ON THE METABOLISM OF ARGININE IN CELLS INFECTED WITH POXVIRUS

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ABSTRACT

Arginine is essential for the replication of vaccinia virus and rabbitpox virus. The importance of arginine lies mainly in its incorporation into structural protein(s), but there is evidence that some arginine may be used for the synthesis of polyamines. Rabbitpox virus and vaccinia virus can utilize citrulline in place of arginine, owing to the virus-directed production in infected cells of the enzymes for the biosynthesis of arginine from citrulline.

A cell system, defective in its ability to synthesize arginine from citrulline, has been developed to enable the investigation of the metabolism of arginine following infection with poxviruses other than those previously studied. This cell system will support the replication of a wide range of poxviruses, but only in the presence of serum. The active component(s) of serum has been shown to have a molecular weight no greater than 700. Evidence is presented that cowpox virus requires arginine for replication and is unable to utilize citrulline instead of arginine. It is shown also that argininosuccinate, an intermediate in the biosynthesis of arginine from citrulline, can replace arginine for the replication of cowpox virus within the defective cell. It has not been determined whether this is a result of cell-directed or virus-directed biosynthesis of arginine from argininosuccinate.

The sensitivity has been improved of a radiochemical system for the assay of the combined activity of the enzymes for the biosynthesis of arginine from citrulline. A spectrophotometric system for the assay of the activity of one of these enzymes has been developed.

CHAPTER 1

INTRODUCTION

Arginine is known to be essential for the replication of poxviruses and many other DNA animal-viruses. The essential nature of arginine for the replication of vaccinia virus was demonstrated by Archard and Williamson (1971) who showed that the requirement for arginine occurs at two distinct stages of the replication cycle. There is an early requirement that is associated with the synthesis of virus DNA and also a late requirement that is associated with the maturation of virus particles and involves the incorporation of arginine into virus proteins. However, while the fate of most of the arginine used for virus replication is to be incorporated as arginine into virus proteins, some of it is metabolized beforehand.

The replication of all viruses is intimately associated with the metabolism of the host-cell and so the requirements for the replication of viruses would be expected to be closely related to the metabolic requirements of the host-cell. This is known to be so in the case of arginine, which is essential not only for the replication of poxviruses, but also for the growth of animal cells in culture. Ornithine and citrulline are both intermediates in the formation of arginine and in many animal cells citrulline, but not ornithine, is able to substitute for arginine. Two such cell species are HeLa and mouse L and it was found (Williamson and Cooke, 1973) that although these cells would not support the replication of vaccinia virus in the presence of ornithine in a medium containing no arginine, the substitution of citrulline for arginine would allow the replication of vaccinia virus in these cells. Furthermore, it was shown that infection with vaccinia virus increased the activities of the enzymes responsible for the biosynthesis of arginine from citrulline. Now, it is possible that the replication of vaccinia virus in such cells in the presence of citrulline is made possible by the action of host-cell enzymes on citrulline to produce arginine. However, there are some cell species, such as mouse sarcoma 180 (S180) and human citrullinaemia, that are unable to use citrulline in place of arginine and it was

shown (Cooke and Williamson, 1973) that rabbitpox virus and vaccinia virus will grow in such a cell system in the presence of citrulline in a medium containing no arginine. Furthermore, citrulline is converted to arginine before incorporation into the poxvirus particle. These studies indicate that rabbitpox virus and vaccinia virus are able to direct the synthesis of enzymes for arginine biosynthesis. This possibility was strongly supported by examination of the biophysical properties of the enzymes induced by infection with vaccinia of HeLa and mouse L cells.

The studies described show that the genome of two poxviruses, rabbitpox virus and vaccinia virus, almost certainly codes for the enzymes effecting the biosynthesis of arginine from citrulline. This thesis is concerned with investigating a wider range of poxviruses to determine the extent of this biosynthetic ability within the poxvirus group. Clearly, in such an investigation it is a great advantage to use cells that cannot synthesize arginine from citrulline. However, initial experiments have shown that the mouse S180 cells previously used are unable to support the replication of more than a small number of poxviruses. It is because of this that a large part of this work is concerned with the development of a cell system that is incapable of synthesizing arginine from citrulline and that can support the replication of a wide range of poxviruses. Further studies are concerned with the development of methods for measuring the activities of the enzymes of arginine biosynthesis.

1(i) Nutritional requirements of cells in culture

It is known (Eagle, 1955, 1959) that cells in culture have an absolute requirement for 13 amino-acids (Table 2.1). All the human or other animal cells examined, whether from normal or malignant tissue, required at least 13 amino-acids for survival and growth. These amino-acids were the same in each cell line, except that in all the human cells examined it was found that citrulline was able to substitute for arginine. However, ornithine was inactive and it was not possible to induce the biosynthesis of citrulline by progressive removal of citrulline from a medium containing ornithine. In contrast to the amino-acid requirement of cells in culture, whole-animal feeding experiments conducted by Rose et al (1955) showed that in man only 8 amino-acids were required to maintain nitrogen balance. Arginine is not one of these eight.

Eagle considered three possible reasons for this difference and tested each experimentally.

The extra requirement could be due to a loss of bioysnthetic mechanisms during prolonged growth in vivo. This was largely discounted by an experiment that showed that the requirement for amino-acids of embryonic material during the first 24 to 48 hours of growth in culture was the same as for other cells established in vitro.

The second possibility was that there was a lack of appropriate precursors or co-factors in the growth medium, but no simple precursors could be found that could be used by cultured cells in lieu of the preformed amino-acids.

Finally, it could be the case that cells have a limited capacity for biosynthesis that is sufficient for survival, but not for growth. Consequently, such cells show a capacity for biosynthesis sufficient for the maintenance of nitrogen-balance in whole-animal feeding experiments, but not adequate for the rapid growth that is characteristic of cell cultures. Such limited synthesis has been demonstrated under certain conditions for glutamine, cyst(e)ine and tyrosine and so this became the favoured theory.

From these experiments a chemically defined medium (MEM) was developed that was suitable for the growth of animal tissues as cell cultures (Eagle, 1959) (Table 2.1.). In addition to the amino-acids, this medium contained 8 vitamins, 6 inorganic salts and glucose. Furthermore, it was found that serum-protein also was required for the growth of mammalian cells in culture. In monolayer cultures, one of the roles of proteins is for the adhesion of cells to the substratum. However, serum is required in cell suspension-cultures and must, therefore, have other functions. Possibly it acts as a carrier for growth factors that are bound to the protein and released slowly into the medium. Later work by Birch and Pirt (1969. See also Materials and Methods) revealed that one of the major requirements for serum for cell growth was for the provision of choline.

As well as the compounds already mentioned, carbon dioxide has been found to be essential for the growth of mammalian fibroblasts (Swim and Parker, 1950). Carbon dioxide is not required merely for maintaining the pH of the medium, although this is an important function, it is also involved in the synthesis of many important metabolites, eg. carbamyl phosphate (Fig 1.1).

It has already been mentioned that in certain cell lines citrulline could substitute for arginine, while ornithine was inactive. Tytell and Neuman (1960) studied the requirement for arginine in a large number of transformed and primary cell lines. They discovered that none of those studied could utilize ornithine in place of arginine and that there was a wide range of ability to utilize citrulline. HeLa cells could use either arginine or citrulline with almost equal facility, as could nearly all the other stable cell lines and the majority of the primary cell cultures. Mouse S180 cells and cells from the chorio-allantoic membrane of the chick embryo are two of the cell lines that were found not to be able to utilize citrulline.

Piez and Eagle (1958) carried out experiments on cultured human cells and found that all those cells examined contained a significant pool of amino-acids, the composition of which did not differ significantly among

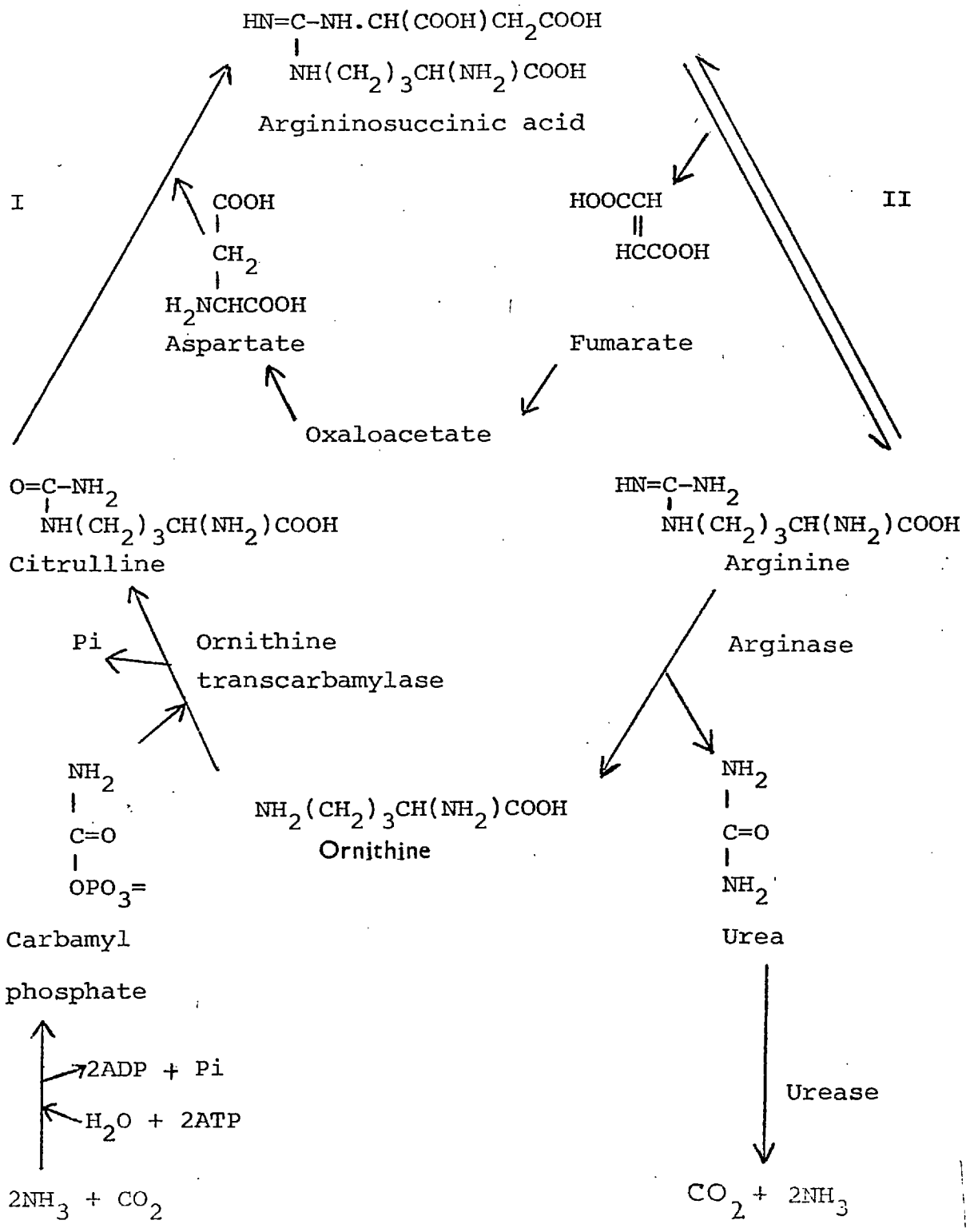
the several strains and was essentially similar to that in animal tissues in vivo. The essential amino-acids, which were supplied in the medium, were concentrated 5-to 11- fold, except cystine, which could not be detected, and lysine and arginine, which were concentrated 2- to 3-times. The non-essential amino-acids, which were not supplied in the medium, had higher distribution ratios, except ornithine which was only slightly concentrated. The existence of this intra-cellularpool is important in that it has to be taken into account in any experiment to investigate the metabolism of an amino-acid contained within the pool.

It was found also that most of the essential amino-acids were metabolized to only a small degree, their primary function apparently being incorporation into proteins.

1(ii) Arginine anabolism in uninfected cells

Arginine is synthesized in the Krebs-Henseleit cycle, also known as the ornithine or urea cycle (Fig 1.1). In this cycle, ornithine reacts with carbamyl phosphate under the influence of the enzyme ornithine transcarbamylase to give citrulline. Citrulline then combines with aspartic acid to give argininosuccinic acid, the reaction being catalysed by argininosuccinate synthetase. Arginine is formed by the breakdown of argininosuccinic acid by argininosuccinate lyase and the cycle is completed by the action of arginase to give ornithine and urea.

It has been shown (Shimke, 1964) that the synthesis of enzymes involved in the conversion of citrulline to arginine is subject to repression by arginine and that both enzymes are repressed to a similar degree. This phenomenon has been demonstrated in HeLa, KB and L cells, although the extent of repression varied between these cells. The effect of limiting-quantities of arginine on the activities of argininosuccinate synthetase and arginino-succinate lyase is specific, as was shown by experiments in which the growth of cells was limited by lack of histidine. This had no effect on the enzyme



I = Argininosuccinate synthetase
II = Argininosuccinate lyase

Fig. 1.1 The Krebs-Henseleit urea cycle

activities associated with arginine biosynthesis. Furthermore, increasing the concentration of arginine increased the activity of arginase.

The substitution of citrulline for arginine, or the addition of citrulline to arginine, did not increase the activities of the biosynthetic enzymes to a level higher than that found in the presence of limiting-concentrations of arginine alone. This shows that the effect being observed was not one of induction by a substrate and that the concentration of arginine controlled the level of enzyme activity.

It was concluded that the phenomenon was one of repression by arginine of the enzymes of arginine biosynthesis, a phenomenon similar to that seen in micro-organisms.

The pathway of arginine biosynthesis is more extensive in micro-organisms than in mammalian cells (Fig. 1.2). The mechanism of control has been well studied in the prokaryotic micro-organism Eschericia coli. In this organism it is known that there are nine structural genes that code for eight different enzymes (Taylor and Trotter, 1972). These genes do not constitute an operon, instead they form what has been called a 'regulon' (Maas and Clark, 1964). The different term was found necessary because while these genes are controlled by the same regulator (the argR gene) they are not all contiguous; four cluster at one locus (ECBH at 79 minutes) while the rest are scattered over the genome (Fig. 1.2). It was found (Jacoby and Gorini, 1969; Kadner and Maas, 1971) that the control of the expression of these genes was not co-ordinate, but parallel, the argR product acting at distinct operator sites differing slightly in affinity for the repressor.

Not all strains of E.coli are controlled in this fashion. With E. coli B, arginine actually induces several biosynthetic enzymes and control is exerted rather by carbon source and growth temperature. However, it was shown (Jacoby and Gorini, 1969) that the difference in control between this strain and the K12 strain is due to one or more mutations in the same argR site.

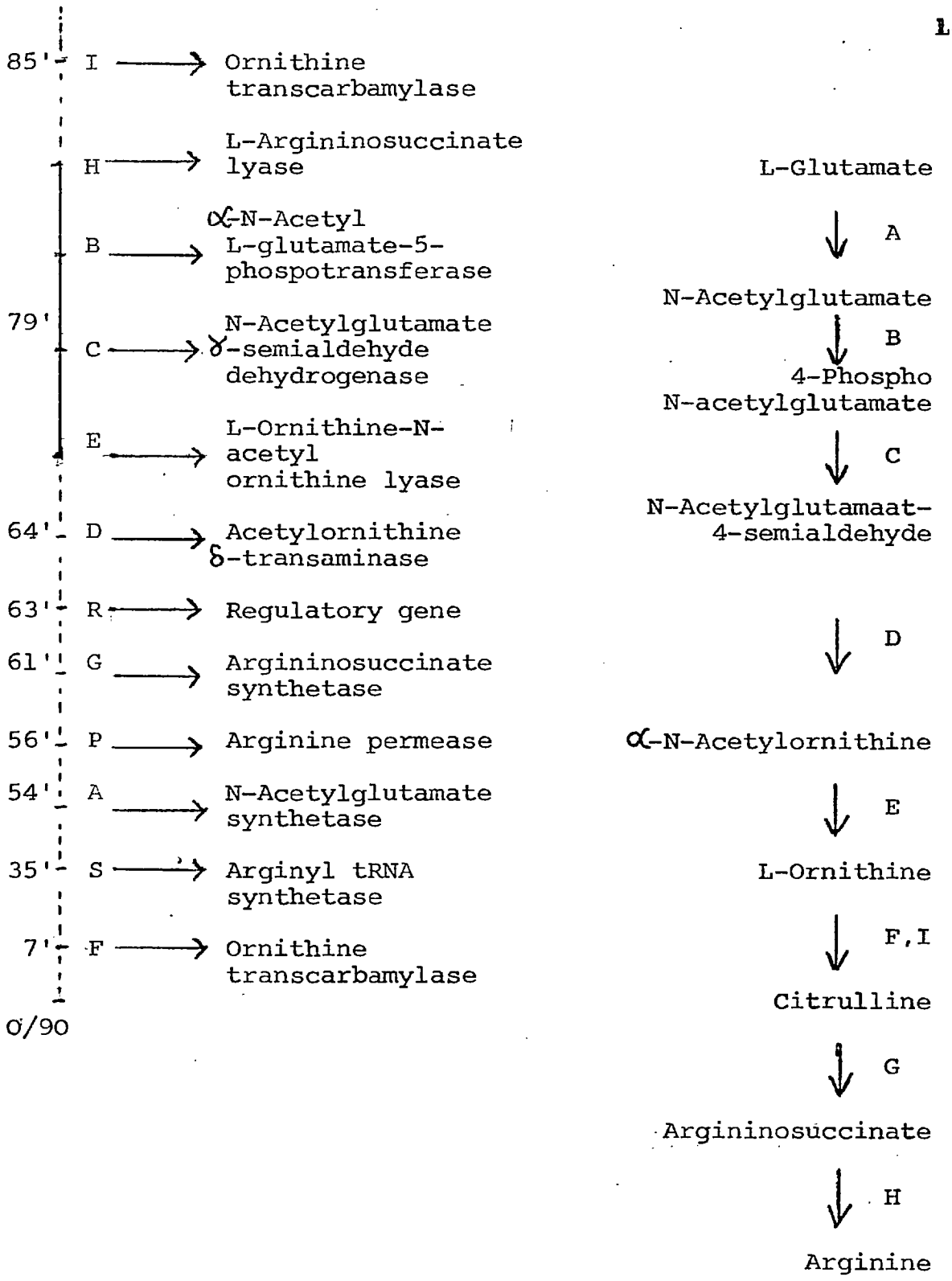


Fig. 1.2 Arginine biosynthesis in Eschericia coli: the arginine regulon

Control of arginine biosynthesis has been studied also in some eukaryotic micro-organisms. In Neurospora crassa, mechanisms of control seem to be broadly similar to those found in E. coli K12 (Barthelmess, Curtis and Kacser, 1974). The structural loci do not form an operon, but are scattered over various chromosomes and it is thought that control is parallel, rather than co-ordinate, with a large number of control sites. The three enzymes ornithine transcarbamylase, argininosuccinate synthetase and argininosuccinate lyase were found to be subject to repression, with arginine, or a simple derivative, acting as a co-repressor. There is some evidence that all the enzymes are subject to unitary control.

However, in this organism there is a second type of regulation, a 'cross-pathway' mechanism (Carsiotis and Jones, 1974; Carsiotis, Jones and Wesseling, 1974). involving the histidine, tryptophan and arginine biosynthetic enzymes. Starvation of any one of these amino-acids derepresses all three sets of enzymes. A similar double system of control has been shown to exist in another eukaryotic micro-organism, Saccharomyces cerevisiae (De forge, Messenguy and Wiame, 1975). It is thought that in this system the argR regulation acts at the DNA level (on operators, for example, although none have been found) and that the general control acts at the translational level.

These studies demonstrate that the control of arginine biosynthesis is broadly similar in all those different organisms that have been studied, in both prokaryotic and eukaryotic micro-organisms and in cultured mammalian cells. In vivo studies (Nuzum and Snodgrass, 1971) also have shown that the enzymes responsible for the conversion of citrulline to arginine are subject to repression.

1(iii) The role of arginine in uninfected cells

An important role of arginine in cultured cells is to be incorporated into proteins. However, there is very little evidence that either ornithine or citrulline is incorporated into proteins. Their importance lies in

their function as intermediates in the formation of arginine.

Arginine is incorporated especially into basic proteins such as histones. Histones are found in chromatin, which is the interphase chromosomal material from eukaryotic cells and which consists of a complex macromolecular association between DNA and protein. The protein is of two types, histone and non-histone. The precise role that this protein plays in genetic regulation is not certain, but the histone chromosomal proteins are, in some way, responsible for the structural integrity of chromatin.

1(iv) Arginine katabolism in uninfected cells

Arginine is broken down by arginase to form ornithine and urea as part of the Krebs-Henseleit cycle (Fig 1.1). The ornithine thus formed can go round the cycle again as previously described, or it can enter different pathways, resulting in the formation of other products. (Dagley and Nicholson, 1970).

Proline, hydroxyproline, glutamate aspartate and possibly asparagine can be formed from ornithine. The initial step in each case is the formation of glutamate semialdehyde (Fig 1.3). This can be converted to proline via L - Δ^1 - pyrroline - 5 - carboxylate, formed by the spontaneous dehydration of the semialdehyde. Proline can undergo oxidation to hydroxyproline, but apparently this occurs only after the incorporation of proline into a peptide.

The semi-aldehyde can be oxidized, possibly by the action of a dehydrogenase, to glutamate. Glutamate can then be converted to aspartic acid by a transamination reaction. The mode of synthesis of asparagine in mammals has not been established, but it could possibly occur by the transfer of the amide-N of glutamine to aspartic acid.

Creatine and creatinine can also be formed from arginine (Fig 1.4). Creatine is formed in a reaction which involves glycine; it proceeds via guanidoacetate and releases ornithine. Methylation of the guanidoacetate

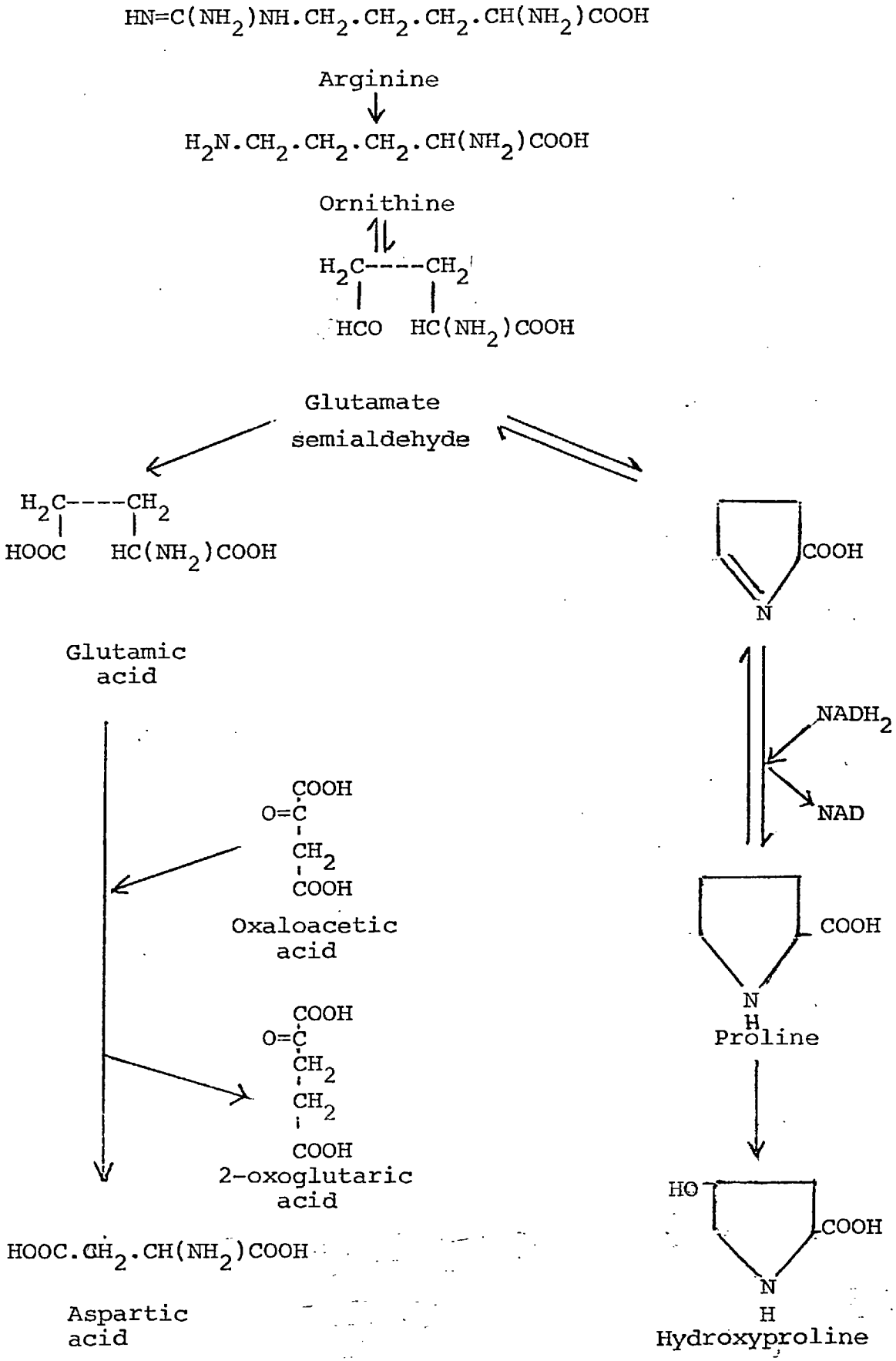


Fig. 1.3 Arginine metabolism leading to the biosynthesis of different amino acids.

