THE ROLE OF ARGININE IN THE REPLICATION OF VACCINIA VIRUS

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ABSTRACT

Arginine is essential for the growth of both HeLa cells and vaccinia virus although depletion of arginine by starvation does not irreversibly affect the ability of either the cells to support virus replication or the virus to direct its replication. Deprivation of arginine results in changes in the pattern of macromolecular synthesis in both infected and control cultures. A marked reduction in the ability of cells to synthesise DNA, RNA and protein and consequently, an inhibition of virus growth is observed. Although virus specific DNA synthesis is completely abolished in arginine deprived, infected cells, the synthesis of at least some virus specific messenger RNA species and the proteins for which they code takes place under these conditions.

The yield of infective virus is dependent on the concentration of arginine in the medium in a dose response relationship, maximum yield being obtained in the presence of 0.09 mM. arginine. Virus yield from cell cultures infected in the presence of suboptimal concentrations of arginine is increased following further addition of arginine at a time after the completion of virus DNA synthesis and arginine is incorporated into mature virus particles. While the early requirement is satisfied by 0.015 mM. arginine, later events show a greater requirement up to a maxiumum of 0.09 mM. arginine.

It is concluded that the virus nutritional requirement is related to at least two functions. One is in early events which precede virus DNA synthesis and on which DNA synthesis depends. Another is in the synthesis of virus structural protein on which the maturation of virus particles eventually depends. These two requirements can be separated chronologically on the basis of their functions although in normally infected cells are probably concurrent. The structural protein(s) involved appears to be a product of early virus expression which would normally accumulate until virus maturation begins in cells infected in complete medium.

CHAPTER 1

INTRODUCTION

In any study of a nutritional requirement for virus replication it is essential that findings are interpreted in relation to the metabolic capacity and the nutritional requirements of the host system. Viruses are intracellular parasites and depend upon the metabolic functions of the host for the elaboration of virus specific products, the synthesis of which is directed by the infecting nucleic acid. As cellular enzymic pathways and biosynthetic mechanisms are utilised, it follows that a cellular nutritional requirement will also affect the replication of an infecting virus. An exception to this generalisation is the virus induced modification of host metabolic function relating to synthesis of nucleic acids. Increased activity of such enzymes is common in animal cells infected with DNA viruses (Green, 1966). The genomes of the larger viruses are capable of coding for the synthesis of many more proteins than are included in mature virus particles. Watson (1965) estimated the size of a bacteriophage gene to be about 10^6 daltons and on this basis the genome of vaccinia virus should correspond to about 150 genes (Sarov

& Becker, 1967). It is probable that some of these genes code for enzymic functions which are expressed during the virus replication cycle (Kit, 1963 ; Kit and Dubbs, 1965). However, in no case yet described has virus infection enhanced the capacity of host cells for synthesis of arginine.

The functions of arginine in cellular metabolism are complex. Apart from incorporation into protein, particularly basic protein, arginine is involved in the Krebs-Henseleit cycle of urea formation (fig. 1). This series of reactions represents the principle route of nitrogen excretion in whole mammals. Arginine is also involved in the synthesis of other amino acids. It is convertible to ornithine by amidinohydrolysis and thence through glutamic acid semialdehyde to glutamate by amino transfer with a 2-oxoacid followed by oxidation of the semialdehyde. Both glutamic acid semialdehyde and glutamate are key intermediates in amino acid metabolism, the former yielding proline by ring closure followed by reduction and the latter yielding aspartate by oxidation (fig. 2 ; Dagley and Nicholson, 1970). These reactions are significant in cultured cells as conventional tissue culture media contain neither proline nor aspartate.

Additionally, the role of arginine derivatives in the synthesis of various polyamines is of interest in the context of the regulatory functions of these compounds, particularly in RNA synthesis (Fox and Weiss, 1964 ; Raina and Cohen, 1966 ; Abraham, 1968). Putrescine can be derived from the amidinohydrolysis of agmatine which is the decarboxylation product of arginine, or conversely, from the decarboxylation of ornithine which is the amidinohydrolysis product of arginine. Spermidine is derived from putrescine by a reaction in which the decarboxylated carbon chain and amino group of methionine is transferred from S-adenosylmethionine (fig. 3). The decarboxylation of these amino acids and the synthesis of polyamines may be significant in the control of pH when cultures become acidic.

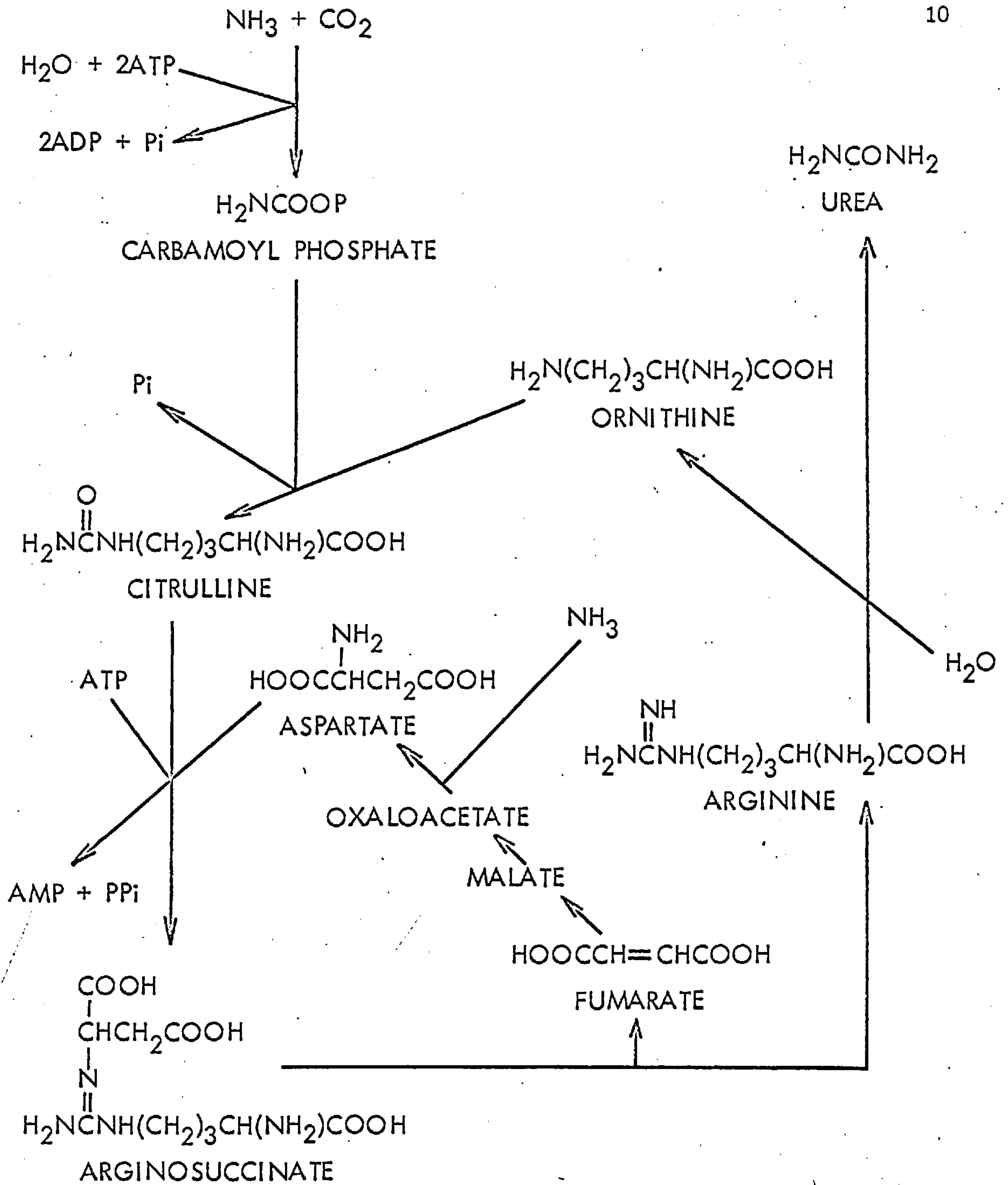

Fig. (1). The Krebs - Henseleit cycle of urea formation.

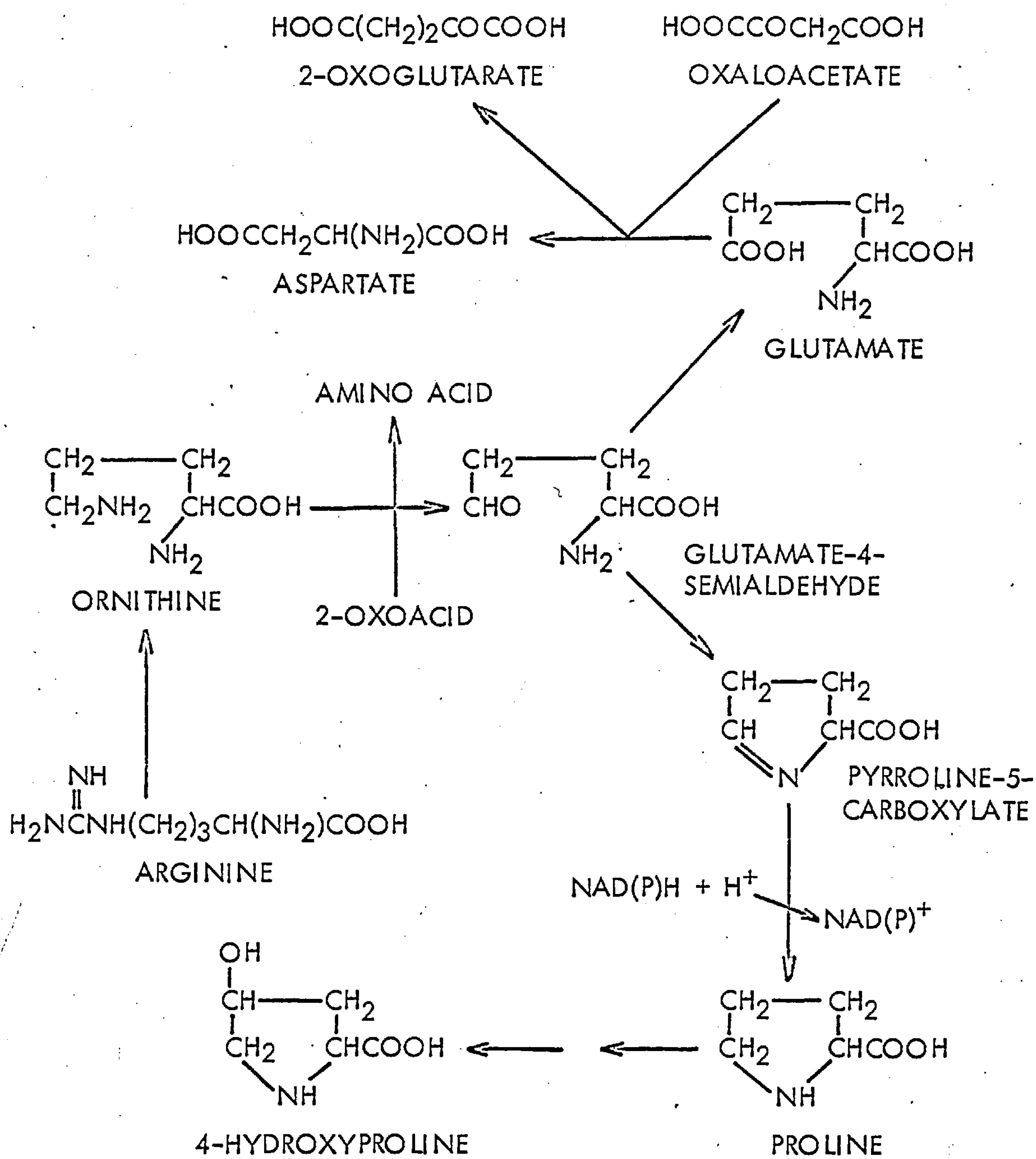

Fig. (2). Some reactions of arginine derivatives in amino acid biosynthesis.

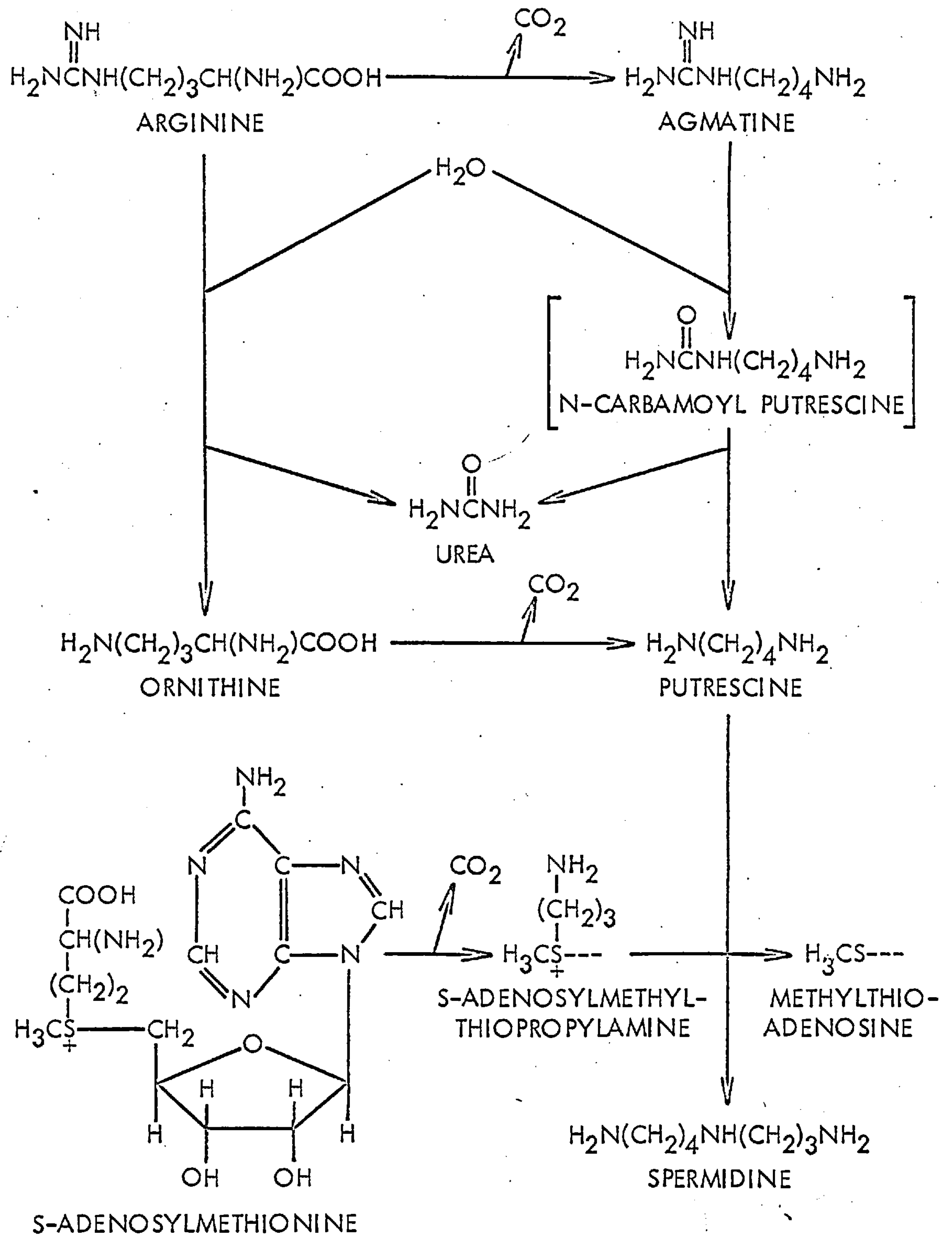

Fig. (3). Some reactions of arginine derivatives in polyamine biosynthesis.

