $1.04 \times 10^{-3} M$ (Figs. 5.7 and 5.8).

b) Uninfected and vaccinia virus-infected mouse L cells

Assay procedures similar to those described above were used. The Michaelis constant for argininosuccinate synthetase-lyase activity in mouse L cells was $0.07 \times 10^{-3} M$ and in vaccinia virus-infected mouse L cells was $1.0 \times 10^{-3} M$ (Figs. 5.5, 5.6, 5.9 and 5.10).

5.7 Arginase activity in HeLa cells infected with vaccinia virus

Since the results presented indicate that there is a stimulation in the activity of the enzyme argininosuccinate synthetase and lyase in both HeLa and mouse L cells infected with vaccinia virus, further investigations were made to determine whether similar changes occurred with other enzymes in the Krebs-Henseleit cycle. Rogers and Moore (1963) have shown increased arginase activity with Shope papilloma virus. Experiments were made to determine the arginase activity in uninfected HeLa cells and after infection with vaccinia virus.

Confluent monolayers of the HeLa cells in 4 oz prescription

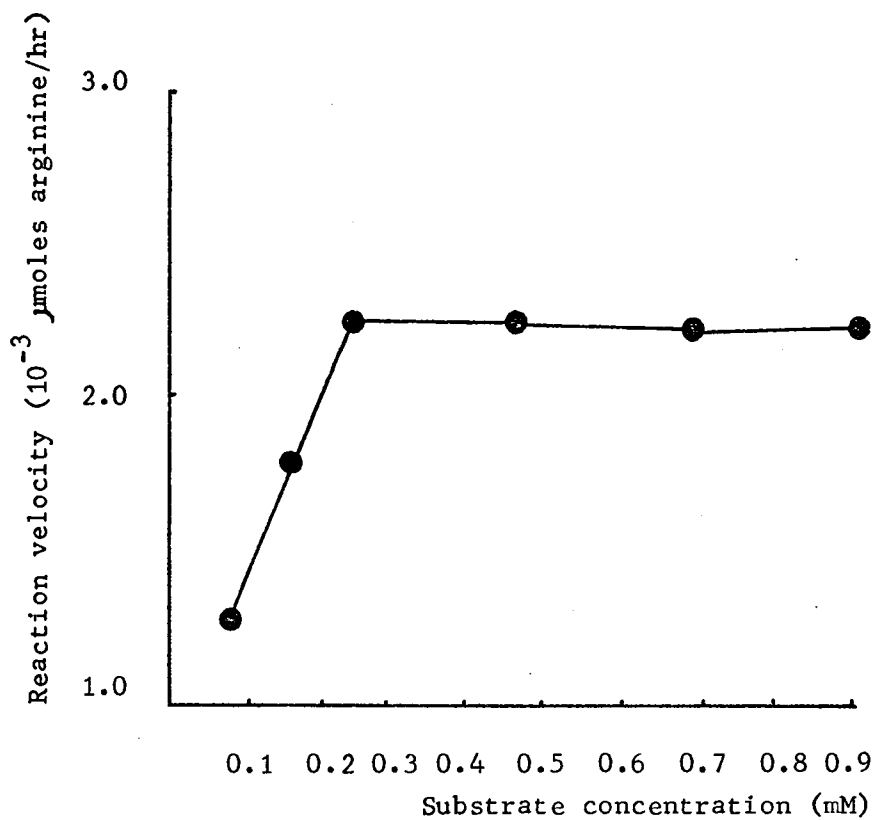


Fig. 5.3 Effect of citrulline concentration on the velocity of argininosuccinate synthetase-lyase activity in uninfected HeLa cells

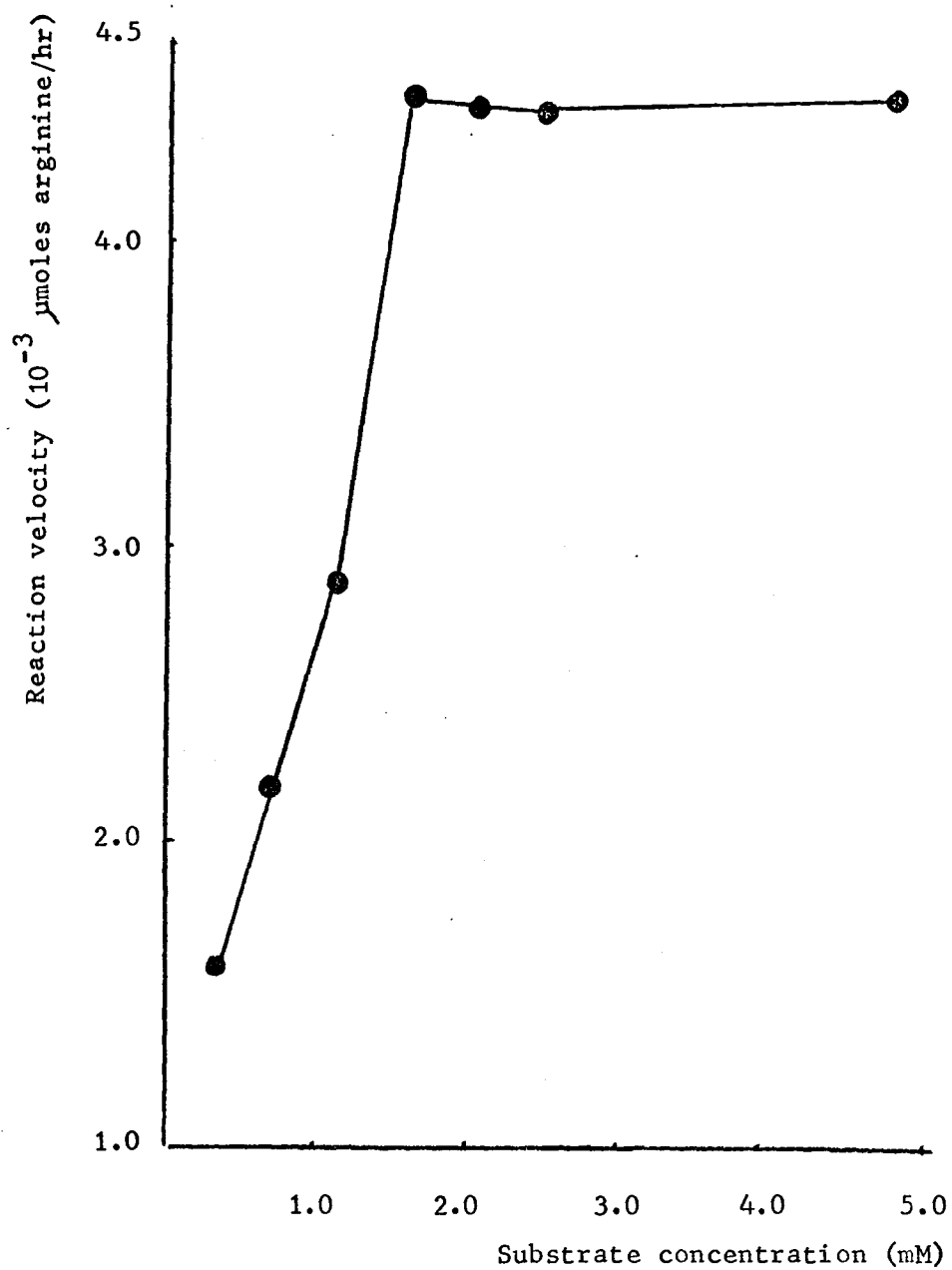


Fig. 5.4 Effect of citrulline concentration on the velocity of arginosuccinate synthetase-lyase activity in vaccinia-virus infected HeLa cells

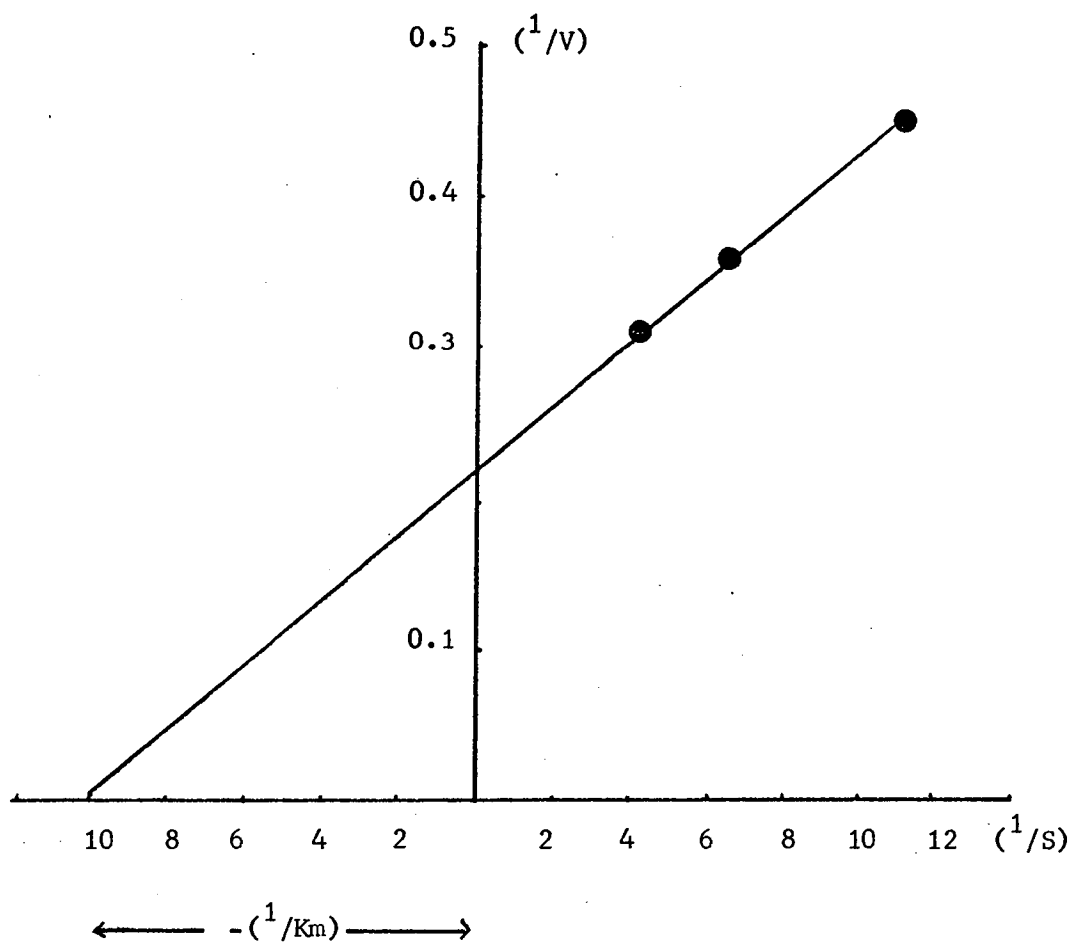


Fig. 5.7 Lineweaver-Burke plot of data presented in Fig. 5.3 (uninfected HeLa cells)