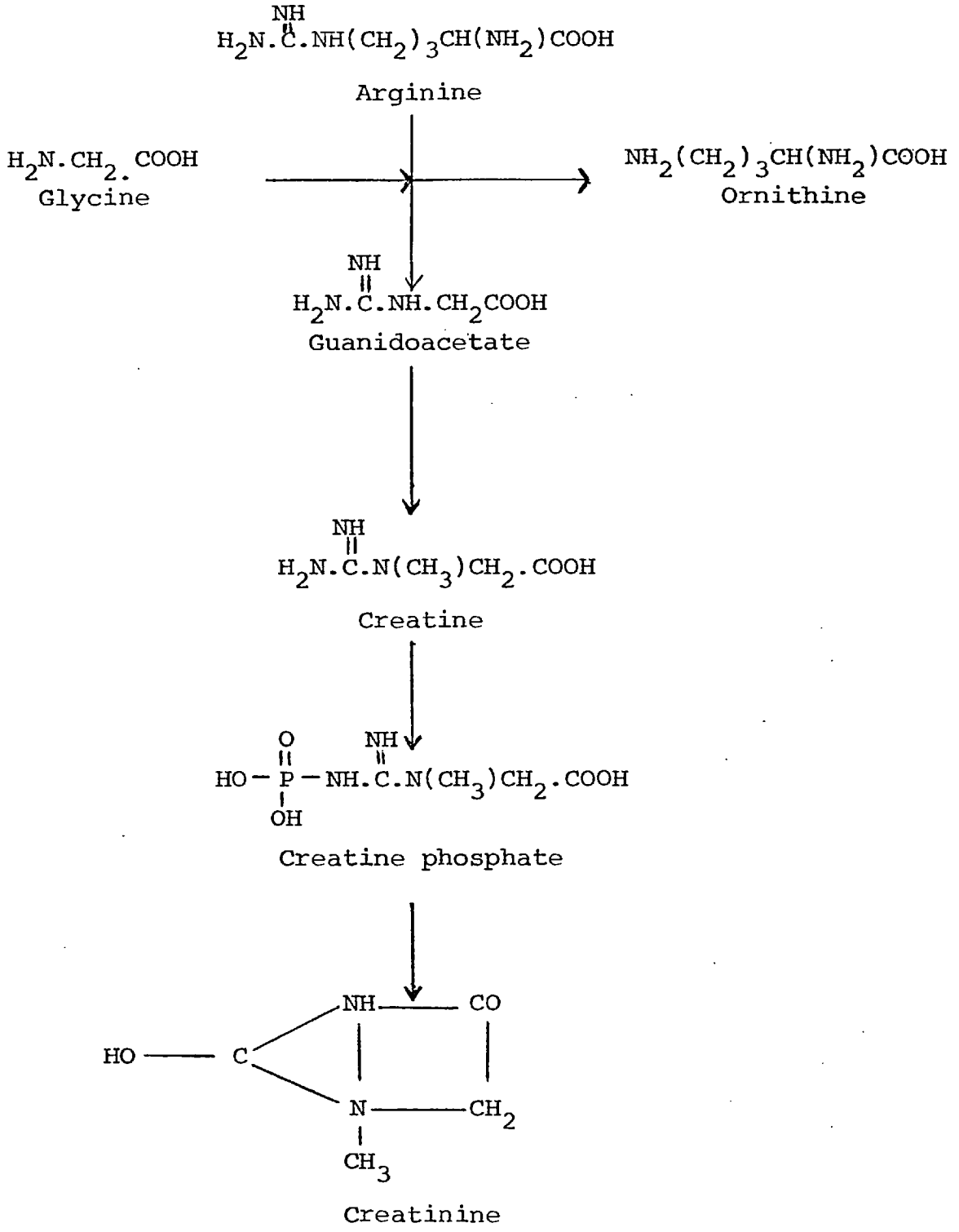


Fig 1.4. Arginine metabolism leading to the biosynthesis of creatine and creatinine

produces creatine which can then be phosphorylated. The phosphate then undergoes a non-enzymic step, involving ring closure, to produce creatinine.

Creatine is important in providing a reservoir of high-energy phosphate which can be readily converted to ATP. Creatinine is a product of creatine metabolism.

Arginine katabolism can also give rise to certain diamines and polyamines, compounds that are widely distributed among living organisms. Examples of this group are putrescine, cadaverine, spermidine and spermine. Cadaverine is produced by decarboxylation of lysine. Putrescine is produced by decarboxylation of ornithine and is involved in the synthesis of spermidine and spermine (Fig. 1.5).

The urea cycle is closely linked to the Krebs cycle (tricarboxylic-acid cycle or citric-acid cycle) which is responsible for carbohydrate metabolism, by virtue of the fact that the amino-acids arginine, ornithine and proline can all be metabolized to produce glutamic acid and hydroxyproline gives alanine. Both of these end products can enter the Krebs cycle (Fig. 1.6).

Thus, the katabolism of arginine gives rise to a wide range of products. It is possible that the requirement for arginine in cell culture stems as much from a need for some or all of the products of arginine katabolism as from a requirement for proteins containing arginine. This may be true also of the requirement for arginine for the replication of viruses that will be discussed in the next section.

1(v) The requirement for arginine for the replication of viruses.

a. Herpes virus

Herpes viruses are DNA viruses that replicate in the cell nucleus. The size of the virus genome varies considerably between different members of the group. The largest is found in equine abortion virus (92×10^6 daltons) and the smallest in rhinotracheitis virus (54×10^6 daltons) (Fenner, 1968).

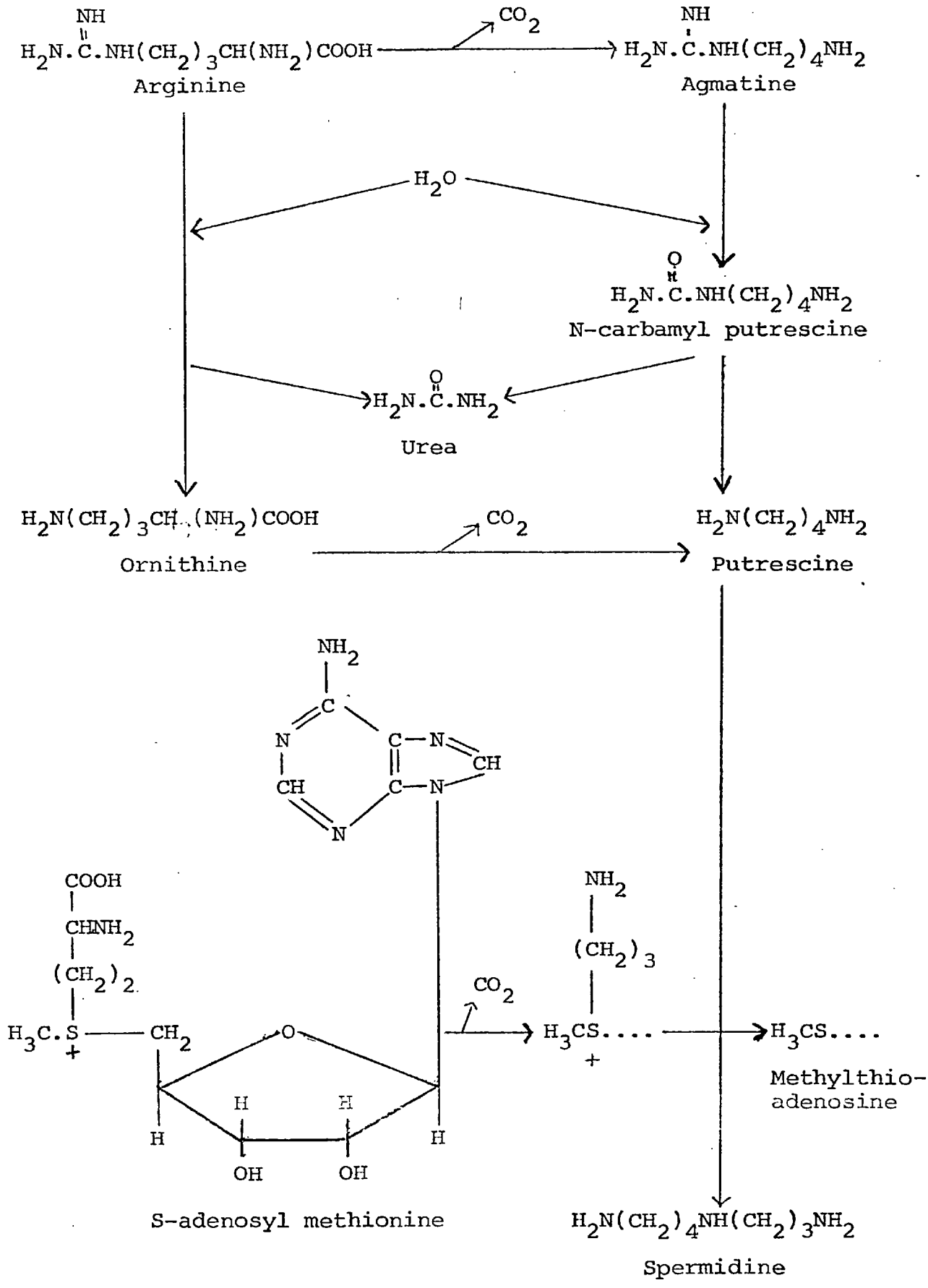


Fig. 1.5 Arginine metabolism leading to the biosynthesis of polyamines.

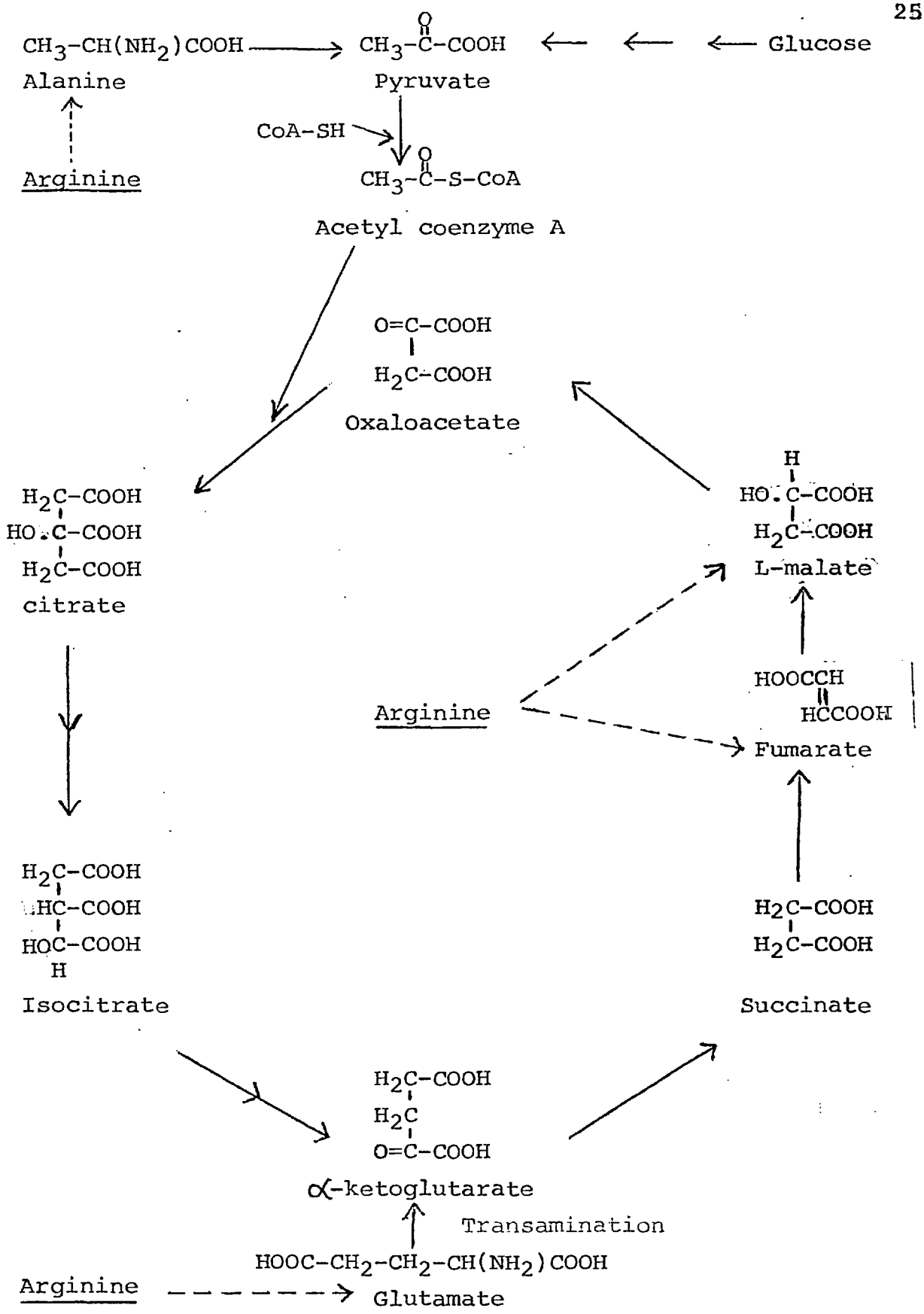


Fig. 1.6 The involvement of arginine metabolism in carbohydrate metabolism: the Krebs tricarboxylic adid cycle.

Arginine is essential for the replication of herpes simplex in Minn EE cells (derived from human oesophageal epithelium) (Tankersley, 1964) and in BSC1 (monkey kidney) cells (Becker, Olshevshy and Levitt, 1967). This requirement of herpes simplex virus for arginine is not uniform (Spring, Roizman and Spear, 1969). The yield of virus from primary cultures of human embryonic fibroblasts, chick embryofibroblasts or of monkey kidney cells is unaffected by arginine deprivation. It is possible that the difference between primary and continuous cell cultures is due to a difference in size of the pools of arginine that are known to be present in some cells (see section 1(i)).

The deprivation of arginine in continous cell-lines infected with herpes virus seems to affect late viral functions. Arginine starvation does not prevent adsorption, penetration and uncoating of virus and replication events occurring during the first hours after infection. The synthesis of virus DNA, virus protein and transport from the cytoplasm to the nucleus are unaffected, but virus particles are not made (Spring et al, 1969). However, subsequent addition of arginine immediately stimulates protein synthesis, which is followed by the formation of virus (Becker et al, 1967).

The late functions affected by the absence of arginine seem to be those concerned with the synthesis of coat-proteins, and arginine is in fact incorporated into the coat-proteins of mature virions

To explain the importance of arginine in the expression of late functions, Spring et al (1969) postulated that the arginine pools present in cells at the onset of arginine starvation are sufficient to allow the synthesis of proteins necessary for the initiation of DNA synthesis. Once the concentration of arginine becomes limiting, the probability that a given protein will be completed depends on the number of arginine residues; thus, given the same frequency of arginine, the shorter proteins have a higher probability of being completed. Consequently, the lack of arginine would be more detrimental to the multiplication of virus than that of other amino-acids because several proteins specified by the virus are both

large and rich in arginine. However, some virus capsid proteins that are rich in arginine are made in the absence of exogenous arginine (Olshevsky and Becker, 1970) suggesting that arginine may be derived by the breakdown of cell proteins.

It is possible that the importance of arginine in herpes simplex virus replication lies not only in the synthesis of structural proteins, but also in the synthesis of polyamines. The presence of both spermine and spermidine has been demonstrated in herpes simplex virus, type 1 (Gibson and Roizman, 1971). Spermidine is bound to the virus envelope and spermine is found in the nucleocapsid. The role of these polyamines is not known, but possibly their function in herpes simplex virus is the same as their presumed function in bacteriophage T_4, that is to neutralize negative charges on DNA. This idea is supported by the fact that there is a nearly constant molar ratio between DNA phosphate and spermine nitrogen in the herpes simplex virion.

b. Adenovirus

Adenoviruses are DNA viruses and, as with herpesvirus, replication occurs in the nucleus of the cell. Virus maturation also occurs in the nucleus, but the synthesis of proteins occurs in the cytoplasm (Fenner, 1968). The DNA of adenoviruses has a molecular weight of between 20×10^6 and 25×10^6 daltons. The replication of adenovirus type 2 has an absolute requirement for arginine. Rouse and Schlesinger (1967) found that the omission of arginine totally abolished the replication of type 2 adenovirus in KB cells, whereas the omission of any of the other amino-acids essential for the growth of cells merely reduced virus replication. In fact, it was found that a medium consisting of salts and glucose supplemented with 0.5mM arginine would support a definite multiplication of virus. These experiments also showed that the requirement for arginine could be satisfied by the use of citrulline in place of arginine, but not by the use of ornithine. Another point of similarity between the replication of

adenovirus and that of herpes virus is that arginine is required for events occurring late in the replication cycle. In cells infected with adenovirus, virus DNA, early viral antigen and limited amounts of structural antigens known to form part of the complete capsid are synthesized during arginine starvation. Arginine is incorporated into protein(s) synthesized immediately before virus maturation and its function is related to the assembly of preformed viral precursors, DNA or capsid protein or both, into complete virions. Later experiments (Russell and Becker, 1968) showed the existence of a factor, called the P-antigen, that was required for maturation. At least one component of this antigen could be an internal protein and the presence of arginine was apparently required for the synthesis of the later, more specific, form of the P-antigen.

However, Rouse and Schlesinger (1972) and Raška, Prage and Schlesinger (1972) found that virus DNA and all the major structural proteins were made during arginine starvation. As in cells infected with herpesvirus, lack of exogenous arginine appears to result in the breakdown of host-cell proteins, releasing arginine. However, when arginine was restored, although virus DNA was incorporated into virus particles, none of the structural proteins made during arginine starvation was available for the assembly of viruses.

As in the replication of herpesvirus, the importance of arginine in the replication of adenoviruses might lie, to some extent, in its conversion to polyamines via ornithine. Shortridge and Stevens (1973) showed that the polyamines spermine and spermidine were present in the virion of adenovirus type 5 in a quantity sufficient to neutralize about 10% of the virus DNA. Pett and Ginsberg (1975) also investigated the presence of polyamines in adenovirus type 5. They found that the incorporation of radioactively labelled ornithine and putrescine into putrescine, spermine and spermidine in infected cells was identical to that in uninfected cells early in infection. However, incorporation into putrescine stopped after 8-12 hours and the rate of incorporation into spermidine was reduced between 12 and 20 hours after

infection, i.e. at the time when structural components are being made. Furthermore, the amount of polyamines found to be associated with purified virus was sufficient to neutralize, at the most, 3-4% of the virus DNA and these small amounts could not be distinguished from non-specific binding of polyamines to virions.

c Papova viruses

This group of viruses,like the other two already discussed, is composed of DNA viruses that replicate in the cell nucleus. The DNA has a molecular weight of about 3×10^6 daltons.

Simian virus (SV) 40, an oncogenic virus, is an example of this group and its requirement for arginine in BSC1 cells has been investigated (Goldblum, Ravid and Becker, 1968). Arginine is essential for the production of complete, infectious, viral progeny. The importance of arginine in this system seems to lie in the synthesis of viral coat proteins. Again, as with the other virus groups examined, it is probably some late step that is affected by the absence of arginine. The T-antigen, a virus antigen isolated from tumours, is synthesized early in infection and is not affected by the lack of arginine.

In the other DNA viruses, the possibility exists that arginine might be important in the synthesis of polyamines. However, this does not appear to be the case with papova viruses. Kass and Knight (1965) performed an analysis of the Shope papilloma virus and found that this virus has little or no polyamine content.

d. Frogvirus

Frogvirus 3 (FV3) is a DNA virus that is generally believed not to replicate in the nucleus, but in the cytoplasm of the infected cell. However, recent evidence suggests that the nucleus plays some part in FV3 replication. Kelly and Atkinson (1975) have demonstrated, by electron microscopy, the presence of virus particles in the nucleus of infected cells. It has been shown also (Goorha, Willis and Grannoff, 1975) that FV3 virus will not replicate in enucleated chick-embryo cells and viral RNA or DNA synthesis could not be detected. Furthermore,

it is known (Manier, Vago, de Vauchelle and Diethoit, 1971) that a virus morphologically similar to FV3 replicates primarily in the nucleus of Paramaecium arcautum cells.

The arginine requirement for the replication of FV3 virus has been demonstrated by Aubertin (1975). In the absence of arginine DNA replication occurs, but the production of progeny virus is inhibited. This is much the same as has been found with the other DNA viruses discussed and like those viruses FV3 virus contains several polypeptides rich in arginine. However, it is not known whether these polypeptides are synthesized in the absence of exogenous arginine. Thus, it cannot be asserted that arginine is essential in FV3 virus replication only for the synthesis of structural polypeptides rich in arginine.

e. Poxvirus

Poxviruses are large, complex viruses; the largest of the true viruses. They are DNA viruses that replicate in the cytoplasm of the cell. Cowpox and vaccinia viruses are two examples of this group. They are brick-shaped entities measuring about 260 x 220 x 220 nm and contain DNA of molecular weight approximately 156 x 10^6 daltons (Allison and Burke, 1962). Taking the probable average size of the bacteriophage gene as being about 10^6 daltons, the poxvirus genome would be expected to contain about 150 genes. The large size of the poxvirus genome means that these viruses would be expected to code for a number of their metabolic requirements. A number of enzymes have shown to be associated with poxvirus infection (Fenner, 1968) and four enzymes are known to be associated with the virus particle: a DNA -dependent RNA-polymerase and a nucleotide phosphohydrolase (Kates and McAuslan, 1967); a protein kinase (Downer, Rogers and Randall, 1973) and a single-strand specific DNase (Pogo and Dales, 1974).

Following infection with poxvirus, synthesis of DNA in the nucleus of the host-cell is suppressed (Hanafusa, 1960). This suppression is almost certainly due to the

virus-associated DNase activity (Pogo and Dales, 1974) which is released from the virus cores in the cytoplasm, enters the nucleus and specifically hydrolyses nascent, single-stranded DNA.

At the same time as host DNA is being suppressed, virus DNA is being synthesized and accumulated in the cytoplasm (Hanafusa, 1960). The synthesis of virus DNA occurs early in the replication cycle and is completed before progeny virus particles appear. Before the synthesis of virus DNA occurs, a number of early proteins appear. These early proteins include DNA polymerase, thymidine kinase and three types of DNase, 'alkaline' DNase, 'neutral' DNase and 'acid' DNase, so called because of the pH values at which optimum activity occurs. The synthesis of these enzymes begins between 1 and 2 hours after infection.

Since poxviruses replicate in the cytoplasm, they would not be expected to be able to use those host-cell enzymes found in the nucleus and so would have to produce their own. There is evidence that some of the early enzymes are virus-coded (Fenner, 1968). The induction by poxviruses of DNA polymerase, thymidine kinase, and the 'alkaline' DNase can be prevented by interferon, which specifically inhibits viral protein synthesis. It has been shown that the host-cell thymidine kinase differs from the enzyme induced by poxvirus infection of mutant cells normally lacking thymidine kinase activity with respect to immunology, kinetics and thermal stability. It has been shown also that the immunological properties of the DNA polymerase induced by vaccinia virus infection are different from those of the enzyme from the uninfected cell. Furthermore, DNA polymerase cannot be induced by vaccinia virus that has been inactivated by ultraviolet radiation. Ultraviolet radiation inactivates virus by damaging the genome. The RNA encoding these early proteins is transcribed from the parental virus DNA by means of the DNA-dependent RNA polymerase associated with the virus particles.