Another aspect of arginine metabolism of great importance in nutritional studies employing cell cultures is the degradation of arginine by pleuropneumonia-like organisms (PPLO ; Mycoplasma sp.) which are common contaminants of secondary and continuous cultures. This aspect of PPLO contamination was reported by Kenny and Pollock (1962, 1963) who noted a retardation of the growth of cultured cells due to depletion of arginine by the contaminants. Schimke and Barile (1963) investigated the mechanism of arginine degradation by five strains of PPLO isolated from tissue cultures and concluded that an arginine dihydrolase pathway involving the enzymes L-arginine iminohydrolase, carbamoyl phosphate:L-ornithine carbamoyl transferase and carbamoyl phosphokinase was employed. This pathway, well known in microorganisms including Streptococcus (Hills, 1940), Clostridium (Schmidt et al., 1952) and Lactobacillus (Walker, 1953) results in the formation of adenosine triphosphate and is in contrast to the minimal breakdown of arginine to ornithine by cellular L-arginine amidinohydrolase (fig. 4). Stalon et al. (1967) have shown that in Pseudomonas at least, the degradation of citrulline to ornithine with the consequent formation of carbamoyl phosphate is not accomplished by a reversal of the action of the biosynthetic carbamoyl phosphate:L-ornithine carbamoyl transferase active in the urea cycle, but by the action of a catabolic enzyme having identical specificity but a different pH optimum. This second enzyme is induced by the presence of arginine whereas the biosynthetic enzyme is repressed. The dihydrolase pathway appears to represent an important source of high energy phosphate for PPLO and other studies support this (Barile et al., 1966 ; Schimke, 1967).

The requirement for arginine in the nutrition of animal cells is well established. Although not strictly necessary for the maintenance of nitrogen balance in whole animals (Madden et al., 1943 ; Rose et al., 1955) omission of arginine from otherwise adequate diets was found to retard growth but not to cause complete cessation (Rose, 1938). Similarly, Eagle (1959) noted that all animal cell

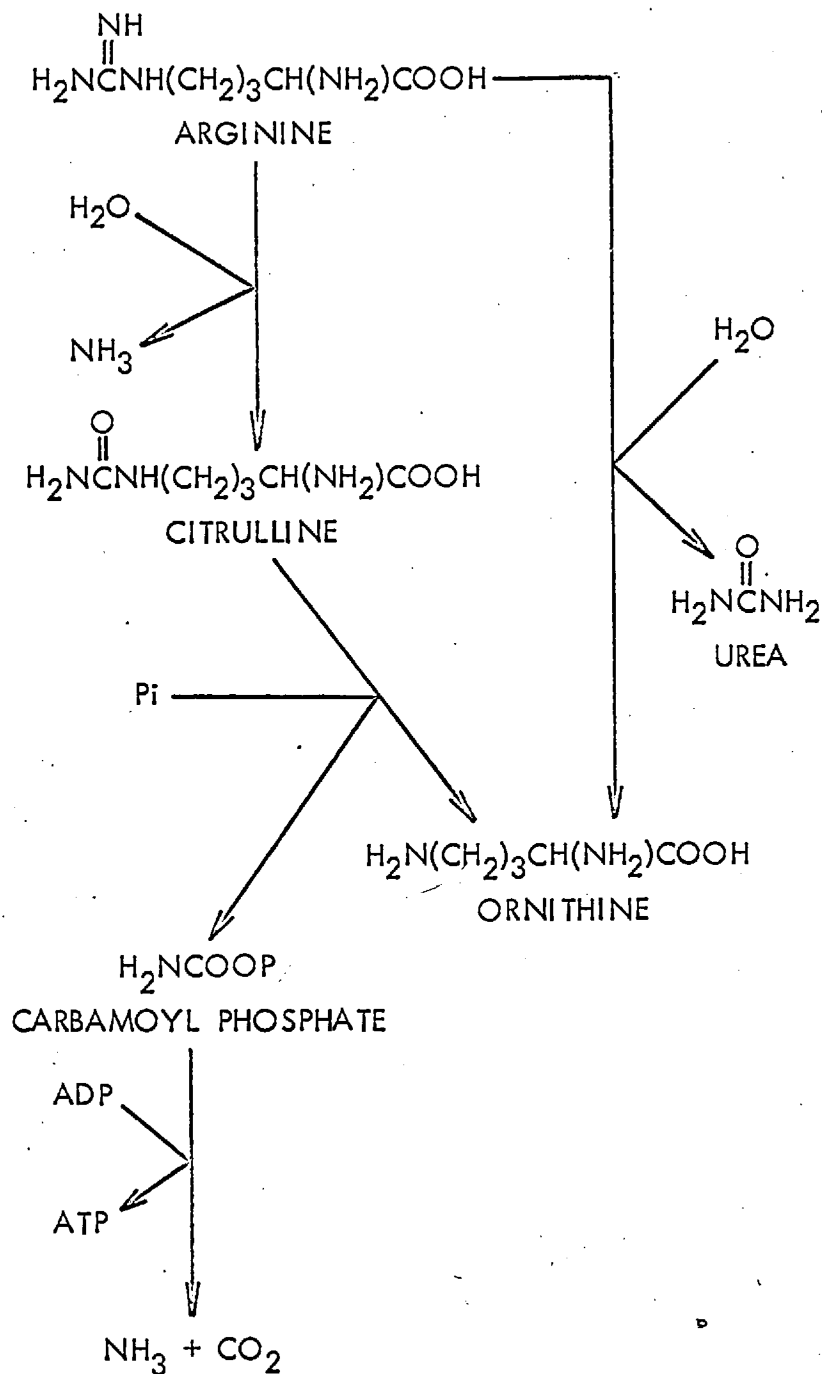

Fig. (4). Degradation of arginine.

cultures examined, whether of normal or malignant derivation, required arginine for survival and propagation. However, in many cultures both of primary and continuous line cells, citrulline but not ornithine can substitute for arginine (Tytell and Neuman, 1960). The presence of the enzymes L-citrulline:L-aspartate ligase and L-arginosuccinate arginine-lyase can be inferred in these cultures although the ability to convert ornithine to citrulline is absent. McCarthy (1962) studied the selective utilisation of amino acids by a variety of mammalian cell cultures and noted an attendant production of ornithine by those cell types having the greatest rates of arginine utilisation. Thus it seems that whilst possessing many of the metabolic functions of whole animals the ability of these cell cultures to synthesise arginine is blocked to some extent, presumably at the level of carbamoyl phosphate: L-ornithine carbamoyl transferase and that under conditions of arginine deprivation significant amounts of arginine are unlikely to be formed. Additionally, the excretion of excess nitrogen as urea will be inhibited although this appears to be of little significance in the growth of cultured cells.

Requirements for arginine in the growth of a number of DNA viruses replicating within the nuclei of their host cells have been reported. The viruses range from the comparatively small Papovaviruses SV40 and polyoma (genome molecular weight $3\text{-}5 \times 10^6$ daltons) through certain Adenoviruses (genome molecular weight $20\text{-}25 \times 10^6$ daltons) to the large and complex Herpesviruses (genome molecular weight $54\text{-}92 \times 10^6$ daltons), herpes simplex virus and cytomegalovirus. The arginine requirements of the adenoviruses and of herpes simplex virus have been extensively investigated and will be considered in detail.

Adenoviruses

Bonifas and Schlesinger (1959) observed an enhancement of the cytopathic effect (CPE) of adenovirus type 2 growing in cultures of KB cells (Eagle, 1955a ; squamous carcinoma of lip) when the concentration of arginine in Eagle's basal medium (BME ; Eagle, 1955b) was raised 4-fold. Using BME containing an elevated concentration of arginine a plaque assay was developed with this system. Rouse et al. (1963) reported an absolute requirement for exogenous arginine for the replication of adenovirus type 2 in KB cell cultures and demonstrated that the reduction in the efficiency of plating of the virus in cultures infected with PPLO was due to the rapid depletion of arginine by these contaminating organisms. Normal growth of the virus could be restored by curing the cultures of PPLO contamination using the antibiotic kanamycin (Pollock et al., 1960) or by raising the concentration of arginine in the medium. The same authors reported a similar arginine effect with adenovirus strains of type 1, 3 and 4.

Bonifas (1967) measured the utilisation of radioactively labelled arginine by adenovirus type 2 infected and control cultures of KB cells. A higher rate of arginine utilisation was established in infected cultures coinciding with the formation of progeny virus in the cells.

Rouse and Schlesinger (1967) measured the yields of adenovirus type 2 from infected cultures of KB cells maintained in media deficient in various single amino acids and found that only arginine deficiency completely prevented virus replication. When arginine was restored to cultures infected for 30 hr. in its absence, infectious virus was produced without lag and at normal rate. The simultaneous addition of 5-fluorodeoxyuridine (FUDR) failed to inhibit subsequent

production of progeny virus and (^{3}H)thymidine supplied only during the period of arginine deprivation resulted in the formation of radioactively labelled virus following restoration of arginine. These results suggest that viral DNA but not complete virions was synthesised in the absence of arginine. Immunofluorescence and haemagglutination techniques indicated that early antigen and proteins associated with the hexon and penton-fibre subunits were also synthesised to some extent in conditions of arginine deprivation. When (^{3}H)arginine was supplied to cultures previously infected in the absence of arginine the radioactive label was associated with the purified yield of virus leading Rouse and Schlesinger to the conclusion that arginine was essential for the synthesis of late protein(s) involved in the maturation of progeny virus.

Hexon, penton and fibre components account for less than the total virion protein (Valentine and Pereira, 1965 ; Green et al., 1967 ; Russell et al., 1967a). Comparison of the amino acid composition of whole virions with that of capsid antigens suggested the presence of additional arginine-rich proteins (Pettersson et al., 1968). Prage et al., (1968) caused sequential disintegration of adenovirus type 2 particles removing penton subunits by treatment with detergent and hexon subunits by repeated freezing and thawing. The resulting cores were extracted with acid and two protein bands were obtained on polyacrylamide gel electrophoresis of the acid extract. These gave lines of precipitation against antiserum to adenovirus type 2 core preparations but not against antisera to hexon, penton or fibre components. There was no serological identity with preparations of either adenovirus type 5 or KB cells. These two basic proteins, immunologically distinct from the known capsid antigens, comprised 30-40% of the core and contained about 20% arginine. Additional proteins associated with the viral DNA could be dissociated by high concentrations of caesium chloride and urea.

Russell and Becker (1968) found that a generally similar situation applied to adenovirus type 5 - infected human embryo kidney cells. Complement fixation, haemagglutination and immunofluorescence tests showed that all the capsid antigens were made in the absence of arginine. Further immunofluorescence tests suggested that the arginine requiring step involved a component of the P antigen. This complex antigen is not related to the capsid antigens but to a component which is thought to be incorporated within the virus particle (Russell and Knight, 1967).

Dubes et al., (1969) have reported an arginine requirement for the replication of human adenoviurus type 1 in the eta line (Chapin and Dubes, 1964) of rhesus monkey kidney cells. Of eleven amino acids tested, only addition of arginine to medium in which cultures had been maintained prior to infection restored the ability of the cells to produce a full yield of virus. No evidence of PPLO contamination was found and it was concluded that the cells themselves utilised arginine at a high rate.

Amino acid analyses of a number of adenovirus strains showed that they contain about twice as much arginine as RNA animal viruses (Polasa and Green, 1967). It seems reasonable to suppose that Adenoviruses contain internal arginine rich basic proteins, perhaps associated with the viral DNA in some specific manner.

Herpesviruses

Tankersley (1964) examined the amino acid requirement for the propagation of herpes simplex virus in cultures of Minn EE cells (Syverton and McLaren, 1957 ; human oesophageal epithelium) maintained in BME, after observing a spontaneous regression of CPE in minimally infected cultures. Eleven amino acids and

glutamine were found to be required to some degree. In the absence of arginine, infected cultures neither showed CPE nor supported virus replication, but restoration of arginine to cultures infected in its absence permitted subsequent appearance of CPE and formation of progeny virus.

Sharon (1966) followed the propagation of herpes simplex virus in cultures of Wish cells (Hayflick, 1961 ; human amnion) maintained in BME and noted an enhancement of the formation of polykaryons on raising the concentration of arginine.

Becker et al., (1967) investigated the nature of the arginine requirement using the HF strain of herpes simplex virus propagated in cultures of BSC_1 cells (Hopps et al., 1963 ; monkey kidney). The yield of virus was found to depend upon the concentration of arginine in the medium. In the absence of arginine, virus replication as determined by both plaque assay and by increase of DNase - resistant DNA was completely inhibited. However, DNA synthesis as followed by incorporation of (^{3}H)thymidine was not affected by deprivation of arginine in either control or infected cultures, although the DNA formed remained susceptible to DNase. Nuclei from cultures previously infected in the presence of (^{3}H)thymidine and (^{14}C)leucine or (^{14}C)arginine were examined by sucrose density gradient centrifugation. In the absence of arginine, coated virus particles were not formed and radioactively labelled proteins were found only in the soluble fraction at the top of gradients. In the presence of arginine however, a band of virus containing both the DNA and protein labels was observed and additionally, two seqarate bands of labelled protein shown to contain either viral capsids or capsomeres (Olshevsky et al., 1967) were present. The same pattern of labelled proteins was observed with either (^{14}C)leucine or (^{14}C)arginine. These results indicate that arginine is essential for the synthesis of viral coat proteins

and is incorporated into them. Deprivation of arginine resulted in a reduction of the rate of protein synthesis in both infected and control cultures. An increase in the rate of protein synthesis in arginine deprived, infected cultures was demonstrated within 2 minutes after the restoration of arginine. Radioactively labelled protein formed in the cytoplasm after addition of arginine to such cultures appeared in coated virus particles in the cell nuclei within 2 hr. This is in accord with the time required under normal circumstances for viral proteins to be transported to the nucleus and the viral DNA coated (Olshevsky et al., 1967). The subsequent increase of DNase-resistant DNA followed the same kinetics as in cultures infected and maintained in complete medium. Thus functional viral messenger RNA was already present in the cytoplasm, but no protein synthesis occurred in the absence of arginine.