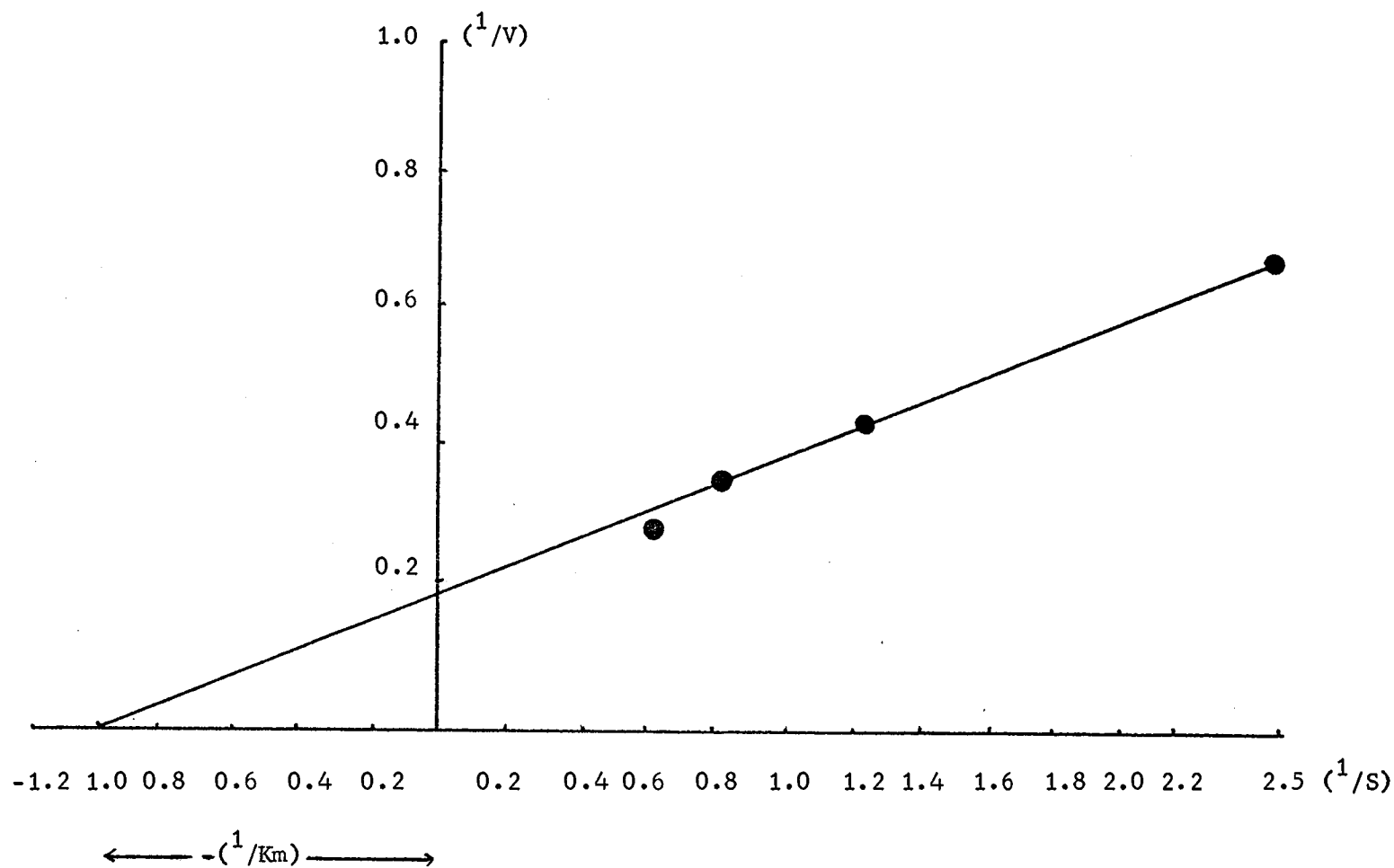


Fig. 5.8 Lineweaver-Burke plot of data presented in Fig. 5.4 (vaccinia-infected HeLa cells)

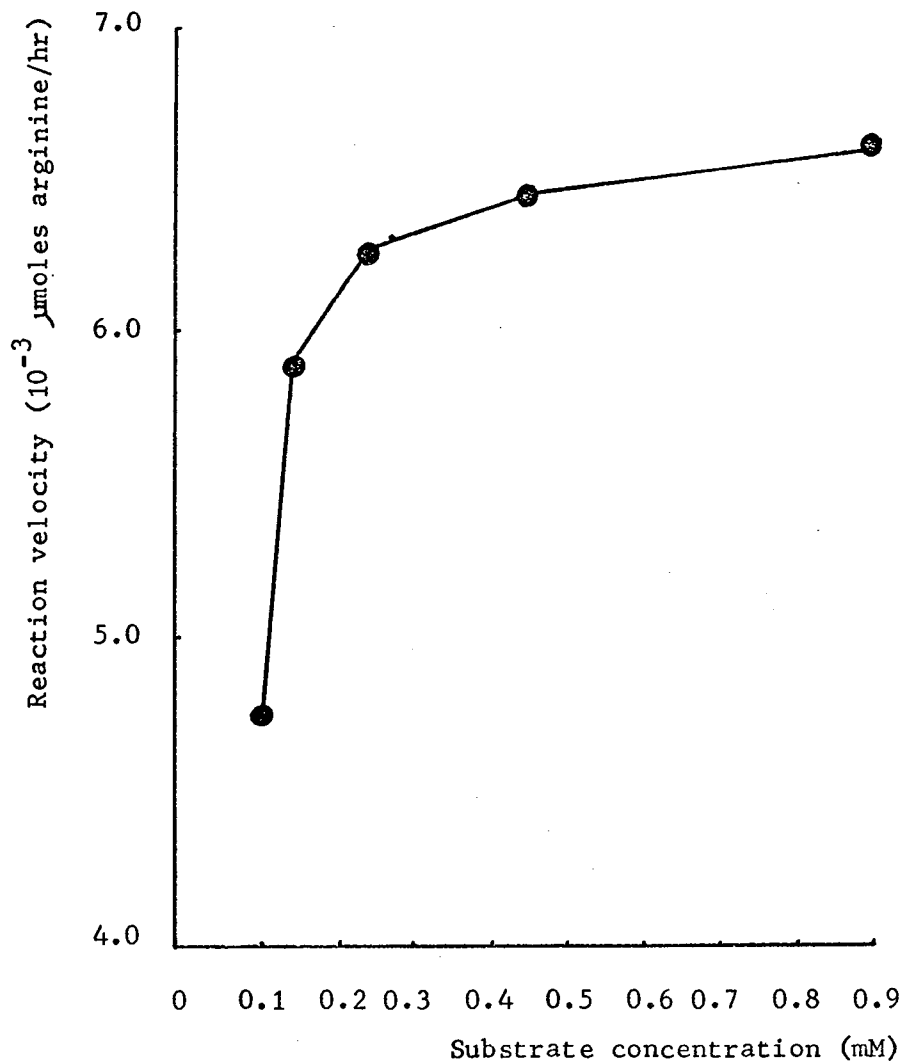


Fig. 5.5 Effect of citrulline concentration on the velocity of argininosuccinate synthetase-lyase activity in uninfected mouse L cells

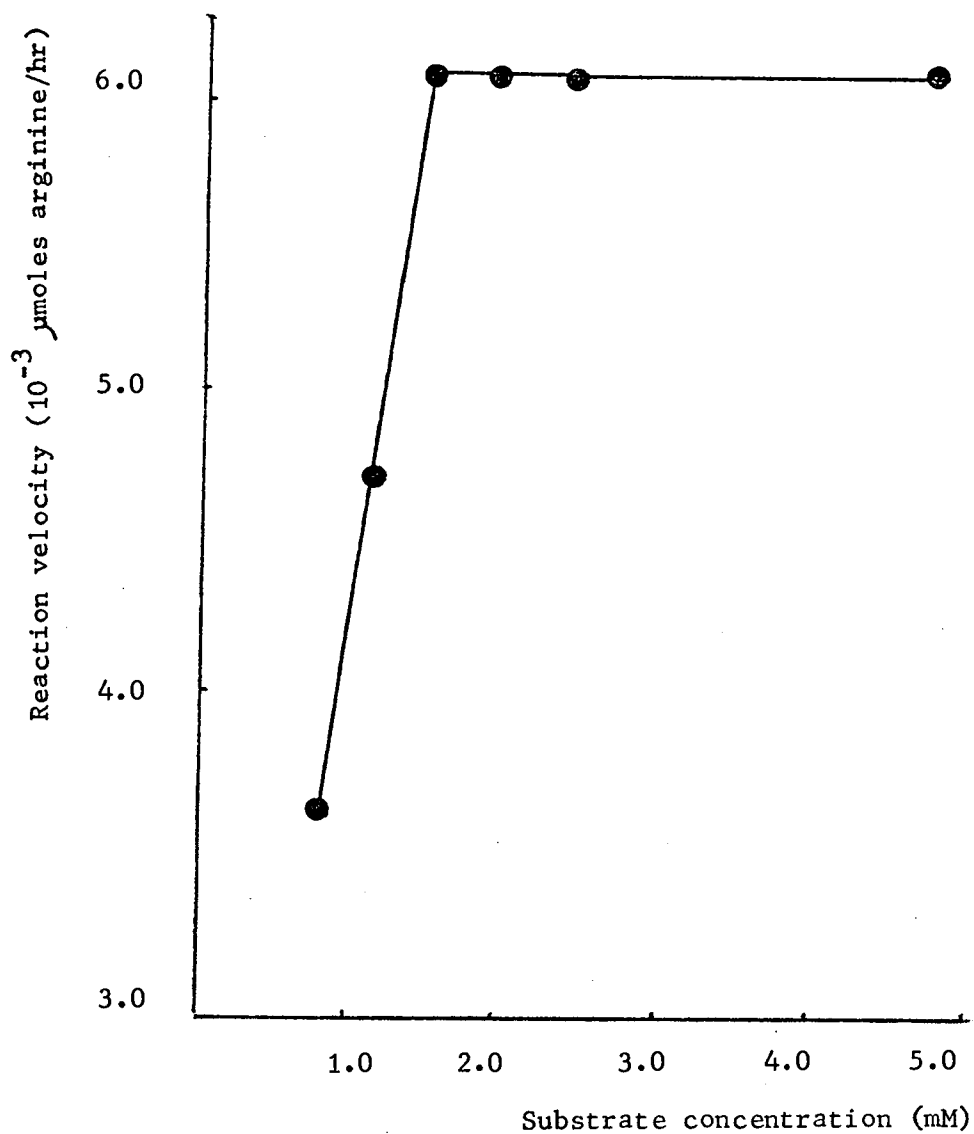


Fig. 5.6 Effect of citrulline concentration on the velocity of argininosuccinate synthetase-lyase activity in vaccinia virus-infected mouse L cells

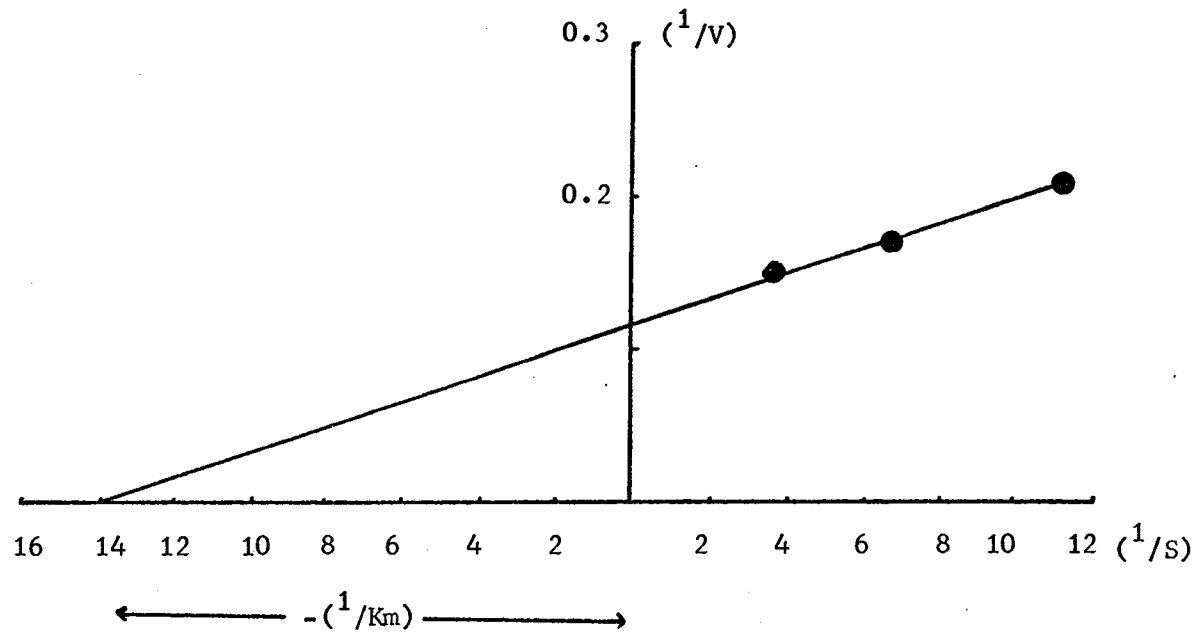


Fig. 5.9 Lineweaver-Burke plot of data presented in Fig. 5.5 (uninfected mouse L cells)