These data show that at least three enzymes associated with the replication of vaccinia virus are encoded in the

virus genome. This independence of vaccinia virus replication from host-specified enzymes has been demonstrated further by Prescott, Kates and Kirkpatrick (1971). They found that uncoating of vaccinia virus and replication of vaccinia virus DNA occurs in L cells that have been enucleated by treatment with cytochalasin B. This suggests that vaccinia virus does not use any host enzymes that are exclusively in the host nucleus before infection. However, subsequent experiments by Pennington and Fallet (1974) on the replication of vaccinia virus in enucleated BSC-1 cells indicated that the presence of the nucleus is required, in some way as yet unknown, for the production of normal yields of mature virions.

The fact that arginine is involved in poxvirus replication was indicated by experiments concerning the growth of variola virus in human embryonic fibroblasts (Wells, 1967). These experiments showed that a requirement for bicarbonate in variola virus replication in these cells could be overcome by the addition of increased concentrations of arginine. Holterman (1969) later showed that the amino-acids that are essential for vaccinia virus replication in Earle's L cells are the same as those required for the growth of L cells. These amino-acids include arginine. A further demonstration of the dependence on arginine of vaccinia virus replication was given by an examination of the effects of mycoplasmas on the growth of virus (Singer, Fitzgerald, Barile and Kirschstein, 1970). The presence in the cells of Mycoplasma arginini decreased the yield of vaccinia virus, which affect could be overcome by the addition of arginine. The presence of M. hyorhinus, a dextrose fermenter, had no affect on the growth of vaccinia virus.

Archard and Williamson (1971) confirmed the dependence of vaccinia virus replication on arginine. They also found that arginine is required for events occurring both early and late in the replication cycle and that arginine is incorporated into the virion. The early requirement occurs before the synthesis of virus DNA . The late requirement is associated with virus maturation, as it is with the other viruses already discussed. Arginine deprivation of HeLa cells infected with vaccinia

virus, in contrast to what is found in cells infected with other DNA viruses, caused a marked suppression of the synthesis of virus DNA, although some virus antigens were detected. Obert, Tripier and Guir (1971) investigated the requirement for arginine of KB cells infected with vaccinia virus In this system, however, DNA replication was not affected significantly by the absence of arginine. Early mRNA was synthesized, but late mRNA was completely inhibited and all the mRNA sequences transcribed in the absence of arginine were found to be identical with early mRNA sequences transcribed in the presence of arginine.

These studies show that the dependence on arginine of the replication of vaccinia virus has in common with the dependence on arginine of the replication of other DNA viruses the fact that arginine is required for events occurring late in the replication cycle and associated with maturation. In addition, HeLa cells infected with vaccinia virus require arginine for events occurring early in the replication cycle and associated with the synthesis of virus DNA. Furthermore, several of the animal viruses described previously have been shown to use arginine for the synthesis of polyamines. Vaccinia virus also contains polyamines; namely spermine and spermidine (Lanzer and Holowczak, 1975). After infection with vaccinia virus, while the synthesis of host-cell protein is being shut off rapidly the synthesis of polyamines still occurs, albeit at a reduced level. This was shown by the use of tritium-labelled ornithine, which continued to be incorporated into polyamines and amino-acids after infection with vaccinia virus. The incorporation of polyamines into the virion seems to be controlled rather than accidental, because spermine and spermidine are found in a constant proportion within the virion. It is suggested that, in addition to basic polypeptides, polyamines might play a role in neutralizing the charges of DNA and could function in the folding and packaging of vaccinia DNA within the core.

1(vi) The biosynthesis of arginine in cells infected with poxvirus

The requirement for arginine for the replication of adenoviruses is known to be satisfied by substituting citrulline for arginine. Thus, there must be present in cells infected with adenovirus the enzymes necessary for the biosynthesis of arginine from citrulline. Either the host-cell enzymes are used or the virus codes for its own. Poxviruses, also, are able to replicate in the presence of citrulline. There is strong evidence that in cells infected with poxviruses, arginine biosynthesis occurs by means of virus-coded rather than host-cell coded enzymes (Cooke and Williamson, 1973; Williamson and Cooke, 1973).

The infection of HeLa cells with vaccinia virus in the presence of citrulline produces an enhanced incorporation of radioactively-labelled citrulline. Analysis of vaccinia particles grown in HeLa cells in the presence of radioactively-labelled citrulline showed that the radioactivity incorporated into the virion was in the form of arginine, not citrulline. Furthermore, the growth in the presence of citrulline of rabbitpox virus in mouse sarcoma 180 (S180) cells and vaccinia virus in HeLa cells was inhibited by canavanine. Canavanine is a homologue of arginine and it inhibits competitively the synthesis of arginine from citrulline. Thus, citrulline is not used directly in the replication of poxviruses, but it is converted to arginine.

The enhancement of the incorporation of citrulline also occurs in mouse S180 cells infected with rabbitpox virus and human citrullinaemia cells will support the replication of vaccinia virus in the presence of citrulline. This occurs despite the fact that mouse S180 cells and human citrullinaemia cells have a metabolic defect such that they are unable to synthesize arginine from citrulline. Consequently, neither cell will grow in a medium in which citrulline is present in place of arginine.

The increase in incorporation of citrulline into mouse S180 cells infected with rabbitpox coincides with

events occurring during the rabbitpox replication cycle that require arginine and it is suppressed by actinomycin D (an inhibitor of RNA synthesis). This indicates that citrulline is being converted to arginine and that this conversion is dependent on the synthesis of new RNA species.

Vaccinia infection of both HeLa and mouse L cells produces an increase in the activity of the enzymes responsible for the conversion of citrulline to arginine to a level higher than that found in uninfected cells. In each case, the enzyme-activity in the presence of citrulline is higher than that in the presence of arginine. The increase in enzyme activity following infection is inhibited by the presence of either puromycin (an inhibitor of protein synthesis) or actinomycin D, indicating that both protein synthesis and RNA synthesis are required for the expression of the increased activities. The inhibition by actinomycin D occurs despite the fact that host-cell mRNA molecules specifying the two enzymes are very stable. They have a half-life in excess of 18 hours and so would be present in the cell during virus replication. This indicates that some change occurs in the transcription of the mRNA, either more molecules of the same species must be produced or different species. Further weight is given to the notion that the virus codes for its own enzymes by differences in the values of the Michaelis constant (Km) of enzymes from infected and uninfected cells. The value for the Km of the combined activity of the two enzymes is the same in HeLa cells infected with vaccinia virus and in L cells similarly infected. However, the value for the Km of this enzyme activity in infected cells is different from that of the enzyme activity in either uninfected cell line. These studies show that vaccinia virus and rabbitpox virus almost certainly direct the synthesis of their own enzymes for the biosynthesis of arginine from citrulline. This thesis is concerned with extending these studies to other poxviruses by investigating their replication in a cell system defective in its ability to utilize citrulline. The mouse S180 system previously used possesses such a defect and so in this respect it is

eminently suitable for use in such studies. However, initial experiments described in this thesis revealed that mouse S180 cells are unable to support the replication of more than a small number of poxviruses. Thus, it became necessary to develop a cell system that is defective in its ability to synthesize arginine from citrulline and that can support the replication of a variety of poxviruses. The development of such a cell system is described and the replication of some poxviruses within this cell system has been investigated. In order to investigate certain biochemical aspects of the role of arginine in the replication of poxviruses it is necessary to be able to measure the activities of the enzymes of arginine biosynthesis. The assay system previously used in this laboratory is a radiochemical method. It suffers from two important short comings. Firstly, there is a high background associated with this method that makes it impossible to measure very low levels of enzyme activity; such levels as result from virus infection of cells unable to use citrulline. Secondly, the nature of the procedure makes undesirable its use for assays of cells infected with dangerous human pathogens such as smallpoxvirus. The rest of this work is concerned with attempts to overcome these difficulties. To this end, the sensitivity of the radiochemical system has been improved by lowering the background activity and another, spectrophotometric, assay system has been investigated.

CHAPTER 2

MATERIALS AND METHODS

2(i) Cell cultures

The cell lines used in this study were a laboratory line of HeLa, mouse sarcoma 180 (S180. Flow Laboratories, Irvine Scotland) Vero (Gibco-Biocult, Glasgow, Scotland) and primary cells prepared from the chorioallantoic membrane (CAM) of chick embryos. Fragments of the CAM of the chick embryo and pieces of the membrane attached to the shell also were used.

S180 and HeLa cells were routinely cultured in 16-ounce, flat, glass bottles in Eagle's minimal essential medium (MEM. Flow Laboratories; Eagle, 1959) (Table 2.1) supplemented with 5% gamma-irradiated calf serum (Gibco-Biocult). Vero cells were grown in 199 medium (Flow Laboratories) supplemented with the same amount of calf serum. The bottles were each seeded with about 5×10^6 HeLa or Vero cells or 3×10^6 mouse S180 cells, in 40 ml. of medium. The cells were grown at a temperature of $37^{\circ}C$ and became confluent in 3 to 4 days. Antibiotics were not used in order that any contamination with bacteria would become evident, thus reducing the risk of a concomitant contamination with mycoplasma going unnoticed.

Cells were removed from the bottles by the following procedure:-
The spent medium was poured off and the cell monolayer washed with about 10 ml. of Dulbecco's PBS (A) (Oxoid Ltd., London, England). About 10 ml. of a solution of ethylene diamine tetra acetic acid (EDTA) at a concentration of 0.04% w/v in PBS (A) was applied to the monolayer for about 5 minutes before it was poured off. After a further 10 minutes, the cells were shaken off into 10 ml. of serum-free medium.

Pieces of chick embryo CAM attached to the shell were prepared according to the method of Fazekas de St. Groth and White (1958).

The embryos were removed from 11-day old eggs and the shell, with attached membrane, was cut into pieces about 5 mm. square. These squares were washed with PBS (A) and single pieces placed into the wells of a divided

plastic tray (Sterilin Ltd., Teddington, Middlesex, England). To each well was added 0.5 ml. of appropriate medium. The tray was sealed and incubated at 37°C with vigorous shaking in a water-bath shaker.

The preparation of fragments of the CAM of the chick was carried out according to the method of Watanabe and Okada (1974). Embryos were used between 11 and 12 days old. The CAM was removed from each embryonated egg and placed in a Petri-dish containing PBS (A). After having been washed, the membranes were minced with sharp scissors and the resulting small fragments were washed several times by allowing them to settle in large volumes of PBS (A). Most of the free erythrocytes were removed in this way. The fragments were spun at 1000g for 5 minutes and the pellet was resuspended in 30 volumes of MEM supplemented with 10% foetal bovine serum. This medium was buffered at pH 7.3 with 14 mM N-2-hydroxyethyl piperazine-N'-2-ethane sulphonic acid (HEPES. Sigma, London Chemical Co. Ltd., London, England; Williamson and Cox, 1968). The cell suspension was dispensed in 2 ml. amounts into 55 mm. glass Petri dishes and incubated at 37°C. After 24 hours, any debris and unattached fragments were removed with Hanks' balanced salts solution (BSS) fresh growth medium added and incubation continued.

Fragments were also maintained in suspension. In this case, the compacted pellet of fragments was suspended in 60 volumes of PE medium (see Media, below) containing no divalent cations. Portions of suspension, 60 ml. in volume, were placed in 250 ml. conical flasks and incubated at 37°C in a water-bath shaker. The PE medium was supplemented or not, as required, with suitable amounts of arginine or citrulline and in all cases with 14 mM HEPES.

The preparation of cells from the CAM of the chick embryo was based on the method of Cursiefen and Becht (1975). Fragments were prepared from the CAM of 11- or 12-day old chick embryos as previously described. These fragments were then resuspended in a solution of 0.01% w/v collagenase and 0.001% w/v hyaluronidase (Sigma, London Chemical Co. Ltd) in Hanks' BSS lacking divalent

cations. The enzyme treatment was carried out at room temperature, with stirring, for 20 minutes before the fragments were washed again in PBS (A). A solution of 0.02% w/v trypsin (Gibco-Biocult) in PBS (A) containing 0.2% EDTA was added to the recovered fragments, which were then stirred at room temperature for 50 minutes. At the end of this time the debris was allowed to settle, the supernatant cell-suspension was collected and centrifuged at about 500g for 4 minutes. The cells were washed with PBS (A) containing 0.2% EDTA and suspended at a concentration of 10^6 cells per ml. in 199 medium or McCoy's 5A medium (Iwokata and Grace modification; Flow Laboratories) supplemented with 10% foetal bovine serum (Gibco-Biocult). This suspension was distributed between 45 mm. glass Petri-dishes in volumes of 5 ml. per dish. The cells were incubated at 37°C in an atmosphere of 6% CO_2 in air saturated with water. After 18 hours any debris and erythrocytes present were removed by washing with Hanks' BSS. Fresh growth medium was added and the cells were incubated as before. Confluent monolayers were obtained in 3 to 4 days.

Sterile procedures were used throughout.

2(ii) Media

In order to examine the effects on cell metabolism and virus growth of the presence or absence of arginine, it was necessary to use a medium in which the concentration of arginine could be accurately defined. Because serum contains variable amounts of free arginine the use of a serum free medium is implied. The basic medium used (Table 2.1) is a modification of Eagle's MEM (PE) described by Birch and Pirt (1969). Experiments with mouse LS cells led these authors to the conclusion that the importance of serum for cells in culture lies mainly in the provision of choline. This PE medium therefore contains a high concentration of choline, which permits cell growth in the absence of serum. In addition, PE medium also contains high concentrations of those amino acids essential for the maximal growth of mouse LS cells

(Griffith and Pirt, 1967). Antibiotics were added as shown in Table 2.1.

This medium was used in experiments involving HeLa and mouse S180 cells and CAM fragments and pieces. In experiments involving CAM cells, various supplements were used (Table 2.2). The supplementation was based on the composition of McCoy's 5A medium (Iwokata and Grace modification).

2(iii) Depletion of intracellular pools of arginine

Before performing virus replication studies it was necessary to deplete the pools of free arginine within the cells. With HeLa and CAM cells and CAM fragments this was done by maintaining the fragments or confluent monolayers of cells in a medium containing no serum or arginine for 18 hours. Mouse S180 cells were starved for only 6 hours. This reduction in starvation time was necessary because longer periods caused the mouse S180 cells to become detached from the glass. Experiments have shown that this starvation time is sufficient to deplete the intracellular pools of free arginine without causing any apparent cytological changes in the cells (Cooke, 1972).

2(iv) Preparation of virus stocks

The viruses used were vaccinia (Lister strain) cowpox (Brighton strain) rabbit pox (Utrecht strain) and one strain each of buffalo pox, elephant pox, and monkey pox (supplied by Professor K. Dumbell). Stocks were prepared by growth in HeLa cells and in the CAM of the chick embryo.

Confluent monolayers of HeLa cells in 40-ounce roller bottles were infected at a multiplicity of infection of between 0.1 and 1.0 pfu per cell with a virus suspension in 20 ml. of MEM supplemented with 1% calf serum. After adsorption for 2 hours at 37°C, 30 ml. of MEM supplemented with 1% calf serum was added. Incubation was continued until confluent cytopathic effect was seen, usually 2 to 3 days, at which time the cells were shaken off into the medium and recovered

Table 2.1 Composition of Eagle's minimal essential medium (MEM) and of Pirt's modified Eagle's medium (PE)

Inorganic salts	mg. per litre		L-amino acids	mg. per litre	
	MEM	PE		MEM	PE
			Arginine	105	See text
NaCl	8,000	8,000	Histidine	31	100
KCl	400	400	Lysine	58	175
$CaCl_2$	140	140	Isoleucine	52	180
$MgSO_4.7H_2O$	100	100	Leucine	52	180
$Na_2HPO_4.12H_2O$	60	60	Methionine	15	30
KH_2PO_4	60	60	Phenylalanine	32	65
$MgCl_2.6H_2O$	100	100	Threonine	48	100
$NaHCO_3$	1,100	1,100	Tryptophan	10	20
			Valine	46	150
Vitamins			Tyrosine	36	70
			Cystine	24	75
			Glutamine	292	200
			Alanine	-	90
i-Inositol	2.0	2.0	Serine	-	20
d-Biotin	-	0.1	Glycine	-	15
Choline	1.0	10.0	Na Glutamate	-	1,530
Nicotinic acid	1.0	1.2			
Pantothenic acid	1.0	1.2	D-Sugars		
Pyridoxal	1.0	1.0	Glucose	2,000	1,000
Thiamine	1.0	2.0			
Riboflavin	0.1	0.2			
Hypoxanthine	-	10.0	Antibiotics		
B_{12}	-	1.0			
Folic acid	1.0	1.0	Penicillin	-	100
			Streptomycin	-	100
			Kanamycin	-	200

Table 2.2

Supplements to Pirt's modified Eagle's medium

Amino acids	Concentration (mg. per litre)	
	P-Mc medium	MPE medium
Asparagine	45.0	45.0
Aspartic acid	20.0	20.0
Proline	17.0	17.0
Hydroxyproline	20.0	20.0
Cysteine	32.0	-
Vitamins		
Ascorbic acid	0.5	-
Nicotinamide	0.5	-
Pyridoxine HCl	0.5	-
p-Aminobenzoic acid	1.0	-
Other compounds		
Glutathione	0.05	-

by low-speed centrifugation (100g for 5 minutes). The pellet of cells was resuspended in 10 ml. of Hanks' BSS and the suspension was subjected to two cycles of freezing and thawing followed by treatment with ultra sonics for 2 minutes. Cell debris was removed by centrifugation (1500g for 5 minutes) and the supernatant was stored frozen at -30°C.

Stocks of virus were prepared from fertile hens' eggs by inoculating 0.1 ml. aliquots of suitable dilutions of virus onto the 'dropped' CAM of 12-day old chick embryos. After incubation at 37°C for 3 days, infected membranes were removed, washed with PBS (A) and placed in a glass Universal bottle containing glass beads. To the membranes was added 1 ml. per membrane of McIlvain's buffer, pH7.3. The viruses were released by agitating the membranes by means of a Rotamixer, freezing and thawing twice and treating with ultrasonics. The coarse debris was removed by centrifugation at 1500g for 5 minutes and the supernatant was stored frozen at -30°C.

2(v) Virus replication

In experiments to measure virus replication in HeLa and mouse S180 cells, confluent monolayers in 6" x ⅝" glass tubes were used. Each tube was inoculated with 0.2 ml. of a suitable dilution of virus in Hanks' BSS and the virus was allowed to adsorb for 1 hour at 37°C. After this time the inoculum was removed, unadsorbed virus was removed by washing with Hanks' BSS and 1 ml. per tube of a suitable medium was added. Replicate tubes were immediately frozen to enable the inoculum recovery to be determined and the remaining tubes were incubated at 37°C for suitable lengths of time before being frozen at -30°C.

In experiments to measure the replication of virus in CAM cells and fragments attached to glass the same procedure was followed, except that the CAM material was grown in 55 mm. or 45 mm. glass Petri-dishes, 1 ml. per dish of virus inoculum was used and 3 ml. per dish of experimental medium was finally added.

A different procedure was used with CAM fragments in suspension. The fragments were centrifuged to produce a compact pellet (1500g for 5 minutes). Virus was then added at 5 x 10^6 pfu per ml. (compacted volume) of fragments in 1 ml. of Hanks' BSS without divalent cations. After adsorption for 2 hours, the fragments were washed twice with Hanks' BSS without divalent cations and 0.2 ml. (compacted volume) portions were placed into 250 ml. conical flasks together with 10 ml. of experimental medium. Incubation was carried out at 37°C in a water-bath shaker.

2(vi) Virus titration

Virus was recovered by subjecting the infected cells to two cycles of freezing and thawing, pooling replicate samples and treating them with ultrasonics for 2 minutes. In the case of CAM fragments, coarse debris was removed by centrifugation at 1500g for 5 minutes. The virus suspension was suitably diluted in Hanks' BSS and 0.2 ml. portions of each dilution were applied to confluent monolayers of Vero cells in 6" x ⅝" glass tubes. Titrations were carried out in triplicate. After adsorption for 1 hour, the inoculum was removed and 1 ml. of 199 medium supplemented with 1% calf serum was added to each tube. Incubation was carried out for two days, at the end of which time the medium was removed and the cells stained with carbol fuchsin (Solmedia Ltd., London, England). The plaques thus revealed were counted and the mean number of plaques from dilutions giving between 30 and 300 plaques was used to estimate the number of infective virus particles present in the original suspension.

2(vii) Radio-incorporation

The overall level of protein synthesis in cells was estimated by measuring the incorporation of ^{3}H-phenylalanine or ^{3}H-leucine. The incorporation of arginine into cell proteins was determined by the use of ^{14}C-(guanido)-arginine. The extent to which citrulline was converted to arginine

in cells was determined by measuring the incorporation into cell proteins of label from ^{14}C-(carbamyl)-citrulline. The radioactive compounds were supplied to cells together with known concentrations of the unlabelled compound. The cells were equilibrated to the same concentrations of unlabelled compounds for 20 hours (6 hours in the case of mouse S180 cells) before adding experimental media containing the appropriate radiochemicals. Incubation was allowed to continue for a suitable length of time. In order to determine the amount of label incorporated, the following procedure was used:-

The cells were subjected to two cycles of freezing and thawing, replicate samples were pooled in centrifuge tubes and treated with ultrasonics for 2 minutes. A known volume of extract was removed for protein estimation. To the remainder of each extract was added trichloroacetic acid (TCA) to a concentration of 10% and the tubes were heated to 56^{o}C for 5 minutes to help the flocculation of protein. The tubes were centrifuged at 1500g for 5 minutes and the precipitates were washed three times with 5 ml. quantities of 5% TCA. The washed precipitates were dissolved in 0.5 ml. of Soluene -350 tissue solubilizer (Packard Instruments Ltd.) and each solution was transferred to a scintillation vial by washing with two 5 ml. portions of Bray's liquid scintillator (Bray, 1960). The radioactivity present in each vial was determined using a Packard three-channel liquid-scintillation spectrometer, model 3310. Quench corrections were made either by a channels-ratio method or by an external standard ratio method.

All radio chemicals used were obtained from the Radiochemical Centre, Amersham, England.

2(viii) Protein determination

Protein was estimated quantitatively by the method of Sutherland et al (1949).

To a solution of 2% w/v sodium carbonate in 0.1N sodium hydroxide was added, to concentration of 2%w/v, a solution consisting of equal volumes of 1% w/v copper

sulphate and 2% w/v sodium potassium tartrate solutions. This solution was added in 5 ml. portions to 0.1 ml. aliquots of protein solution dissolved in 0.9 ml. of 1N sodium hydroxide and left for 10 minutes. After this time, 0.5 ml. of Folin-Ciocalteau's reagent was added. After 30 minutes the absorbance was measured with an EEL colourimeter (Evans Electroselenium Ltd., Halstead, Essex, England) using a red (607) filter. Bovine serum albumen solutions of known concentration were used as standards from which a calibration curve could be drawn.

2(ix) Chromatography

One-dimensional thin-layer chromatography and molecular exclusion chromatography were employed. Thin layer chromatography was carried out using Merck Kieselgel plates (Anderman and Co. Ltd., London, England). The solvent system used was butan - 1 - ol, acetic acid and water in the proportions 12:3:5 respectively. Samples and standards were applied to the plates in 0.01 ml. volumes. After the applied solutions had dried, the plates were placed in a glass tank (Shandon Chromatank, Shandon Ltd., London, England) containing a quantity of solvent sufficient to immerse the bottom of the plates below the line of the applied samples. The tank was sealed with Apiezon grease (Edwards High Vacuum Ltd., Crawley, Sussex, England). After development, the plates were dried and sprayed with Ehrlich's reagent to develop the chromatogram. Ehrlich's reagent consists of a mixture of 1 volume of p-methylamino benzaldehyde, 10% in concentrated hydrochloric acid, and 4 volumes acetone, prepared immediately before use. Maximum colour development occurs in about 30 minutes. Urea is seen as a bright yellow colour and appears immediately. Citrulline also shows up as a yellow colour, but this appears slowly. Spots were identified by comparison with standards run at the same time.