Jeney et al., (1967) reported the arginine requirement for the replication of a genital strain of herpes simplex virus in PPLO-free cultures of the continuous cell lines HeLa (Gey et al., 1952 ; cervical carcinoma), HEp_2 (Fjelde, 1955 ; laryngeal epithelioma) and KB. Arginine was not required for virus replication in primary cultures of human or chick embryonic fibroblasts or of monkey kidney cells. Adsorption of the virus to HeLa cells was not inhibited in the absence of arginine although the development of CPE and of specific viral antigens detectable by immunofluorescence were blocked. Further, Gonczol et al., (1967) demonstrated that deprivation of arginine inhibited DNA synthesis in uninfected HeLa cell cultures but not in cultures of human embryonic fibroblasts. Infection of HeLa cell cultures in complete medium resulted in an enhancement of incorporation of the radioactive label from (^{32}P)disodium hydrogen phosphate into the DNA fraction of acid-precipitable material. In the absence of arginine however, incorporation into both infected and control cultures was reduced and the stimulation of DNA synthesis

normally observed on infection in the presence of complete medium was absent.

These results are in contrast to those of Becker et al., (1967) and apparent anomalies must be explained in terms of cellular metabolism. The primary cultures studied by Jeney et al., (1967) are capable of synthesising both protein and functional DNA in conditions of experimental arginine deprivation implying some availability of endogenous arginine. Of the continuous cell lines, HeLa cells at least, have greatly impaired ability to synthesise both DNA (Gonczol et al., 1967) and protein (Archard, results to be presented) in the absence of arginine. The evidence as to whether the reduction of DNA synthesis under these conditions depends on reduction of protein synthesis or whether arginine has some direct function in the synthesis of DNA is inconclusive. Kates and McAuslan (1967a) demonstrated that synthesis of viral DNA in rabbit poxvirus infected HeLa cells requires concurrent synthesis of protein(s) associated stoichiometrically rather than catalytically with the formation of the nucleic acid. Similarly, Shimono and Kaplan (1969) suggested that in polyoma virus-infected mouse embryo cells histone synthesis was stimulated in parallel with the stimulation of DNA synthesis. However, Hare (1969) showed that in cultures of mouse embryo cells in which DNA synthesis was inhibited by the presence of the arginine structural analogue canavanine, patterns of histone synthesis were unaltered up to the time required for 50% inhibition of DNA synthesis. Synthesis of RNA was also inhibited in the presence of canavanine but the rate of inhibition did not correlate with experimentally induced changes in the rate of inhibition of DNA synthesis. These results suggest that inhibition of DNA synthesis is related to an event not directly involving a block in the synthesis of either RNA or histones. This event was thought to be the synthesis of unspecified abnormal

canavanyl protein. The BSC_1 line of monkey kidney cells employed by Becker et al., (1967) has reduced ability to synthesise protein in arginine free medium but synthesis of DNA appears to be unaffected. These results may be related to experimental conditions. Without depletion of the intracellular pool of arginine by maintenance of cultures in arginine free medium prior to infection sufficient arginine may be available to permit synthesis of protein necessary for the initiation of viral DNA synthesis. When the arginine concentration became limiting the ability of the cells to synthesise further proteins would depend upon the nature of the proteins. A selective failure of synthesis would occur, related to the number of arginine residues specified by the messenger RNA. Large protein molecules would have a reduced probability of completion, as would proteins rich in arginine. Such an effect has been observed by Spring et al., (1969) who used amino acids radioactively labelled with different isotopes to demonstrate changes in the proteins synthesised in herpes simplex virus infected HEp_2 cultures on deprivation of arginine. Mixtures of lysates of infected cultures maintained in the presence and absence of arginine were subjected to polyacrylamide gel electrophoresis. The extent of labelling of proteins synthesised in the absence of arginine compared to that in the presence of arginine was inversely proportional to the protein molecular weight, as determined by migration in the gels. Additionally, a marked decrease in the labelling of protein bands at five positions in the gels indicated that these proteins were arginine rich. Similarly, Olshevsky and Becker (1970a) have demonstrated that of the seven major structural proteins detected by polyacrylamide gel electrophoresis of enveloped herpes simplex virions the two capsid proteins and at least some of the glycoproteins of the envelope were synthesised in BSC_1 cultures infected in the absence of arginine. The two viral core proteins were not. The capsid proteins and one of the glycoproteins have been shown to have the lowest arginine to leucine ratios among the herpes simplex virus structural proteins (Oshevsky and

Becker, 1970b). Virus specified protein synthesised in the absence of arginine was utilised in the formation of progeny virus when arginine was restored to deprived cultures. This is supported by the results of Courtney et al., (1970) who demonstrated that in herpes simplex virus infected cultures of BHK_{21} cells (MacPherson and Stoker, 1962 ; continuous baby hamster kidney) maintained in the absence of arginine, immunofluorescent and complement-fixing antigens were confined mainly to the cytoplasm. Restoration of arginine to the cultures at 10 hr. post infection resulted in the appearance of normal amounts of these antigens in the cell nuclei within 6 hr. The results of Inglis (1968) however, are in contrast. No formation of either immunofluorescent or complement-fixing antigens was detected in herpes simplex virus infected cultures of RK_{13} cells (Beale et al., 1963 ; continuous rabbit kidney) maintained in the absence of arginine. This discrepency may be a reflection of the different host systems as a variability in the capacity of cell lines to produce complement-fixing antigens has been reported (Geder et al., 1968).

It is of interest that proteins synthesised in cultures of primary rabbit kidney cells infected with the Herpesvirus pseudorabies virus contain more arginine relative to leucine than do proteins synthesised in uninfected cultures (Kaplan et al., 1970).

A requirement for arginine in the replication of another herpesvirus, cytomegalovirus, has been reported (Minashima and Benyesh-Melnick, 1969). Cultures of human embryonic lung fibroblasts infected with strains of either human or monkey cytomegaloviruses in the absence of arginine developed no CPE and produced neither complement-fixing antigen nor progeny virus. When arginine was restored to such cultures CPE appeared within 2 days in contrast to the 4-5 days required in cultures infected

in complete medium, and progeny virus was produced.

The results obtained with Herpesvirus infected cultures are more difficult to interpret than those obtained with Adenovirus infected cultures owing to variability in cellular function. However, an overall similarity with the Adenoviruses is apparent in that certain arginine rich proteins associated with the viral cores are not produced in the absence of arginine although early viral functions are expressed to some degree. Additionally, the accumulation of viral antigens outside the nuclear membranes of arginine deprived, infected cells suggests that arginine may be involved in a transport phenomenon.

Papovaviruses

Goldblum et al., (1968) reported an arginine requirement for the replication of Simian virus 40 (SV40) in cultures of BSC_1 cells. No mature progeny virus was produced in infected cultures deprived of arginine. Immunofluorescent techniques indicated that whilst synthesis of the early tumour antigen was only slightly diminished, synthesis of late viral coat proteins decreased tenfold. Restoration of arginine to deficient cultures reversed the inhibition. The continued synthesis of tumour antigen whilst the vegetative replication cycle is inhibited by arginine deprivation is of interest. Rogers and Moore (1963) have demonstrated that the genome of the oncogenic Shope papilloma virus codes for an arginase present in infected cells. The expression of late viral function in this case may be regulated by the concentration of arginine in the cells, leading to a state of latency and possibly to oncogenesis.

The effect of arginine deprivation on the replication of polyoma virus in cultures of primary mouse embryo cells has been reported (Winters and Consigli, 1969). Although production of

mature progeny virus was inhibited by lack of arginine, DNA synthesis measured by the incorporation of (^{3}H)thymidine was unaffected. However, the DNA remained susceptible to degradation by nucleases as determined by release of the radioactive label. Protein synthesis measured both by immunofluorescence and by incorporation of (^{3}H)valine was also unaffected. Thus arginine deprivation prevented maturation of polyoma virus, a situation resembling the effect on adenovirus replication.

From the results presented it seems that a requirement for arginine in a late synthetic step may be a feature common to the replication of many intranuclear DNA viruses. The work presented in this thesis deals with the role of arginine in the replication of a DNA virus in which the synthesis and assembly of virus specific macromolecles is entirely confined to the cytoplasm. In this context a number of reports concern the requirement for arginine in the growth of poxviruses.

Poxviruses

Wells (1967) described the dependence of haemagglutinin production by variola infected human embryonic fibroblast cultures upon the availability of bicarbonate. Raising the concentration of arginine in the medium partially overcame this effect which was ascribed to the biosynthtic relationship between bicarbonate, carbamyl phosphate and arginine presumed to hold in primary cell cultures.

Holtermann (1969) investigated the amino acid requirement for the growth of the WR strain of vaccinia virus in cultures of Earle's L cells (Sanford et al., 1948 ; continuous mouse fibroblast). After depletion of the intracellular pool of amino acids by incubation of the cultures in a balanced salts solution prior to infection it was found that the amino acids essential for growth of the virus

included arginine.

The results of Singer et al., (1970) are of special interest, relating both to the results of Rouse et al., (1963) and to those of Jeney et al., (1967). The growth of vaccinia virus in cultures of primary hamster embryonic fibroblasts and in cultures of FL cells (Fogh and Lund, 1957 ; continuous human aminion) was studied. Superinfection of the cultures with an arginine utilising species of Mycoplasma resulted in a reduction of virus yield of 1.7 log.10 from the continuous cell cultures but of only 1.1 log 10 from the primary cell cultures. Superinfection with a dextrose fermenting (non arginine utilising) species of Mycoplasma had no effect on virus yield from either type of cell culture. Raising the concentration of arginine in the medium 4-fold nullified the effect of the arginine utilising contaminant. Thus Mycoplasma infection emphasised the viral arginine requirement which was greater in the continuous cell cultures than in the primary cell cultures.

The experiments to be described were designed to investigate the nature of the arginine requirement for the replication of vaccinia virus. This was examined by the biological parameter of infectivity and by the biochemical parameter of changes in patterns of macromolecular synthesis in cultures infected in the presence and absence of arginine. The fate of arginine supplied during the replication cycle was also studied.

CHAPTER 2

MATERIALS AND METHODS

2.1 Virus and cell culture

The growth of the Lister strain of vaccinia virus in monolayer cultures of HeLa cells maintained in complete or arginine deficient media was studied. Stock HeLa cultures were grown in a commercial Eagle's minimum essential medium (MEM ; Eagle, 1959 ; Flow Laboratories Ltd., Irvine, Scotland) supplemented with 5% (v/v) whole calf serum (Fraburg Ltd., Maidenhead, Berkshire, England.) in either 12 oz. prescription bottles containing 35 ml. of medium or 40 Oz. roller bottles containing 150 ml. of medium. All media routinely contained 200 I.U. per ml. penicillin, 200 µg. per ml. streptomycin, and 50 µg. per ml. nystatin. Bottles were seeded at a cell density of 2.5 x 10^5 cells per ml. and confluent monolayers were removed from culture bottles and redistributed as cell suspension as follows. After decanting the spent medium and washing the

monolayers with two changes of phosphate buffered saline solution A (PBSA ; Dulbecco, 1954 ; Oxoid Ltd., London.) the cultures were incubated at 37° for 5 min. with a volume of 0.02% (w/v)ethylene diamine tetracetic acid (EDTA ; British Drug Houses Ltd., Poole, Dorset, England.) solution in PBSA sufficient to cover the cells. The detached cells were recovered by low speed centrifugation (100g, 2 min.) and resuspended without serum by repeated pipetting in a small volume of medium. After estimating the cell density of a sample of the suspension using a standard haemocytometer, the remainder was diluted as appropriate with medium containing serum and distributed into bottles.

The importance of avoiding Mycoplasma contamination of cell cultures has already been emphasised. The cultures were periodically tested for contamination by attempting to isolate PPLO on suitable media. Samples of cell suspension were inoculated into PPLO broth (Morton and Lecee, 1954 ; Difco Laboratories, Detroit, Michigan, U.S.A.) supplemented with 10% (w/v)yeast extract (Oxoid Ltd.) and 20% (v/v) horse serum (Oxoid Ltd.). After incubation at 37° for 7 days, any intact HeLa cells were disrupted by ultrasonic treatment (Headland Electrosonic bath ; Surgical Supplies Ltd., London.) of the broths for 30 sec. and samples were inoculated onto PPLO agar (Morton et al., 1951 ; Difco Laboratories) plates supplemented in the same manner as the broths. The plates were reincubated at 37° and examined microscopically for the presence of PPLO colonies at intervals between 7 and 14 days after inoculation. Identification was aided by the use of Dienes stain which is selectively taken up by these colonies. Results were consistantly negative although this culture system was capable of supporting the growth of PPLO as demonstrated by the isolation from another continuous cell line of colonies typical in morphology and staining. Additionally, HeLa

cells were occasionally cultured in antibiotic free medium to eliminate the possibility of static contamination by microorganisms.

Virus stocks were prepared by infecting confluent monolayers of HeLa cells in 40 oz. bottles (approximately 1×10^8 cells per bottle) at low multiplicity (0.1 - 1.0 pfu per cell) using a virus inoculum suspended in 20 ml. of serum free MEM. After adsorption at 37° for 2 hr., the inoculum was replaced with growth medium. Confluent CPE, taking the form of rounding and partial detachment of the cells, was observed after incubation 37° for 2 days. Infected cells were recovered by shaking into the medium which was then subjected to low speed centrifugation (100g, 5 min.). The spent medium was discarded and the cell pellet resuspended in 5 ml. of arginine deficient medium (see below). After disruption of the cells by two cycles of freezing and thawing and ultrasonic treatment for 2 min., the cell debris were removed by centrifugation (1,500g, 5 min.) and the virus containing supernatant was retained. The pellet was again resuspended, the suspension ultrasonicated and the debris removed by centrifugation. The supernatents were pooled and constituted the virus stock which was stored frozen at -50° in small aliquots. Virus yield was about 2×10^9 pfu per 40 oz. bottle. No estimate of the particle to infectious unit ratio was made.