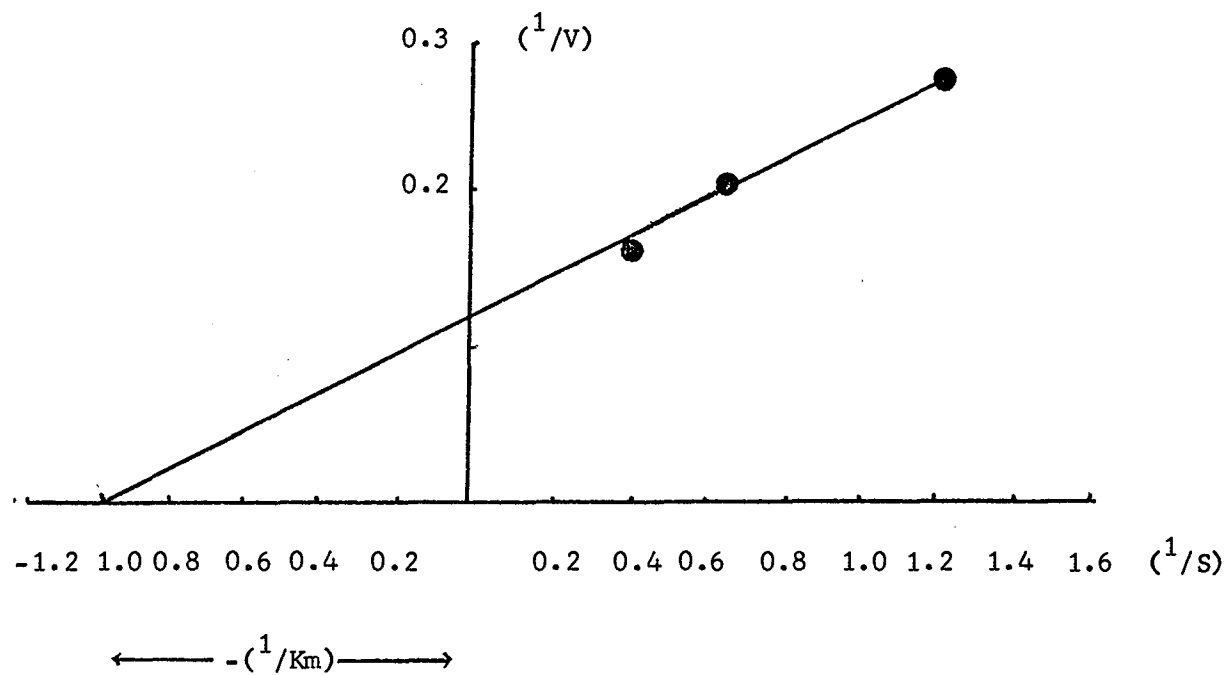


Fig. 5.10 Lineweaver-Burke plot of data presented in Fig. 5.6 (vaccinia infected mouse L cells)

bottles were maintained in PE medium containing 0.6mM L-arginine for 18 hr before infecting with 5 p.f.u./cell vaccinia virus in similar medium. After adsorption for 30 minutes the monolayers were incubated for 20 hr at 37° in 10 ml of similar medium containing 0.1 μ C/ml (¹⁴C)-guanido-labelled arginine. Uninfected cell cultures were treated in the same way. After this time aliquots of media from uninfected and vaccinia virus-infected cell cultures were used to determine the amounts of urea liberated into the medium by methods previously described. Since one mole of arginine is catabolised to one mole of urea this gave an indirect measurement of the arginase activity from the cells. The results were expressed as the number of μ moles of urea released per mg cell protein. There was no significant increase in the amount of urea formed in vaccinia virus-infected HeLa cells compared to uninfected cells (Table 5.4).

5.8 Discussion

Citrulline can substitute for arginine for the growth of poxviruses in a number of continuous cell lines (see Chapters 3 and 4). Experiments have shown that when citrulline substitutes for arginine it is converted to arginine prior to incorporation into mature virus particles. In mouse S180 and human citrulline cells, which are deficient in the mechanisms for converting citrulline to arginine, citrulline can substitute for arginine in virus-infected cells. In HeLa and mouse L cells infected with vaccinia virus host-directed mechanisms may be used to convert citrulline to arginine. In mouse

Table 5.4 Arginase activity from uninfected and vaccinia virus-infected HeLa cells

Medium	μ M urea synthesised after 20hr/mg cell protein	
	Uninfected cell cultures	Infected cell cultures
0.6mM L-arginine	0.172	0.187

S180 and human citrullinaemia cells similar mechanisms may be used but it would be necessary to suggest that these functions are quantitatively insufficient to support cell growth but sufficient to support virus growth. Alternatively, these functions are completely absent in these cells. In the latter case, virus infection must result in the derepression of the host cell genome or the viral genome codes for the necessary enzyme activity.

It was not possible to produce sufficient quantities of human citrullinaemia cells necessary for assays and with the mouse S180 cells the background activity of the assay system prevented definitive experiments. Additionally, calculations concerning the amounts of arginine required to produce the number of virus particles obtained in a single cycle of infection in mouse S180 cells provided a basis for a theoretical determination of the degree of enzyme activity required to produce this amount of arginine. This amount of enzyme activity would not be measurable with the assay system described without either reducing the background activity of the assay system or scaling up the production of virus-infected cell extracts by at least 100-fold.

In view of these limitations experiments were carried out in HeLa and mouse L cells. Both these cell lines will grow in the presence of citrulline and are therefore competent in argininosuccinate synthetase and lyase activity. Using these systems experiments were designed to look for relative changes in enzyme activity of uninfected and vaccinia virus-infected cells rather than absolute differences that might have

been expected with human citrullinaemia and mouse S180 cells. In both HeLa and mouse L cells there was a marked stimulation in argininosuccinate synthetase-lyase activity following infection with vaccinia virus. Schimke (1964) has shown that these enzyme activities are determined by the extracellular concentration of citrulline and arginine. Enzyme activity is elevated in the presence of citrulline and suppressed in the presence of arginine. A similar pattern of results were observed in virus-infected cells. However, the enzyme activity in virus-infected cells maintained with arginine was greater than in uninfected cells maintained with citrulline. Also, the enzyme activity from infected cells was greater than that from uninfected cells maintained in similar medium. These changes in argininosuccinate synthetase-lyase activity following virus infection could arise either from the synthesis of increased amounts of enzymes or from changes in the turnover rate of these enzymes following virus infection.

The expression of the invading viral genome in vaccinia virus infection takes place at two different times in the replication cycle (Salzman & Sebring, 1967). The first expression occurs immediately after infection when transcription of the invading viral genomes takes place leading to the production of early mRNA. The second stage of expression occurs after the replication of the invading viral genomes and progeny virus DNA is transcribed producing late mRNA. These two species of mRNA differ in their base sequence and therefore certain functions of the virus genome are expressed either before or after

virus-specified DNA replication. Expression of late functions can be prevented by inhibiting DNA synthesis. Some increased enzyme activity was observed following virus-infection of HeLa cells in the presence of a concentration of FUdR known to inhibit virus replication. These results show that the changes in enzyme activity demonstrated are virus-directed and that these changes are early events controlled by the invading viral genome. Since arginine has been shown to play an important role in events leading to virus-specified DNA synthesis it would be expected that these enzyme activities would be early functions.

Jungwirth and Joklik (1965) demonstrated that the "switch off" mechanisms controlling the expression of early functions in vaccinia virus-infected cells was dependent upon virus-specified DNA synthesis. If DNA synthesis is inhibited with FUdR control of early functions failed to operate leading to a continued expression of enzyme functions. Argininosuccinate synthetase-lyase activity in virus-infected cells in the presence of FUdR was two-fold higher than that from cells infected in the absence of this metabolic inhibitor. These results indicate that the metabolic control mechanisms for argininosuccinate synthetase-lyase activity in vaccinia virus-infected cells are dependent on the synthesis of viral nucleic acid and that there is a switch-off mechanism operable in infected cells which regulates the activities of these enzymes.

Inhibition of transcription by actinomycin D in virus-infected

cells led to a marked reduction in the increase in enzyme activity compared with cells infected in the absence of this inhibitor.

These results indicate that mRNA synthesis is necessary for increased enzyme activity. However, enzyme activity of uninfected cells was not affected by treatment with actinomycin D. Additional experiments showed that the mRNA species of uninfected HeLa cells coding for argininosuccinate synthetase-lyase activity have long half lives in excess of 24 hr. This indicated that host-specified mRNA coding for argininosuccinate synthetase and lyase should be available in virus-infected cells. The necessity for synthesis of new mRNA for increased enzyme activity indicates that there is either increased transcription of the host-cell genome or transcription of the invading viral genome.

Failure to demonstrate increased enzyme activity in virus-infected cells treated with puromycin shows that protein synthesis is necessary for increased activity. The level of enzyme activity detected in such cells was considerably lower than that of uninfected cells similarly treated with puromycin. Additionally, in cells infected with vaccinia virus in the presence of puromycin the level of enzyme activity was lower than that from uninfected cells treated similarly. Thus, although the mRNA species for argininosuccinate synthetase-lyase activity in uninfected HeLa cells appear to have long half lives, the data from these experiments indicate that there is a rapid turnover of enzymic protein. Furthermore, the turnover rate of these enzymes appears to be increased in cells infected with vaccinia virus.

Although the results obtained from experiments using metabolic inhibitors provide some evidence that the argininosuccinate synthetase-lyase activity is virus-specific, the results may be interpreted on the basis of virus-directed changes in the expression of the host cell genome. If the enzymes in virus-infected cells are host-specific they would be expected to show the same kinetic characteristics as those from uninfected cells. A comparison of Michaelis constants (K_m) of enzymes from HeLa and mouse L cells shows differences in K_m values between uninfected and vaccinia virus-infected cells (Table 5.5). These results indicate the appearances of enzymes peculiar to virus-infected cells. This could be explained by derepression of pre-existing host cell iso-enzymes. However, although the K_m value in both species of infected cells was $1.0 \times 10^{-3} M$ the K_m values from uninfected cells were different, being $0.07 \times 10^{-3} M$ for mouse L cells and $0.1 \times 10^{-3} M$ for HeLa cells. Thus, although the characteristics of the enzymes in the uninfected cells as determined by their K_m values were different, enzymes of identical characteristics were produced following virus-infection. These results provide strong evidence for the virus-specific nature of argininosuccinate synthetase-lyase activity in vaccinia virus-infected cells.