Molecular exclusion chromatography was carried out using Sephadex G-10 (Pharmacia Fine Chemicals AB, Uppsala, Sweden) packed in a column 1.6 cm. in diameter to a height

of about 30 cm. The gel was equilibrated to PBS (A) before use. The void volume was determined using Dextran Blue, a high molecular weight dye that is totally excluded from the gel.

2(x) Enzyme assays

Two methods were used for the measurement of the activity of argininosuccinate synthetase and argininosuccinate lyase.

(a) Radiochemical assay

This assay relies on the action of the two enzymes on ^{14}C-(carbamyl) - citrulline to produce ^{14}C - (guanido) - arginine. The two enzymes are subject to feed-back inhibition by arginine, so the labelled arginine must be removed as it is formed. This is done by using excess arginase, which results in the formation of ^{14}C-urea and ornithine. The ^{14}C-urea is then treated with urease to produce $^{14}CO_2$ and NH_3. The evolved $^{14}CO_2$ is collected and the radioactivity is measured. The procedure is based on that of Carulli, Kaihara and Wagner (1968) and was developed by Cooke (1972).

The apparatus consists of two glass scintillation vials joined at the neck by means of a rubber collar (Plate 2.1). Through the side of the collar is a metal canula by means of which liquid can be added to the lower vial. The canula is attached to a piece of plastic tubing which can be closed by means of a roller clamp. In the upper vial is a cylinder, 4 cm. x 8 cm. of Whatman No. 1 filter paper soaked with 0.4 ml. of 0.1 N potassium hydroxide to trap carbon dioxide as potassium bicarbonate. The lower vial contains the reaction mixture.

The apparatus was kept upright in a metal rack and the reactions were carried out at37^{o}C in a water-bath shaker. At the end of each experiment the upper vials were dried *in vacuo* over phosphorus pentoxide and to each vial was added 25 ml. of a toluene-based liquid scintillator consisting of:-

A) Filter paper soaked with KOH

B) Plastic tubing for the addition of reagents

C) Reaction mixture

Plate 2.1 The apparatus for the radiochemical assay of enzyme activity

2, 5-diphenyl oxazole (PPO) 4 gm.

1, 4-bis-(2-(4-methyl, 5-phenyl oxazolyl)) - benzene (POPOP) 50 gm.

Toluene 1 l.

PPO and POPOP were obtained from Packard Instruments Ltd. Toluene was obtained from British Drug Houses Ltd.

The efficiency of this system has been determined (Cooke, 1972). It was found that the quantitative recovery of $^{14}CO_2$ was greater than 95% and that maximum recovery was achieved after absorption for 1 hour. The efficiency of counting was found to be 60%.

This method was used to measure the combined activity of the two enzymes involved in the conversion of citrulline to arginine.

The reaction mixture for each sample consisted of:-

Aspartic acid	2.5 µmoles
Adenosine 5'-triphosphate	1.2 µmoles
Phospho(enol)pyruvate	5.0 µmoles
Pyruvate kinase	40 µgm.
KCl	10 µmoles
$MgCl_2$	1.2 µmoles
Citrulline	20 µmoles
^{14}C-(carbamyl)-citrulline (specific activity 0.1 µ Ci/ µmole)	1 µmole

The reagents were dissolved in 0.5 ml. Tris-HCl buffer, 0.05 M, pH 8.0. To this mixture was added 0.5 ml. of a solution of arginase at an activity of about 400 units/ml.

^{14}C-(carbamyl)-citrulline was obtained from the Radiochemical Centre, Amersham, at a specific activity of 53 to 55 µ Ci/µmole. The specific activity was adjusted by the addition of 'cold' citrulline. All the other organic reagents were obtained from Sigma, London Ltd.

1. Purification of arginase

Arginase was purified according to the method of Bach, Hawkins and Swaine (1968). Partially purified arginase (Sigma, London Ltd.) at a specific activity of 42 units/mg.

was used. This enzyme preparation was dissolved in 100 mg. amounts by stirring for 2 hours in 10 ml. of 'manganese - maleate' buffer, pH 6.8 (8.9 gm. $MnSO_4$ and 1.8 gm. disodium maleate in 1 litre of glass distilled water. The pH was adjusted by the addition of 1N - hydrochloric acid).

The enzyme solution was centrifuged at 1500g for 5 minutes to sediment any undissolved material and the supernatant was dialysed at 4°C for 20 hours, against 1 litre volumes of 'manganese-maleate' buffer. The buffer was changed once during this period. The dialysed material was treated with sodium hydroxide to raise the pH to 7.3, warmed to 37°C and heated at 65°C for 3 minutes. The resulting precipitate was removed by centrifugation at 1500g for 5 minutes, the supernatant recovered and stored at -30°C.

2. Assay procedure

The activity of the arginase preparation was measured as follows:-

The assay apparatus already described was used. Into the lower vial was placed 0.5 ml. of a solution containing 0.32 M arginase and 0.012 mM $MnCl_2$ in 0.05 M Tris -HCl, pH 8.0. To this was added 0.1 μ Ci ^{14}C-(guanido) arginine of specific activity 55 to 60 μ Ci μmole and 0.5 ml. of a diluted sample of the arginase preparation. The apparatus was immediately sealed and shaken at 37°C for 1 hour. After this time, the reaction was stopped by boiling for three minutes and the pH adjusted to 7.0 by the addition of 0.5 ml. of 1 N hydrochloric acid followed by 1.0 ml. of 1.6 M Na_2HPO_4. Five units of urease (type C-3. Sigma, London Ltd.) in 0.5 ml. of 0.1M phosphate buffer, pH 7.0, were added, the apparatus resealed and shaken at 37°C for 20 minutes. At the end of this time 1.0 ml. of 1 N hydrochloric acid was added and the apparatus was shaken at 37°C for 1 hour. The upper vial was then dried and the radioactivity measured as previously described.

The combined activity of argininosuccinate lyase and

argininosuccinate synthetase was measured in exactly the same way, except that the reaction mixture used was that previously described, containing ^{14}C-(carbamyl)-citrulline.

(b) Spectrophotometric assay

This assay is based on a blood urea nitrogen test that detects ammonia (Manoukin and Fawaz, 1969; Fig. 2.1).

The reaction mixture for each sample was as follows:-

β-NADH	25	μmoles
L-glutamate dehydrogenase	15	units
Urease	1	unit
Arginase	50	units
Argininosuccinic acid	2.5	μmoles

All in a total volume of 3.0 ml.

Arginase was used as a solution, purified as previously described, at a concentration of 400 units/ml in 'manganese-maleate' buffer, pH 7.3. Urease was used as a solution at a concentration of 10 units/ml in a phosphate buffer at pH 7.0. All other reagents were dissolved in a sodium-Tris-maleate buffer, pH 7.4, which consists of a mixture of two solutions:-

A. 6.05gm Tris buffer + 5.8gm. maleic acid in 100 ml. of distilled water

B. 0.5 N NaOH

The appropriate buffer is prepared by the addition of 25 ml. of A to 27 ml of B and the volume is made up to 100 ml. with distilled water.

Cell extracts were assayed in quantities of 2 mg. cell protein in 0.5 ml. buffer. Before the addition of the extract, the optical density at 340 nm of the reaction mixture was measured against a buffer blank in a PYE Unicam SP 1800 spectrophotometer using a cell having a light path of 1 cm. The extract was then added and the reaction allowed to proceed at 30°C for 60 minutes. At the end of this time the reaction was stopped by boiling for 3 minutes and the optical density of the solution again measured after removing any precipitate by centrifugation. Controls consisted of:-

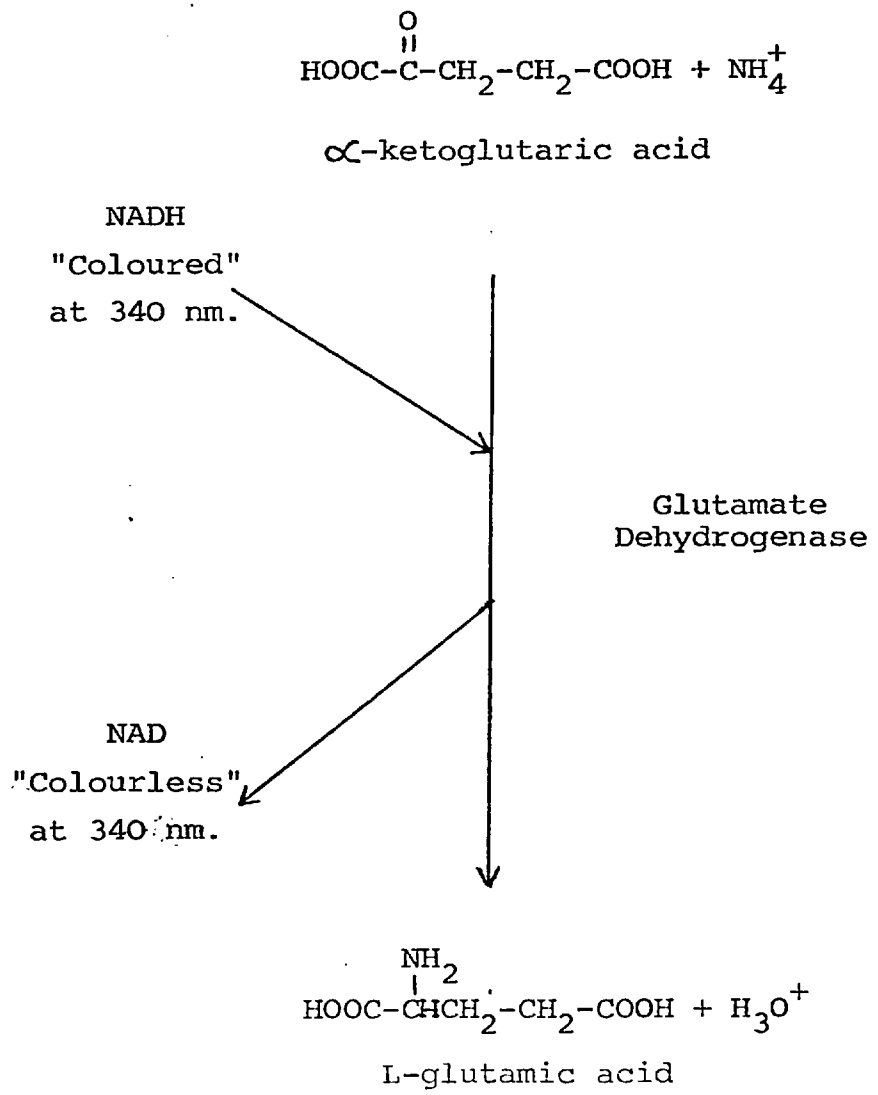


Fig. 2.1 The spectrophotometric detection of ammonia in solution.

buffer replacing extract; boiled extract replacing extract; buffer replacing substrate; buffer replacing arginase.

CHAPTER 3

STUDIES ON THE REPLICATION OF POXVIRUSES IN MOUSE SARCOMA 180 CELLS

3(i) Introduction

Studies by Cooke and Williamson (1973) have shown that rabbitpox virus will grow in the presence of citrulline in cells defective in their ability to synthesize arginine from citrulline (ASSL-defective). It was shown further (Williamson and Cooke, 1973) that this ability of rabbitpox virus to grow in such cells in the presence of citrulline is almost certainly due to the production of virus-coded enzymes for the biosynthesis of arginine from citrulline.

The proposed study is an attempt to extend these observations to other poxviruses. Initial experiments involved the screening of a number of poxviruses for their ability to replicate in the presence of citrulline. Clearly, such screening is facilitated by the use of ASSL-defective cells. Furthermore, in the presence of citrulline the metabolism of such cells will be reduced drastically, allowing the effects of poxvirus infection to be observed more clearly. It will be possible also in ASSL-defective cells to measure in absolute terms the activities of the enzymes of arginine biosynthesis from citrulline following infection with poxvirus, rather than relative increases in activity.

The ASSL-defective cells used by Cooke and Williamson (1973) were mouse sarcoma 180 cells (Tytell and Neuman, 1960). Consequently, the suitability of mouse S180 cells for biological studies with poxviruses other than rabbitpox virus was investigated.

3(ii) The growth of mouse S180 cells

The growth of S180 cells was examined in the presence of 0.5mM citrulline or 0.5mM arginine in PE medium containing 5% calf serum and in the absence of either amino-acid in the same medium. The results, presented in Table 3.1, show that in the presence of citrulline the number of viable cells present after 6 days was no greater than the number of cells seeded originally. Furthermore, in a medium containing neither arginine nor citrulline, no

Table 3.1

Multiplication of mouse S180 cells in the presence of citrulline or arginine

Medium	Viable cell count ($x10^{-5}$) at 6 days
0.5mM citrulline	0.8
0.5mM arginine	13.0
Arginine free	No viable cells recovered

Viable cell count at time, t=0 $0.9x10^{5}$

viable cells could be recovered. All the cells originally present had died and had come off into the medium. However, in the presence of arginine the number of viable cells increased almost 15-fold. These data show that while mouse S180 cells are capable of growing in the presence of arginine, they will not grow in the presence of citrulline. Furthermore, these cells will not survive in the absence of both amino acids. This indicates that some biosynthesis of arginine from citrulline occurs, but at a rate that is sufficient only for maintenance of basal metabolism, not for cell multiplication. It can also be seen that the rate of growth of mouse S180 cells is quite low in this medium, the doubling time being about 36 hours.

The level of protein synthesis in mouse S180 cells in the presence of 0.5mM citrulline or 0.5mM arginine was determined by measuring the incorporation of radioactively labelled amino-acids. The radiochemicals used were ^{14}C-(carbamyl)-citrulline at a specific activity of 62 mCi/m.mole, ^{14}C-(guanido)-arginine at a specific activity of 60mCi/m.mole and ^{3}H-phenylalanine at a specific activity of 406 mCi/m.mole. Each ^{14}C compound was used at a concentration of 0.1 μCi/ml. the ^{3}H compound at a concentration of 0.5 μCi/ml. Phenylalanine is quite widespread amongst proteins and so the level of its incorporation gives a good estimate of the overall level of protein synthesis. Arginine is incorporated directly into some proteins, but citrulline is not. Therefore the amount of ^{14}C-label incorporated from ^{14}C-citrulline in the presence of citrulline is a measure of the extent of bioysnthesis of arginine from citrulline.

Cells were exposed to the appropriate radiochemicals over a 24-hour period. The results, given in Table 3.2, show that the level of incorporation of label from citrulline is much lower than the level of incorporation of label from arginine. In fact, the amount of citrulline utilized is 18% that of arginine. However, the reduction of incorporation of phenylalanine in medium containing citrulline as compared to that in medium containing arginine is not so drastic, only 54%. Thus, these results show that the reduction of the incorporation of

Table 3.2

Incorporation of radioactivity available as ^{14}C-(guanido)-arginine, ^{14}C-(carbamyl)-citrulline and ^{3}H-phenylalanine into TCA-precipitable material in mouse S180 cells

Medium	Radioactivity incorporated* (DPM x 10^{-3}/mg. cell protein)		
	Citrulline	Arginine	Phenylalanine
0.5mM Citrulline	3.15	-	6.10
0.5mM Arginine	-	17.94	13,33

* Amounts of radioactivity are expressed on the basis of identical specific activities (0.1μCi/μmole).

^{14}C-label in the presence of citrulline is only partly due to the general reduction of incorporation of amino-acids into protein. Thus, the reduction is due specifically to a low level of synthesis of arginine from citrulline. These studies confirm that mouse S180 cells are defective in their ability to utilize citrulline. Thus, these cells have been shown to be a suitable system for studying the metabolism of arginine following infection with poxvirus.

3(iii) The replication of poxvirus in mouse S180 cells

Initial experiments were performed to investigate the replication in mouse S180 cells of vaccinia virus, buffalopox virus, monkeypox virus, rabbitpox virus, elephantpox virus and cowpox virus. Monolayers of mouse S180 cells in 6" x ⅝" tubes were infected with about 10^6 pfu per tube of virus in the presence of 1.0mM citrulline or 1.0mM arginine as described in Materials and Methods. Virus yields were measured after 24 hours (Table 3.3). The yield was estimated by dividing the number of virus recovered by the initial inoculum recovery. It can be seen that rabbitpox virus gave good yields in the presence of either 1.0mM arginine or 1.0mM citrulline, although the yield in the presence of citrulline was slightly lower. However, no yield was obtained in the absence of both amino acids. Cowpox virus gave a small yield in the presence of arginine, but no yield was obtained in the presence of citrulline or in the absence of both citrulline and arginine. No yields were obtained of any of the other poxviruses even in the presence of 1.0 mM arginine.

The results show that in mouse S180 cells no replication occurs within 24 hours of buffalopox virus, elephantpox virus or monkeypox virus and that these cells are defective in their ability to support the replication of cowpox virus. However, rabbitpox virus will grow well in these cells after 24 hours. It is shown also that while cowpox virus produces low yields of infectious virus, growth appears to be inhibited in the presence of citrulline. This indicates that cowpox virus has a defect in its ability to utilize citrulline.

Table 3.3

Poxvirus replication in mouse S180 cells in the presence of arginine or citrulline

Virus	Virus yield (log_{10})		
	1.0mM arginine	1.0mM citrulline	Arginine free
Buffalopox	NY*	NY	NY
Monkey pox	NY	NY	NY
Vaccinia	NY	NY	NY
Cowpox	0.3	NY	NY
Elephantpox	NY	NY	NY
Rabbitpox	2.1	1.6	NY

* No yield

This conclusion was confirmed by further experiments. The results, in Table 3.4, show that in the presence of 1.0 mM arginine a significant, but small, yield of cowpox virus is obtained (about 13-fold) whereas in the presence of 1.0 mM citrulline, 2% calf serum or in the absence of arginine and citrulline no yield is obtained. In fact, the virus recovered is less than the inoculum recovery. This negative yield is most probably the result of the decay of the initial background of adsorbed virus. In the absence of any virus replication this would give a decrease with time in the amount of virus recovered. No virus replication would be expected in the presence of unsupplemented PE medium, but the fact that the virus recovered in the presence of citrulline is less than that recovered in unsupplemented PE medium indicates that cowpox virus is defective in its ability to utilize citrulline. It is shown also that the batch of calf serum used, at a concentration of 2%, does not contain sufficient free arginine to allow the replication of cowpox virus.

The experimental limitations imposed by the low yields of cowpox virus in the presence of arginine led to attempts to adapt cowpox virus to mouse S180 cells. This was done by serial passage of cowpox virus in these cells. However, it was not found possible to improve by this means the growth characteristics of cowpox virus in mouse S180 cells.

To determine whether or not increasing the amount of arginine available would give higher yields, mouse S180 cells were infected with cowpox virus at a m.o.i. of 10pfu/cell and yields were measured after incubation for 24 hours in the presence of different concentrations of arginine or citrulline. Rabbitpox virus gives good yields in mouse S180 cells (Cooke, 1972) and so was used as a control in these studies. From Table 3.5a it can be seen that low yields of rabbitpox virus and cowpox virus were obtained even in the presence of 1 mM or 2 mM arginine. The fact that low yields of rabbitpox virus were obtained after 24 hours indicates that mouse S180 cells will support only a low rate of poxvirus

Table 3.4

The replication of cowpox virus in mouse S180 cells in the presence of arginine or citrulline

Medium	Infectivity titre (pfu/ml. x 10^{-4})
1.0mM arginine	120
1.0mM citrulline	0.4
2% calf serum	1.2
Arginine, serum free	1.2

Inoculum recovery 9 x 10^4 pfu/ml.

replication. To test this possibility, yields of rabbitpox virus and cowpox virus were measured at later times after infection. In order to magnify any difference that might have been obtained in yields with the different media, a low inoculum of virus (m.o.i. 0.01 pfu/cell) was applied to the cells so that multiple cycles of virus replication could occur. The results are presented in Tables 3.5b and 3.5c. It can be seen that good yields of rabbitpox virus were obtained after 48 hours and 72 hours in the presence of either arginine or citrulline. However, no improvement in the yield of cowpox virus was obtained even after 72 hours. After 48 hours (Table 3.5b) the yields of cowpox virus obtained in 1.0mM arginine and 2.0mM arginine were about five-fold. However, in the presence of citrulline, from 1.0mM up to 4.0mM, no yield of virus was obtained. This indicates, again, that cowpox virus is unable to utilize citrulline.

The results in Table 3.5c show that after 72 hours the yield of cowpox virus obtained in the presence of 2.0mM arginine was about the same (3-fold) as in the presence of 1.0mM citrulline. Furthermore, the yield of cowpox virus rose with increasing concentration of citrulline. At a concentration of 6.0mM citrulline a 17-fold increase was obtained. These results indicate that over a prolonged period the inability of cowpox virus to grow in mouse S180 cells in the presence of citrulline is overcome to an extent that increases with the concentration of citrulline.

3(iv) Discussion

The initial experiments on uninfected mouse S180 cells confirmed the lack of ability of these cells to utilize citrulline in place of arginine. In the presence of citrulline no growth was observed and overall protein synthesis was reduced to 46% of that observed in the presence of arginine. Furthermore, the utilization of labelled citrulline in the presence of citrulline was very much reduced, to 18% of the incorporation of labelled arginine in the presence of arginine. The cell

Table 3.5

Virus replication in mouse S180 cells in the presence of arginine or citrulline

a. Yields of infective virus particles at 24 hours post-infection

Medium	Infectivity titre (pfu/ml. x 10^{-5})	
	Rabbitpox virus	Cowpox virus
0.05mM arginine	4.9	4.6
1.0mM arginine	49	-
2.0mM arginine	-	31
1.0mM citrulline	16	14
2.0mM citrulline	15	9
Inoculum recovery	0.3	3
Inoculum*	500	500

* To each cell monolayer was applied 0.2ml. of virus suspension

Table 3.5

Virus replication in mouse S180 cells in the presence of arginine or citrulline

b. Yields of infective virus particles at 48 hours post infection

Medium	Infectivity titre (pfu/ml. x 10^{-3})	
	Rabbitpox virus	Cowpox virus
0.05mM arginine	-	2.8
1.0mM arginine	44	14
2.0mM arginine	60	16
1.0mM citrulline	58	3
2.0mM citrulline	22	6
4.0mM citrulline	-	3
Inoculum recovery	0.3	3
Inoculum*	50	50

* To each cell monolayer was applied 0.2ml of virus suspension.

Table 3.5

Virus replication in mouse S180 cells in the presence of arginine or citrulline

c. Yields of infective virus particles at 72 hours post infection

Medium	Infectivity titre (pfu/ml. x 10^{-3})	
	Rabbitpox virus	Cowpox virus
2.0mM arginine	67	1.0
1.0mM citrulline	>250	1.0
2.0mM citrulline	-	1.5
4.0mM citrulline	-	4.6
6.0mM citrulline	-	5.2
Inoculum recovery	0.3	0.3
Inoculum*	50	50

* To each cell monolayer was applied 0.2 ml. of virus suspension

growth experiments showed also that while the cells did not grow in the presence of citrulline they did not survive in the absence of both citrulline and arginine. This is probably due to the fact that the enzymes for the biosynthesis of arginine from citrulline are present in these cells, but their substrate affinity is very low (J.D. Williamson, Personal Communication). Thus sufficient arginine can be synthesized from citrulline to maintain a basal rate of metabolism, but insufficient to allow the cells to grow. These studies show that mouse S180 cells appear to be eminently suitable for the investigation of arginine metabolism following infection with poxvirus.

However, it was shown that the usefulness of the mouse S180 cell system is severely limited by the fact that of the number of poxviruses studied only rabbitpox virus gave good yields of progeny virus in the presence of arginine.

Cowpox virus was the only other virus of those studied that gave a significant, albeit small, yield in the presence of arginine. Furthermore, cowpox virus seems able to replicate only in the presence of arginine, not in the presence of citrulline. This indicates that cowpox virus is defective in its ability to utilize citrulline. However, the interpretation of these results is made difficult owing to the low yields obtained. Attempts to improve the yields by "adaptation" met with no success.

Further studies on the replication of rabbitpox virus and cowpox virus in mouse S180 cells showed that a significant yield of rabbitpox virus could be obtained from infected cells only after incubation for 48 hours or longer. This is probably a reflection of the fact that the mouse S180 cells used in these experiments were themselves slow growing and also of the fact that mouse S180 cells are very sensitive to arginine deprivation (Cooke, 1972). Good yields of cowpox virus in mouse S180 cells could not be obtained even after incubation for 72 hours. However, the yields obtained after 48 hours were higher in the presence of 1.0mM arginine than in the presence of an equimolar concentration of citrulline.