Experiments following the production of progeny virus were performed with HeLa cell cultures grown in 6"x⅝" test tubes using a modified Eagle's medium (table 1). The medium was based on that employed by Birch and Pirt (1969) who, from a study of the choline and serum protein requirements of mouse LS cells in culture, concluded that many widely used media are choline deficient and that a major role of serum in such media is to provide choline. As many of the experiments to be described depended on the presence of known low concentrations of arginine

Inorganic salts	mg. per L.
NaCl	8,000
KCl	400
$CaCl_2$	140
$MgSO_4.7H_2O$	100
$Na_2HPO_4.12H_2O$	60
KH_2PO_4	60
$MgCl_2.6H_2O$	100
$NaHCO_3$	1,100

Vitamins	mg. per L.
i-Inositol	2.0
d-Biotin	0.1
Choline	10.0
Nicotinic acid	1.2
Pantothenic acid	1.2
Pyridoxal	1.0
Thyamine	2.0
Riboflavin	0.2
Hypoxanthine	10.0
B_{12}	1.0

L-amino acids	mg. per L.
Histidine	100
Lysine	175
Isoleucine	180
Leucine	180
Methionine	30
Phenylalanine	65
Threonine	100
Tryptophan	20
Valine	150
Alanine	90
Serine	20
Glycine	15
Tyrosine	70
Cystine	75
Glutamine	200
Na glutamate	1,530

D-sugars	mg. per L.
Glucose	1,000

Table (1). Defined experimental medium.

in the medium it was considered desirable to exclude serum, a possible source of amino acids and consequently a medium containing a relatively high concentration of choline was chosen. It will be shown that under conditions of partial arginine deprivation the presence of serum in the medium has a virus growth promoting effect. The medium also contained elevated concentrations of amino acids as shown to be required for optimal growth of mouse LS cells (Griffiths and Pirt, 1967) and glutamic acid partially replaced the labile constituent glutamine. The pH of the medium at 37° was maintained at 7.3 by the addition of 14 mM N-2-hydroxyethyl piperazine-N'-2-ethanesulphonic acid (HEPES ; Williamson and Cox, 1968 ; Calbiochem Ltd., London.). Kanamycin sulphate (Bristol Laboratories, Feltham, England.) was added to the medium at a final concentration of 200 µg. per ml. as an additional precaution against Mycoplasma contamination. Tubes were seeded with 1 ml. of medium supplemented with 5% calf serum and containing $4x10^{5}$ cells. Confluent monolayers were obtained after incubation at 37° for 1-2 days and consisted of approximately 10^{6} cells. Cultures were depleted of arginine or equilibrated to a required concentration of arginine by incubation in appropriate medium without serum for 18 hr. prior to use. The maintenance of cultures for up to 48 hr. in medium lacking both serum and arginine resulted in no visible cytological changes (plates 1 and 2). Cultures were infected at a multiplicity of 5 pfu per cell by adsorption at 37° for 1 hr. from a virus inoculum suspended in 0.2 ml. per tube of medium containing an appropriate concentration of arginine. At the end of the adsorption period, designated time zero, the inocula were removed, the cultures washed once with Hanks' balanced salt solution (BSS ; Hanks, 1949) to reduce unadsorbed virus and medium lacking serum and containing an appropriate concentration of arginine was added. Samples consisting of 5 tubes from each series of cultures were taken at various times post infection and immediately frozen at -50 to await titration of infective virus.

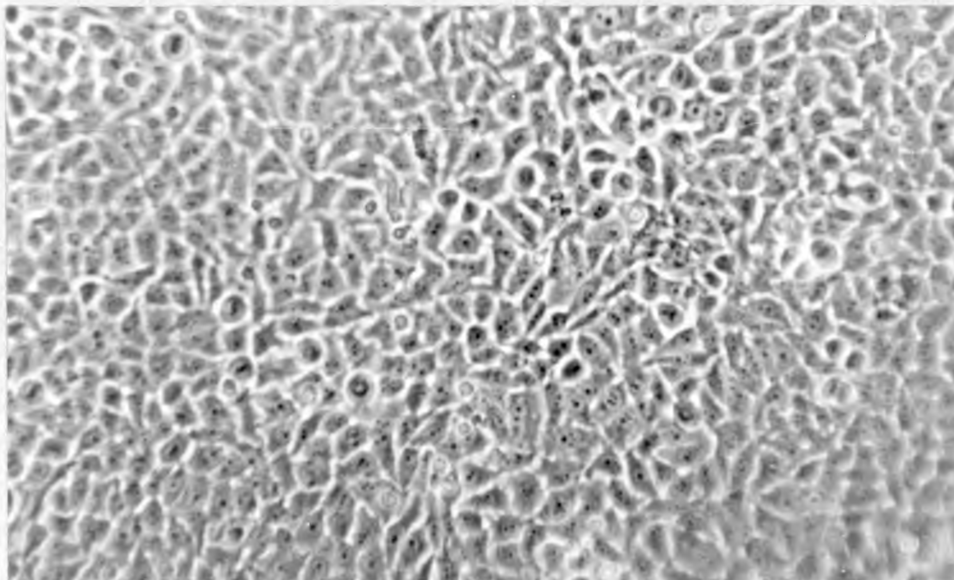

Plate (1). HeLa cell cultures grown in experimental medium containing 1mM. arginine and supplemented with 5% (v/v) calf serum.

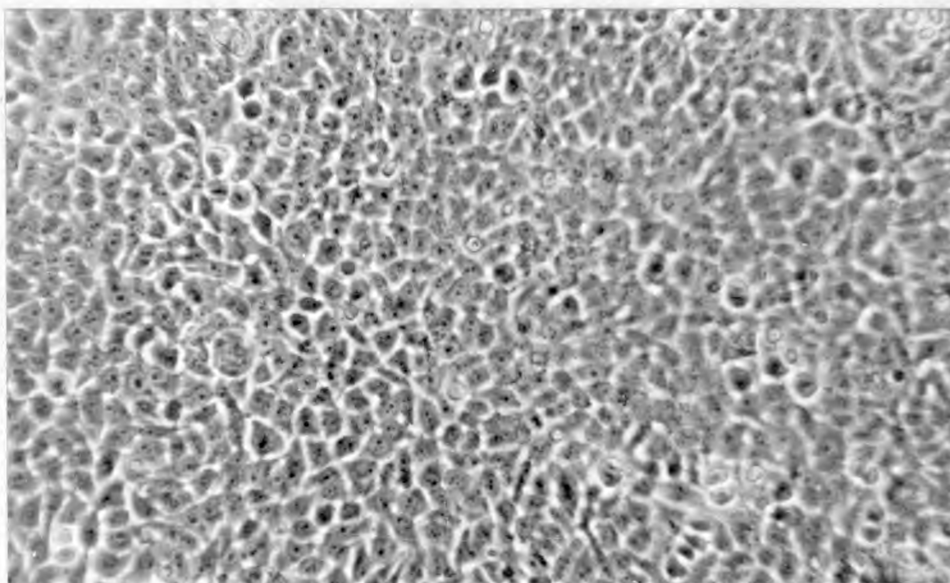

Plate (2). Similar cultures maintained for a further 48hr. in medium lacking both arginine and serum.

Infectivity titres were determined by plaque formation in monolayers of RK_{13} cells prepared in 6"x⅝" test tubes. Stock cultures of RK_{13} cells were grown in 40 oz. roller bottles containing 150 ml. of a commercial 199 medium (Morgan et al. 1950, Flow Laboratories Ltd., Irvine, Scotland) supplemented with 10% calf serum. Bottles were seeded at a cell density of $2.5x10^5$ cells per ml. and confluent monolayers were obtained after incubation at 37° for 5-6 days. Monolayers were removed from culture bottles and redistributed as cell suspension by a method similar to that used for HeLa cultures except that the 0.02% solution of EDTA in PBSA also contained 0.05% (w/v)trypsin (Baird and Tatlock Ltd., London.). Tubes were seeded with 1 ml. of medium 199 supplemented with 10% calf serum and containing $4x10^5$ cells. Confluent monolayers were obtained after incubation at 37° for 4-5 days.

After two cycles of freezing and thawing, the cells from HeLa cultures awaiting infectivity assay were scraped into the medium using a Pasteur pipette and the cell suspensions from tubes constituting a sample were pooled. Cell disruption was completed by treatment with ultrasonics for 2 min. Serial tenfold dilutions of the ultrasonicates were prepared in medium 199 without serum and dilutions over a range of 3 log_{10} were plated in triplicate onto RK_{13} cell cultures in tubes. Inocula of 0.2 ml. per tube were allowed to adsorb at 37° for 1 hr. The inocula were then removed, the monolayers washed once with Hanks' BSS and 1 ml. aliquots of medium 199 supplemented with 5% calf serum were supplied. The titration cultures were then reincubated at 37° pending plaque formation. At 40 hr. post infection plaques were 0.5-1.0 mm. in diameter and were counted after removing the medium and staining the monolayers (carbol fuchsin ; Solmedia Ltd., London.). Additional plaques, readily distinguishable by their smaller size began to appear by 48 hr. post infection. These have been shown to be due to the establishment of secondary foci of infection rather

than to the late development of primary foci (Baxby and Rondle, 1967). Infectivity titres were computed from the arithmetic means of replicate determinations at dilutions where 30 to 300 plaques per monolayer were present. Replicate determinations were usually within 10% of the mean.

This assay was compared to plaque and haemadsorption assays in HeLa cell cultures. Plaque formation was detected as described above. Haemadsorbing foci were detected by flooding the infected cell sheets of titration tubes with 0.2 ml. aliquots of a 2% (v/v) suspension of vaccinia sensitive fowl red blood corpuscles (Salisbury Laboratory Ltd., Salisbury, Wiltshire, England.) in complement fixation test diluent (Meyer et al, 1946 ; Oxoid Ltd.). After incubation at 37° for 30 min., unadsorbed fowl cells were removed by washing the monolayers with diluent, the adsorbed fowl cells fixed with 5% (w/v) formaldehyde solution in water and the haemadsorbing foci counted.

2.2 Use of radioactively labelled precursors.

Experiments involving the use of radioactively labelled precursors were also performed with HeLa cell cultures in test tubes. The synthesis of DNA, RNA and protein in infected and control HeLa cell cultures in the presence and absence of arginine was followed by the incorporation into acid precipitable material of suitable precusors labelled with the radioactive isotopes ^{3}H or ^{14}C. DNA synthesis was followed by the incorporation of (^{3}H)thymidine (specific activity 5 Ci per mMole). RNA synthesis by the incorporation of (^{14}C)uridine (specific activity 492 mCi per mMole) and protein synthesis by the incorporation of (^{3}H)leucine (specific activity 150 mCi per mMole) and (^{14}C)phenylalanine (specific activity 475 mCi per mMole). The incorporation of arginine into infected and control cultures and into preparations of purified progeny virus was examined using uniformly labelled (^{14}C)arginine (specific activity 312 mCi per mMole) and guanido labelled (^{14}C)arginine (specific activity 33.4 mCi per mMole). All labelled compounds were obtained from the Radiochemical Gentre, Amersham, Buckinghamshire, England. Labelled compounds were usually supplied to cultures in media containing a background concentration of the unlabelled compound, cultures having been equilibrated in such media for 18 hr. prior to use. Tube cultures of HeLa cells were prepared and infected as previously described. After removal of unadsorbed virus, 1 ml. aliquots of experimental medium containing the appropriate labelled precursor were supplied. At this stage similar medium was added to uninfected control cultures, all proceedures being carried out at 37°. Samples consisting of 5 tubes from each series of cultures were taken at various times post infection and immediately frozen at -50°. After two cycles of freezing and thawing cells were scraped into the medium and cell suspensions from tubes constituting a sample were pooled. Cell disruption was completed by treatment for 2 min. with ultrasonics. Aqueous trichloracetic acid solution was added to the ultrasonicates

to 10% (w/v) final concentration. The resulting precipitates were flocculated by heating at 56° for 5 min. and collected by centrifugation at 1,500 for 5 min., the supernatants being discarded. The pellets were thoroughly washed by resuspension and centrifugation in three changes of distilled water and dissolved in 1 ml. aliquots of Soluene 100 (Packard Instruments Ltd., Wembley, Middlesex, England.). Each solution was quantitatively transfered to a scintillation vial by washing with a total volume of 10 ml. of Brays scintillation liquid (Bray, 1960) and the incorporated radioactivity was determined by counting in a Packard three channel liquid scintillation spectrometer, model 3310. Additionally, a procedure for fractionation of the trichloracetic acid precipitable material was examined. The washed precipitates were resuspended in 10 ml. aliquots of 10% trichloracetic acid and heated at 90° for 20 min., under which conditions nucleic acid material is redissolved (Schneider, 1945). The remaining precipitates were removed by centrifugation at 1,500g for 5 min. and the supernatants retained. The pellets, which contained the protein fractions, were redissolved in 2 ml. aliquots of 0.5N aqueous sodium hydroxide solution and any insoluble residue removed by centrifugation as above. The high molecular weight material was then reprecipitated by the addition of 5 ml. aliquots of 20% trichloracetic acid, the precipitates flocculated by heating at 56° for 5 min. and removed by centrifugation. The supernatants were tested for the presence of unprecipitated material by the further addition of trichloracetic acid solution and discarded. Lipid material was removed by washing the pellets once with 2 ml. aliquots of ethyl alcohol/diethyl ether (1:1) and once with 2 ml. aliquots of diethyl ether. The remaining material was regarded as the protein fraction.

Individual quench corrections for samples containing a single isotope were made from the efficiencies of counting as determined by an internal channels ratio method. Optimum gain settings for each isotope were

determined by plotting the counts recorded for a suitably quenched standard against the value of the gain setting applied to counting channel of the instrument with the discriminators set at their upper and lower limits to include emissions at all detectable pulse-heights. Discriminator settings are designated 50 - 1000 and correspond to the lowest and highest detectable energy of emission respectively. The gain setting may be regarded as the degree of amplification applied to that portion of the spectrum delineated by discriminator settings. A narrow quench monitoring channel viewing only a portion of the emission spectrum was then established within the range of the counting channel, both channels counting at the optimum gain setting. The ratio of the number of events detected in the quench monitoring channel to that detected in the counting channel is a measure of the spectral shift resulting from sample quenching. For each isotope the relationship of the efficiency of counting to the value of the channels ratio was determined by counting a series of standards containing a known constant amount of radioactivity but quenched to different degrees. Curves of the calculated efficiency of counting versus channels ratio were plotted and quench monitoring channel discriminator settings resulting in a linear standard curve were determined. The constants of the equations describing the linear standard curves were computed and used to determine the efficiency of counting and thus the radioactivity expressed as disintegrations per minute from the ratio of the counts of experimental samples recorded simultaneously in two channels.