Table 5.5 Michaelis constants for argininosuccinate synthetase-lyase activity from uninfected and vaccinia virus-infected HeLa and mouse L cells

Cell line	Michaelis constants for argininosuccinate synthetase-lyase activity ($\times 10^{-3}\text{M}$)	
	Uninfected cell cultures	Infected cell cultures
HeLa	0.105	1.04
mouse L	0.07	1.0

CHAPTER 6

GENERAL DISCUSSION

The results of the present study confirm and extend previous observations on the role of arginine in poxvirus replication. Arginine is essential for the growth of poxviruses in a number of continuous cell lines (Holterman, 1969; Obert et al, 1971) and there is a quantitative relationship between the concentration of arginine supplied in the medium and the yield of infectious virus (Archard, 1971). Arginine has been shown to be present in the mature virion (Turner & Kaplan, 1968; Archard & Williamson, 1971) and one function, therefore, is in the formation of virus structural protein(s). However, there is evidence of some metabolism of arginine before incorporation into the virus particle (Archard, 1971). Products of arginine metabolism, histones and polyamines, for example,

have important structural and functional roles in both prokaryotes and eukaryotes.

Before discussing the possible roles of arginine in poxvirus replication it is pertinent to consider the sequence of events in cells infected with these viruses. Joklik (1963a) has shown that the uncoating of phagocytosed poxvirus particles is a two stage process. The first stage commences immediately after infection and is mediated by pre-existing, host-cell lysosomal enzymes. The second stage requires both mRNA and protein synthesis and results in viral DNA becoming sensitive to DNAases (Joklik, 1963b). Throughout the replication cycle of poxviruses there is an essential participation of virus-specific RNA. The requirement for RNA synthesis commences shortly after infection and continues until about 6 hr p.i. (Loh, 1964). Early mRNA species have been detected from $\frac{1}{2}$ hr p.i. (Becker & Joklik, 1964). Kates and McAuslan (1967) have demonstrated a DNA-dependent RNA-polymerase activity associated with purified core preparations of rabbitpox virus. This enzyme activity is presumably associated with the transcription of the parental viral genome producing early mRNA species concerned with the elaboration of events leading to virus-specified DNA synthesis. Since poxviruses replicate in the cytoplasm of infected cells (Cairns, 1960), whereas host-cell DNA synthesis takes place essentially in the nucleus, it is likely that specific enzymes will be involved in viral DNA replication. Jungwirth and Joklik (1964) have shown that there are marked increases

in DNA polymerase, thymidine kinase and DNAase activities in the cytoplasm of cells infected with vaccinia virus, commencing about $1\frac{1}{2}$ hr p.i. Protein synthesis is required for production of these early proteins; their formation is subject to "switch-off" by progeny virus DNA. Additionally, the synthesis of at least three viral structural proteins commences shortly after infection and their synthesis continues in the absence of DNA synthesis (Holowzak & Joklik, 1967). Two of these proteins are constituents of the viral core.

In HeLa cells infected with vaccinia virus, virus-specified DNA synthesis normally begins at $1\frac{1}{2}$ - 2 hr p.i. and is complete by 4 hr p.i. (Joklik & Becker, 1964). Kates and McAuslan (1967) have shown that concurrent protein synthesis is required during the period of DNA synthesis and that certain protein(s) formed is associated stoichiometrically rather than catalytically with newly synthesized DNA. This protein is unusual in that it is coded for by a relatively unstable messenger. Jungwirth and Joklik (1965) have shown that the mRNA species for most early proteins have long half lives. At about $3\frac{1}{2}$ hr p.i. the synthesis of early proteins is "switched-off" (Jungwirth & Joklik, 1965). The accumulation of progeny genomes following virus-specified DNA synthesis induces a second RNA polymerase (Pitkanen et al, 1968), which is responsible for the formation of late mRNA species transcribed from progeny genomes. This activity is found exclusively in maturing virus particles and is concerned with the synthesis of the majority of structural proteins (Salzman & Sebring, 1967).

Previous reports have indicated that both nucleic acid and protein synthesis have significant roles throughout the replication cycle of poxviruses. This is apparent from the earliest events leading to the release of functional viral nucleic acid (Joklik, 1963b) up to the time of maturation (Salzman & Sebring, 1968). It has been established that cells in culture require arginine (Eagle, 1959) and that this amino acid is also required for the replication of vaccinia virus (Holterman, 1969). Since both DNA and RNA synthesis are dependent on enzymic protein, one of the earliest requirements for arginine, immediately after infection may be involved in the translation of early mRNA species, transcribed by the virus-associated RNA-polymerase, for events leading to virus-specified DNA synthesis. Additionally, there may be a requirement for arginine in the synthesis of the early structural proteins described by Holowzak and Joklik (1967). Two of these structural proteins are constituents of the virus core and by analogy with other systems may be expected to be rich in arginine. The presence of such arginine-rich proteins associated with the virus-core rather than with capsid components have been demonstrated in adenovirus (Prage et al, 1968; Becker & Russell, 1968), and in herpes simplex virus (Olshevsky & Becker, 1970a and b). As well as the importance of arginine as a constituent of ribosomal protein, metabolic derivatives of arginine have been shown to have an important role in other systems. In L.leichmanii the guanido carbon of arginine may serve as a source of purines and pyrimidines for nucleic acid biosynthesis (Hutson & Downing, 1967). It is unlikely that arginine serves a similar function for

nucleotide biosynthesis in cells infected with poxviruses because identical yields of vaccinia virus are produced with equimolar concentrations of arginine both in the absence and presence of a full complement of nucleotides (Archard, 1971).

At the time of virus-specified DNA synthesis there may be a further requirement for arginine for enzymic protein. Also, arginine-containing proteins of a histone nature may be involved structurally as stabilizers of newly synthesized viral DNA. Kates and McAuslan (1967) have shown that one requirement for protein synthesis in cells infected with rabbitpox virus at the time of DNA synthesis is for a structural rather than an enzymic protein, since it is involved in stoichiometric amounts with the newly synthesized DNA. A similar requirement for protein synthesis has been described in cells infected with adenovirus (Polassis & Green, 1965), herpes virus (Becker et al, 1967) and polyoma virus (Shimono & Kaplan, 1969).

The major structural proteins of poxviruses are synthesized after virus-specified DNA replication. This involves the transcription of late mRNA species and their subsequent translation to structural protein. It is unlikely that arginine will be involved directly in the synthesis of mRNA but it is possible that polyamine derivatives of arginine are involved as stabilizers of ribosomal protein (Cohen & Lichtenstein, 1960). Additionally, it is likely that the major requirement for arginine will be involved in the synthesis of both enzymic and structural protein for events leading to maturation.

Archard (1971) has demonstrated that arginine is essential for the growth of vaccinia virus in HeLa cells and that there is a quantitative relationship between the concentration of arginine in the growth medium and the yield of infectious virus. In HeLa cells protein, DNA and RNA synthesis are markedly inhibited in the absence of arginine. However, deprivation of cell cultures prior to infection does not irreversibly block their ability to support virus replication, or for the virus to direct its replication when arginine is added immediately after infection. The arginine requirement in vaccinia virus-infected HeLa cells can be divided into at least two distinct events. One is an early requirement concerned with events leading to virus-specified DNA synthesis. This proceeds maximally with a concentration of 0.03mM arginine. The second requirement, which determines quantitatively the yield of infectious virus, is concerned with later events leading to the elaboration of virus-specified structural proteins and maturation. A concentration of 0.09mM arginine is required for the maximal yield of infectious virus to be produced (Archard, 1971).

Experiments involving the rates of incorporation of (^{14}C)-labelled arginine into vaccinia virus-infected HeLa cells have shown that there is a peak in the rate of incorporation immediately after infection. At 3 hr and 5 hr p.i. there are further peaks. These peaks in the rate of incorporation of arginine presumably reflect the time-course of different arginine-dependent events (Archard, 1971). The

major virus-specific events occurring immediately after infection are the uncoating of the virus particle, formation of early mRNA and enzymes associated with DNA synthesis. Events at 3 hr and 5 hr p.i. are the synthesis of progeny virus DNA and the formation of late mRNA and virus structural proteins, respectively.

Although arginine is essential for vaccinia virus-DNA replication in HeLa cells (Archard, 1971), in similar infected KB cells virus-specified DNA synthesis is not inhibited in the absence of arginine (Obert et al, 1971). However, there is a requirement for arginine in these cells after DNA synthesis for events leading to the synthesis of late mRNA species and formation of progeny virions. Similarly, in cells infected with herpes simplex virus, virus-specified DNA synthesis is not affected in cultures deprived of arginine but maturation is inhibited (Becker et al, 1967). A similar requirement has been shown in adenovirus-infected cells (Rouse et al, 1967). With reference to both herpes simplex and adenovirus it is pertinent that these species are nuclear replicating viruses. Thus, it is likely that they will be less dependent on the synthesis of enzymic protein concerned with DNA replication than poxviruses which replicate in the cytoplasm. The difference between the arginine requirements for vaccinia virus growth in HeLa cells and those for nuclear replicating viruses indicate differences in host-cell directed metabolism and virus-directed metabolism. The degree of vaccinia virus-DNA synthesis in the absence of arginine in KB cells compared to HeLa cells presumably reflects differences in

the susceptibility of these latter cells to arginine deprivation and suggests that in KB cells some endogenous arginine is available.

Concerning the effect of arginine deprivation on host-cell macromolecular synthesis, Gonczol et al (1967) have reported that in HeLa cells deprived of arginine, DNA synthesis is reduced by about 61%. Thus, the requirement for arginine for vaccinia virus-DNA replication may be a reflection of the host-cell system. In cells infected with vaccinia virus, although overall protein synthesis is markedly reduced in the absence of arginine, some early proteins have been detected (Archard, 1971). However, since virus-specified DNA synthesis is inhibited this suggests that arginine is required for enzymic proteins concerned with this event and that their synthesis is impaired in these conditions. Alternatively, deprivation of arginine at the time of DNA synthesis may inhibit the synthesis of protein required for the stability of newly synthesized DNA (Kates & McAuslan, 1967). Such a protein has been shown to be associated with DNA in herpes simplex virus particles (Olshevsky & Becker, 1970a and b).

In other species arginine may be utilized as a source of ATP (Schimke, 1964; Barke & Roon, 1967). Since DNA replication is an endergonic process a possible role of arginine in poxvirus replication could be for the provision of energy. In S.cerevisiae aerobic metabolism, and thus the availability of ATP, can be induced by replacing glucose with galactose in the growth medium (Polakis & Partley, 1964).

Continuous cell lines in culture metabolize glucose anaerobically. The results of the present study shows that in cultures of HeLa cells in medium containing galactose the yield of vaccinia virus with different concentrations of arginine was identical to that of infected cultures in the presence of the same concentration of glucose. These results indicate that arginine does not serve as a source of ATP in vaccinia virus replication.

As far as RNA metabolism is concerned in vaccinia virus-infected cells, it is unlikely that arginine is essential for the synthesis of early mRNA species because a DNA-dependent RNA-polymerase has been associated with purified virus particles (Kates & McAuslan, 1967). However, the synthesis of late mRNA species requires the synthesis of a second DNA-dependent RNA-polymerase (Pitkanen et al, 1968). Since the accumulation of progeny genomes is a necessary criterion for the induction of this second polymerase, DNA synthesis and protein synthesis are prerequisite. Arginine may be involved directly in the synthesis of this enzyme or alternatively in a structural role affecting its activity and stability. Again, it is unlikely that arginine is involved in the synthesis of nucleotides or in the provision of ATP for reasons previously mentioned.

Polyamine derivatives of arginine, however, may have a significant role in later events concerned with the elaboration of virus-specific late mRNA. Obert (personal communication) has demonstrated that the requirement for arginine for late mRNA synthesis in KB cells infected

with vaccinia virus can be overcome by the addition of spermine or spermidine. In bacterial cells polyamines are necessary as stabilizers of ribosomal RNA (Cohen & Lichtenstein, 1960). Becker and Joklik (1964) have demonstrated the appearance of new populations of polyribosomes in vaccinia virus-infected HeLa cells. Thus, polyamines may be involved in the stability of polyribosomes during the transcription of virus-specific mRNA.