This gives a further indication that cowpox virus is defective in its ability to utilize citrulline. It was shown also that after 72 hours the yield of cowpox virus in the presence of citrulline increased with the concentration of citrulline. A possible explanation for this phenomenon is that supplying increasing concentrations of citrulline overcomes the inability of mouse S180 cells to synthesize arginine from citrulline. It is known (Tedesco and Mellman, 1967) that the inability of human citrullinaemia cells to grow in the presence of citrulline is due to the low substrate affinity of the enzymes for arginine biosynthesis from citrulline within these cells. Previous experiments (Williamson, J.D., Personal Communication) have shown that this is true also of mouse S180 cells. A sufficiently high concentration of citrulline will compensate for the low substrate affinity of the enzymes, thus the cells will produce sufficient arginine from citrulline to allow poxvirus replication to occur. This means that increasing the concentrations of citrulline will produce an increase in the yield of cowpox virus from mouse S180 cells.

These studies show that while mouse S180 cells are defective in their ability to utilize citrulline they are defective also in their ability to support the replication of poxviruses except rabbitpox virus. In addition, Cooke (1972) has shown that the IHD strain of vaccinia vitus will give good yields in these cells. Thus, of the poxviruses examined only two are known to give good yields in mouse S180 cells. This means that these cells have a very limited usefulness in any studies on arginine metabolism following infection with poxvirus.

However, experiments performed using mouse S180 cells have indicated that cowpox virus is defective in its ability to utilize citrulline.

CHAPTER 4

THE REPLICATION OF POXVIRUS IN CAM CELLS

4(i) Introduction

Previous experiments have shown that while mouse S180 cells are defective in their ability to utilize citrulline they are defective also in their ability to support the replication of many poxviruses. Of the other cell lines known to be ASSL-defective only two are stable cell lines, viz Walker carcinoma 256 cells (Tytell and Neuman, 1960) and human citrullinaemia cells (Tedesco and Mellman, 1967). Obviously, it is an advantage to use a stable cell line because it allows the easy production of large numbers of cells. However, Walker carcinoma cells are derived from the rat, thus their use would be expected to present difficulties similar to those experienced with the use of mouse S180 cells, derived from a closely related species. Furthermore, the availability of Walker carcinoma cells is very limited. Because of these considerations the use of Walker carcinoma cells was not investigated. The other available stable cell line with the required metabolic defect, the human citrullinaemia line, has been used in previous studies on the replication of poxviruses (Cooke, 1972) but those available were of such a high passage number that further growth proved impossible.

In the absence of a suitable stable cell line it was necessary to consider the possibility of using a primary cell culture. Primary cells found to be unable to utilize citrulline (Tytell and Neuman, 1960) include chick-embryo cells derived from the heart, the lung and the chorio-allantoic membrane (CAM). The chick CAM *in ovo* will support the replication of a large number of poxviruses and it is reasonable to propose that the cells derived from the CAM will retain this characteristic *in vitro*. Therefore, the cultivation *in vitro* of these cells and their suitability as a system for studying arginine metabolism in poxvirus infection were investigated.

4(ii) The preparation of CAM cells by trypsinization

Tytell and Neuman (1960) prepared cells from the chick CAM by treating the tissue with 0.2% trypsin in Earle's BSS at 37°C for 2 to 6 hours.

Attempts were made to use this method, but very low yields of cells were obtained. In a typical experiment, about 5×10^6 cells were recovered from the CAM of 40 11-day old chick embryos. The few cells that were recovered were incubated at 37°C in 6" x ⅝" glass tubes at a concentration of 5×10^5 cells/tube in a medium similar to that used by Tytell and Neuman (1960). This medium was McCoy's 5A medium (Iwokata and Grace modification) supplemented with 10% calf serum and 7.5% of an extract obtained from chick embryos

The chick embryo extract was prepared from 11-day old embryos, which were decapitated and expressed through a 10 ml. sterile plastic syringe. After washing with Hanks' BSS, an equal volume of Hanks' BSS was added. The mixture was stirred, left at room temperature for 30 minutes and centrifuged at 2000g for 20 minutes. The supernatant was collected and stored frozen at -30°C. Before use, the frozen extract was thawed slowly and spun at 2000g for 10 minutes.

After 18 hours incubation, the cells were examined microscopically and it was found that some cells had stuck to the glass. Fresh growth medium was added and the cells examined after a further 24 hours incubation at 37°C. It was seen that most of the cells had come off the glass into the medium. The use of higher concentrations of serum, up to 30%, did not improve the viability of the cells. It was concluded that the extensive trypsin treatment required to remove cells from the CAM affects adversely the viability of the CAM cells.

In view of the difficulty experienced in preparing cells from the CAM, the possibility was investigated of using small pieces of CAM tissue as a substitute for a cell monolayer.

4(iii) Preparation of CAM fragments

Fragments were prepared by the method of Watanabe and Okada (1974) according to whom membrane explants applied to a suitable substratum will stick and grow out. This peripheral cell growth allows direct cytological examination, which is not possible with pieces of membrane. The method of preparation is described in Materials and Methods. It was found that very few fragments stuck to the surface of the Petri-dish. However, those that did stick produced extensive growth from the periphery of the tissue explant. Thus, if sufficient fragments could be made to stick a sufficiently large cell sheet would be produced to allow this system to be used in studies on virus replication.

In an attempt to increase the number of fragments adhering to the substratum, the fragments were incubated under various conditions; in glass Petri-dishes, in plastic dishes obtained from different commercial sources, in plastic dishes of which the surface had been roughened and in the presence of carbon dioxide instead of HEPES. However, the numbers of fragments adhering remained small and variable. Despite this, attempts were made to measure protein synthesis in the cells of these fragments under various conditions, using radioactively labelled amino acids. However, because of the small amount of cellular material present the amount of protein was too small to be measured with any degree of accuracy. Thus, the results obtained from the radioincorporation experiments were of little significance. Virus growth experiments also were unsuccessful owing, again, to the fact that the yields of virus obtained depended on the numbers of fragments present.

Because of the difficulties involved in getting fragments to stick to the substratum, fragments were maintained in suspension using PE medium as described in Materials and Methods. An experiment was performed on such a suspension culture to determine the effect of different concentrations of arginine or citrulline on the incorporation of ^{14}C-(carbamyl)-citrulline (specific

activity 53 μCi/μmole, concentration 0.14 μ Ci/ml.) ^{14}C-(guanido) - arginine (specific activity 55.3μ Ci/μmole, concentration 0.12 μ Ci/ml.) and ^{3}H-leucine (specific activity 1Ci/m.mole, concentration 0.5 μ Ci/ml.). The results presented in Table 4.1. show that in the presence of citrulline, overall protein synthesis, as indicated by the incorporation of leucine, reaches a maximum at 0.2mM citrulline. However, the incorporation of citrulline appears to rise with increasing concentration up to 1.0 mM citrulline. In the presence of arginine, overall protein synthesis rises to a peak at 0.5 mM as does the incorporation of arginine. Thus, 0.2 mM citrulline or 0.5 mM arginine apparently allow optimal metabolic activity in the cells of CAM fragments and so these concentrations were used in the subsequent incorporation experiment (Table 4.2). It can be seen that the overall level of protein synthesis in the presence of citrulline is 36% that in the presence of arginine. However, the incorporation of label from citrulline is 2% that from arginine. In both experiments incorporation was allowed to occur for 40 hours. If the comparable figures in Table 4.1 are examined it will be seen that the overall level of protein synthesis in 0.2 mM citrulline is about 83% that in the presence of arginine. However, the incorporation of label from citrulline is 13% that from arginine. Thus, both sets of results show that the cells of CAM fragments are defective in their ability to utilize citrulline. However, the difference between the figures obtained from the two experiments indicates that the cells of the fragments used in each experiment differ in the amounts of intracellular free arginine present, despite the fact that the fragments were treated in an identical fashion in each experiment. This indicates that one difficulty involved in the use of CAM fragments is that of depleting the intracellular pools of free arginine.

When the growth of rabbitpox virus in CAM fragments was examined in the presence of PE medium containing 1 mM arginine or 1 mM citrulline or neither amino acid, no yield of virus could be obtained even after incubation

Table 4.1

Incorporation of radioactivity available as ^{14}C-(carbamyl)-citrulline, ^{14}C-(guanido)-arginine and ^{3}H-leucine into TCA-precipitable material in CAM fragments maintained in suspension in the presence of different concentrations of arginine or citrulline

Medium	Radioactivity incorporated* (DPM x 10^{-3}/mg. cell protein)		
	Leucine	Citrulline	Arginine
0.1mM citrulline	30.9	2.7	-
0.2mM citrulline	44.1	4.7	-
1.0mM citrulline	41.2	7.8	-
0.1mM arginine	27.64	-	13.9
0.2mM arginine	35.3	-	20.0
0.5mM arginine	52.9	-	37.2
1.0mM arginine	31.8	-	24.2
No citrulline, arginine	21.2	-	-

* Amounts of radioactivity are expressed on the basis of identical specific activities (0.1 μCi/μmole)

Table 4.2

Incorporation of radioactivity available as ^{14}C-(carbamyl)-citrulline, ^{14}C-(guanido)-arginine and ^{3}H-leucine into TCA-precipitable material in CAM fragments maintained in suspension in the presence of arginine or citrulline

Medium	Radioactivity incorporated* (DPM x 10^{-3}/mg. cell protein)		
	leucine	citrulline	arginine
0.5mM arginine	40.6	-	57.6
0.2mM citrulline	14.7	1.2	-

* Amounts of radioactivity are expressed on the basis of identical specific activities (0.1μCi/μmole).

for 72 hours (Table 4.3). The results show that after 24 hours the number of infectious viral particles is less in each case than the original applied inoculum, probably the result of the decay of adsorbed virus particles. After 48 hours in the presence of 1.0 mM arginine or 1.0 mM citrulline, the number of infectious particles rose 3-fold to 4-fold. In the absence of either amino acid the rise is 2-fold. These results indicate that some virus replication has occurred. The increase in infectious virus particles recovered in the absence of citrulline and arginine indicates that the arginine pools of the cells of these fragments had not been entirely depleted, allowing limited virus replication. After 72 hours the numbers of infectious virus particles recovered in the presence of arginine or citrulline remained the same as those obtained after 48 hours. This shows that under the conditions used the cells of these fragments are capable of supporting only a limited amount of virus replication. The decrease of infectious virus particles in the absence of either amino acid is probably the result of the eventual depletion of the arginine pools stopping the replication of virus, together with the decay of the non-replicating virus.

These results show that CAM fragments in suspension are defective in their ability to utilize citrulline, but they appear to be defective also in their ability to support the replication of poxvirus. This, together with the apparent difficulty involved in the depletion of the intracellular pools, led to the investigation of a different system.

4(iv) The replication of virus in CAM pieces attached to the shell.

The technique of growing viruses in pieces of chick-embryo CAM attached to the shell was developed by Fazekas de St. Groth and White (1958) for use with influenza virus. The technique was developed to overcome certain short-comings involved in the use of fertile hens' eggs or mice for the measurement of infectivity of influenza virus.

Table 4.3

The replication of rabbitpox virus in CAM fragments in suspension in the presence of citrulline or arginine.

Time (hours)	Infectivity titre (pfu/ml. x 10^{-6})		
	1.0mM Arginine	1.0mM Citrulline	No citrulline No arginine
24	0.5	0.6	0.3
48	2.0	2.0	0.6
72	2.0	2.0	0.3

Inoculum recovery 2 x 10^6 pfu/ml.

Pieces of CAM membrane were prepared as described in Materials and Methods. To each piece was added 0.5 ml. of medium containing 2 x 10^4 pfu/ml. of buffalopox virus. This virus was used because it is one of those poxviruses that gave no yield of progeny virus in mouse S180 cells and so provides a test for the suitability of the CAM system for these studies. Incubation was carried out for 72 hours, at the end of which time the pieces of membrane were removed from the shell. Replicate pieces were pooled, together with the medium in which they had been incubated. The pooled replicates were frozen, thawed and treated with ultrasonics for 10 minutes. The debris was removed by centrifugation and the supernatant virus suspension was titrated as previously described. The inoculum recovery was estimated by freezing and thawing some of the original medium containing virus and titrating as before. The results are given in Table 4.4. It can be seen that high yields were obtained both in a complete growth medium and in PE medium containing citrulline in place of arginine. The results in Table 4.3 show that no yields of rabbitpox virus were obtained 72 hours after infection of CAM fragments maintained in suspension in PE medium containing arginine or citrulline. These results show that CAM cells in pieces of membrane are capable of supporting the growth of at least one poxvirus, but that CAM cells in small fragments of minced tissue allow very little replication of virus. This defect of cells in CAM fragments may well be a result of the severe treatment to which the CAM is subjected to produce fragments. Alternatively, this difference between cells in CAM pieces attached to the shell and cells in CAM fragments may be a reflection of the difference in intracellular pools of nutrients.

Fazekas de St. Groth and White (1958) reported that influenza virus will replicate in pieces of CAM attached to the shell in a medium consisting solely of inorganic salts and a source of carbon. Thus, these pieces of membrane must contain intracellular pools of essential nutrients. Previous experiments have shown that CAM fragments also contain intracellular pools of arginine

Table 4.4

The replication of buffalopox virus in CAM pieces attached to the shell.

Medium	Virus yield (log_{10})
MEM + 5% calf serum	3.1
PE (arginine free) + 1.0mM citrulline	2.2

which prove difficult to deplete. Thus, there is presented a potential difficulty in the use of CAM pieces attached to the shell, a difficulty involved in depleting the intracellular pools of arginine. However, the publication of a method for the preparation of cells from the chick embryo CAM made unnecessary the further use of this technique.

4(v) The growth of CAM cells

During the course of this work, Cursiefen and Becht (1975) reported a method for the preparation of cells from the CAM of the chick embryo. The basic difference between this method and that of Tytell and Neuman (1960) is that before trypsinization the minced tissue is treated with a mixture of hyaluronidase and collagenase. It was reported that this initial, double enzyme treatment is a prerequisite for getting individual cells into suspension by trypsin and that no cells can be removed from the CAM by conventional trypsinization alone. The two enzymes, hyaluronidase and collagenase, remove the mucin layer that is known to cover the whole CAM of the chick embryo and especially the epithelioid cells.

The details of the preparation of CAM cells using the method described by Cursiefen and Becht (1975) are given in Materials and Methods. In a typical preparation, about 1.5×10^8 cells were obtained from the CAM from 24 11-day old embryos. This is far in excess of the yields obtained using trypsin alone (1.5×10^6 cells from 40 membranes). The cells were seeded at a concentration of 5×10^6 cells per dish in 45 mm. diameter glass Petri-dishes and monolayers were formed in about 3 to 4 days. The monolayers consist mainly of cells of epithelioid appearance with large, round nuclei. Fibroblasts grew around islands of epithelioid cells.

In these studies, which concern metabolism within infected and uninfected cells, it was necessary to use a chemically defined medium. Cursiefen and Becht (1975) used 199 medium, which is a very complex medium. However,

McCoy's medium, used by Tytell and Neuman (1960), is much simpler and so this medium was used initially for the growth of CAM cells. The use of either McCoy's or 199 medium appeared to make no difference to the culture of CAM cells as far as growth rate, cytological appearance and longevity were concerned.

CAM cells were grown under a variety of conditions in order to determine their requirements for growth. It was found that optimal growth occurred in glass Petri-dishes using McCoy's 5A medium, or 199 medium, supplemented with 10% foetal bovine serum (FBS) in an atmosphere of 6% CO_2 in air saturated with water. Penicillin, streptomycin and kanamycin were used to minimize the risk of contamination with bacteria or, more importantly, with mycoplasmas. It was not found possible to grow CAM cells in MEM medium supplemented with FBS, although these cells could be maintained in this medium. The use of 199 or McCoy's medium supplemented with 10% calf serum or newborn-calf serum would not allow the growth of CAM cells. None of the plastic Petri-dishes tried, obtained from different commercial sources, gave results as good as those obtained using glass dishes. The presence of HEPES appeared to be detrimental, as did that of Nystatin, a fungicide. Thus, CO_2 was used to buffer the medium and no fungicide was used.

PE medium used in previous studies is chemically defined but is less complex than McCoy's 5A medium. The presence in PE medium of higher concentration of choline permits the use of lower concentrations of serum. Because of these factors, attempts were made to measure the growth of CAM cells in PE medium containing either 0.5 mM arginine or 0.5 mM citrulline and supplemented with 10% FBS. No growth occurred, indicating that the medium lacked some vital component(s). As these cells willgrow in McCoy's 5A medium, the basic PE medium was supplemented with those amino acids exclusive to McCoy's medium (see Materials and Methods). The use of this supplemented medium (MPE) containing 0.5 mM arginine and with added serum allowed these cells to grow and produce confluent monolayers, so MPE medium was used as the basis for the experimental media used in subsequent incorporation experiments.

4(vi) Radio-incorporation into CAM cells

The incorporation into CAM cells of ^{14}C-(carbamyl)-citrulline (of specific activity 53 mCi/m.mole and concentration 0.24 μ Ci/ml.) ^{14}C-(guanido) - arginine (of specific activity 55.3 m Ci/m.mole and concentration 0.21 μ Ci/ml.) and ^{3}H-phenylalanine (of specific activity 12 Ci/m.mole and concentration 0.7 μ Ci/ml.) was measured as previously described. The results, presented in Table 4.5, show that the incorporation of phenylalanine in the presence of citrulline is 39% that in the presence of arginine. However, the incorporation of label from citrulline is 3.0% that from arginine. These results show that CAM cells are deficient in their ability to synthesise arginine from citrulline, confirming the results obtained with CAM fragments in the present study and the previous observations of Tytell and Neuman (1960). The results show also that the incorporation of phenylalanine from 199 medium containing no serum is lower than that from MPE medium supplemented with arginine. The incorporation of arginine is much the same in both media. This is probably a reflection of the fact that while 199 medium is more complex than MPE medium, the concentrations of the various components of MPE medium are much higher than those of 199. This difference in concentrations may be sufficient to affect the overall rate of metabolism.

Having shown that uninfected CAM cells are deficient in their ability to utilize citrulline, the ability of poxviruses to utilize citrulline in these cells was investigated.

Table 4.5

Incorporation of radioactivity available as ^{14}C-(carbamyl)-citrulline, ^{14}C-(guanido)-arginine and ^{3}H-phenylalanine into TCA precipitable material in CAM cells

Medium	Radioactivity incorporated* (DPM x 10^{-3}/mg. cell protein)		
	Phenylalanine	Citrulline	Arginine
0.5mM arginine	10.00	-	4.28
0.5mM citrulline	3.88	0.13	-
No arginine No citrulline	2.44	-	-
199 (serum free)	4.85	-	4.88

* Amounts of radioactivity are expressed on the basis of identical specific activities (0.1μCi/μmole).

4(vii) The replication of poxvirus in CAM cells

Confluent monolayers of CAM cells were maintained for 18 hours in arginine-free MPE medium. The cells were infected with monkeypox virus, buffalopox virus, cowpox virus, elephantpox virus or vaccinia virus and the yields were measured after incubation for 24 hours in serum-free MPE medium containing 0.5mM arginine or 0.5mM citrulline. No virus was recovered in excess of the initial inoculum. It was possible that this lack of yield was due to deprivation of arginine causing some damage to the cells, making them incapable of supporting virus replication. Another possible explanation is that virus replication occurs slowly in these cells and that longer than 24 hours is required to obtain good yields of virus. A third possible explanation is that the medium used was deficient in some way, that it lacked some factor(s) essential for the replication of viruses.

Experiments were performed to test the possibility that arginine starvation had impaired the ability of the cells to support virus replication. Cells that had been starved for various times, or not starved, were infected with vaccinia virus in different media and virus yields were measured after 24 hours. The results given in Table 4.6 a. and b. show that similar yields were obtained from both sets of infected cells, indicating that starvation for 1 day had not affected the ability of the cells to support the growth of virus. The results presented in Table 4.7 show that there is no significant difference between the yields obtained from cells starved for 1 day and from cells starved for 2 days. This confirms that arginine starvation, even for 2 days, does not impair the ability of CAM cells to support the replication of vaccinia virus.

Table 4.6a

The replication of vaccinia virus in CAM cells starved of arginine

Medium	Virus yield (log_{10})
199 + 5% FBS	1.5
MPE (arginine free) + 5% FBS	0.6
MPE (arginine free)	0.8

Table 4.6b

The replication of vaccinia virus in CAM cells not starved of arginine

Medium	Virus yield (log_{10})
199 + 5% FBS	1.4
MPE (arginine free) + 5% FBS	0.9
MPE (arginine free)	0.7

Table 4.7

The replication of vaccinia virus in CAM cells starved of arginine for different lengths of time

Medium	Virus yield (log_{10})	
	1 day starvation	2 day starvation
199 + 5% FBS	1.8	1.9
MPE (arginine free)	1.0	0.9

It is shown also that 24 hours is sufficient time to allow good yields of virus to be obtained in 199 medium supplemented with FBS. The fact that a small yield of virus is obtained in MPE without arginine and supplemented, or nor, with serum shows that the intracellular pools of essential nutrients had not been entirely depleted.

These results eliminate the possibilities that the failure to obtain yields of vaccinia virus is due to cell-damage as a result of arginine starvation or to a low rate of replication of the virus.

This left the possibility that MPE is lacking some component(s) essential for the replication of virus. The missing component(s) might have been present in a more complex medium such as McCoy's 5A or in the serum.

Experiments were carried out to investigate the possibility that the virus growth factor missing from MPE is present in more complex medium. To this end a medium was used that consists of PE medium supplemented with all those components, except bactopeptone, exclusive to McCoy's 5A medium. This medium was designated P-Mc medium and its formulation is given in Materials and Methods. It consists of MPE medium supplemented with four vitamins (ascorbic acid, nicotinamide, pyridoxine and p-aminobezoic acid) and glutathione. Starved CAM cells were infected with vaccinia virus, cowpox virus and elephantpox virus and incubated for 48 hours in P-Mc medium containing 0.5 mM arginine and supplemented with 5% FBS, or 199 medium containing 5% FBS. The results are presented in Table 4.8. It can be seen that good yields of all the viruses were obtained and that the yields obtained in P-Mc medium were similar to those obtained in 199 medium. This shows that P-Mc medium containing arginine and serum will allow the replication in CAM cells of the three poxviruses used.

As indicated previously these studies are greatly facilitated by the use of medium containing no serum, because serum contains a variable amount of free arginine. However the possibility still remained that

Table 4.8

The replication of elephantpox virus, cowpox virus and vaccinia virus in CAM cells

Medium	Virus yield (log_{10})		
	Elephant virus	Cowpox virus	Vaccinia virus
199 + 5% FBS	2.3	1.9	3.4
P-Mc (0.5mM arginine) + 5% FBS	3.1	2.3	3.3

the essential virus-replication factor(s) was added with the serum. To resolve this, starved CAM cells were infected with vaccinia virus and incubated for 48 hours in 199 medium supplemented, or not, with 5% FBS or in P-Mc medium containing 0.5 mM arginine, but no serum. The results presented in Table 4.9 show that only the medium supplemented with serum gave a good yield of virus. Thus, the presence of FBS seems to be essential for the replication of vaccinia virus in CAM cells.

Having established the existence of a requirement for serum for the replication of vaccinia virus, the qualitative nature of this requirement was investigated in terms of molecular weight. If the requirement is for high molecular weight compound(s), then the low molecular weight constituents of the medium, particularly the concentrations of arginine or citrulline, may be readily defined. Dialysis will remove low molecular weight components from a solution, therefore FBS was fractionated by this process using Visking dialysis tubing. Dialysis was carried out for 20 hours at $4^{\circ}C$ using 2 ml. quantities of serum against 500 ml. quantities of Hanks' BSS, with one change of dialysis medium. The material remaining within the dialysis sac was sterilized by filtering through a 450 nm. Millipore filter.

CAM cells were infected with vaccinia virus and incubated for 48 hours in media supplemented with 5% dialysed serum or 5% untreated serum. The yields obtained are presented in Table 4.10. It can be seen that good yields were obtained only in the presence of untreated serum, indicating that dialysis had removed from the serum the factor(s) essential for the replication of vaccinia virus in CAM cells.