Individual quench corrections for samples containing both ^{3}H and ^{14}C isotopes were made by a similar internal channels ratio method employing three channels, the activities being determined simultaneously. The emission spectrum of each isotope was determined by plotting the counts recorded for an appropriate standard at optmum gain against the base line discriminator

setting of a narrow channel corresponding to 4% of the detection range and viewing small portions of the spectrum at various positions. As the spectra of the two isotopes overlap, it is not possible to detect separately the events due to each isotope solely on the basis of discriminator settings. Discriminator settings for a ^{14}C counting channnel were chosen in such a way that at the optimum gain setting for these conditions effectively no events due to ^{3}H emissions were detected in this channel. A ^{14}C quench monitoring channel having the same gain setting was then established within the range of the counting channel. The remaining channel, set to count ^{3}H at optimum gain, occupied the lower energy portion of the detection range below the ^{3}H threshold point and thus counted all detectable events due to ^{3}H emissions together with some events due to low energy ^{14}C emissions. Using known constant amounts of radioactivity a series of standards quenched to different degrees was prepared for each isotope in such a way that analogous members of each series were identically quenched. Whole calf serum dissolved in Soluene 100 was used as a quenching agent in order to simulate the type of quenching produced in experimental samples. Each standard was then counted simultaneously in the three channels. From the ^{14}C channnels ratio and the calculated efficiency of counting of the ^{14}C quenched standards in the ^{14}C counting channel a quench correction curve was prepared as described for single isotope counting. Additionally, the percentage of the known ^{14}C disintegrations per minute detected as counts per minute in the ^{3}H counting channel was plotted against ^{14}C channels ratio for each member of this series. Above a certain degree of quenching these three calibration curves were approximately linear and the constants of the equations describing the curves were computed as for the quench correction curves used for single isotope counting. The activities of a sample containing both isotopes could thus be determined absolutely in the following way. After simultaneous counting in

the three channels, the ^{14}C channels ratio was determined and used to compute the efficiency of ^{14}C counting and thus the ^{14}C activity expressed as disintegrations per minute. From this value the number of counts due to ^{14}C emissions detected in the ^{3}H counting channel was computed and on subtraction from the total counts in this channel the number of counts due to ^{3}H emissions alone was obtained. Consideration of the ^{3}H efficiency of counting at that value of ^{14}C channels ratio gave the ^{3}H activity expressed as disintegrations per minute.

2.3 Production and purification of radioactively labelled virus.

Confluent monolayers of HeLa cells in 40 oz. bottles were infected with vaccinia virus suspended in 20 ml. aliquots of serum free experimental medium at a multiplicity of 5 pfu per cell. After adsorption at 37° for 2 hr., the inoculum was replaced with medium containing either uniformly labelled (^{14}C)arginine or guanido (^{14}C)arginine. After further incubation at 37° for 24 hr. the infected cells were shaken into the medium and recovered by low speed centrifugation (100g, 5 min.). Virus was extracted and purified by a method essentially similar to that described by Joklik (1962). Cell pellets were resuspended in 10 ml. volumes of 0.01 M tris(hydroxymethyl)aminomethane (Tris)buffer, pH 8.6 and after disruption of the cells by two cycles of freezing and thawing followed by ultrasonic treatment for 2 min. cell debris were removed by centrifugation at 1,500g for 5 min. and the virus containing supernatents were retained. All subsequent manipulations were performed at 4°. The pellets were resuspended and ultrasonicated in similar buffer and after centrifugation supernatants from the same samples were pooled. The supernatants were then emulsified with equal volumes of Arcton 113 (Imperial Chemical Industries Ltd., Mond Division, Runcorn, Cheshire, England) and the phases reseparated by centrifugation at 1000g for 5 min. The denser organic phases containing extracted cell material were discarded and the upper aqueous phases were centrifuged at 15,000g for 30 min. in a Spinco model L ultracentrifuge. The resulting virus -containing pellets were resuspended in 0.2 ml. aliquots of 0.001 M Tris buffer, pH 8.6 by ultrasonic treatment and centrifuged through 36% (w/v)sucrose solution in similar buffer in a Spinco SW50L rotor at 23,000g for 90 min. The low ionic strength of the buffer was intended to reduce aggregation of virus particles. The pellets were then similarly resuspended, centrifuged through 50% sucrose solutions at 130,000g for 90 min. and finally resuspended

in 0.5 ml. volumes of the dilute Tris buffer. Aliquots of 0.1 ml. of these suspensions were layered onto preformed potassium tartrate density gradients (20-60%(w/v) in 0.001 M Tris buffer., pH. 8.6) prepared by allowing diffusion at 4° for 18 hr. of 5 layered solutions, total volume 4.5 ml. and centrifuged at 130,000g for 90 min. The gradients were fractionated by upward displacement using 70% potassium tartrate solution in the dilute Tris buffer and collecting 12 fractions of 0.4 ml. from each gradient. An apparatus was constructed for this purpose in which a rack and pinion mechanism, driven through reduction gearing by an electric motor, operated a syringe and supplied the displacing solution at a rate of approximately 15 ml. per hr. to a 23g x ⅜" needle inserted in the base of the centrifuge tube (plate 3). Thus, gradients were completely displaced in about 20 min., the fractions being collected through Teflon adaptors placed in the necks of the tubes and having conical internal sections to minimise fraction mixing. This method was found to result in better resolution than did simple dropwise collection through the punctured base of the tube. 0.1 ml. aliquots of the fractions were assayed for infectivity and for incorporated radioactivity by the methods previously described.

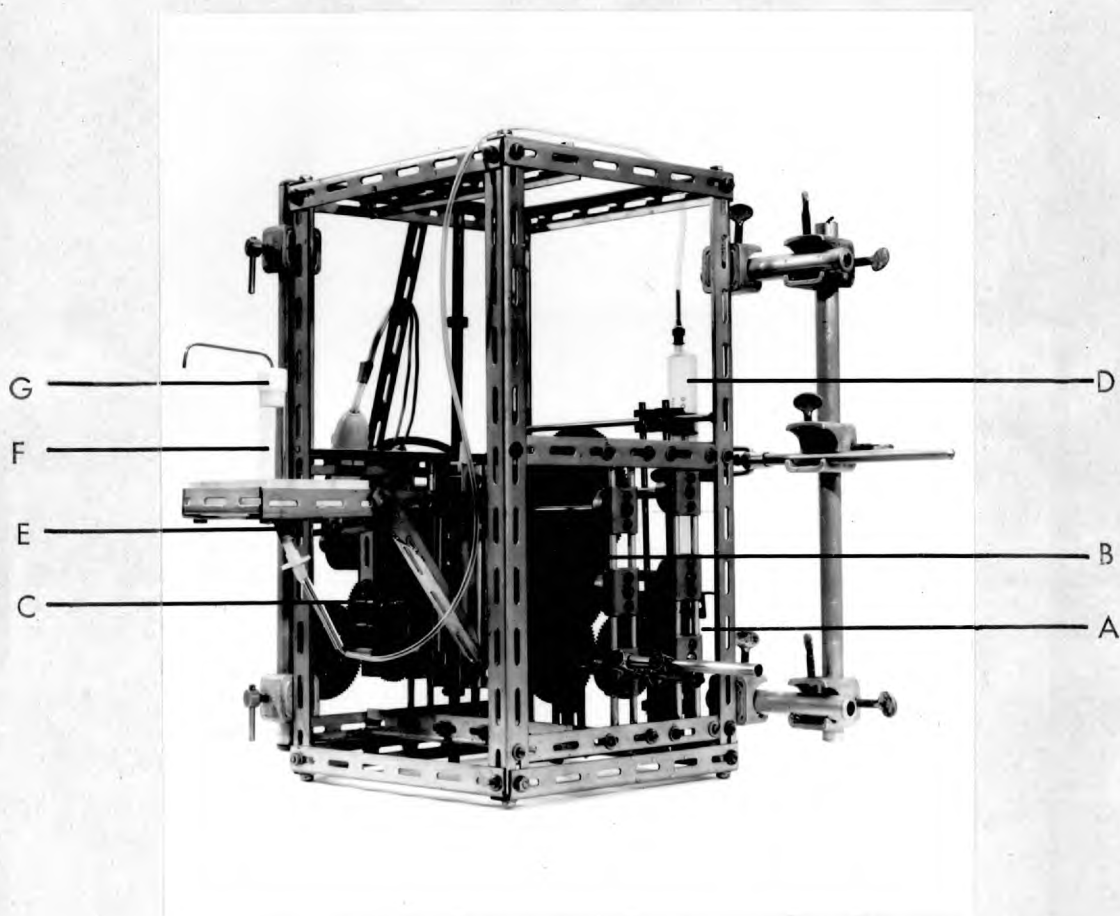

Plate (3). Apparatus constructed for the fractionation of density gradients.

A - rack and pinion; E - 23g. x ⅜" needle;

B - reduction gearing; F - centrifuge tube;

C - electric motor; G - teflon adaptor.

D - 20ml. syringe;

2.4 Diffusion in gel tests.

Petri dishes of 10 cm. diameter were filled to a depth of approximately 3 mm. with 20 ml. of diffusion medium consisting of (w/v) 1% agar, 0.9% sodium chloride and 0.001% Merthiolate. Reagent wells 9 mm. in diameter and 14 mm. between well centres were cut in isometric patterns using a hollow punch guided by a perspex die and superfluous diffusion medium was removed using a pipette. The antigen wells contained extracts of vaccinia infected HeLa cell cultures grown in 4 oz. prescription bottles containing 10 ml. of medium. The extracts were prepared at 24 hr. post infection by subjecting cultures to two cycles of freezing and thawing followed by ultrasonic treatment for 2 min. and clarification of the ultrasonicates by centrifugation at 1,500g for 5 min. The supernatants were then centrifuged at 10,000g for 60 min. to remove virus particles and the resulting soluble antigen preparations were concentrated approximately 25-fold by ultrafiltration at 4° against PBSA. The antiserum wells contained an antivaccinia serum prepared by hyperimmunisation of rabbits, convalescent from vaccinia infection, with three to five intravenous injections of partially purified vaccinia virus suspensions. These suspensions were prepared from vaccinia-infected rabbit skin material to avoid interspecies antibody production. Rabbits were bled 8-10 days after the final injection. After the reagent wells had been filled, the plates were developed at room temperature in a sealed perspex box containing moistened tissue paper to maintain the humidity. The plates were usually fully developed in 2-3 days and the precipitation patterns were recorded photographically after visual examination. No line pattern component visible by eye was absent from the final photographic print.

CHAPTER 3

EVALUATION OF TECHNIQUES USED IN INFECTIVITY AND RADIOACTIVITY ASSAYS

3.1 Comparison of infectivity assays in monolayer cultures of RK_{13} and HeLa cells.

Monolayer cultures of RK_{13} cells and of HeLa cells were prepared in tubes as described. A series of tenfold dilutions of a suspension of vaccinia virus grown in HeLa cells was prepared and plated onto the monolayers. At various times post infection counts of plaques in RK_{13} and HeLa cell monolayers and of haemadsorbing foci in HeLa cell monolayers were made. Infectivity titres were computed and are shown in table 2.

At 20 hr. post infection plaques in both RK_{13} and HeLa cell monolayers were very small and impossible to count accurately. Haemadsorbing foci were countable when viewed against a dark background by reflected rather than direct

Time post infection (hr.)	Infectivity titres		
	pfu/ml. (RK_{13})	pfu/ml. (HeLa)	haf/ml. (HeLa)
20	NA	NA	2.6×10^7
40	5.6×10^6	3.6×10^7	5.3×10^7
60	6.5×10^6	4.0×10^7	2.4×10^8

Table (2). Comparison of infectivity assays.

NA = not applicable.

illumination. At 40 hr. post infection plaques in RK_{13} monolayers were round, well defined, of constant size and easily countable. Plaques in HeLa monolayers at this time were smaller, irregular in shape and difficult to count while the number of haemadsorbing foci in HeLa monolayers had approximately doubled and some variability in the size of foci was apparent. The efficiency of plating of the virus appeared to be 6 to 10 fold greater in HeLa cell monolayers than in RK_{13} cell monolayers. At 60 hr. post infection plaque counts in monolayers of both RK_{13} and HeLa cells had increased by 10-15% due to the appearance of smaller, presumed secondary plaques and the number of haemadsorbing foci in monolayers of HeLa cells had increased approximately 5 fold. Thus, in spite of the greater efficiency of plating of the virus on HeLa cell monolayers, plaques in these cultures were unsuitable for counting and in addition to requiring a more elaborate detection system the haemadsorption assay appeared to detect sites of secondary infection not yet sufficiently advanced to form plaques. This latter observation is supported by the comparatively early appearance of haemadsorbing foci at 20 hr. post infection. It is concluded that plaque formation in RK_{13} cell monolayers gave the more reliable estimate of infectivity and this method was used for all subsequent titrations.

3.2 Calibration data relating to liquid scintillation counting.

The optimum gain setting for each isotope was determined as described and was 55% for ^{3}H and 11% for ^{14}C (fig. 5 and 6). Linear single isotope quench correction curves for ^{3}H and ^{14}C quenched series are shown in fig. 7 and 8. The quench monitoring channels are set at the lower energy end of the detection range on the counting channels and increased quenching producing reductions in the energy of emissions results in an inrease in the value of the channels ratio. The maximum efficiency of counting was about 45% for ^{3}H and about 85% for ^{14}C.

The spectra of emissions resulting from ^{3}H and ^{14}C disintegrations from similarly quenched standards determined under conditions of optimum gain are shown in fig. 9 and completely overlap. The spectra derived from the same standards under the conditions chosen for simultaneous assay are shown in fig. 10, together with the positions of the counting and quench monitoring channels. The gain setting of 25% applied to the ^{14}C counting and quench monitoring channels increases the number of ^{14}C counts detectable in these channels and reduces the number of ^{14}C counts appearing as ^{3}H counts in the ^{3}H counting channel. The number of ^{3}H counts detected in the ^{14}C counting channel under these conditions is negligible. For dual isotope counting the quench monitoring channel is set at the upper energy end of the detection range of the ^{14}C counting channel and increased quenching results in a decrease in the value of the channels ratio. The linear portions of the quench correction curves for dual isotope counting are shown in fig. 11 and under these conditions the maximum efficiency of counting was about 12% for ^{3}H and about 30% for ^{14}C.