It was not possible to demonstrate any sparing effect on the utilization of arginine in vaccinia virus-infected HeLa cells by the addition of polyamines to culture medium at the time of infection. Thus, if there is a requirement for these compounds this must represent only a small fraction of the total arginine available to virus-infected cells. In cells infected with herpes simplex virus (^{14}C)-labelled ornithine has been detected in polyamines in mature virus particles when added to cultures prior to infection but not if it is added at the time of infection (Gibson & Roizman, 1971), indicating in this instance that polyamines are synthesized from endogenous products of host-cell metabolism.

In mammalian cells polyamines are synthesized from ornithine by a pathway involving ornithine decarboxylase, a pyridoxal-dependent enzyme (Russell & Snyder, 1968). The activity of cellular arginase was unaffected following infection of HeLa cells with vaccinia virus. Thus, ornithine is available endogenously. In vaccinia virus-infected HeLa cells, virus replication was inhibited by canaline, a structural

analogue of ornithine Kekomakis et al (1969) have shown that canaline is a potent inhibitor of all pyridoxal-dependent enzymes, including all those mediating decarboxylation and transamination reactions. However, these enzymes will be non-essential in cell cultures because the products are either supplied in the growth medium or are not associated with this type of cell. It may be concluded that canaline inhibits vaccinia virus replication either by direct competition with ornithine as a substrate for specific enzymes or by inhibiting ornithine decarboxylase activity by association with pyridoxal phosphate. It has been shown that there is an increase in ornithine decarboxylase activity in HeLa cells following infection with vaccinia virus (J.D. Williamson, personal communication). Canaline inhibited the growth of vaccinia virus at a molar ratio canaline : arginine of 1 : 50, suggesting that if inhibition is the result of equimolar competitive inhibition of the formation of a metabolic product of arginine, this represents only 2% of the total arginine available to virus-infected cells.

It may be concluded that in HeLa cells infected with vaccinia virus it is unlikely that arginine is involved directly either in the synthesis of nucleotides for DNA or RNA synthesis, or a source of energy. Previous data indicate that arginine is involved in events leading to the elaboration of early enzymes concerned with virus-specified DNA synthesis (Jungwirth & Joklik, 1965; Archard, 1971) and possibly in the synthesis of functional protein(s) necessary as a stabilizer for

newly synthesized viral DNA (Kates & McAdam, 1967). With respect to later events following DNA synthesis it is likely that arginine is involved in the synthesis of enzymic protein necessary for the transcription of late mRNA species. Additionally, in KB cells infected with vaccinia virus it has been shown that polyamine derivatives of arginine are required for events leading to the production of late mRNA (G. Obert, personal communication). In both T-even bacteriophage (Hershey, 1960) and herpes simplex virus (Gibson & Roizman, 1971) polyamines have been detected as structural components of mature virions. Furthermore, the incorporation of different (^{14}C)-labelled arginine preparations into mature vaccinia virus particles have suggested that products of arginine metabolism may be involved as structural components of poxviruses (Archard, 1971).

Archard (1971) has shown that the major requirement for arginine in cells infected with vaccinia virus is for the synthesis of virus-specified structural proteins. Additionally, since there is evidence that there is some metabolism of arginine prior to the incorporation into protein (Archard, 1971) it is possible that arginine serves as a source of other aminoacids. Both proline and asparagine could be synthesized from arginine and proline has been shown to be present in structural proteins of poxvirus particles (Turner & Kaplan, 1968). However, there was no sparing effect on the requirement for arginine in HeLa cells infected with vaccinia virus by the addition of either of these aminoacids. These results indicate that arginine does not

make any significant contribution to the synthesis of these amino-acids. However, since proline and asparagine are non-essential aminoacids (Eagle, 1959) the results indicate that they are synthesized by alternative pathways.

The essential role of arginine for protein synthesis in vaccinia virus replication was demonstrated by the inhibition of virus replication by canavanine or octopine, both structural analogues of arginine. Canavanine inhibits protein synthesis (Hare, 1969) by binding to arginyl-tRNA for incorporation into protein (Neurath et al, 1970). The nature of inhibition by canavanine was demonstrated by the decrease in the amount of (^{14}C)-labelled arginine incorporated into virus-infected cells treated with a growth inhibitory concentration of this compound. Although incorporation of (^{14}C)-labelled arginine was reduced by only 17%, there was no production of infectious virus. These results suggest that either canavanine specifically inhibits the synthesis of certain proteins or is incorporated into all proteins containing arginine rendering these proteins non-functional.

Canavanine also inhibits the replication of Semliki Forest virus if added to cultures within the first 2 hr p.i., due to inhibition of virus-specified RNA synthesis (Ranki & Kaariainen, 1970). There is no inhibition of virus production if canavanine is added at 3 hr p.i. or later. Similarly, in adenovirus-infected cells DNA synthesis and the synthesis of capsid components and maturation is inhibited if canavanine is added to cultures immediately after infection. However, if

canavanine is added at a later time, virus maturation only is impaired (Neurath et al, 1970) possibly by the inhibition of synthesis of an arginine-rich internal component (Prage et al, 1968). These results indicate that inhibition of virus replication by canavanine arises from impairment of both enzymic and structural proteins.

In summary, the results of supplementation and structural analogue experiments indicate that the prime role of arginine in poxvirus replication is in the formation of virus-specific protein(s). Some of these proteins constitute structural components of the virus particle (Turner & Kaplan, 1968; Archard & Williamson, 1971) but other arginine-containing proteins may be involved in other events in the virus replication cycle. A further quantitatively minor role is served by products of arginine metabolism. Proline constitutes 5% of the amino-acid composition of vaccinia virus particles (Turner & Kaplan, 1968) but no evidence was obtained from supplementation experiments that proline is derived by metabolism of arginine. However, evidence was obtained to indicate that arginine serves as a source of polyamines. It is not possible to ascribe a functional role to polyamines in the system described but comparison with other systems suggests involvement in the formation of late mRNA and as a structural component of the virus particle.

Since the essential role of arginine in vaccinia virus replication is for incorporation into protein and for the formation of essential

metabolic derivations, total substitution of arginine in the medium supplied to infected cells could be effective only if the substituent satisfied all these requirements. Tytell and Neuman (1960) investigated the growth of 31 stable cell lines in medium containing equimolar concentrations of ornithine, citrulline or arginine. In each instance, there was no cell growth when ornithine was supplied in place of arginine but many of the cell lines tested grew in the presence of citrulline. Furthermore, citrulline biosynthesis could not be induced by progressive removal of citrulline from culture medium, indicating that this is not a simple induction-repression mechanism but that there is some defect in the enzyme ornithine transcarbamoylase (Eagle, 1959).

Neither cell growth nor vaccinia virus replication occurred with HeLa cells in medium containing ornithine: both cell growth and virus replication occurred in medium containing citrulline. Additionally, there was a quantitative relationship between the concentration of citrulline and the yield of infectious virus from these cells, maximum yield being obtained at a concentration of 0.1mM citrulline. These results indicate that virus infection does not facilitate citrulline biosynthesis and suggest the ability to utilize ornithine or citrulline for virus replication reflects the enzyme complement of the host cell. HeLa cells lack ornithine transcarbamoylase activity but possess argininosuccinate synthetase-lyase activity.

It is pertinent at this point to consider other pathways of

arginine metabolism operable in uninfected cells which appear to remain functional following infection with vaccinia virus. Although mammalian cells can only incorporate L-isomers of aminoacids (Eagle, 1959), the growth of HeLa cells in medium containing 0.6mM D-arginine was similar to that of cultures maintained with the same concentration of the L-isomer. These results suggest that HeLa cells can convert D-arginine to the L-isomer. This conversion could be mediated either by a D-aminoacid oxidase followed by transamination or by the action of cellular racemase. In HeLa cells infected with vaccinia virus and maintained in medium containing a suboptimal concentration of 0.05mM L-arginine the yield of virus was enhanced by the addition of D-arginine. Maximal yield was reached by the addition of 0.5mM D-arginine, indicating that this form of arginine is utilized less efficiently than L-arginine. Additionally, there was a dose response relationship between the concentration of D-arginine supplied in the growth medium and the yield of virus. Again, maximum yield of virus required a concentration of 0.5mM D-arginine. These results infer that the pathway which converts D-arginine to L-arginine in uninfected HeLa cells is operable following virus infection. Since D-arginine is utilized less efficiently than L-arginine in vaccinia virus-infected cells this may reflect the formation of a racemic mixture by a D-aminoacid oxidase-transamination mechanism. Alternatively, arginine racemase activity may be weakly expressed in continuous cell cultures. Eagle (1959) has shown that D-cyst(e)ine can replace the L-isomer in cell culture by mobilizing L-cyst(e)ine residues bound to serum protein.

However, since serum was omitted from experimental media it is unlikely that D-arginine acts in a similar way in the system described.

The growth of vaccinia virus in cells capable of utilizing citrulline for cell growth could be mediated either by pre-existing host-cell enzymes or by virus-directed enzymes. However, in two cell lines, which have metabolic defects in the Krebs-Henseleit urea cycle such that they have an absolute requirement for arginine for cell growth, poxvirus replication was permitted in media containing citrulline. In mouse S180 cells there was a quantitative relationship between the concentration of citrulline supplied in the medium and the yield of rabbitpox virus. A similar relationship was found between the concentration of citrulline and the replication of vaccinia virus in human citrullinaemia cells. In each instance, maximal yields of virus were obtained at a concentration of 0.5mM citrulline.

Tedesco and Mellman (1967) have shown that in human citrullinaemia cells the enzyme argininosuccinate synthetase has markedly reduced substrate specificity. This enzyme has zero velocity at concentrations of citrulline up to 1.0mM. The site of the metabolic lesion in mouse S180 cells has not been thoroughly investigated. However, since these cells did not grow in medium containing citrulline (Tytell & Neuman, 1960) or argininosuccinic acid, it may be concluded that there is at least some defect in the enzyme argininosuccinate lyase.

Despite the inability of uninfected mouse S180 cells to synthesize arginine from citrulline the pattern of the rates of incorporation of (^{14}C)-carbamoyl-labelled citrulline into virus-infected cells was parallel to the incorporation of (^{14}C)-guanido-labelled arginine into infected cells. Additionally, these rates of incorporation into rabbitpox virus-infected mouse S180 cells at different times in the virus replication cycle were almost identical to the patterns of incorporation of both citrulline and arginine (Archard & Williamson, 1971) into vaccinia virus-infected HeLa cells. These results may infer either that in poxvirus-infected cells citrulline is utilized in place of arginine or that citrulline is converted to arginine prior to its incorporation into virus-specified products. In HeLa cells infected with vaccinia virus the mechanism by which citrulline is utilized in virus-infected cells must involve its conversion to arginine because the radioactive label from (^{14}C)-carbamoyl-citrulline was detected as arginine in purified virus particles. Furthermore, both canavanine and β -methyl aspartic acid inhibited virus replication in HeLa cell cultures maintained with citrulline. Since these analogues inhibit the enzymes argininosuccinate synthetase and lyase (Walker, 1953; Bayer & McMurray, 1969) these results infer that argininosuccinic acid is the intermediate for arginine biosynthesis in poxvirus-infected cells. Thus, it may be concluded that citrulline is not incorporated per se in events for which arginine is required in poxvirus replication but that citrulline is converted to arginine in virus-infected cells.