Since dialysis will remove from a solution any compound of molecular weight 20,000 or less, the serum factor(s) must have a molecular weight less than that value. In order to obtain more precise information about the serum factor(s), FBS was passed through a column of Sephadex G-10 (See Materials and Methods). This resin will totally exclude any molecule with a molecular weight of 700 or greater and so material eluted immediately

Table 4.9

The replication of vaccinia virus in CAM cells in the presence or absence of serum

Medium	Virus yield (log_{10})
199 + 5% FBS	1.9
199 (serum free)	0.6
P-Mc (0.5mM arginine)	0.8

Table 4.10

The replication of vaccinia virus in CAM cells in the presence of arginine and a high molecular weight fraction obtained by dialysis of foetal bovine serum.

Medium	Virus yield (log_{10})
199 + 5% dialysed FBS	0.7
199 + 5% whole FBS	1.9
P-Mc (0.5mM arginine) + 5% dialysed FBS	0.7
P-Mc (0.5mM arginine) + 5% whole FBS	1.6

after the void volume contains no compound with a molecular weight less than this value. FBS was applied to a column of Sephadex G-10, the void volume of which had been determined as previously described, and eluted with PBS(A). After the void volume of PBSA had eluted, fractions were collected and the protein content determined by absorption of light at 280 nm. The serum fractions containing the major part of the protein were pooled and sterilized by filtration through a 450 nm. Millipore filter. Passage through the column of Sephadex caused a dilution of about 2-fold, thus in virus growth experiments the eluted protein fraction (G-10FBS) was used at a concentration of 10% and whole serum at a concentration of 5%.

CAM cells were infected with vaccinia virus and incubated for 48 hours in media supplemented with appropriate concentrations of whole serum or Sephadex-treated serum. The results are given in Table 4.11. It can be seen that the yields obtained using media containing G-10FBS were much lower than those obtained using media containing untreated serum and were no higher than yields obtained using a medium containing no arginine or serum. This shows that Sephadex treatment had removed from FBS the factor(s) required for virus replication. It can be concluded that the molecular weight of the serum factor(s) required for the replication of vaccinia virus in CAM cells is 700 or less.

These studies have shown that the use of FBS is essential for the replication of poxvirus in CAM cells. It is known that FBS contains free arginine, thus it was necessary to determine whether the arginine content of FBS is sufficient to support poxvirus replication. To this end, starved CAM cells were infected with vaccinia virus and incubated for 48 hours in P-Mc medium containing no arginine and supplemented with 5% FBS. In order to determine the minimum requirement for serum for vaccinia virus replication, starved CAM cells infected with vaccinia virus were incubated for 48 hours in P-Mc containing 0.5 mM arginine and supplemented with various concentrations of FBS. The results are presented in Tables 4.12 a. and b.

Table 4.11

The replication of vaccinia virus in CAM cells in the presence of arginine and a high molecular weight fraction obtained by Sephadex G-10 molecular exclusion chromatography of foetal bovine serum

Medium	Virus yield (log_{10})
199 + 5% FBS	2.2
199 + 10% G-10 FBS	1.4
P-Mc (0.5mM arginine) + 5% FBS	2.3
P-Mc (0.5mM arginine) + 10% G-10 FBS	1.3
P-Mc (arginine free) (serum free)	1.3

Table 4.12

The replication of vaccinia virus in CAM cells in the presence of 0.5mM arginine and different concentrations of foetal bovine serum.

a.

Medium	Virus yield (log_{10})
199 + 5% FBS	1.5
P-Mc (0.5mM arginine) + 5% FBS	1.5
P-Mc (0.5mM arginine) + 1% FBS	1.0
P-Mc (0.5mM arginine) + 0.5% FBS	1.0
P-Mc (arginine free) (serum free)	1.1

Table 4.12

The replication of vaccinia virus in CAM cells in the presence of 0.5mM arginine and different concentrations of foetal bovine serum.

b.

Medium	Virus yield (log_{10})
P-Mc (0.5mM arginine) + 5% FBS	1.9
P-Mc (0.5mM arginine) + 4% FBS	1.9
P-Mc (0.5mM arginine) + 2% FBS	1.9
P-Mc (arginine free) + 5% FBS	1.0
P-Mc (arginine free) (serum free)	0.7

It can be seen that good yields of virus were obtained in the presence of serum concentration of 2% or greater. The yields obtained in the presence of concentrations of serum of 1.0% or less were no higher than the yields obtained in the absence of serum and arginine.

Furthermore, it is shown that the yield of vaccinia virus obtained using P-Mc medium containing 5% FBS, but no arginine was only slightly higher than the yield obtained in the absence of both arginine and serum and was much lower than that obtained in a complete medium.

These data show that the minimum requirement for serum lies between 1% and 2% and that FBS of the particular batch used, at a concentration of 5%, does not contain sufficient free arginine to support the replication of vaccinia virus. Thus, in subsequent experiments to measure virus replication all media were supplemented with 2% FBS.

4(viii) The replication of vaccinia virus and cowpox virus in the presence of arginine or citrulline

CAM cells were infected with cowpox virus or vaccinia virus and incubated for 48 hours in P-Mc medium containing 0.5 mM citrulline, 0.5 mM arginine or neither amino acid and supplemented with 2% FBS, or in 199 medium supplemented with 2% FBS. The results are presented in Table 4.13. It can be seen that the yield of vaccinia virus in the presence of citrulline or arginine is very similar to the yield obtained in 199 medium. However, while in the presence of arginine the yield of cowpox virus is similar to the yield of vaccinia virus, in the presence of citrulline the yield of cowpox virus is much lower than the yield of vaccinia virus. Furthermore, the yield of cowpox virus in the presence of citrulline is about the same as the yield in P-Mc medium containing serum, but no arginine. These results indicate that cowpox virus is defective in its ability to utilize citrulline for replication.

Table 4.13

The replication of vaccinia virus and cowpox virus in CAM cells in the presence of arginine or citrulline.

Medium	Virus yield (log_{10})	
	Cowpox virus	Vaccinia virus
199 + 2% FBS	1.8	1.8
P-Mc (0.5mM arginine) + 2% FBS	2.1	1.9
P-Mc (arginine free) + 0.5mM citrulline + 2% FBS	1.1	2.0
P-Mc (arginine free) + 2% FBS	1.0	1.0
P-Mc (arginine free) (serum free)	0.8	0.7

4(ix) The replication of cowpox virus and vaccinia virus in the presence of argininosuccinic acid

Argininosuccinic acid is an intermediate in the formation of arginine from citrulline. Thus, in an attempt to identify the point in the metabolic pathway at which cowpox virus is defective in its ability to utilize citrulline for replication, starved CAM cells were infected with cowpox virus or vaccinia virus and incubated for 48 hours in P-Mc medium containing 0.5mM argininosuccinic acid or 0.5 mM arginine and supplemented with 2% FBS. The results of this experiment, presented in Table 4.14, show that in the presence of argininosuccinic acid the yield of cowpox virus is about the same as that of vaccinia virus. Furthermore, the yield of cowpox virus in the presence of argininosuccinic acid is only slightly lower than that in the presence of arginine. This shows that arginine biosynthesis from argininosuccinic acid occurs in CAM cells infected with cowpox virus.

4(x) Discussion

To facilitate studies into the metabolism of arginine in cells infected with poxviruses it is necessary to use a cell system that is unable to synthesize arginine from citrulline. The available continuous cell lines were found not to be suitable; the mouse S180 cells were defective in their ability to support the replication of more than a few poxviruses and the human citrullinaemia cells could not be grown. Therefore, attention was turned to the possible use of a primary cell line. Tytell and Neuman (1960) found that a number of primary cell lines, including cells isolated from the CAM of the chick embryo, were defective in their ability to utilize citrulline. The chick-embryo CAM cells were of especial interest because it is known that the CAM of the chick-embryo will support the growth *in ovo* of a large number of poxviruses. However, it was not found possible to prepare these cells by the method of Tytell and Neuman and so the suitability for these

Table 4.14

The replication of vaccinia virus and cowpox virus in CAM cells in the presence of arginine or argininosuccinic acid.

Medium	Virus yield ($\log_{10}$)	
	Cowpox virus	Vaccinia virus
P-Mc (0.5mM arginine) + 2% FBS	1.6	1.5
P-Mc (arginine free) + 0.5mM argininosuccinic acid + 2% FBS	1.3	1.4

studies of CAM fragments and CAM pieces attached to the shell was investigated. According to Watanabe and Okada (1974) explants of CAM tissue applied to a suitable substratum will adhere and cells will grow out from them. When this technique was tried, it was found that the number of pieces of tissue that would adhere was too low to be of any use. However, the maintenance of CAM fragments in suspension allowed radioactive incorporation experiments to be performed and these showed that the cells of the CAM were unable to utilize citrulline in place of arginine. However, it proved impossible to grow virus in this system. There are at least two possible reasons for this. It may be that the adsorption of virus to cells in pieces of membrane occurs more slowly than adsorption to isolated cells, perhaps owing to an extracellular covering present in tissue. If this is the case, then the 2 hours allowed for adsorption might not have been sufficient. Another possible explanation is that the medium lacked some component essential for the growth of poxviruses. The use of CAM pieces attached to the shell gave good yields of buffalopox virus, a virus that is apparently unable to replicate in mouse S180 cells. However, radio-incorporation experiments performed with CAM fragments in suspension show that these fragments contain intracellular pools of nutrients that are difficult to deplete. Furthermore, Fazekas de St. Groth and White (1958) were able to grow influenza virus in CAM pieces attached to the shell in a medium consisting of salts and a source of carbon. Thus, the use of CAM pieces attached to the shell for studies concerning poxvirus replication would be expected to present some difficulties. The difficulty of depleting the intracellular pools of nutrients in CAM fragments and pieces attached to the shell is probably a consequence of the fact that such fragments and pieces consist of a large number of cells in close association. Thus, many cells would not be in direct contact with the medium. It would be expected that the use of individual cells in suspension or in a monolayer would not present such difficulties concerned with

depleting the intracellular pools because the cytoplasm of each of the cells is separated from the medium only by the cell membrane. This facilitates the transport of nutrients between the cytoplasm and the medium.

No further work was carried out using CAM pieces attached to the shell because of the availability of a method for the preparation and cultivation of cells from the CAM of the chick embryo (Cursiefen and Becht, 1975). This method was tried with great success. The method tried initially, that of Tytell and Neuman (1960) involved the treatment of the tissue with trypsin. However, the method of Cursiefen and Becht (1975) involves a double enzyme treatment. This is necessary because the cells of the CAM, especially the epithelioid cells, are covered with a layer of mucin that is unaffected by the action of trypsin and that prevents the trypsin coming into contact with the cells. Thus, a preliminary treatment is necessary to remove this layer. This preliminary treatment involves the use of a mixture of hyaluronidase and collagenase. These enzymes act upon the mucin layer to destroy its structural integrity, thus allowing the trypsin to reach the cells and release them from the membrane structure. The fact that Tytell and Neuman were able to isolate CAM cells by treatment with trypsin alone can only be explained by assuming that the trypsin preparation they used was a crude one that contained also hyaluronidase and collagenase or similar enzymes.

Growth experiments with CAM cells showed that these cells require the presence of amino acids besides the essential amino acids found in PE medium and MEM. The additional amino acids required (see Materials and Methods, Table 2.2) are all linked with the metabolism of arginine (Fig 1.3) so this requirement is probably a reflection of the defect that these cells are reported to possess with respect to the metabolism of arginine (Tytell and Neuman, 1960). Radioincorporation experiments showed that the incorporation into proteins of label from radioactively labelled citrulline in the presence of citrulline is very low compared with the incorporation of

label from radioactively labelled arginine in the presence of arginine. A comparison of the levels of incorporation of labelled phenylalanine in the presence of citrulline and in the presence of arginine show that in this case the reduction is much smaller. Thus, the low level of utilization of citrulline is not merely due to a reduction of overall protein synthesis in the presence of citrulline, but is due specifically to a low level of synthesis of arginine from citrulline. This confirms the findings of Tytell and Neuman (1960) that CAM cells are incapable of utilizing citrulline in place of arginine and shows that this metabolic defect is concerned specifically with the pathway of arginine biosynthesis.

Initial virus growth experiments show that three poxviruses, viz elephantpox virus, vaccinia virus and cowpox virus, none of which is able to replicate satisfactorily in mouse S180 cells, will replicate well in CAM cells in a growth medium containing FBS. This finding, together with the fact that buffalopox virus will replicate in CAM pieces attached to the shell, gives strong support to the expectation that this cell system will allow the replication of a wide range of poxviruses.

Further experiments showed that the presence of FBS is required for the production of good yields of infective vaccinia virus particles in CAM cells. It is this serum requirement that is probably one of the reasons for the lack of growth of vaccinia virus in CAM fragments in suspension, since these suspension cultures were maintained in the absence of serum.

It was not found possible to substitute for whole serum either dialysed serum or serum that had been subjected to molecular exclusion chromatography using Sephadex G-10. Thus, the virus-growth factor present

in foetal bovine serum must have a molecular weight of 700 or less. This finding excludes the possibility that the growth factor is a polypeptide or similar large molecule, although it could be an amino acid, vitamin or other nutrient. However, this is unlikely in view of the fact that a very complex growth medium was used and that poxviruses can replicate in other animal cells in a minimal essential medium containing no serum. The possibility exists that the growth factor is a steroid hormone or similar compound.

Experiments to determine the quantitative nature of the serum requirement have shown that 2% serum is sufficient to allow the growth of vaccinia virus. Furthermore, it has been shown that the free arginine content of the batch of serum used is such that 5% serum does not provide sufficient free arginine to support the growth of vaccinia virus in the absence of exogenous arginine. Thus, experiments to investigate the requirement for arginine for the replication of poxviruses are not precluded by the need for the presence of serum. Such experiments carried out with vaccinia virus and cowpox virus showed that while vaccinia virus is able to replicate just as well in the presence of citrulline as in the presence of arginine, cowpox virus is less able to replicate in the presence of citrulline than in the presence of arginine. This means that cowpox virus is defective in its ability to utilize citrulline for the synthesis of arginine. Thus, the indications given by initial experiments concerning the growth of cowpox virus in mouse S180 cells are confirmed. Furthermore, it has been shown that arginino-succinic acid is utilized for virus replication in CAM cells infected with cowpox virus.

Previous experiments (Cooke and Williamson, 1973; Williamson and Cooke, 1973) have given strong evidence that the enzymes for the synthesis of arginine from citrulline in cells infected with rabbitpox virus or vaccinia virus are virus-coded. It is known also (Fenner, 1968) that soon after infection with vaccinia virus, host-cell macromolecular synthesis is turned off.

Thus, the ability of cowpox virus to replicate in CAM cells in the presence of argininosuccinic acid may be due to the presence in infected cells of virus-coded or host-cell coded argininosuccinate lyase, or both.

There are only four possible ways to account for the failure of cowpoxvirus to utilize citrulline for replication. The virus might be unable to synthesize both of the enzymes for the biosynthesis of arginine from citrulline or it might be able only to synthesize a defective form of either or both of these enzymes. The data available allow one of these possibilities to be eliminated, that the virus codes for a fully functional form of argininosuccinate synthetase and a defective form of the lyase. This can be demonstrated as follows:-

Cowpox virus and CAM cells can each be argininosuccinate synthetase defective (ASS^-) argininosuccinate lyase defective (ASL^-) or both ($ASSL^-$). This gives nine possible combinationsof defect in an infected cell. The theoretical ability of each combination to give yields of infectious virus particles in the presence of argininosuccinic acid or citrulline is given in Tables 4.15 a and b. The results previously presented show that cowpox virus will not replicate in CAM cells in the presence of citrulline, but they will replicate in the presence of argininosuccinic acid. Thus, those combinations can be eliminated that will allow replication in the presence of citrulline and that will not allow replication in the presence of argininosuccinic acid. This leaves the possibilities that:-

1 Both CAM cells and cowpox virus are ASS^-

2 CAM cells are ASS^- and cowpox virus is $ASSL^-$

3 CAM cells are $ASSL^-$ and cowpox virus is ASS^-

Thus, cowpox virus produces a defective form of argininosuccinate synthetase, a defective form of both argininosuccinate synthetase and lyase or does not code for either enzyme. This reasoning assumes that infection with cowpox virus does not turn off host-cell macromolecular synthesis. If this assumption proves not to be valid, then possibility (2) can be eliminated, which means that cowpox virus is able to produce only a defective form of argininosuccinate synthetase.

Table 4.15

The theoretical effect of different defects in the ability of the host-cell to utilize citrulline on the replication of poxviruses with similar defects

a. Replication in a medium containing citrulline

Cell \ Virus	ASS^-	ASL^-	$ASSL^-$
ASS^-	-	+	-
ASL^-	+	-	-
$ASSL^-$	-	-	-

\+ = Replication possible
\- = Replication not possible

Table 4.15

The theoretical effect of different defects in the ability of the host-cell to utilize citrulline on the replication of poxviruses with similar defects

b. Replication in a medium containing argininosuccinic acid

Virus / Cell	ASS^-	ASL^-	$ASSL^-$
ASS^-	+	+	+
ASL^-	+	-	-
$ASSL^-$	+	-	-

\+ = Replication possible
\- = Replication not possible

The exact nature of the arginine metabolic lesion in cowpox virus can be determined by further investigation into the nature of that lesion in CAM cells.

CHAPTER 5

ENZYME ASSAYS

5(i) Introduction

The biosynthesis of arginine from citrulline occurs in the Krebs-Henseleit cycle (Fig 1.1) by the action of the two enzymes, argininosuccinate synthetase and argininosuccinate lyase. The synthetase converts citrulline to argininosuccinic acid and the lyase breaksdown this compound to form arginine. Arginine may be estimated colourimetrically using the Sakaguchi reaction. However, the two enzymes are subject to feed-back inhibition by arginine and so it is necessary to remove the arginine as it is formed by using excess arginase. This enzyme converts arginine to ornithine and urea, both of which compounds may be estimated colourimetrically. However, the available tests are not specific. This lack of selectivity can be overcome by separating the reaction products by chromatographic techniques that do not breakdown compounds during separation. The products can be identified by comparison with standards and estimated quantitatively by colourimetry. However, this procedure is tiresome and time consuming. Furthermore, the enzyme activity found in cells is relatively low and colourimetric techniques are relatively insensitive. Thus, obtaining an appreciable quantity of reaction products would require a large quantity of cell extract.

A simpler method by which the activity of these enzymes can be determined is by treating the urea formed with urease, to produce CO_2 and NH_3. Either of these two compounds can be readily assayed.

Evolved CO_2 can be measured volumetrically, but this method is not sufficiently sensitive. A much more sensitive method involves the use of radiochemicals. Citrulline labelled with ^{14}C in the carbamyl moiety will be converted by the action of argininosuccinate synthetase and lyase to arginine containing the ^{14}C-atom in the guanido moiety The action of arginase on the ^{14}C-(guanido)- arginine removes the guanido group to give ^{14}C-urea and ornithine. The action of urease will give $^{14}CO_2$ and ammonia. The evolved $^{14}CO_2$ can be collected and the amount of radioactivity present measured. An assay based on this principle has been developed (Cooke, 1972) and the proce-

dure is described in Materials and Methods. However, this technique has certain limitations, not least of which is the fact that its use with material from cells infected with virus carries a risk of releasing virus that is unacceptably high in the case of a dangerous pathogen such as variola virus. There is also a high background level of activity associated with this method, probably due to the use of commercial preparations of enzymes and other chemicals that are relatively impure. This background is acceptable in the measurement of relatively high enzyme activities, such as are found in infected and uninfected HeLa cells. However, the enzyme activities in cells that are defective in their ability to convert citrulline to arginine would be expected to be very low. Even upon infection with virus, the activities would probably be too low to be measured against the background (Cooke, 1972). Another limitation imposed by the use of this method is the need for radioactively labelled substrates. The lack of availability of radioactively-labelled argininosuccinic acid means that it is only possible to measure the combined activity of the two enzymes argininosuccinate synthetase and lyase using ^{14}C-(carbamyl)-citrulline.

Spectrophotometric techniques have certain advantages over the radio-chemical assay. One of these advantages is the fact that there is less risk of releasing infective virus. Furthermore, specially prepared intermediates are not required and so the use of argininosuccinic acid instead of citrulline allows the activity of argininosuccinate lyase to be measured independently of that of the synthetase.

The results are presented here of attempts to overcome the problems associated with the radiochemical assay. A spectrophotometric assay system has been investigated and experiments have been performed to identify the causes of the high background activity associated with the radio-chemical method and to try to reduce this as far as possible.

5(ii) Development of a spectrophotometric assay system

The assay system used involves a reaction sequence similar to that involved in the radiochemical method, but it is ammonia in solution rather than evolved carbon dioxide that is detected. This method is based on a clinical technique for the measurement of blood-urea nitrogen. The basic reaction (Fig 2.1) and experimental details are given in Materials and Methods. In the reaction, the ammonia released by the action of urease on urea is combined with α-ketoglutarate to produce L-glutamic acid by the action of glutamate dehydrogenase. This reaction is coupled to the oxidation of NADH to NAD, which results in a decreased absorption of light at 340nm. Thus, in this system the amount of ammonia present can be detected by measuring the absorbance at 340nm.

Initial experiments were performed to determine the optimal amounts of the reagents. The sensitivity of the method was established by carrying out assays using solutions containing known quantities of ammonia or urea. It was found possible to measure amounts of ammonia as low as 0.02 μmoles per cuvette containing 3.5 ml. of solution and amounts of urea as low as 0.01 μmoles per cuvette containing 3.5 ml. with an accuracy better than 90 per cent. This apparently greater sensitivity in the measurement of urea is due to the fact that 1 mole of urea will produce 2 moles of ammonia, thus the two results are in good agreement. Cooke (1972) has shown that the combined activity of argininosuccinate synthetase and argininosuccinate lyase in an extract of HeLa cells maintained in the presence of arginine, conditions in which the synthesis of these enzymes is repressed, is just over 0.01 μmoles of arginine synthesized/mg. of cell protein/hour. In the spectrophotometric assay procedure 1 mole of arginine will produce, after treatment with arginase and urease, 2 moles of ammonia. The amount of cell protein available from a 40-ounce bottle of HeLa cells is in the order of 20 mg. Thus, this assay system is sufficiently sensitive to measure the activities of argininosuccinate synthetase and argininosuccinate lyase

even in extracts of HeLa cells maintained in conditions in which the synthesis of these enzymes is repressed.

Since this assay system is sensitive to extremely small amounts of ammonia, great care was taken during the course of these experiments, and in subsequent assays, to ensure that no extraneous ammonia entered the system. Freshly distilled water was used to make all solutions and glassware was thoroughly washed in freshly distilled water before use.

Having established that this assay was sufficiently sensitive to measure the lowest levels of enzyme activity expected to be found in HeLa cells, an assay of the activity of argininosuccinate lyase from cell extracts was carried out using argininosuccinic acid as the substrate as described in Materials and Methods.

Cell extracts were prepared from confluent monolayers of HeLa cells or mouse L cells maintained in the presence of arginine in 40-ounce roller bottles. The cells from each bottle were resuspended in 5.0 ml. of buffer and the cell contents were extracted by subjecting the cells to two cycles of freezing and thawing and to ultrasonics for 2 minutes. The debris was sedimented by centrifugation (1500 g for 5 minutes) the supernatant extract was collected and aliquots were used in the assays. Table 5.1 gives the results of an assay on an extract of HeLa cells maintained in the presence of 0.5 mM arginine. It can be seen that all the controls showed an appreciable level of enzyme activity. The decrease in optical density (OD) in control 1, containing no extract, and control 3, containing boiled extract, indicates the presence in the enzyme preparations of significant amounts of argininosuccinate lyase, although not as much as is found in the cell extract. The decrease in optical density in control 2, containing no argininosuccinate, and control 4, containing no arginase, indicatesthe presence of these compounds in the cell extract. Control 3, containing boiled extract, was chosen as a blank because it was identical to the complete assay in every respect except for the lack of argininosuccinate lyase activity.