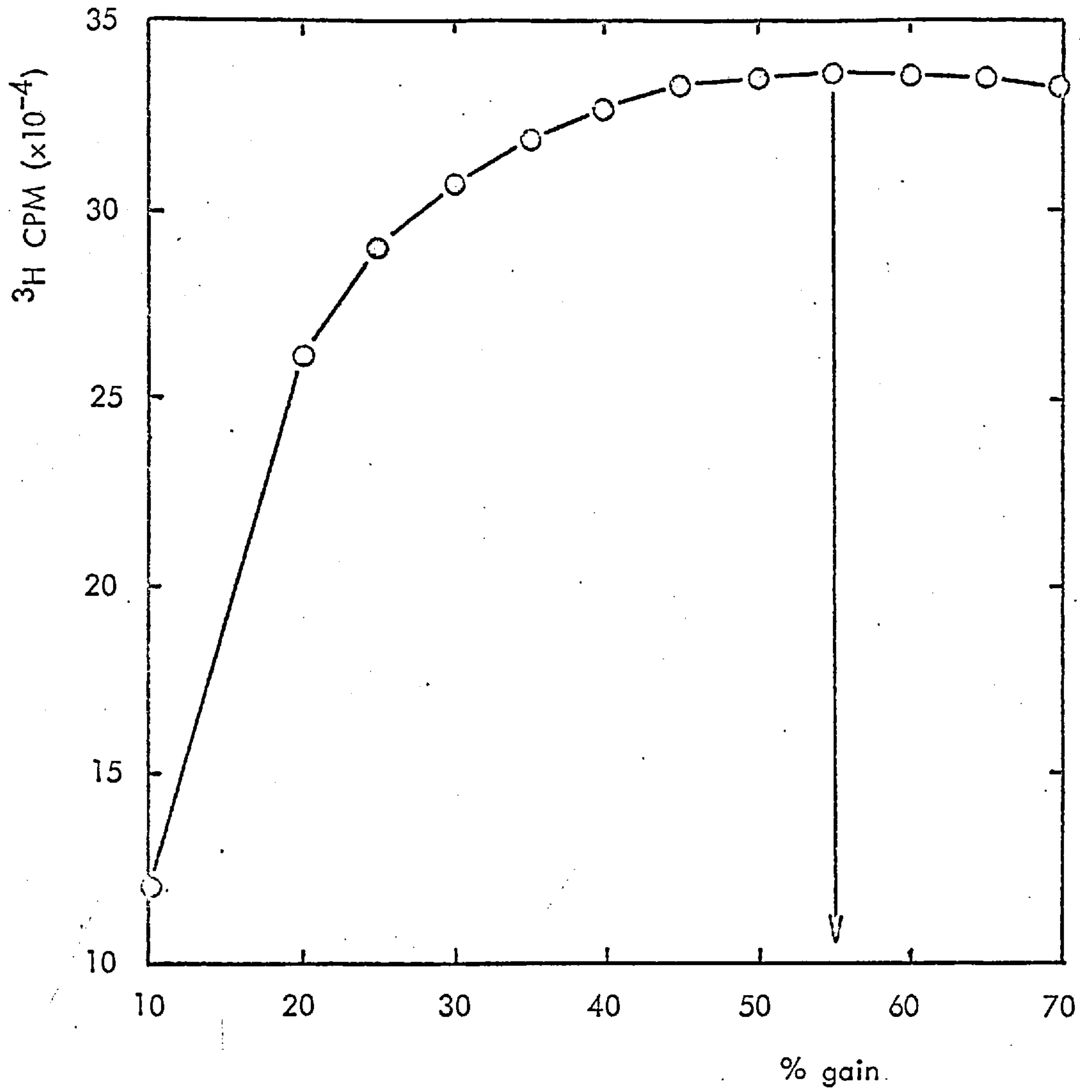

Fig. (5). Determination of optimum gain for ^{3}H counting.

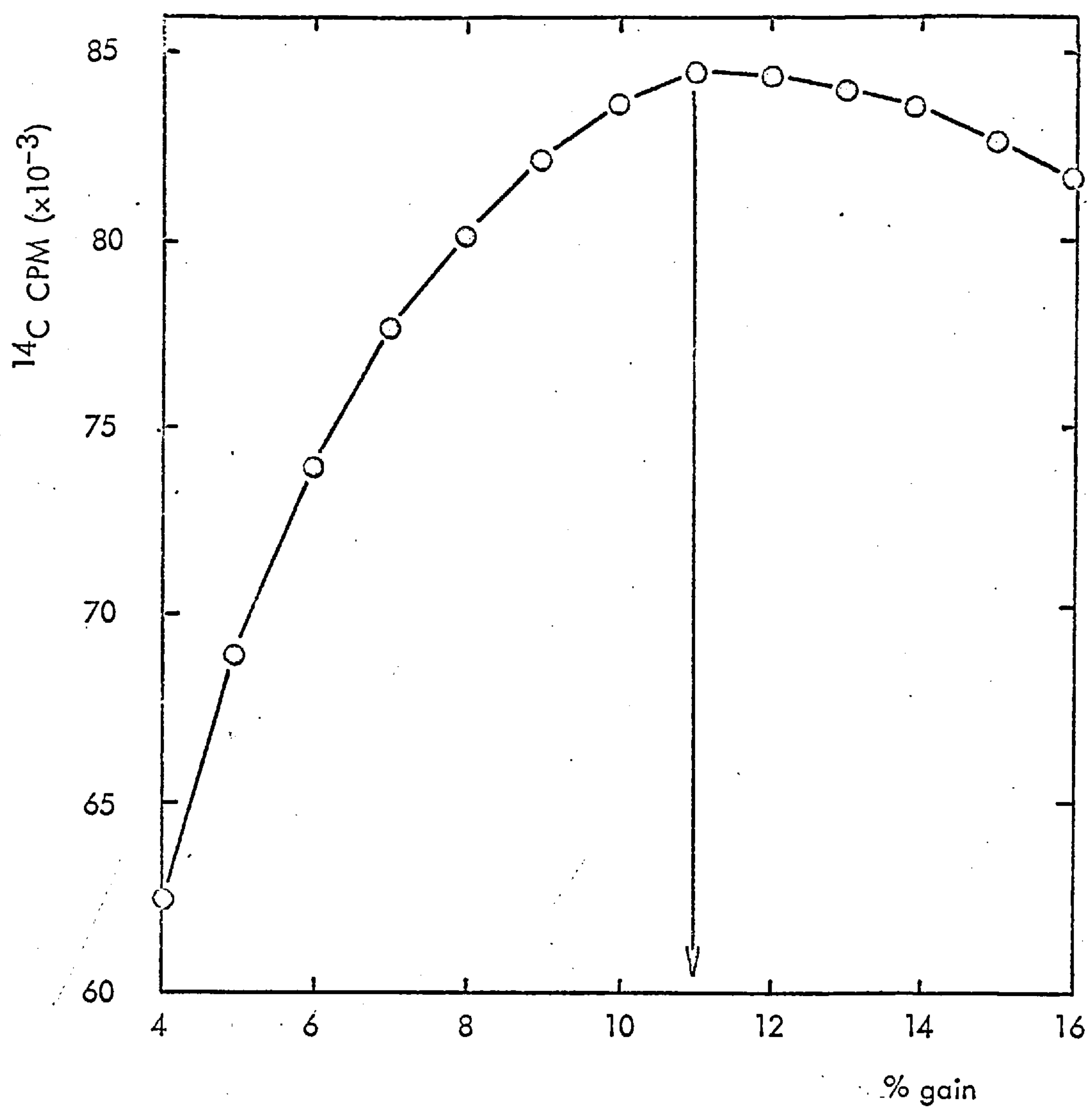

Fig. (6). Determination of optimum gain for ^{14}C counting.

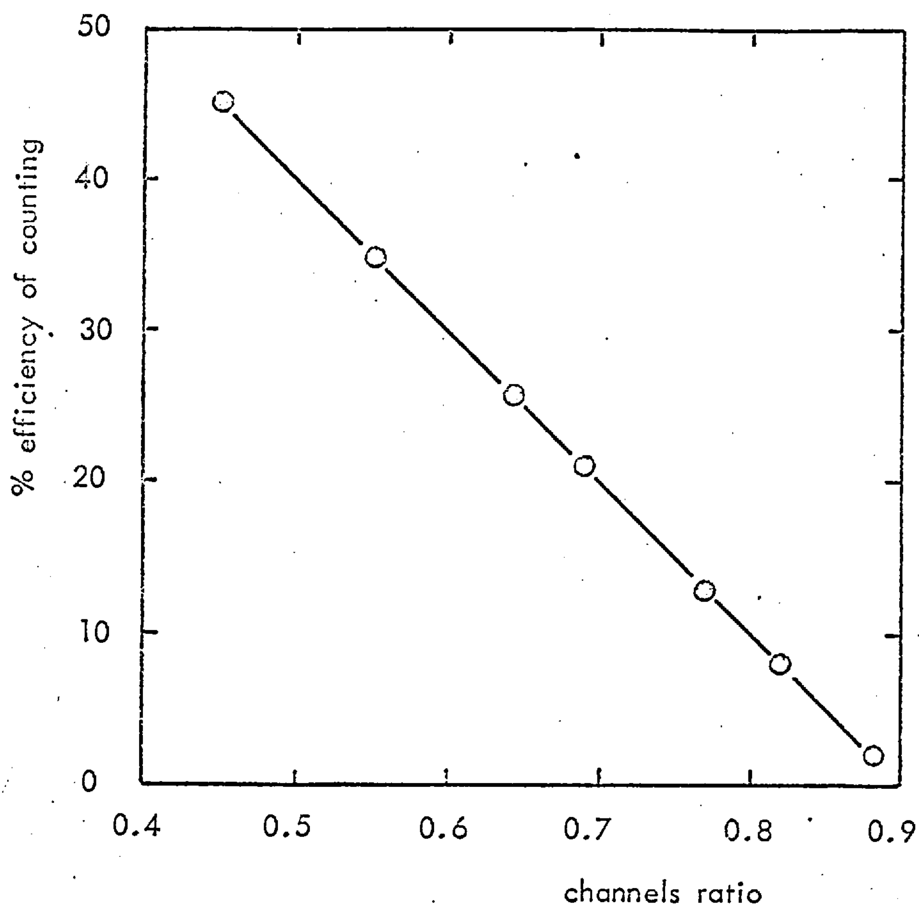

Fig. (7). ^{3}H quench correction curve.

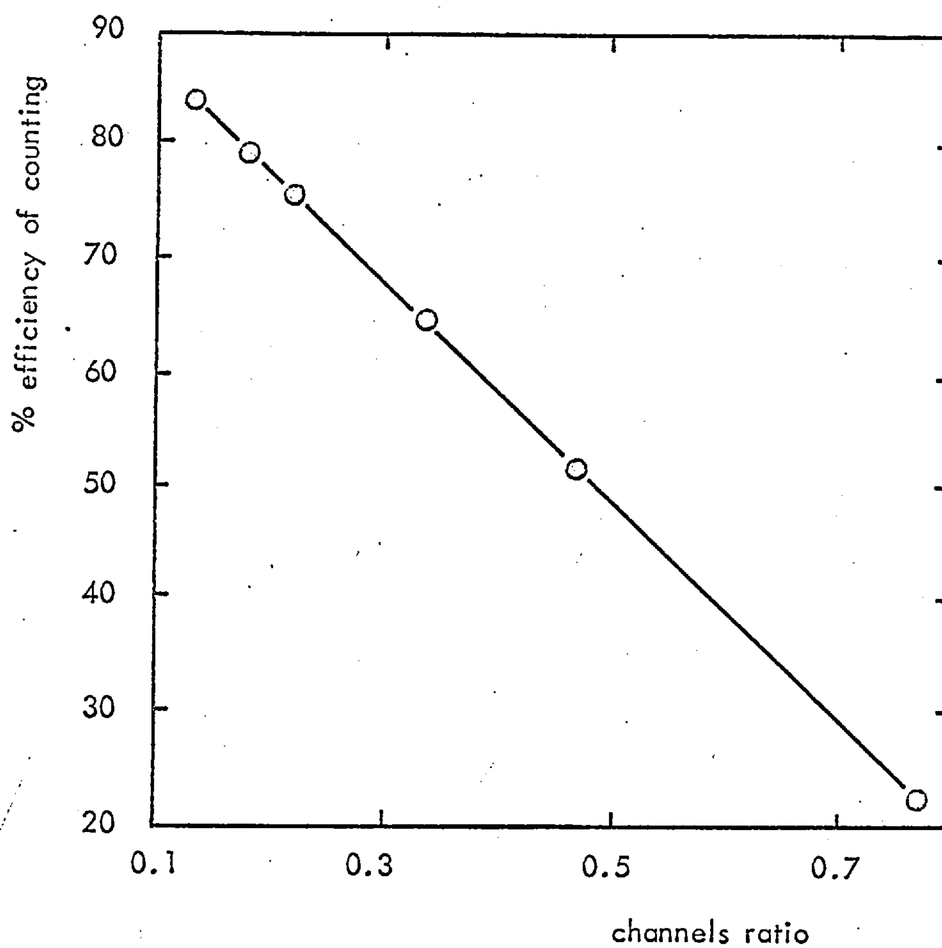

Fig. (8). ^{14}C quench correction curve.

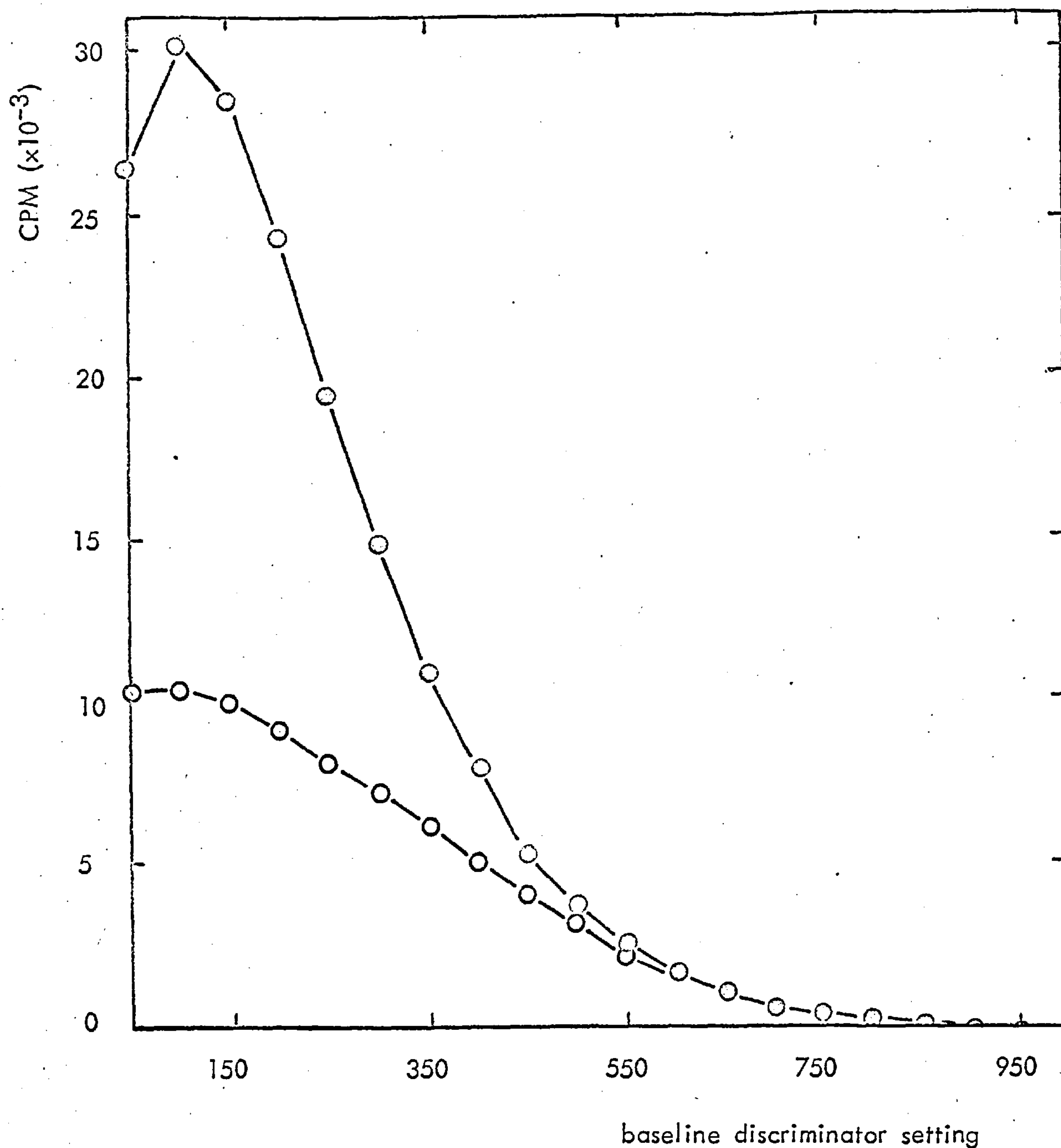

Fig. (9). ^{3}H and ^{14}C spectra under conditions of optimum gain.