Further information concerning the mechanism for arginine biosynthesis in poxvirus-infected cells may be inferred from the data that in HeLa cells the mRNA species coding for the enzymes argininosuccinate synthetase and lyase have long half lives, in excess of 24 hr. However, when vaccinia virus-infected HeLa cells were treated with actinomycin D at the time of infection the initial peak in the incorporation of (^{14}C)-carbamoyl-labelled citrulline was reduced and the peaks at 3 hr and 5 hr p.i. were abolished. These results suggest that there is a requirement for the synthesis of new mRNA species in poxvirus-infected cells before citrulline can be successfully metabolized to arginine.

Schimke (1964) has shown that the activity of the enzymes argininosuccinate synthetase and lyase is dependent on the extracellular concentration of arginine or of citrulline. When arginine is supplied in the culture medium these enzymes are repressed, whereas in the presence of citrulline they become induced. Argininosuccinate synthetase-lyase activity in both HeLa and mouse L cells maintained in media containing either citrulline or arginine increased markedly following infection with vaccinia virus. However, in virus-infected cell cultures maintained with arginine the activity of these enzymes was greater than that from uninfected cell cultures maintained with citrulline. This enhanced activity cannot be explained simply on the basis of an induction-repression mechanism of pre-existing host-cell enzymes, but suggests that poxvirus-infected cells have an enhanced

capacity for arginine biosynthesis.

It is pertinent at this point to consider other enzyme activities which have been shown to increase following virus infection and to consider the criteria which have been employed to determine the mechanisms underlying these changes in enzyme activity. During the last decade biochemical techniques have facilitated numerous studies concerned with the replication of viral nucleic acid. The activities of several enzymes associated with DNA metabolism, DNA polymerase, thymidine kinase and several DNAases, are increased in the cytoplasm of vaccinia virus-infected cells shortly after infection (Jungwirth & Joklik, 1963). In each instance, transcription of new mRNA species and protein synthesis are required for their formation. McAuslan (1963) has demonstrated that the later repression of thymidine kinase requires three events, viz. the synthesis of progeny viral DNA, the synthesis of mRNA and of a repressor protein. The repression of DNA polymerase, thymidine kinase and DNAase formation is prevented when progeny viral DNA synthesis is inhibited with FUDR and enzyme activity continues for at least a further 5 hr. The mRNA species coding for these early enzymes appear to be very stable in contrast to the marked lability of the bulk of viral mRNA (Shatkin, 1963). These observations suggest that progeny viral cistrons code for the synthesis of proteins which block the function of the mRNA for early enzymes. No explanation has been found to indicate why parental viral cistrons do not "switch off" early enzyme activity, but similar

mechanisms are operable in bacteriophage infected-cells (Dirksen, Wiberg, Koerner & Buchanan, 1960).

In the present study, the increased argininosuccinate synthetase-lyase activity in HeLa cells infected with vaccinia virus required both mRNA and protein synthesis. There was no increase in enzyme activity following infection in the presence of actinomycin D. However, the half life of mRNA coding for these enzymes in uninfected cells was in excess of 24 hr and their expression continued for at least a similar period of time. These results indicate again that argininosuccinate synthetase-lyase activity requires the synthesis of new species of mRNA. In addition, increased enzyme activity was not observed in infected cells treated with purumycin. On the contrary, enzyme activity of both uninfected and infected cells in which protein synthesis had been arrested was lower than that of control cells. The remaining enzyme activity of uninfected cells, however, was significantly greater than that of virus-infected cells. This indicates a more rapid turnover of host-cell argininosuccinate synthetase-lyase enzymes following virus infection. These results show that the host-cell enzymes are catabolised following virus infection and that both transcription and translation are required for the formation of enzymes present in virus-infected cells.

There are five possible mechanisms which may be proposed to explain increased enzyme activity following virus infection. These are :-

- (1) Increased expression of a pre-existing host-coded function.
- (2) Derepression of a host-coded function.
- (3) Derepression of a host-coded isoenzyme.
- (4) Virus-modification of a host-coded function.
- (5) Direct expression of a virus-coded function.

The increased argininosuccinate synthetase-lyase activity in vaccinia virus-infected cells requires both transcription and translation. All the mechanisms for virus-induced enzyme activity are similarly dependent. The appearance of argininosuccinate synthetase-lyase activity in human citrullinaemia and mouse S180 cells following infection with poxviruses may occur by mechanisms 1 to 4 if there are host-coded functions for these enzymic activities in uninfected cells. However, it is unlikely that utilization of citrulline in virus-infected cells is associated with an increased expression of pre-existing host-cell enzymes because these enzymes are defective in both mouse S180 and human citrullinaemia cells. Similarly, it is unlikely that mechanisms for 2 or 3 are operable because they would offer no selective advantage to these cells. The Michaelis constant for argininosuccinate synthetase in human citrullinaemia cells is $10 - 100 \times 10^{-3}$ (Tedesco & Mellman, 1967), a value 10-fold higher than argininosuccinate synthetase-lyase activity from vaccinia virus-infected cells. In view of these observations, it seems probable that the enzyme activity in vaccinia virus-infected cells is due either to a virus-modification of host-coded functions or a direct

expression of virus-coded functions.

It would be expected that if increased argininosuccinate synthetase-lyase activity is a result of an increased expression of pre-existing host-coded functions, that the enzymes from virus-infected cells would retain the properties of the uninfected, host-cell enzymes. The Michaelis constant values from HeLa and mouse L cells were $0.1 \times 10^{-3} \text{ M}$ and $0.07 \times 10^{-3} \text{ M}$ respectively, reflecting differences in properties of enzymes from different species; the values from vaccinia virus-infected cells of both species were identical and had a value of $1.0 \times 10^{-3} \text{ M}$. These results reflected the expression of different genetic functions. This is further evidence that mechanisms 1, 2 or 3 are not operable in virus-infected cells and indicates that this activity is dependent on alternatives 4 or 5. In HeLa cells infected with adenovirus there is an increased expression of aspartate transcarbamoylase activity during the time of virus-specified DNA synthesis (Consigli & Ginsberg, 1964a). This increase in activity is controlled by a feed-back mechanism and can be inhibited by the addition of pyrimidines to the culture medium. The enzyme from virus-infected cells has different physico-chemical properties to that from the uninfected cells. However, if the virus-induced enzyme is heated its properties are similar to that from uninfected cells (Consigli & Ginsberg, 1964b), suggesting that a virus-modification of a pre-existing, host-coded function is responsible. It may be suggested that the stimulation of DNA synthesis at

the time of viral DNA replication depletes the cell pool of pyrimidines and thus removes feed-back control mechanisms. Once virus-specified DNA synthesis has finished the accumulation of pyrimidines in the cell pool will effectively inhibit further enzyme activity.

On the basis of the Michaelis constant values in HeLa and mouse L cells infected with vaccinia virus and the data from mouse S180 and human citrullinaemia cells, it is not possible to determine whether virus-induced argininosuccinate synthetase-lyase activity is a virus-induced modification of host-cell enzymes or specific virus-coded functions. Furthermore, this would be difficult to resolve because if this activity is dependent on pre-existing host-coded functions, the active complex will consist of at least two components. Enzymatically active proteins from the host-cell may combine with activating protein(s) specified by the invading viral genome. The physico-chemical properties of the complex may be different to the enzymes from uninfected cells. Additionally, the complex would contain immunologically identifiable virus-specified components. However, inhibition of enzyme activity by the association of antiviral antibody with the virus-specified portion of the complex may alter the tertiary structure of the complex and result in the loss of enzymic activity specified by the host moiety.

It may be concluded that the increase in argininosuccinate synthetase-lyase activity in cells infected with poxviruses is not

due to enhanced expression or derepression of a host-coded function and that the expression of the viral genome is essential for this activity in virus-infected cells.

It is possible that the increased argininosuccinate synthetase-lyase activity associated with poxvirus replication is a reflection of the state of evolution of these viruses at this time. It has been shown by nearest neighbour base sequence analyses that vaccinia virus DNA has properties more closely allied to bacterial DNA than to mammalian DNA (Subak-Sharpe, Bürk, Crawford, Morrison, Hay & Keir, 1966), whereas other viruses, particularly oncogenic species, have DNA similar to that from the host organism. This may infer that the poxviruses originated from a free-living species which has become parasitic during the course of time. Alternatively, continued expression of argininosuccinate synthetase-lyase activity may have some selective advantage and may be a reflection of evolutionary adaption.

It has been shown that citrulline is excreted through the skin of all adults at a concentration of 0.5mM (Coltman, Rowe & Atwell, 1966) the concentration at which poxvirus growth was maximal in both human citrullinaemia and mouse S180 cells which are unable to utilize this amino-acid for cell growth. Additionally, arginine is excreted through the skin in lesser amounts and is not always present (Coltman et al, 1966). Since poxviruses of animals are dependent on the Dissemination of virus through skin lesions (Fenner, 1947) the

ability of virus-infected cells to utilize citrulline may be a selective advantage to assist the spread of these diseases.

REFERENCES

ABRAHAM, K.A. (1968).

Studies on the DNA-dependent RNA polymerase from Escherichia coli.

I. The mechanism of polyamine induced stimulation of enzyme activity.

European Journal of Biochemistry 5 143-146

APPLEYARD, G., HUME, V.B.M., WESTWOOD, J.C.N. (1965).

The effect of thiosemicarbazones on the growth of rabbitpoxvirus in tissue culture.

Annals New York Academy of Science 130 92-104

ARCHARD, L.C. (1971).

Ph.D. Thesis. The role of arginine in the replication of vaccinia virus.

ARCHARD, L.C., WILLIAMSON, J.D. (1971).

The effect of arginine deprivation on the replication of vaccinia virus.

Journal of General Virology 12 249-258

BACH, S.J. , HAWKINS,R.A., SWAINE,D.(1963)

A short method for the purification of arginase from ox liver.

Biochemical Journal 89 263

BALANDIN,I.G., KOZLOVA,I.A., LUSHINOV,A.A., PETERSON,O.A.,

CHERBUKINA,N.B.(1971)

Studies on RNA polymerase inhibitor in virus infected cells.III

Induction of cellular nucleic acid synthesis in vaccinia virus infected cells.

Acta Virology 15 1-6

BAYER,S.M., McMURRAY,W.C.(1969)

Inhibition of urea cycle enzymes by aspartic acid analogues.

Canadian Journal of Biochemistry 47 361-369

BEATY,E.,HODES,M.E.(1967)

Induction of arginase in E.coli spheroplasts by the Shope Papilloma virus.

Journal of Cell Biology 35 11

BECKER,Y.,JOKLIK,W.K.(1964)

Messenger RNA in cells infected with vaccinia virus

Proceedings of the National Academy of Science 51 577-585

BECKER,Y.,OLSHEVSKY,U, LEVITT,J.(1967)

The role of arginine in the replication of Herpes simplex virus.

Journal of General Virology 1 467-474

BECKER, Y. WEINBERG, G. (1970)

Effect of arginine deprivation on macromolecular processes in
Burkitt's lymphoblasts.

Experimental Cell Research 60 470-474

BECKER, Y., WEINBERG, G. (1971)

Burkitt's lymphoblasts and their Epstein-Barr virus.

Israel Journal of Medical Sciences 7 561-567

BEER, S. V., KOSUGE, T. (1970)

Spermidine and spermine components of turnip yellow mosaic virus.

Virology 40 930-938

BIRCH, J. R. PIRT, S. J. (1969)

The choline and serum protein requirements of mouse fibroblasts
(strain LS) in culture.

Journal of Cell Science 5 135-142

BONIFAS, V. M. SCHLESINGER, R. N. (1959)

Nutrient requirements for plaque production by Adenovirus.

Federation Proceedings 18 560.

BONIFAS, V.M. (1967)

Time-course and specificity of the arginine requirement for Adenovirus.