Substracting the OD decrease of control 3 from the OD

Table 5.1

Spectrophotometric assay of the activity of argininosuccinate lyase in an extract of HeLa cells

Test	Optical density at 340 nm		
	Initial	Final	Initial-Final
Complete reaction mixture	0.77	0.25	0.52
Without cell extract (Control 1)	0.76	0.37	0.39
Without substrate (Control 2)	0.74	0.29	0.45
With boiled extract (Control 3)	0.75	0.26	0.49
Without arginase (Control 4)	0.75	0.37	0.38

decrease of the complete assay (test) gives the OD decrease (D) due to the activity of argininosuccinate lyase in the cell extract. It is known that the OD at 340 nm. with a 1 cm. light path of 1 μmole/ml. of NADH is 6.22, thus the amount of NADH/ml. oxidized to NAD is D/6.22 μmole/ml. The volume in the cuvette is 3.5 ml. so the total amount of NADH oxidized is 3.5 D/6.22 μmole. In this system 1 μmole of argininosuccinate will produce 1 μmole of arginine by the action of argininosuccinate lyase. The action of arginase on 1 μmole of arginine produces 1 μmole of urea, which is broken down by urease to produce 2 μmoles of ammonia. The ammonia evolved reacts with α-ketoglutarate to give glutamic acid and during this reaction NADH is oxidized to NAD. In this reaction 1 μmole of ammonia causes the oxidation of 1 μmole of NADH. Thus, in this system 1 μmole of argininosuccinic acid causes the oxidation of 2 μmoles NADH. Therefore, the amount of arginine produced from argininosuccinate is 3.5D/6.22 x 2. The assay was run for 1 hour and an amount of extract was used that contained 2 mg. cell protein, thus the enzyme activity can be calculated using the following formula:

$$\text{Enzyme activity} = \frac{3.5D}{6.22 \times 2 \times 2} \text{ μmole arginine/mg. cell protein/hour.}$$

where D = (OD decrease of complete assay) − (OD decrease of blank).

Using the figures in Table 5.1 this formula gives the activity of argininosuccinate lyase as being:-

$$\frac{3.5(0.52-0.49)}{6.22 \times 2 \times 2} = 0.004 \text{ μmole arginine per mg. cell protein per hour.}$$

Table 5.2 gives the results of a similar assay carried out using an extract of mouse L cells also maintained in the presence of 0.5 mM arginine. As with the HeLa assay, the controls show a considerable decrease in OD, presumably for the same reasons as obtained in the case of the HeLa assay. Using the figures presented in Table 5.2 in the above formula, the activity of argininosuccinate lyase in mouse L cells is found to be:-

Table 5.2

Spectrophotometric assay of the activity of argininosuccinate lyase in an extract of mouse L cells

Test	Optical density at 340 nm		
	Initial	Final	Initial-Final
Complete reaction mixture	0.95	0.23	0.72
Without cell extract (Control 1)	0.94	0.40	0.54
Without substrate (Control 2)	0.92	0.26	0.66
With boiled extract (Control 3)	0.93	0.40	0.53
Without arginase (Control 4)	0.94	0.40	0.54

$$\frac{(0.72 - 0.53)}{6.22 \times 2 \times 2} \times 3.5 = 0.03 \text{ μmoles arginine produced/mg. cell protein/hour.}$$

Cooke (1972) has determined the combined activity of argininosuccinate lyase and argininosuccinate synthetase in extracts of HeLa cells and mouse L cells both maintained in the presence of 0.6 mM arginine. The activity in HeLa cells was found to be 0.0115 μmoles arginine produced/mg. cell protein/hour, and that in mouse L cells was found to be 0.0325 μmoles/mg/hour. Thus it can be seen that, using this spectrophotometric assay, the activity of argininosuccinate lyase in HeLa cells maintained in arginine was found to be about one third of the combined activity of argininosuccinate lyase and argininosuccinate synthetase in these cells maintained in similar conditions. The activity of the lyase in mouse L cells maintained in arginine was found to be just less than the combined synthetase-lyase activity in these cells maintained under similar conditions.

However, the fact of the high level of activity in the controls casts some doubt on the accuracy of the results obtained. This high background is most probably due to contamination with argininosuccinate lyase of one or all of the enzyme preparations used. In order to get around this difficulty, an attempt was made to measure directly, by spectrophotometry, one of the products of the action of argininosuccinate lyase. The advantage of this is that it obviates the need for additional enzymes. The only requirements are for a source of the activity to be measured and a substrate. Fumarate is a byproduct of the action of argininosuccinate lyase on argininosuccinic acid and a solution of fumarate will absorb light at a wavelength of 280 nm. Thus, this system was investigated.

However, the presence of fumarase in the cell extract made it impossible to obtain meaningful results. Because of these factors, attention was turned to improving the sensitivity of the radiochemical system.

5(iii) The radiochemical assay system

The radiochemical assay system is described in detail in Materials and Methods. Using this method, an assay was

performed on a 0.5 ml. sample of extract prepared from HeLa cells maintained in arginine as previously described. A control was used that was identical in every respect except that it contained buffer in place of extract. The results (Table 5.3) show that a considerable amount of activity is present in the control. The urease preparation used was a crystallised preparation of high specific activity and the arginase was purified before use (see Materials and Methods). However, it was possible that these preparations were contaminated with sufficient quantities of the arginine biosynthetic enzymes to give rise to the high background. In order to test this possibility, the urease and arginase were assayed in the absence of cell extract for the presence of contaminating enzyme activity. The assay was performed in exactly the same way as for cell extracts.

Two parallel sets, A and B, of assay apparatus were set up, each in duplicate, as for an enzyme assay. To the reaction vessels in each set were added, instead of cell extract, 0 5 ml quantities of urease at a concentration of 10 units/ml., 0.5 ml. quantities of arginase at a concentration of 200 units/ml., 1.0 ml. quantities of a 1:1 mixture of urease and arginase, or 0.5 ml. quantities of buffer. The volume of each vessel was made up to 2.0 ml. with buffer.

Reaction was allowed to continue for 1 hour before being stopped by boiling. The pH was adjusted to 7.0 as usual and 0.5 ml. quantities of urease (10 units/ml.) were added to the reaction vessels of set A. To the vessels of set B were added 0.5 ml. quantities of phosphate buffer, pH 7. Both sets of vessels were then treated as in a normal enzyme assay.

The results are presented in Table 5.4. The figures obtained from set B show that in the absence of both urease and arginase very little activity is detected. Thus, the high background is not due to the non-enzymic breakdown of the labelled citrulline to release $^{14}CO_2$. In the presence of arginase alone the activity is appreciably higher, indicating that the arginase does contain some contaminating activity of the enzymes of arginine

Table 5.3

Assay of argininosuccinate synthetase-lyase activity in an extract of uninfected HeLa cells by measurement of $^{14}CO_2$ evolved from ^{14}C-(carbamyl)-citrulline

Enzyme activity*	
Cell extract	Control
1100	800

* Radioactivity (DPM) evolved/hour

Table 5.4

Assay of argininosuccinate synthetase-lyase activity in preparations of urease and arginase by measurement of $^{14}CO_2$ evolved from ^{14}C-(carbamyl)-citrulline

Enzymes initially present	Enzyme activity*	
	A Urease added after incubation for 1 hour	B No urease added after incubation for 1 hour
Arginase	1000	400
Urease	1800	1700
Arginase + urease	2500	2200
None	700	100

* Radioactivity (DPM) evolved/hour

biosynthesis and some urease activity. However, in the presence of urease alone a considerable amount of activity is seen. This must be due to either the presence of relatively large amounts of arginine biosynthetic enzymes in the urease preparation or to the presence of radioactivity subject to the action of urease to release $^{14}CO_2$. These conclusions are confirmed by the figures obtained from set A. In each case an activity is detected in set A that is higher than the corresponding activity in set B. In view of the high degree of purity of the urease preparation, these results are unlikely to be due to some contaminating enzyme activity. However, it is possible that these results are due to the presence in ^{14}C-(carbamyl)-citrulline of some ^{14}C-urea.

Citrulline labelled with ^{14}C in the carbamyl moiety is prepared from sodium cyanate-^{14}C and copper L-ornithine by the method of Smith (1955). These two compounds are heated together to form copper L-^{14}C-(carbamyl)-citrulline. Copper is used to protect the α-amino group of the amino-acid and it is removed by treatment with H_2S in acid to give L-^{14}C-(carbamyl)-citrulline. Now, heating cyanate produces urea, thus the ^{14}C-(carbamyl)-citrulline will be contaminated with ^{14}C-urea.

The presence of ^{14}C-urea in the preparation of ^{14}C-(carbamyl)-citrulline used in the enzyme assays was tested by treating with urease for 2 hours a sample of the citrulline preparation at the same specific activity and concentration as in the enzyme assays and measuring evolved $^{14}CO_2$ in the usual way. A control containing no urease was tested in an identical manner. The results in Table 5.5 show that while very little activity is evolved in the absence of urease, a considerable amount was evolved in the presence of urease. This indicates that the ^{14}C-citrulline preparation contained an appreciable quantity of ^{14}C-urea.

Samples of the ^{14}C-citrulline were subjected to thin-layer chromatography as described in Materials and Methods. After the chromatogram had been developed, the areas containing citrulline and urea were identified by comparison with standards and the material within each area was removed

Table 5.5

Amount of $^{14}CO_2$ evolved from a preparation of ^{14}C-(carbamyl)-citrulline by the action of urease.

Radioactivity evolved (DPM)	
Urease present	Urease absent
3300	330

and placed into scintillation vials containing Bray's liquid scintillator. The radioactivity was measured as already described. The results (Table 5.6) show that the radioactivity associated with urea was about 3.0% that associated with citrulline. This confirms that the ^{14}C-(carbamyl)-citrulline was contaminated with ^{14}C-urea.

Citrulline has a molecular weight of 175 and urea has a molecular weight of 60. Thus, these two compounds can be separated by means of molecular-exclusion chromatography (gel filtration) a technique that separates compounds on the basis of differences in molecular weight.

A solution in PBS(A) of ^{14}C-(carbamyl)-citrulline of specific activity 0.1mCi/m.mole and known concentration was passed through a column of Sephadex G10 as described in Materials and Methods. Fractions of volume 1 ml. were collected and 0.1 ml. samples of each fraction were placed into 5 ml. quantities of Bray's scintillation liquid and the radioactivity of each was measured as previously described. A graph of the results was made (Fig 5.1a) with the fraction numbers plotted along the abscissa. The ordinate consists of the total radioactivity within each sample expressed as a percentage of the total radioactivity applied to the column. Two peaks can be seen on this graph. The larger peak (A1) contains the greater part of the radioactivity and this eluted before the much smaller peak (B1). Since urea has a much lower molecular weight than citrulline it would be expected to be retained by the Sephadex gel to a greater extent than citrulline and thus elute after the citrulline. On this basis, the smaller peak obtained should represent urea and the larger one citrulline. The major fractions of each peak were pooled and a sample of the presumptive citrulline pool (A1) was passed through a Sephadex G10 column. This was done in order to find out whether the first elution had completely separated urea from citrulline. Fractions were collected again and the radioactivity of each was measured. The results were plotted on a graph as before (Fig. 51b) It can be seen

Table 5.6

Separation of ^{14}C-urea from a preparation of ^{14}C-(carbamyl)-citrulline using thin-layer chromatography

Area on TLC plate identified as:	Radioactivity recovered (DPM x 10^{-4})
Citrulline	73
Urea	2.2

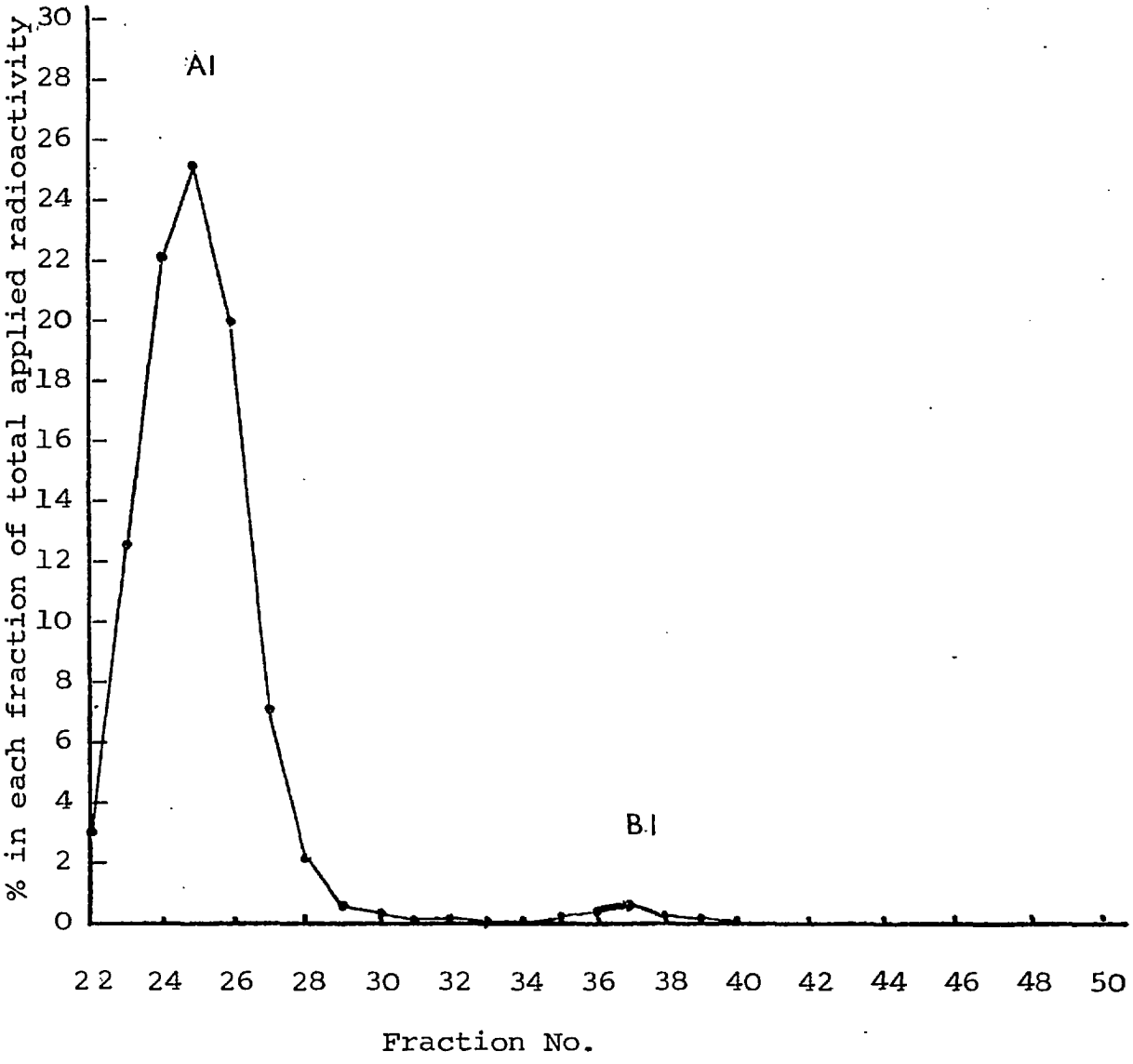


Fig. 5.1 The separation of ^{14}C-citrulline from ^{14}C-urea by molecular exclusion chromatography using Sephadex G-10.

a. Treatment of a crude preparation of ^{14}C-citrulline.

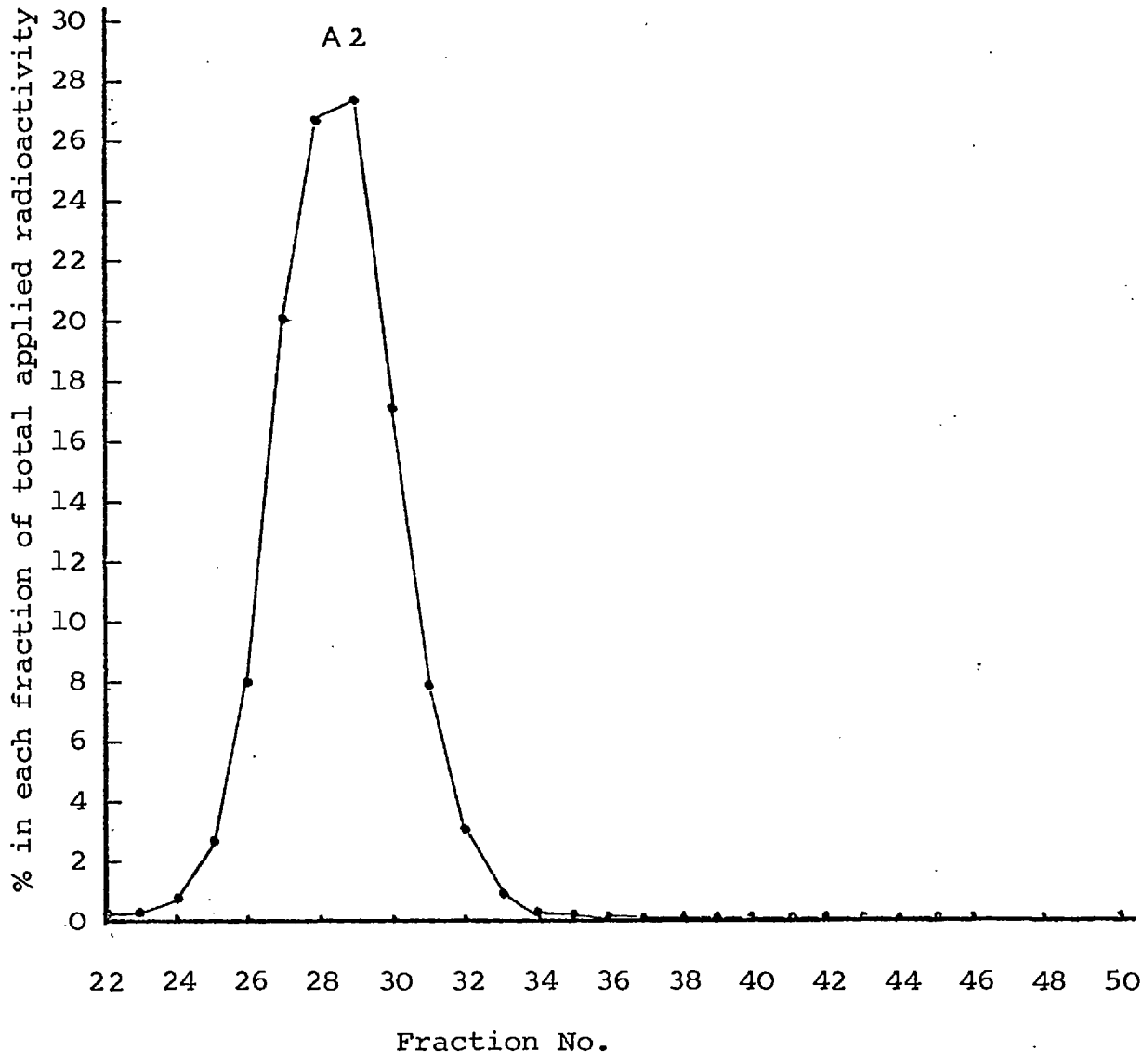


Fig. 5.1 The separation of ^{14}C-citrulline from ^{14}C-urea by molecular exclusion chromatography using Sephadex G-10.

b. The further treatment of ^{14}C-citrulline purified by elution of the crude preparation.

that this time a smaller peak is not obtained. These data indicate that the first treatment with Sephadex G10 had removed the urea contamination of the citrulline.

In order to confirm this, the major fractions of the peak were pooled (A2), and samples of known radioactive concentration of A2, A1 and B1 were treated with urease for 2 hours. Evolved $^{14}CO_2$ was measured as described previously. The results (Table 5.7) give, in each case, the percentage of the activity present in each sample that is released as $^{14}CO_2$ by the action of urease. Thus, the figures give an indication of the proportion of radioactivity within each pool that was present in the form of ^{14}C urea. It can be seen that the radioactivity released as $^{14}CO_2$ from B1 is 40% of the total. However, the radioactivity released as $^{14}CO_2$ from A1 is only about 0.01% of the total present. A similar figure is given by A2. These data confirm that the ^{14}C-(carbamyl)-citrulline is contaminated with an appreciable quantity of ^{14}C-urea and show that this contamination is almost entirely removed by passing once through Sephadex G-10.

In order to test the effect on the background of the enzyme assay system of the removal of ^{14}C-urea from the ^{14}C-citrulline, an assay was performed with the purified citrulline preparation, using Jack-bean meal as a source of argininosuccinate synthetase and lyase. The results (Table 5.8) show that a considerable reduction in background activity has been achieved (cf Table 5.3).

5(iv) Discussion

The investigation into a spectrophotometric system for assay of the enzymes of arginine anabolism has shown that this system can measure readily the levels of enzyme activity that are found within extracts of uninfected cells. Assays were carried out using extracts of HeLa and mouse L cells maintained in the presence of arginine, conditions under which the formation of the enzymes of arginine biosynthesis is repressed. The results obtained with mouse

Table 5.7

Amount of $^{14}CO_2$ evolved by the action of urease on separate fractions collected after Sephadex G-10 molecular exclusion chromatography of a preparation of ^{14}C-(carbamyl)-citrulline

Fraction	Radioactivity released by urease (% DPM originally present)
Urea (B1)	40
Citrulline (First elution. A1)	0.01
Citrulline (Second elution. A2)	0.015

Table 5.8

Assay of argininosuccinate synthetase-lyase activity in an extract of Jack-bean meal by measurement of $^{14}CO_2$ evolved from ^{14}C-(carbamyl)-citrulline purified by Sephadex G-10 molecular exclusion chromatography

Enzyme activity*	
Jack-bean meal extract	Control
1100	400

* Radioactivity (DPM) evolved/hour

L cells show that the activity of argininosuccinate lyase in these cells is close to the combined activity of argininosuccinate synthetase and lyase measured in these cells by Cooke (1972) using a radiochemical assay procedure. The activity of the lyase measured in HeLa cells by the spectrophotometric assay is about one third of the combined activity of the synthetase and lyase measured by Cooke. A possible explanation for this is that in HeLa and mouse L cells the specific activity of argininosuccinate synthetase is very much higher than that of the lyase. Thus, the rate-determining step in the conversion of citrulline to arginine is the breakdown of argininosuccinate to arginine by argininosuccinate lyase. Therefore, an assay of the combined activity of argininosuccinate synthetase and lyase will give the activity of the lyase. That the lyase activity measured in HeLa cells by the spectrophotometric assay is lower than the combined synthetase-lyase activity measured by the radioassay system is probably a result of the high background in the spectrophotometric assay. The lyase activity is higher in L cells than in HeLa cells, thus measurements of lyase activity in L cells will be less affected by the high background.

The controls used in these assays also showed the presence in the cell extracts of appreciable quantities of the substrate, argininosuccinate. Clearly, the amount of argininosuccinate in cell extracts will be variable and so the effect of this exogenous substrate on the reaction kinetics of the assay will introduce an unknown factor into the assay. In order to get around this problem it would be necessary to dialyse cell extracts before performing assays. This will remove small molecules from the extract and thus eliminate the unknown factor from the assay.

However, the high background activity imposes severe limitations on the ability of this assay system to measure low levels of enzyme activity. A possible reason for the high background is that a crude preparation of urease was used that was prepared from Jack-bean meal, which is known to be a source of the enzymes of arginine biosynthesis from citrulline. This urease was used because the preparation of high specific-activity used in the radio-

chemical assay was not available at the time at which work on the spectrophotometric assay system was being carried out. It is possible that this preparation of urease will prove to be sufficiently free of argininosuccinate lyase to allow the potential of this assay system to be realized.

It has been shown that the high background associated with the radiochemical assay system is largely due to the contamination with ^{14}C-urea of the commercially available ^{14}C-(carbamyl)-citrulline. The figures obtained of the amount of contamination (2% to 3%) agree quite closely with the manufacturers figures for the purity of this preparation (98% to 99%) While the amount of contamination is very small, it is highly significant in the context of the enzyme assay system. The amount of enzyme activity present in a typical extract of HeLa cells is such that in the order of 1% of the ^{14}C-citrulline available is converted to $^{14}CO_2$. Now, a large excess of urease is used in this assay so any urea present will be almost entirely hydrolysed. Thus, contamination with only 1% ^{14}C urea will yield a background level of activity comparable to the activity released by the action of the enzymes being assayed. The presence of ^{14}C urea in ^{14}C-(carbamyl)-citrulline is a consequence of the method of synthesis.

Most of this ^{14}C urea is removed by chromatography during commercial preparation, but not all of it since a purity of 98% to 99% is adequate for most purposes. Citrulline can be separated from urea by the use of Sephadex chromatography by virtue of the difference in molecular weights. The advantage in this case of using Sephadex rather than other techniques of separation, such as ion-exchange chromatography, is that Sephadex can be used at physiological pH. Thus, the citrulline will not be broken down to any great extent and upon elution it can be used in the assay without altering the pH of the eluted solution.