○—○ ^{3}H, 55% gain; ○—○ ^{14}C, 11% gain.

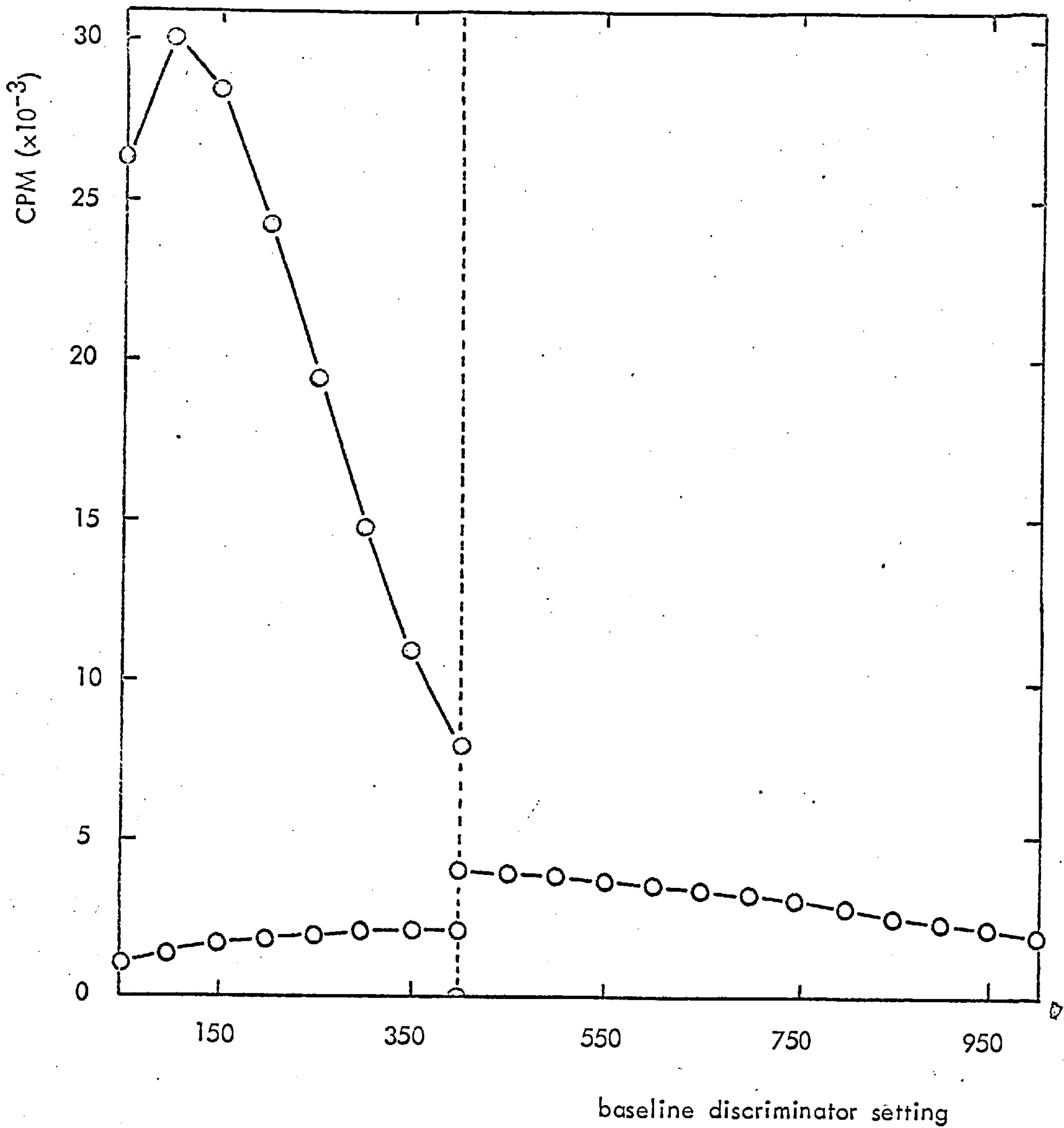

Fig. (10). ^{3}H and ^{14}C spectra under conditions for dual isotope counting.

O—O ^{3}H, 55%gain; O—O ^{14}C, 55% or 25% gain.

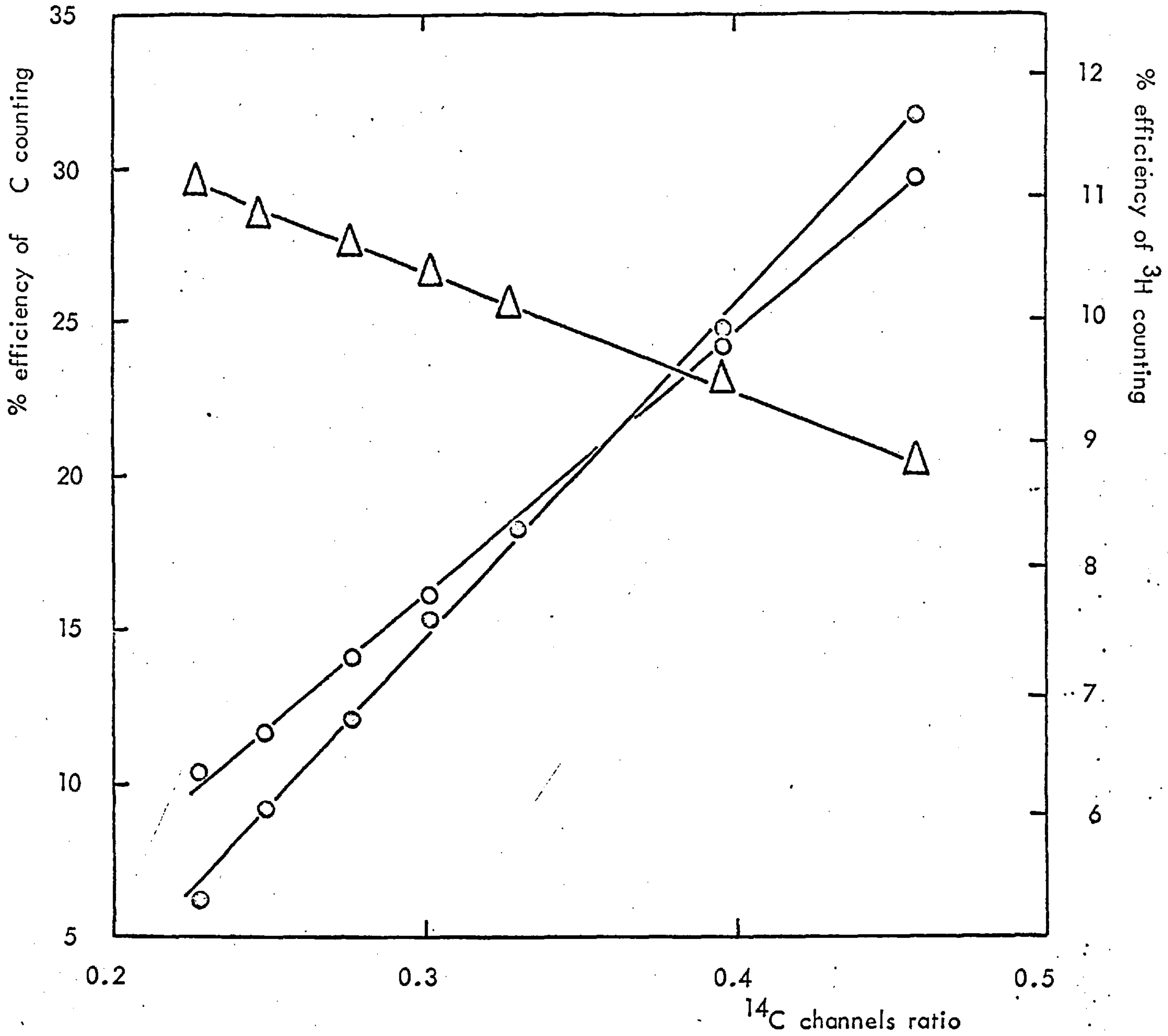

Fig. (11). Quench correction curves for dual isotope counting.

O——O % efficiency of ^{14}C counting in ^{14}C channel;

Δ——Δ % efficiency of ^{14}C counting in ^{3}H channel;

O——O % efficiency of ^{3}H counting in ^{3}H channel.

3.3 Distribution of incorporated radioactivity between fractions of trichloracetic acid precipitable material.

HeLa cell cultures were equilibrated in serum free experimental medium containing 1 mM. arginine. After equilibration, cultures were reincubated for 24 hr. with similar medium containing either 0.5 μCi. per ml. (^{3}H)thymidine or 0.5 μCi. per ml. (^{3}H)leucine. Samples were taken from each set of cultures and trichloracetic acid precipitable material prepared. Aliquots of the precipitates were assayed for total incorporated radioactivity or fractionated as described and the nucleic acid, protein and insoluble residue fractions similarly assayed. The radioactivity incorporated into whole trichloracetic acid precipitates and the distribution of radioactivity between fractions, corrected for sample volume, is shown in table 3.

Of the total radioactivity of whole trichloracetic acid precipitates, 8-9% was lost during the manipulations of the fractionation proceedure. No significant activity was associated with the lipid fractions. Only 7% of the activity incorporated by cultures incubated in the presence of (^{3}H)thymidine was recovered in fractions other than the nucleic acid fraction and only 6.3% of the activity incorporated by cultures incubated in the presence of (^{3}H)leucine was recovered in fractions other than the protein fraction. In view of the loss of activity incurred during fractionation and the high specificity of labelling, only whole trichloracetic acid precipitates were assayed for radioactivity in subsequent incorporation experiments.

Labelled precursor	DPM whole precipitate	DPM nucleic acid fraction	DPM protein fraction	DPM insoluble residue	Total DPM in fractions
(^{3}H)-thymidine	342,872	287,834	19,302	4,781	311,917
(^{3}H)-leucine	391,227	17,662	335,394	7,018	360,074

Table (3). The distribution of incorporated radioactivity between fractions of trichloracetic acid precipitable material.

3.4 Comparison of density gradient fractionation methods

The fractionation of gradients by upward displacement as described was compared to the fractionation of similar gradients by collection through the pierced base of the centrifuge tubes. Discontinuous sucrose gradients each consisting of 1 ml. volumes of 10% (w/v) and 20% aqueous sucrose solutions floated on a 2 ml. volume of 30% sucrose solution were prepared. The gradients were fractionated by either method within 30 min. of preparation collecting each 4 ml. volume in 25 fractions. As a measure of the sucrose concentration, the refractive index of each fraction was determined using a Hilger refractometer. The refractive indices were plotted against fraction number and the resulting profiles are shown in fig. 12.

Collection by upward displacement preserved the steps in the discontinuous gradients. The small degree of mixing of the individual solutions can be partly attributed to diffusion. In gradients collected through the base of the tubes however, a much greater degree of mixing was evident and the step due to the band of 20% sucrose solution was almost completely removed. This mixing is probably due to surface tension and swirling effects during the passage of the liquid through the puncture. Dense material retained by surface tension effects around the rim of the puncture would be mixed with lighter material passing through, whereas in the upward displacement method, light material is continually removed by denser material behind it.

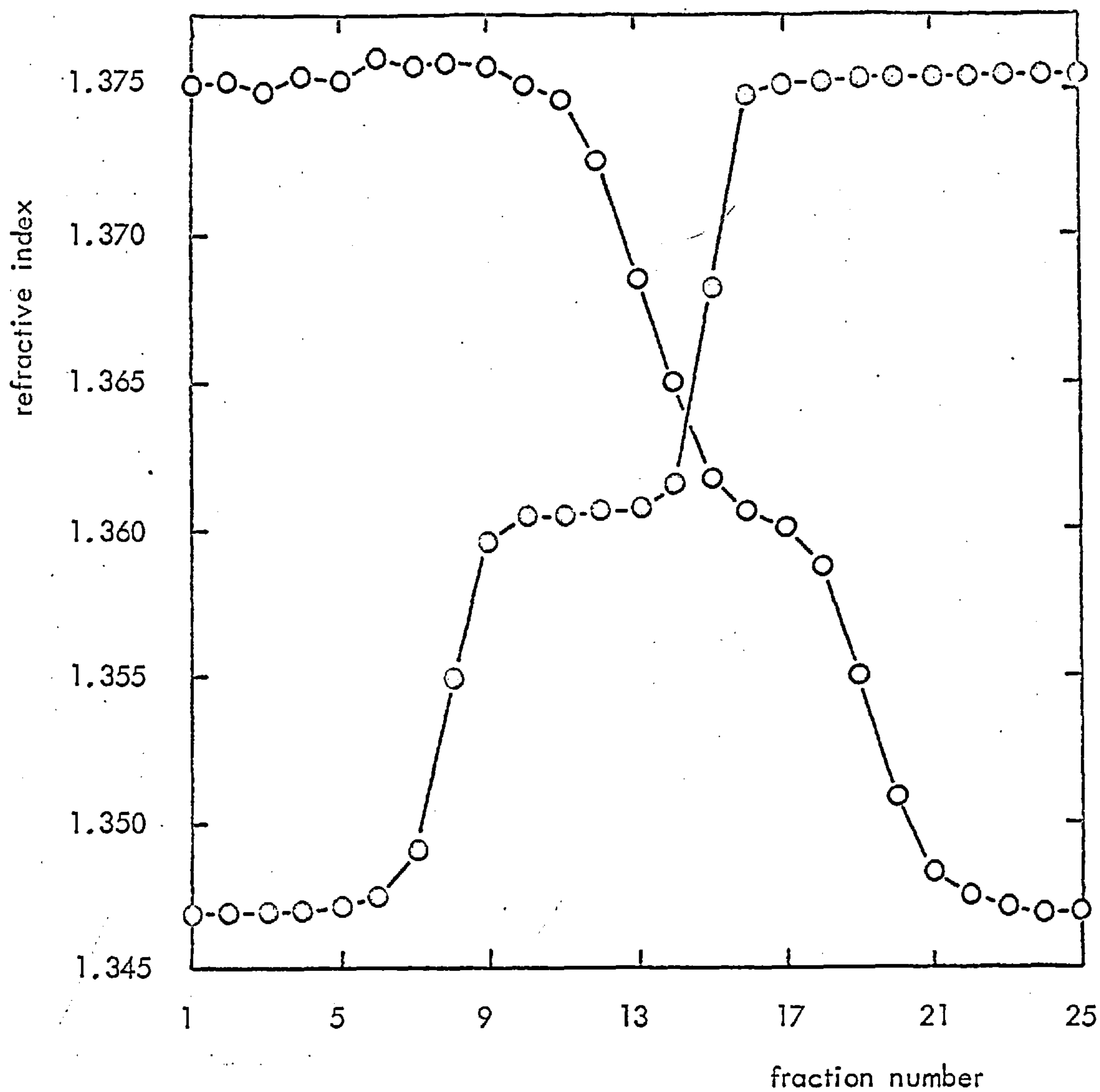

Fig. (12). Comparison of density gradient fractionation methods.