Archiv fur die gesamte Virusforschung 20 20-28

BRAY, G.A. (1960)

A simple efficient liquid scintillator for counting aqueous solutions in a liquid scintillation counter.

Analytical Biochemistry 1 279.

CAIRNS, J. (1960)

The initiation of vaccinia virus infection.

Virology 11 603-623

CARRULLI, N., KAIHARA, S., WAGNER, H.N. Jnr. (1968)

Radiosiotopic assay for arginase activity

Analytical Biochemistry 24 515-522

CHANG, R.S., LIEPINS, H., MARGOLISH, M. (1961)

CO₂ requirement and nucleic acid metabolism of HeLa and human conjunctiva cells.

Proceedings of the Society for Experimental Biology and Medicine

106 149-152

CHARLES, H.P., BROADBENT, J.A. (1964)

Carbon Dioxide mutants of Neurospora

Nature. 201 1004-1006

CHARLES, H.P., ROBERTS, G.A. (1968)

CO₂ as a growth factor for mutants of E.coli.

Journal of General Microbiology. 51 211-224

COHEN, S.S., FLAKS, J.G., BARNER, H.D., LOEB, M.R., LICHTENSTEIN, J. (1958)

The mode of action of 5'-fluoro-uracil and its derivatives.

Proceedings of the National Academy of Science (US) 44 1004-1012

COHEN, S.S., LICHTENSTEIN, J. (1960)

Polyamines and ribosome structure.

Journal of Biological Chemistry 235 2112-2116

COHEN, S.S. (1961)

Virus induced acquisition of metabolic function.

Federation Proceedings 20 641-649

COLTMAN, C.A., ROWE, N.J., ATWELL, R.J. (1966)

The amino-acid composition of sweat in normal adults.

American Journal of Clinical Nutrition 18 373-378

CONSIGLI, R.A., GINSBERG, H.S. (1964a)

Control of aspartate transcarbamoylase activity in type-5 adenovirus infected HeLa cells.

Journal of Bacteriology 87 1027-1033

CONSIGLI, R.A., GINSBERG, H.S. (1964b)

Activity of aspartate transcarbamoylase in uninfected and type-5 adenovirus infected HeLa cells.

Journal of Bacteriology 87 1034-1043

CPURTNEY, R.J., McCOOMBS, R.M., BENYESH-MELNICK, M. (1970a)

Antigens specified by Herpes virus. I Effect of arginine deprivation on antigen synthesis.

Virology 41 379-386

COURTNEY, R.J., McCOOMBS, R.M., BENYESH-MELNICK, M. (1970b)

Antigens specified by Herpes virus. II Effect of arginine deprivation on the synthesis of cytoplasmic and nuclear proteins.

Virology 43 356-365

DAGLEY, S. NICHOLSON, D.E. (1970)

An introduction to Metabolic Pathways.

Blackwell Scientific Publications.

DIRKSEN, M.L., WIBERG, J.S., KOERNER, J.F., BUCHANAN, J.M. (1960)

Proceedings of the National Academy of Science (US) 46 1425-1430

EAGLE, H. (1959)

Amino-acid metabolism in mammalian cell cultures

Science 130 432-437

EAGLE, H. (1965)

Metabolic controls in cultured mammalian cells.

Science 148 42-51

EAGLE, H., BARBAN, S., LEVY, M., SCHULZE, H.O. (1959)

The utilization of carbohydrates by human cell cultures.

Journal of Biological Chemistry 233 551-558

ERON, L.J., MCAUSLAN, B.R. (1966)

The nature of Poxvirus-induced Deoxyribonucleases.

Biochemistry Biophysics Acta. Research Communications 22 518-523

FENNER, F. (1947)

Australian Journal of Biological Medical Science. 25 257 & 327.

FLAKS, J.G. COHEN, S.S. (1957)

The enzymic synthesis of 5'-hydroxymethyldeoxycytidylic acid.

Biochemistry Biophysics Acta 25 667-668

FOLEY, G.E., DROLET, B.P., MCCARTHY, R.E., GOULET, K.A., DOKOS, J.A.,

FILLER, D.A. (1960)

Isolation and serial propagation of malignant and normal cells in semi-defined media.

Cancer Research 20 930.

FOX, C.F., WEISS, S.B. (1964)

Enzymatic synthesis of RNA. ii) Properties of the Deoxynucleic acid-primed reaction with Micrococcus lysodeikticus RNA polymerase.

Journal of BIOlogical Chemistry 239 175-185

GEYER, R.P., CHANG, R.S. (1958)

Bicarboate as an essential for human cells in vitro

Archives Biochemistry and Biophysics 73 500-506

GEYER, R.P., NEIMARK, J.M. (1958)

Response of CO₂-deficient human cells in vitro to normal cell extracts.

Proceedings of the Society for Experimental Biology and Medicine

99 599-601

GEY, G.U., COFFMAN, W.D., KUBICEK, M.T. (1962)

Tissue culture studies of the proliferative capacity of cervical carcinoma and normal epithelium.

Cancer Research 12 264.

GIBSON, W., ROIZMAN, B. (1971)

Compartmentalization of spermine and spermidine in the Herpes simplex virion.

Proceedings of the National Academy of Science (US) 68 2818-2821

GOLDBLUM, N., RAVID, Z., BECKER, Y. (1968)

Effect of withdrawal of arginine and other amino-acids on the synthesis of tumour and viral antigens of SV40 virus.

Journal of General Virology 3 143-146

GOLD, P., DALES, S. (1968)

Localization of the nucleotide phosphohydrolase activity in vaccinia virus.

Proceedings of the National Academy of Science (US) 60 845-852

GONCZOL, E., JENEY, E., VACZI, L. (1967)

Replication of Herpes simplex virus in arginine-free media. II) DNA synthesis in infected and non-infected human embryonic fibroblasts and HeLa tissue culture cells.

Acta microbiologica Academiae Scientiarum Hungaricae 14 39-43

GREEN, M., PINA, M. (1962)

Stimulation of the DNA synthesising enzymes of cultured human cells by vaccinia virus infection.

Virology 17 603-604

GRIFFITHS, J. B., PIRT, S. J. (1967)

The uptake of amino-acids by mouse cells (strain LS) during growth in batch culture and chemostat culture ; the influence of cell growth rate.

Proceedings of the Royal Society (B) 168 421.

GWATKIN, R.B.L., SIMINOVITCH, L. (1960)

Multiplication of single mammalian cells in a non-bicarbonate medium.

Proceedings of the Society for Experimental Biology and Medicine

103 718-721

HANKS, J.H., WALLACE, R.E., (1949)

Relation of oxygen and temperature in the preservation of tissues by refrigeration.

Proceedings of the Society for Experimental Biology and Medicine (N.Y)

71 196-200

HARE, J.D. (1969)

Reversible inhibition of DNA synthesis by the arginine analogue canavanine in Hamster and Mouse cells in vitro

Experimental Cell Research 58 170-174

HARGREAVES, F.D., LEACH, R.H. (1970)

The influence of mycoplasma infection on the sensitivity of HeLa cells for the growth of viruses.

Journal of Medical Microbiology 3 259-265

HENLE, W., HENLE, H. (1968)

Effect of arginine deficient media on the Herpes type virus

associated with cultured Burkitt's Tumour cells.

Journal of Virology 2 182-191

HERSHEY, A.D. (1957)

Some minor components of T₂ bacteriophage.

Virology 4 237-264

HIRSCHFIELD, I.N., ROSENFELD, H.J., LEIFER, L., MAAS, W.K. (1970)

Isolation and characterisation of a mutant of E.coli blocked in the synthesis of putrescine.

Journal of Bacteriology 101 725-730

HOLLOWZAK, J.A., JOKLIK, W. (1967)

Studies on the proteins of vaccinia virus. I Structural proteins of the virions and the cores.

Virology 33 717-725

HOLTERMAN, O.A. (1969)

Amino-acid requirement for the proagation of vaccinia virus in Earle's 'L' cells.

Journal of General Virology 4 585-591

HUTSON, J.Y., DOWNING, M. (1968)

Pyrimidine biosynthesis in Lactobacillus leichmanii

Journal of Bacteriology 96 80-93

INGLIS,V.(1968)

Requirements of arginine for the replication of Herpes simplex virus.

Journal of General Virology 3 9-17

INOUE,M.,PARDEE,A.B.(1970)

Requirements of polyamines for Bacterial Division.

Journal of Bacteriology 101 770-776

JENEY,E.,GONCZOL,E.,VACZI,L.(1967)

Replication of Herpes simplex virus in arginine-free media.I) Effect of arginine deficiency in different tissue culture cells.

Acta Microbiologica Academiae Scientiarum Hungaricae 14 31-37

JOKLIK,W.(1962)

Some properties of poxvirus DNA.

Journal of Molecular Biology 5 265-274

JOKLIK,W.(1963a)

The intracellular uncoating of poxvirus DNA.I) The fate of radioactively labelled Rabbitpox virus.

Journal of Molecular Biology 8 263-276

JOKLIK,W.(1963b)

The intracellular uncoating of poxvirus DNA.II) The molecular basis

of the uncoating process.

Journal of Molecular Biology 8 277-288

JOKLIK, W., BECKER, Y. (1964)

The replication and coating of vaccinia virus.

Journal of Molecular Biology 10 452-474

JOKLIK, W. (1966)

The Poxviruses.

Bacteriological Reviews 30 33-67

JUNGWIRTH, C., JOKLIK, W. (1965)

Studies on 'Early' enzymes in HeLa cells infected with vaccinia virus.

Virology 27 80-93

KATES, J. R., BEESON, J. (1970)

RNA synthesis in vaccinia virus I) Mechanism of synthesis and release of RNA in vaccinia cores. 50 19-33

II) Synthesis of polyriboadenylic acid.

50 1-18

KATES, J. R., MCAUSLAN, B. R. (1967)

Poxvirus DNA-dependent RNA-polymerase.

Proceedings of the National Academy of Science (US) 58 134-141

KEKOMAKI, K., JANNA, J., RAHIALA, E.L., RAINA, A., RAIHA, N. (1969)

Canaline: a potent inhibitor of pyridoxal enzymes.

Scandinavian Journal of Investigation 23 (Supplement 108) 30.

KENNY, G.E., POLLOCK, M.E. (1963)

Mammalian cell cultures contaminated with PPLO.

Journal of Infectious Disease 112 7-16

KIT, S. (1963)

Aquisition of DNA synthesising enzymes by animal cells infected with poxviruses.

Experimental Cell Research Supplement 9 270-275

KIT, S. DUBBS, D.R., PIEKARSKI, L.J. (1963a)

Inhibitory effect of puromycin and fluorophenylalanine on the induction of thymidine kinase by vaccinia virus-infected 'L' cells.

Biochemistry Biophysics Research Communications 11 176-181

KIT, S., PIEKARSKI, L.J., DUBBS, D.R. (1963b)

Induction of thymidine kinase by vaccinia virus-infected mouse fibroblasts.

Journal of Molecular Biology 6 22-27

KORNBERG, A., ZIMMERMAN, S.B., KORNBERG, A.J. (1961)

Glucosylation of Deoxyribonucleic acid by enzymes of bacteriophage.

Journal of Biological Chemistry 236 1487-1493

LAVER, W.G. (1970)

Isolation of arginine rich protein from particles of Adenovirus type II.

Virology 41 488-500

LOH, P.C. (1964)

The role of RNA in the synthesis of vaccinia virus.

Proceedings of the Society for Experimental Biology and Medicine

116 789-792

LWOFF, A., MONOD, J. (1947)

Attempt to analyse the role of CO₂ in the microbial environment

Annals de L'institut Pasteur 73 323-347

MacPHERSON, I.A., STOKER, M.G.P. (1962)

Polyoma transformation of Hamster cell clones: an investigation

of genetic factors affecting cell competence.