Sephadex G-10 has an exclusion limit of 700, thus molecules with a molecular weight greater than this will be totally excluded and will pass straight through the column. Citrulline has a molecular weight of 175 and urea

has a molecular weight of 60, therefore citrulline will be retained by the gel to a lesser extent than urea and will elute before urea. The experiments described show that one passage through the column of Sephadex G-10 produced an almost total separation of citrulline from urea. This separation could not be improved by subsequent passage through the gel.

That the high background of this assay system was largely due to ^{14}C-urea contaminating the ^{14}C-citrulline was shown by the two enzyme assays performed; one on a HeLa cell extract using unpurified citrulline and one on a Jack-bean meal extract using purified citrulline. While the activities obtained from the extracts were the same, the activity in the control using purified citrulline was half that in the control using unpurified citrulline. Thus, the sensitivity of this assay system has been greatly improved.

CHAPTER 6

GENERAL DISCUSSION

Arginine is known to be essential for the growth of animal cells in culture and for the replication of many DNA viruses. An important role of arginine in cultured cells is to be incorporated into proteins, especially basic proteins such as histones. Histones, together with other proteins, form a complex with DNA that is known as chromatin. Arginine is involved also in intermediary metabolism. This occurs in the Krebs-Henseleit cycle in which arginine is katabolized to ornithine with the production of urea. The urea may be excreted from the cell, thus this cycle is a means of disposing of nitrogenous waste. The ornithine may be converted back to arginine via citrulline and argininosuccinic acid. Alternatively, ornithine may be decarboxylated to produce putrescine, a diamine that is a precursor of the polyamines spermidine and spermine.

Experiments described within this thesis have shown that arginine is essential for the replication of cowpox virus. Previous experiments have demonstrated that arginine is essential also for the replication of vaccinia virus (Archard and Williamson, 1971) and rabbitpox virus (Cooke, 1972). Archard and Williamson (1971) have demonstrated also that the requirement for arginine for the replication of vaccinia virus occurs at two distinct stages in the replication cycle. The first requirement occurs just before DNA synthesis begins. The second is concerned with virus maturation and involves the incorporation of arginine into the virion. These studies give a strong indication that the requirement for arginine for replication is a common feature of all poxviruses. It is indicated also that part of this requirement involves the synthesis of arginine-rich proteinsthat are incorporated within the mature virion.

Arginine-rich histones have been shown to be important in maintaining the geometry of DNA within uninfected cells (Vandegrift et al, 1974). These authors selectively removed all histones except those rich in arginine (III and IV) under conditions that did not allow

redistribution of protein along the DNA during extraction. The circular dichroism (CD) spectrum was then compared with the spectrum of native chromatin and it was found that the spectra were essentially identical. This indicated that the arginine-rich histones were capable of maintaining the tertiary structure of the DNA.

X-ray studies by Pardon et al (1967) and Pardon and Wilkins (1972) showed that the DNA of chromatin has a super-helical tertiary structure (100 A diameter, 120 A pitch) imposed upon a B-form secondary structure. It has been shown also that histone IV alone is capable of maintaining the super-helical form of DNA in chromatin in the presence of divalent cations.

Vandegrift et al constructed a computer model of super-helical DNA of pitch 120A and diameter 100A and calculated the CD spectrum of this model. They found that it agreed quite closely with the CD spectrum obtained experimentally and they concluded that the unique CD spectrum of the DNA component of native chromatin is the result of supercoiling of chromatin DNA by arginine-rich histones, probably histone IV specifically, helped by the presence of calcium ions.

Thus, the importance of arginine in animal cells consists in its incorporation into proteins that stabilize the tertiary structure of DNA.

An arginine-rich protein has been found associated also with vaccinia virus DNA (Pogo, Katz and Dales, 1975). Vaccinia virus DNA, which is synthesized early in infection in viroplasmic matrices or "factories", can be isolated, before it is incorporated into virus particles, in the form of a DNA-protein complex. The protein component consists of basic protein together with some of the virus-induced activities that are eventually incorporated into virions. The factories contain about 10% DNA and 90% protein and the arginine-rich protein (AEP) contains about 10% of all the leucine and 45% of all the arginine in the proteins of the factories. AEP is synthesized early in the replication cycle and is not formed in appreciable quantities in the absence of the synthesis of virus DNA. This protein complexes with progeny DNA by

means of ionic bonds and it is the predominant protein in the complex. The role of AEP is unknown. It may have a function similar to that of the basic polypeptides of T-even bacteriophages, interacting with specific DNA sequences before or during the incorporation of DNA into virus cores, or similar to that of the histones of eukaryotic cells or perhaps both.

Proteins rich in arginine have been found associated also with other animal DNA-viruses that require arginine for replication.

Herpes simplex virus contains many such proteins (Spring et al,1969). Adenoviruses also require arginine for replication. Arginine is incorporated into adenoviruses immediately before virus maturation (Rouse and Schlesinger, 1967). Russell and Becker (1968) showed the existence of a maturation factor (the P-antigen) the synthesis of one form of which apparently required the presence of arginine. Furthermore, it is known that adenoviruses contain twice the amount of arginine found in RNA viruses (Polasa and Green, 1967). Thus the possibility arises that adenoviruses contain an internal protein rich in arginine that is associated with DNA and that has properties similar to those of histones. Laver (1970) showed that adenoviruses contain a single arginine-rich protein that is the major constituent of the core proteins. This protein is probably the P-antigen of Russell & Becker (1968). Thus it is indicated that the importance of arginine in the replication of adenoviruses lies in its incorporation into a protein component that is essential for the maturation of the virus, possibly by stabilizing the tertiary structure of DNA as is the case with histones in cellular chromatin.

From these studies it can be seen that proteins rich in arginine play an important role both in animal cells and in animal viruses containing DNA. Furthermore, this role is probably the same in each case; to maintain the tertiary structure of DNA.

Arginine is synthesized in uninfected cells in the Krebs-Henseleit cycle from citrulline via argininosuccinic acid. In this thesis are presented experiments performed to extend previous investigations into the

metabolism of arginine in cells infected with poxviruses by the use of a wider range of these viruses. Previous work on this subject (Cooke, 1972) has involved the use of mouse sarcoma 180 (S180) cells, which have a metabolic lesion such that they are defective in their ability to utilize citrulline (Tytell and Neuman, 1960). Such cells were used because they allow the direct observation of the metabolism of arginine due to virus replication unobscured by host-cell metabolism of arginine.

Initial experiments confirmed that mouse S180 cells are defective in their ability to utilize citrulline for growth. However, further experiments showed that very few poxviruses could replicate within these cells to give adequate yields. Of the several viruses examined only rabbitpox virus gave good yields. Cooke (1972) has shown that the IHD strain of vaccinia also will give good yields in mouse S180 cells. Cowpox virus gave low yields in mouse S180 cells, but despite this, experiments have indicated that this virus is defective in its ability to utilize citrulline Further experiments demonstrated that the yields increased with increasing citrulline concentration. Probably, this is due to the fact that the enzymes of arginine biosynthesis from citrulline present in mouse S180 cells have a low substrate affinity (J.D. Williamson, Personal Communication). Thus, the increase in yields of cowpox virus with increasing concentration of citrulline was probably the result of the host-cell enzymes synthesizing greater amounts of arginine.

This apparent failure of mouse S180 cells to support the replication of the majority of poxviruses investigated may be due to one of a number of reasons. It is possible that mouse S180 cells lack specific receptor sites so that some types of poxvirus fail to adsorb to the cell surface. Possibly, the genome of some types of poxvirus is more susceptible to host-cell DNase enzymes, perhaps in some manner analogous to the restriction and modification system of bacteria. Another possible explanation is that some types of poxvirus require for replication certain nutrients that are not present in the media used.

The apparent inability of cowpox virus to utilize citrulline for replication indicated by experiments with mouse sarcoma 180 cells was investigated further using CAM cells. These cells have been reported to be defective in their ability to utilize citrulline for growth (Tytell and Neuman, 1960). This finding was confirmed by radio-incorporation experiments carried out using both CAM cells in small fragments of tissue maintained in suspension and confluent monolayers of CAM cells.

However, it was found that no yields of poxvirus could be obtained in CAM cells in fragments or in monolayers in media containing no serum. The fact that good yields of virus could be obtained using the CAM in ovo and in vitro as small pieces attached to the shell showed that CAM cells were not intrinsically incapable of supporting the replication of poxviruses. Further experiments using monolayers of CAM cells demonstrated that serum was required for the replication of poxviruses in these cells. It was also shown by the use of media containing serum fractionated by molecular exclusion chromatography, using Sephadex G-10, that the poxvirus replication factor(s) present in serum has a molecular weight of 700 or less. It is unlikely that the factor(s) is a nutrient such as an amino acid or vitamin in view of the fact that the media used are complex, containing a very large number of 'essential' and 'non-essential' nutrients. However, serum is known to contain a large number of components, of both high and low molecular weight, a number of which have been identified as cell-growth factors.

Since virus replication and host cell metabolism are intimately associated it would be expected that such cell-growth factors could affect also virus replication.

Gospodorowicz (1974) investigated the initiation of DNA synthesis in resting 3T3 cells (mouse embryo fibroblasts) and he isolated a fibroblast growth factor (FGF) that is a polypeptide found in the brain and pituitary. When glucocorticoids (dexamethasone and hydrocortisone) were present FGF stimulated DNA synthesis as effectively as serum. Holley and Kiernan (1974) also investigated the initiation of DNA synthesis in 3T3-4A cells in Dulbecco-Vogt

modified Eagle's medium containing 10% calf serum and supplemented with those components exclusive to McCoy's medium and Ham's F-12 medium. Four growth factors were isolated from serum, three of which were found to initiate DNA synthesis in quiescent cells. These three factors could be replaced by known substances, viz the FGF of Gospodorowicz, insulin and dexamethasone (9 α-fluoro 16α-methyl prednisolone). An additional heat-stable factor was isolated from serum that produced an enhanced initiation of DNA synthesis in the presence of a protein fraction of serum. It was found that a combination of FGF, dexamethasone, insulin and the heat-stable fraction of mouse serum gave a response about 80% of that given by 4% calf serum.

These studies have shown that dexamethasone (a steroid hormone) is one of the factors required for the growth of 3T3-4A cells. Since the poxvirus replication factor(s) has a molecular weight of 700 or less and since steroid hormones fall within this range of molecular weights it is possible that this factor is a steroid hormone.

The failure of poxvirus to replicate in CAM cells in the absence of serum may be due to failure of the virus to adsorb to the cell surface, to penetrate the cell membrane or to replicate once within the cytoplasm.

The method of preparation of CAM cells from tissue involved the use of trypsin. Now, this enzyme is known to act on the surface of cells, thus it is possible that the exposure of CAM cells to trypsin altered the cell surface in such a way as to affect the adsorption or penetration of virus. It is known that steroid hormones can induce changes at the cell surface. Ballard and Tomkins (1969) investigating hepatoma tissue culture (HTC) cells found that dexamethasone and other steroids induce a modification of the cell surface producing an increase in adhesiveness for glass. This induction is inhibited by actinomyin D, puromycin and cycloheximide, indicating the necessity for RNA and protein synthesis. It was considered possible that the hormone induces the incorporation of a basic protein in the cell surface structure or that a surface component may be modified by steroid-inducible enzymes. Thus, it is possible that similar steroid-induced changes to the surface of CAM cells are required, perhaps to repair damage caused by

trypsin, to allow adsorption of poxvirus or to allow the uptake of the virus through the cell membrane.

However these authors have shown also that steroid hormones regulate the synthesis of tyrosine aminotransferase within HTC cells. If a similar mechanism operates within CAM cells, then in the absence of serum poxvirus replication may be inhibited by the inability to metabolize one or more aminoacids.

It is possible also that the poxvirus replication factor is an oligopeptide, since such compounds are known to affect cell growth and DNA synthesis. Thus, they may affect also virus replication.

Eagle (1960) grew cells in a medium separated from serum by 'Cellophane' and he found that no growth occurred unless proteases were added to the serum. This indicated that some low molecular weight polypeptides or oligopeptides were required for cell growth. Furthermore, Pickart and Thaler (1973) have isolated a tripeptide from human serum that prolongs the survival of normal liver cells and stimulates the growth of neoplastic liver cells (Morris rat hepatoma). This tripeptide has a molecular weight of about 300 and consists of glycine, lysine and histidine.

It has been found (Pohjanpelto and Raina, 1972) that putrescine stimulates the division of non-embryonic human fibroblasts. This stimulation is produced also by the polyamines spermidine, spermine and cadaverine, but to a lesser extent. Putrescine was found to be effective within a very small range of concentration, the optimum being 3×10^{-8}M. A concentration greater than 10^{-3}M was found to be inhibitory.

Ham's F12 medium contains putrescine and it is reported to be able to support the growth of hamster cells without serum in cloning experiments. However, Lambert and Pirt (1975) using MRC-5 cells, found that the serum growth factor could not be replaced by a mixture of over 60 vitamins, co-enzymes, hormones and other organic and inorganic compounds, including putrescine and hydrocortisone, considered to be possible growth factors.

Spermidine is produced from putrescine by S-adenosyl methionine (SAM) decarboxylase. It has been shown (Heby,

Marton, Wilson and Martinez, 1975) that increased proliferation of rat brain-tumour cells is accompanied by increased levels of both ornithine decarboxylase and SAM decarboxylase. It was shown further that the spermidine content of the cells varied during the culture cycle in a manner similar to that of the enzyme activities and showed a high, positive correlation with the specific growth rate of the cells. Putrescine showed only a low, positive correlation and spermine a higher, but negative correlation. The high correlation between specific growth rate and polyamine synthesis indicates that these events are primarily associated with cell replication.

Earlier experiments carried out by Fillingame and Morris (1973) showed that lymphocytes stimulated to divide by transformation with Concanavalin A show large increases in cellular levels of putrescine, spermidine, spermine, RNA and protein. The use of methylglyoxal bis (guanyl hydrazone) (MGBG) completely prevented the intracellular accumulation of spermidine and spermine in cultures of transforming lymphocytes. However, RNA accumulation was unaffected and the absolute rate of RNA synthesis increased in the presence of Con A with or without the accumulation of spermine and spermidine. Furthermore, all classes of RNA were synthesized and the rate of processing of rRNA precursors remained unaltered. These results indicate that increases in the levels of spermidine or spermine or both are not essential for mediating the large increases in cellular RNA occurring during lymphocyte transformation. However, MGBG partially inhibited DNA synthesis in such a way that the number of lymphocytes in which DNA synthesis was initiated remained unaltered after Con A stimulation, but progress through S phase to mitosis was impaired (Fillingame et al, 1975). These results suggest that increased levels of spermine or spermidine might be necessary for normal progress from DNA synthesis to cell division. However, blocking SAM decarboxylase with MGBG produces an increase in putrescine levels. Thus it is possible that the increased amount of putrescine could substitute for spermine and spermidine for the synthesis of RNA and protein, but not for the synthesis of DNA.

Mamont, Böhlen, McCann, Bey, Schuber and Tardif (1976) investigated the role of polyamines in rat hepatoma tissue culture (HTC) cells in suspension. These cells were induced to proliferate, by diluting the cell suspension, in the presence of DL-α-methyl ornithine, which is a potent competitive inhibitor of ornithine decarboxylase. In these cells DL-α-methyl ornithine blocked the usual increase in the levels of putrescine and spermidine, which resulted in a rapid decrease in the levels of putrescine followed by a striking decrease of spermidine. Incorporation of ^{3}H-thymidine into DNA and cell proliferation were inhibited. The addition of putrescine, spermidine or spermine produced an immediate resumption of cell proliferation. Cell proliferation was restored also by L-ornithine, probably by relieving the competitive inhibition of ornithine decarboxylase.

The data do not exclude the participation of putrescine in DNA replication, but these authors suspect that spermidine plays the major role because its level is maintained for at least 7-8 hours, which correlates with the initial maintenance of DNA synthesis. Furthermore, MGBG, which blocks the accumulation of spermidine, not of putrescine, appears to exert specific inhibition of DNA replication in lymphocytes stimulated by Con A.

The role of spermidine in DNA synthesis in animal cells is not known, but there is some evidence that it is concerned with stabilizing DNA folds in bacteria (Flink and Petijohn, 1975). There is also evidence that spermidine stimulates the activity of DNA-dependent DNA-polymerases of the rat brain (Chiu and Sung, 1972) and that its presence is required for the formation <u>in vitro</u>, of duplex, replicative forms of bacteriophage DNA from single-stranded viral templates (Wickner, Shekman, Geider and Kornberg, 1973).

Thus, the available data suggest strongly that polyamines, especially spermidine, are required for the replication of cellular DNA. Furthermore, it is likely that the involvement of polyamines in DNA replication is concerned with establishing or maintaining the conformation of DNA prior to cell division.

Polyamines have been found associated also with animal viruses containing DNA. Spermine and spermidine are both present within vaccinia virus (Lanzer and Holowczak, 1975). These authors showed also that radioactivity from tritium-labelled ornithine continued to be incorporated into polyamines and aminoacids in HeLa cells after infection with vaccinia virus. This continued incorporation was probably due to the presence in cells infected with vaccinia virus of a unique ornithine decarboxylase (Hodgson and Williamson, 1975). Small amounts of spermine and spermidine have been found also within the adenovirus (Shortridge and Stevens, 1973; Pett and Ginsberg, 1975). In herpes simplex virus, spermine was found to be present in the nucleocapsid with a nearly constant ratio between DNA phosphate and spermine nitrogen (Gibson and Roizman, 1971). This allows the possibility that the function of spermine is to neutralize the negative charges on the virus DNA to enable it to be packed into the virion.

It has been shown that diamines and polyamines, especially spermidine, are important for the replication of DNA in some animal cells. The polyamines spermine and spermidine are known to be present in poxviruses and other DNA viruses, thus it is not impossible that these compounds play some role also in virus replication.

Archard and Williamson (1971) have shown that arginine is required for vaccinia virus replication for events occurring early and late in the replication cycle. The early requirement is for events concerned with DNA synthesis. The late requirement is concerned with virus maturation and involves the incorporation of arginine into structural proteins. However, it was shown also that some arginine is metabolized before incorporation into the virion. Thus, it is possible that the late requirement for arginine for vaccinia virus replication involves not only the synthesis of polypeptides, but also the synthesis of polyamines. Such a requirement for polyamines could explain the failure to obtain yields of infectious progeny virus from infected CAM cells in the absence of serum if CAM cells were unable to synthesize polyamines.

Normal sera have been shown to contain putrescine (Pohjanpelto and Raina, 1972) which is a precursor of spermidine and spermine.

If CAM cells were unable to synthesize spermine or spermidine and if these polyamines were required for poxvirus replication, then it would be expected that these cells would not support the replication of poxviruses in the CAM *in ovo*. However, the CAM *in ovo* can support the replication of a wide range of poxviruses. This means that polyamines would have to be provided by other cells of the chick embryo via the vascular system or the amniotic or chorionic fluid. Raina (1963) has investigated the metabolism of spermidine and spermine in the embryos of White Leghorn chicks. It was found that while no spermine or spermidine was present in unincubated eggs, both were present in the embryo. Different amounts of polyamines were found in different tissues and the amounts found in extra-embryonic parts, such as the yolk sac, were in general much lower than in the embryo. In the amniotic and allantoic fluids both polyamines were found only in trace amounts. These results indicate that much of the polyamine synthesis occurs in the cells of embryonic tissue and that the extra-embryonic parts synthesize comparatively little, giving some support to the idea that CAM cells are defective in their ability to synthesize spermidine.

The data presented here constitute *prima facie* evidence that the serum factor, or one of the serum factors, required for poxvirus replication in CAM cells is spermine, spermidine or a precursor. However, the possibility has not been eliminated that a hormone or oligopeptide is required for poxvirus replication in CAM cells. The requirement for a serum factor(s) for the replication of poxviruses in CAM cells may also explain the failure of poxviruses to replicate in mouse S180 cells. These two cell lines are similar in respect of their ability to utilize citrulline and so it is not impossible that they are similar in other respects. In mouse S180 cells also the replication of virus was investigated in media containing no serum. Rabbitpox virus and the IHD strain of vaccinia

virus will give good yields in mouse S180 cells, thus it is possible that the metabolic competance of these viruses is different from that of other poxviruses, enabling them to overcome the presumed metabolic defect of the host-cell. However, since the replication of these two viruses was not investigated in CAM cells no firm conclusions can be drawn about the nature of their ability to replicate in mouse S180 cells.

The need for the presence of serum for the replication of poxviruses in CAM cells did not preclude further studies, since it was shown that a concentration of serum higher than the minimum required for virus replication did not contain sufficient free arginine to allow the replication of virus without the further addition of arginine. Further virus replication experiments, carried out using media supplemented with the minimum concentration of serum, showed that while the replication of vaccinia virus was unaffected by substituting citrulline for arginine, the replication of cowpox virus was strongly inhibited. This showed that cowpox virus was defective in its ability to utilize citrulline. It was shown further that the replication of cowpox virus was unaffected by substituting argininosuccinic acid for arginine. This demonstrated that either CAM cells or cowpox virus or both code for the enzyme required to synthesize arginine from argininosuccinic acid. From theoretical considerations it was deduced that if infection with cowpox virus does not turn off macromolecular synthesis within the CAM cell, then cowpox virus must be able to code for neither of the enzymes of arginine biosynthesis from citrulline, for a defective form of both enzymes, or a defective form of argininosuccinate synthetase. If, on the other hand, host-cell macromolecular synthesis is turned off following infection with cowpox virus, then it can be concluded that cowpox virus is able to produce a defective form only, of argininosuccinate synthetase.

These questions can be resolved most easily by analysis of the enzymes of arginine biosynthesis from citrulline within cowpox virus-infected and uninfected CAM cells. In order to perform such an analysis a sensitive assay system is required, since the activities of any

defective enzymes would be expected to be very low. In this thesis has been described an improvement of the sensitivity of the radiochemical assay system used in previous studies. Furthermore, the development has been described of an assay system that will allow the separate measurement of the activity of argininosuccinate lyase rather than the combined activity of argininosuccinate synthetase and lyase. This assay is not yet sufficiently sensitive to measure very low levels of enzyme activity, but the use of purified enzyme preparations as already discussed should improve markedly the sensitivity.

It has been proposed that poxviruses originated from a free-living organism. This proposition is supported by nearest-neighbour base-frequency analysis of vaccinia virus DNA, which shows that vaccinia virus DNA resembles more closely bacterial DNA than mammalian DNA (Subak-Sharpe et al, 1966). Other viruses have DNA similar to that from the host organism. Cooke (1972) proposed that the ability to synthesize arginine from citrulline was retained by poxviruses because it conferred some selective advantage. The basis of this proposition is that the eccrine sweat of the human adult contains citrulline at a concentration of 0.5mM (Coltman et al, 1966) which is the concentration that gives maximal yields of poxvirus in human citrullinaemia and mouse S180 cells. Neither of these cells is able to utilize citrulline for growth. Furthermore, arginine is not always present in human eccrine sweat and when it is present it is at a lower concentration than citrulline. Citrulline occurs also in protein of human hair, in the cells of both the medulla and the inner root sheath. It is not known whether it is citrulline qua citrulline that is incorporated or whether arginine is incorporated and subsequently converted to citrulline. This is one of the very few instances of citrulline occurring in protein. Cooke (1972) concluded that the ability of poxviruses to utilize citrulline might have conferred a selective advantage owing to the dissemination of poxvirus through skin lesions (Fenner, 1947).

If this thesis is accepted, then the inability of cowpox virus to utilize citrulline for replication implies

that the cells of bovine skin provide sufficient free arginine to allow the replication of cowpox virus. It is unlikely that the source of free arginine is eccrine sweat since, in general, only primates possess large numbers of true eccrine glands in their hairy skin. Cows rely partially on apocrine glands for dissipating heat and these glands secrete only small amounts and only at long intervals. Free amino-acids have not been detected in apocrine secretions (Montagna and Parakkal, 1974). It is perhaps significant that in infected cows the appearance of pocks is limited mainly to the udder, few appearing in the hairy skin. It is possible that the skin of the udder contains active eccrine sweat glands and that their secretions contain free arginine.

The fact that cowpox virus differs from vaccinia virus, rabbitpox virus and possibly other poxviruses in being unable to utilize citrulline for replication provides a relatively simple means of differentiating cowpox virus and vaccinia virus, which are otherwise very similar and also very similar to variola virus. Hitherto, these viruses could be distinguished only immunologically or on the basis of pock morphology.

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