Virology 16 147-151

MAGEE, W. (1962)

DNA polymerase and Deoxyribonucleotide kinase activities in cells

infected with vaccinia virus.

Virology 17 604-607

MARK, G.E., KAPLAN, A.S. (1971)

Synthesis of proteins in cells infected with Herpes virus. VII) Lack of migration of structural viral proteins to the nucleus of arginine deprived cells.

Virology 45 53-60

McAUSLAN, B.R., KATES, J.R. (1967)

Poxvirus-induced Deoxyribonucleases: Regulation of synthesis, control of activity in vivo. Purification and properties of the enzymes.

Virology 33 709-716

McCOY, T., MAXWELL, M.D., KRUSEP, F. (1961)

Carbohydrate metabolism in the Jensen and JA sarcomas in vitro.

Cancer Research 21 97-100

MINAMISHIMA, Y., BENYESH-MELNICK, M. (1969)

Arginine dependent events in cytomegalovirus infection.

Bacteriological Proceedings 170.

MORGAN, J.F., MORTON, H.J., PARKER, R.C. (1950)

Nutrition of animal cells in tissue culture. I) Initial studies on a synthetic medium.

Proceedings of the Society for Experimental Biology and Medicine.

73 1-7

MORRIS, D.R., PARDEE, A.B. (1967)

Multiple pathways of putrescine biosynthesis in E.coli.

Journal of Biological Chemistry 241 3129-3135

MORTON, H.E., LEECE, J.G. (1953)

Selective action of thallium acetate and crystal violet for
pleuropneumonia like organisms of human origin.

Journal of Bacteriology 66 646-649

MORTON, H.E., SMITH, P.S., LEBERMAN, P.R. (1951)

Investigation of the cultivation of pleuropneumonia-like organisms
from man.

American Journal of Syphilis, Gonorrhea and Venereal Diseases.

MOSS, B.J. (1968)

Inhibition of HeLa cell protein synthesis by vaccinia virus.

Journal of General Virology 2 1028-1035

McMURRAY, W.C., MOHYUDDIN, F., BAYER, S.M., RATHBUN, J.C. (1964)

Proceedings of the International Congress on the Scientific study
of Mental Retardation. Denmark. p.117

MUNYON, W., PAOLETTI, E., GRACE, J.T. (1967)

RNA polymerase activity in purified infectious vaccinia virus.

Proceedings of the National Academy of Science (US) 58 2280-2287

NEURATH, A.R., WEINER, F.P., RUBIN, B.A., HARTZELL, R.W. (1970)

Inhibition of Adenovirus replication by canavanine.

Biochemistry Biophysics Acta 41 1509-1517

NUZUM, C.T., SNODGRASS, P.J. (1971)

Urea cycle adaptations to dietary protein in primates.

Science 172 1042-1043

OBERT, G., TRIPIER, F., GUIR, J. (1971)

Arginine requirement for late mRNA transcription of vaccinia virus
in KB cells.

Biochemistry biophysics Research Communications 44 362-367

OLSHEVSKY, U., BECKER, Y. (1970a)

Synthesis of Herpes simplex structural proteins.

Nature 226 851-853

OLSHEVSKY, U., BECKER, Y. (1970b)

Herpes simplex structural proteins.

Virology 40 948-956

PETERFORSKY, B., UNDERFRIEND, S. (1963)

Oxidation of proline on the ribosomes of E.coli.

Biochemistry Biophysics Research Communications 12 257.

PITKANEN, A., McAUSLAN, B.R., HEDGPETH, J., WOODSON, B. (1968)

Induction of poxvirus ribonucleic acid polymerase.

Journal of Virology 2 1363-1367

POGO, B.G.T., DALES, S. (1969)

Two Deoxyribonuclease activities within purified vaccinia virus.

Proceedings of the National Academy of Science (US) 68 820-827

POLAKIS, E.S., BARTLEY, W. (1965)

Changes in the enzyme activity of Saccheromyces cerevisiae during aerobic growth on different carbon sources.

Biochemical Journal 97 284-302

POLAKIS, E.S., BARTLEY, W. (1966)

Changes in the intracellular concentrations of adenosine phosphates and nicotinamide nucleotides during aerobic growth cycle of yeast on different carbon sources.

Biochemical Journal 99 521-533

PRAGE, L., PETTERSON, U., PHILIPSON, L. (1968)

Internal proteins of Adenovirus.

Virology 36 508-511

RAINA, A., COHEN, S.S. (1966)

Polyamine and RNA synthesis in a polyauxotrophic strain of E.coli.

Biochemical Journal 55 1587-1593

RANKI, M., KAARIAINEN, L. (1970)

The effect of canavanine on Semliki Forest virus RNA synthesis.

Annals Medicinae Experimentalis et Biologiae Fenniae 48 238-245

RATNER, S., ANSLOW, O., PETRACK, B. (1953)

VI) Enzymic cleavage of argininosuccinic acid to arginine and fumaric acid.

Journal of Biological Chemistry 204 115-125

REICH, E., FRANKLIN, R. M., SHATKIN, A. J., TATUM, E. L. (1961)

Effect of actinomycin D on cellular nucleic acid synthesis and virus production.

Science 134 556-557

ROGERS, S., MOORE, M. (1963)

Studies on the mechanism of action of Shope papilloma virus. I)

Concerning the nature of the induction of arginase in infected cells.

Journal of Experimental Medicine 117 521-542

ROGERS, P., KRZYZEK, R., KADEN, T. M., ARFMAN, E. (1971)

Effect of arginine and canavanine on arginine mRNA synthesis.

Biochemistry Biophysics Research Communications 44 1220-1227

ROON, R. J., BARKE, H. A. (1971)

II) Putrescine transcarbamoylase from Streptococcus faecalis.

Federation for the American Society of Experimental Biology

Part II.No.2

ROSE,W.C.,WIXON,R.L.,LOCKHART,H.B.,LAMBERT,G.F.(1955)

The amino-acid requirements of man XV) The valine requirement:
summary and findings.

Journal of Biological Chemistry 217 987-995

ROUSE,H.C.,BONIFAS,V.H.,SCHLESINGER,R.W.(1967)

Dependence of Adenovirus replication on arginine and inhibition of
plaque formation by PPLOs.

Virology 20 357-365

ROUSE,H.C.,SCHLESINGER,R.W.(1967)

An arginine dependent step in the maturation of type-2 Adenovirus.

Virology 33 513-522

ROWE,B.W.,MILLER,L.L.(1971)

Inhibition of urea biosynthesis and conversion of L-citrulline-
 ^{14}C to ^{14}C urea by α -methyl aspartic acid and β -methyl aspartic acid.

Proceedings for the Society for Experimental Biology and Medicine

138 357-360

RUSSELL,D.,SNYDER,S.H.(1968)

Amine synthesis in rapidly growing tissues; Ornithine decarboxylase

activity in regenerating rat liver, chick embryo and various tumours.

Biochemistry 60 1420-1426

RUSSELL, W. C., BECKER, Y. (1968)

A maturation factor for adenovirus.

Virology 35 18-27

RUSSELL, W. C., LAVER, W. G., SANDERSON, P. J. (1968)

Internal components of adenovirus.

Nature 219 1127-1130

RUSSELL, W. C., BRODATY, E., ARMSTRONG, J. A. (1971)

A cytochemical study of basic proteins in adenovirus infected-cells.

Journal of General Virology 11 87-93

RYSER, H. J.-P. (1968)

Uptake of protein by mammalian cells; an under-developed area.

Science 159 390-396

SAROV, I., BECKER, Y. (1967)

Studies on vaccinia virus DNA.

Virology 33 369-375

SALZMAN, N. P. (1960)

The formation of vaccinia virus DNA and vaccinia virus.

Virology 10 150-152

SALZMAN, N.P., SEBRINGE, D. (1967)

Sequential formation of vaccinia virus proteins and viral
Deoxyribonucleic acid replication.

Journal of Virology 1 16-23

SCHIMKE, R.T. (1963)

Methods in Enzymology 17a. 310-313. Arginase deiminase (Mycoplasma)

SCHIMKE, R.T., BARILE, M.F. (1963)

Arginine metabolism in pleuropneumonia-like organisms isolated
from mammalian cell cultures.

Journal of Bacteriology 86 195-206

SCHIMKE, R.T. (1964)

Enzymes of arginine metabolism in mammalian cell cultures. I)
Repression of argininosuccinate synthetase and argininosuccinate
lyase.

Journal of Biological Chemistry 239 136-145

SCHIMKE, R.T. (1967)

Studies on the metabolism of arginine by mycoplasma.

Annals New York Academy of Science 143 573-577

SHATKIN, A. (1963)

Actinomycin D and vaccinia virus infection of HeLa cells.

Nature 199 357-358

SHIMONO, H., KAPLAN, A. S. (1968)

Correlation between the synthesis of DNA and histones in polyoma infected mouse embryo cells.

Virology 37 690-694

SLETTON, K., AAKESSON, I., ALUSAKER, J. O. (1971).

Presence of ornithine in the urate binding α_1 - α_2 globulins.

Nature New Biology 231 118.

SINGER, S. H., FITZGERALD, E. A., BARILE, M. F., KIRSCHSTEIN, R. L. (1970)

Effect of mycoplasma on vaccinia virus growth: requirement for arginine.

Proceedings for the Society for Experimental Biology and Medicine
133 1439-1442

STANBRIDGE, E. (1971)

Mycoplasma and cell cultures.

Bacteriological Reviews 35 206-227

STEINART, P. M., HARDING, M. W. J., ROGERS, G. E. (1969)

The characteristics of protein bound citrulline.

Biochemistry Biophysics Acta 175 1-9

SWIM, H.E., PARKER, P.F. (1958)

Role of CO₂ as an essential for six permanent strains of fibroblasts.
Journal of Biophysics and Biochemical Cytology 4 525-528

TANKERSLEY, R.W. (1964)

Amino-acid requirements of Herpes simplex in human cells.
Journal of Bacteriology 87 609-613

TEDESCO, G.A., MELIMAN, W.J. (1967)

Argininosuccinate synthetase activity and citrulline metabolism
in cells cultured from a citrullinaemic subject.
Proceedings of the National Academy of Science 57 829-834

TURNER, G.S., KAPLAN, C. (1970)

Photoinactivation of vaccinia virus with Rose Bengal.
Journal of Virology 3 433-443

TYTELL, A.A., NEUMAN, R.E. (1960)

Growth response of stable and primary cell cultures to L-ornithine,
L-citrulline and L-arginine.
Experimental Cell Research 20 84-91

WALKER, J.B. (1953)

An enzyme reaction between canavanine and fumarate.

Journal of Biological Chemistry 204 139-146

WATSON, J.D. (1965)

Molecular Biology of the Gene . p.281.

W.A. Benjamin Inc. New York

WELLS D.G. (1967)

Ph.D. thesis. Studies on Variola virus.

WESTALL, R.G. (1960)

Argininosuccinicacidaemia; identification and reaction of the abnormal metabolite in a newly described form of mental disease with some preliminary metabolic studies.

Biochemical Journal 77 135-144

WILLIAMSON, J.D., COX, P. (1968)

Use of a new buffer in the culture of animal cells.

Journal of General Virology 2 309-312

WINTERS, A.L., CONSIGLI, R.A. (1969)

Arginine requirement of polyoma virus in primary mouse embryo cells.

Bacteriological Proceedings 182.

YASAMUR, J., KAWAKITA, A. (1963)

Vero cells.

Nippon Rinsho 21 1201.

YASHNE, J., GORINI, L. (1965)

Phosphorylation in vivo and in vitro.

Journal of Biological Chemistry 240 1681-1686