O——O collection by upward displacement;

O——O collection by gravity.

CHAPTER 4

THE EFFECT OF ARGININE DEPRIVATION ON THE FORMATION OF INFECTIVE VIRUS

4.1 Growth curves in the presence and absence of arginine.

HeLa cell cultures were equilibrated in serum free experimental medium which either lacked arginine or contained 1 mM. arginine. After virus infection the cultures were reincubated with media similar to those used for equilibration. Samples were taken from each series of cultures at various times post infection and titrated for infective virus (fig. 13).

In cultures infected and maintained in the absence of arginine no increase in the titre of infective virus above that recoverable at zero time was noted. In additional experiments no increase in the titre of infective virus was noted even after incubation for a total of 48 hr., indicating that the virus

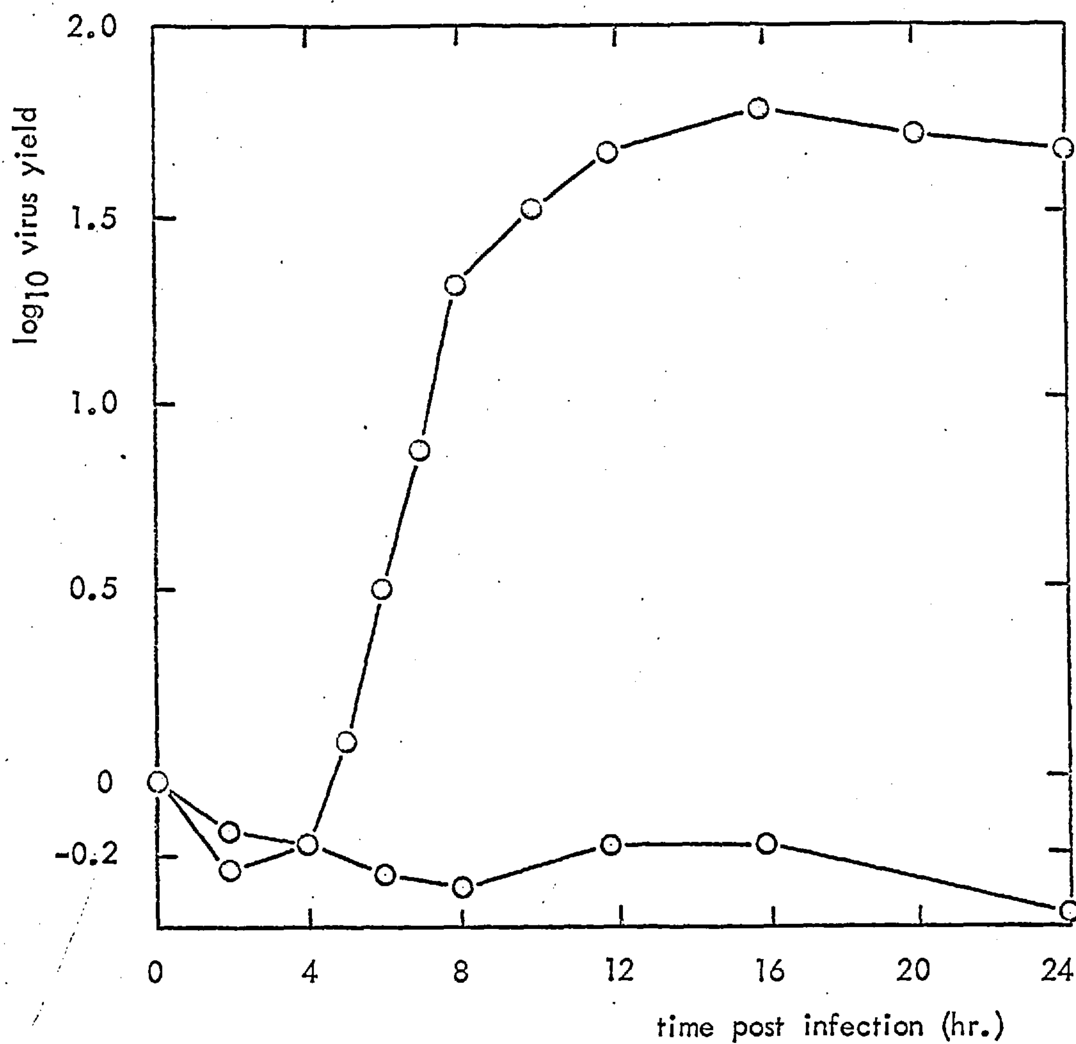

Fig. (13). The effect of arginine deprivation on the formation of infective virus.

○—○ cultures infected in the presence of 1mM. arginine;

○—○ cultures infected in the absence of arginine.

replication cycle was not merely delayed by deprivation of arginine. However, in cultures infected and maintained in the presence of 1 mM. arginine infective progeny virus was detected by 5 hr. post infection and the virus titre rose rapidly to a maximum yield of approximately 1.8 $\log_{10}$ at 16 hr. post infection, thereafter declining slightly. In further experiments a similar production of infective virus was observed in cultures maintained in the absence of arginine for 18 hr. prior to infection and in the presence of 1 mM. arginine thereafter, indicating that arginine deprivation did not irreversibly affect the ability of the cells to support virus replication.

The very rapid production of progeny virus and the comparatively low yields obtained are slightly atypical of the growth of vaccinia virus strains (Joklik, 1966) and may be a charactaristic of this cell/virus system.

4.2 Dependence of virus yield on arginine concentration.

HeLa cell cultures were equilibrated in serum free experimental media containing various concentrations of arginine. After virus infection the cultures were reincubated with media similar to those used for equilibration. Samples were taken from each series of cultures at 18 hr. post infection and titrated for infective virus (table 4).

Below a limiting value, the yield of infective virus was dependent on the concentration of arginine supplied in the medium. An arginine concentration of 0.09 mM. was found to be sufficient for maximum yield of virus.

These results establish the dependence of virus yield on arginine concentration but fail to distinguish between two alternative phenomena. These are (i) that a dose response relationship exists in which the total production of progeny virus is quantitatively related to an arginine requiring event such as synthesis of a macromolecular product or (ii) that a dose delay relationship exists in which the rate of production of progeny virus is related to an arginine requiring event such as the synthesis of a specific enzyme. The nature of the relationship of virus yield to arginine concentration was resolved by further experiments in which HeLa cell cultures were equilibrated, infected and maintained in a similar manner to those described above but were sampled at various times after infection and titrated for infective virus (fig. 14).

These results show that the rate of production of progeny virus under conditions of partial arginine deprivation is independent of the arginine concentration at any arginine concentration which permits virus growth. However, the yield of virus at any time after the first appearance of progeny virus is proportional to the concentration of arginine supplied in the

Arginine concentration (mM)	Log_{10} virus yield
0	0
0.015	0
0.03	0.71
0.06	1.66
0.09	1.92
0.12	1.90
0.26	1.89
0.52	1.80

Table (4). The dependence of virus yield on arginine concentration.

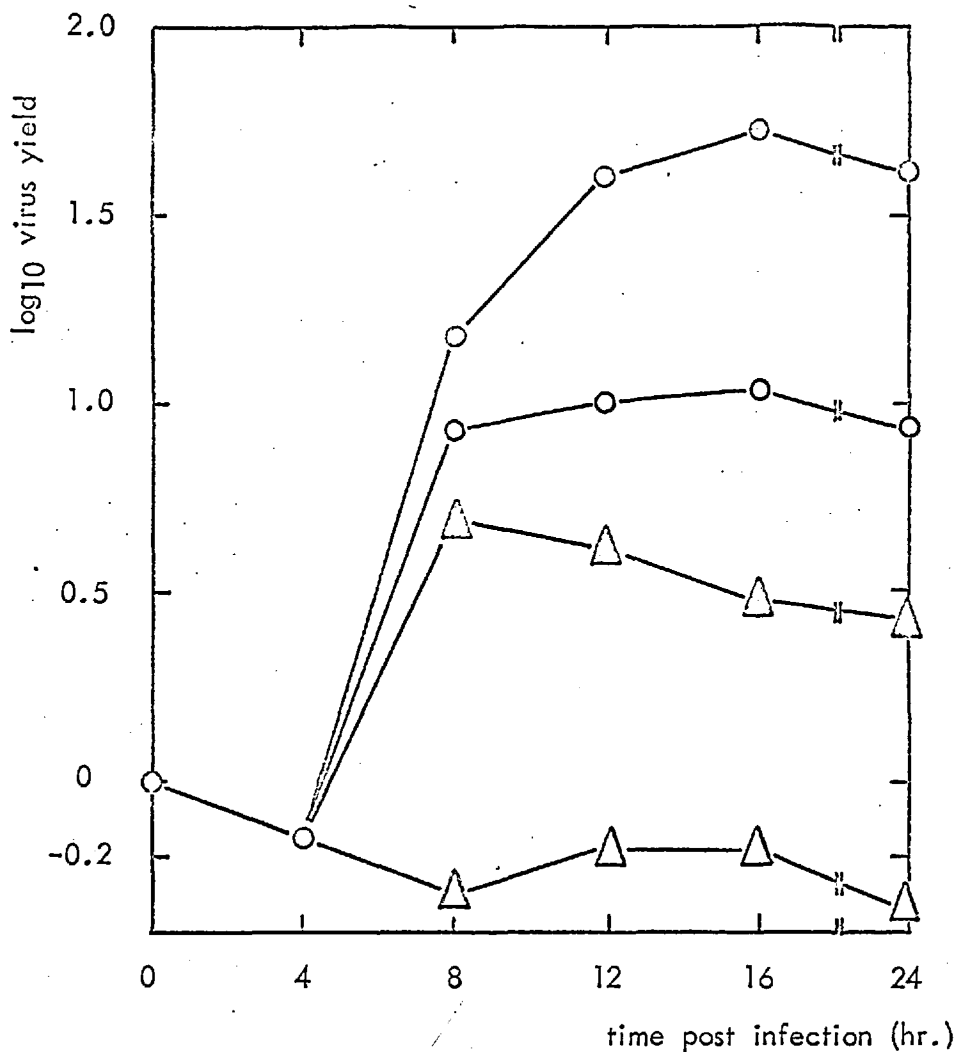

Fig. (14). The dependence of virus yield on arginine concentration.

△—△ cultures infected in the absence of arginine;

△—△ cultures infected in the presence of 0.03mM. arginine;

○—○ cultures infected in the presence of 0.05mM. arginine;

○—○ cultures infected in the presence of 0.10mM. arginine.

medium, favouring the first hypothesis. Additionally, these results exclude the possibility that the requirement for arginine is a threshold phenomenon in which arginine is required for an all or nothing event upon which continuation of the virus replication cycle depends.

4.3 The effect of serum under conditions of partial arginine deprivation.

HeLa cell cultures were equilibrated in serum free experimental medium containing a virus growth limiting concentration of 0.03 mM. arginine. After virus infection some cultures were reincubated with similar medium and some with media containing the same concentration of arginine but supplemented either with 2% (v/v) unheated calf serum or with 2% calf serum which had previously been heated at 56° for 2 hr. Samples were taken from each set of cultures at 18 hr. post infection and titrated for infective virus (table 5).

Under these conditions of partial arginine deprivation the presence of 2% unheated calf serum had a virus growth promoting effect equivalent to approximately 0.03 mM. arginine. This effect was reduced using calf serum which had previously been heated. It is suggested that calf serum contains available arginine which is destroyed, perhaps enzymatically, on heating or is complexed with another serum component.

Sample	Infectivity titre (log_{10} pfu/ml.)
Inoculum recovery	5.15
Without serum	5.76
+2% heated calf serum	6.08
+2% unheated calf serum	6.34

Table (5). The effect of serum under conditions of partial arginine deprivation.

CHAPTER 5

THE SYNTHESIS OF DNA, RNA AND PROTEIN IN INFECTED AND CONTROL CULTURES IN THE PRESENCE AND ABSENCE OF ARGININE

The nature of the relationship of the production of progeny virus to the concentration of arginine supplied in the medium suggests that under conditions of arginine deprivation virus specific events are quantitatively limited rather than rate limited. Consequently, changes in the patterns of macromolecular synthesis resulting from infection and arginine deprivation of cell cultures were investigated.

5.1 Synthesis of DNA

HeLa cell cultures were equilibrated in serum free experimental medium which either lacked arginine or contained 1 mM. arginine. After virus infection of some cultures from each set, all cultures were reincubated with media similar to those used for equilibration but containing 0.5 μCi. per ml. (^{3}H)thymidine. Samples were taken from each series of cultures of various times after the addition

of media containing the labelled precursor and assayed for incorporated radioactivity. The time course of incorporation of (^{3}H)thymidine into infected and control cultures maintained in the presence and absence of arginine is shown in fig. 15

The rate of incorporation into uninfected control cultures maintained in the presence of arginine declined after 6 hr. In infected cultures under these conditions, the initial rate of incorporation was greater than into controls but this too declined after 6 hr. Very little activity was incorporated into either type of culture between 12 and 22 hr. In the absence of arginine incorporation of (^{3}H)thymidine was greatly reduced in both, although incorporation into infected cultures was still greater than into controls.

The time course of incorporation of a nucleic acid precursor into control cultures maintained in complete medium was expected to be linear. It is concluded that the rapid initial rates of incorporation into those cultures represented an artificial stimulation caused by the availability of an exogenous source of a nutrient not normally supplied in the culture medium. In cell cultures maintained in media not containing nucleic acid precursors, the synthesis of purine and pyrimidine derivatives is probably rate limiting in the elaboration of nucleic acids.

Consequently, similar experiments were performed in which HeLa cell cultures were equilibrated in serum free experimental medium which either lacked arginine or contained 1 mM. arginine but which also contained 0.02 mM. concentrations of the nucleosides thymidine, uridine, cytidine, adenosine and guanosine. After virus infection of some cultures from each set, all cultures were reincubated with media similar to those used for equilibration but containing 0.2 μCi. per ml. (^{3}H)thymidine. Each series of cultures was sampled and assayed for incorporated radioactivity as before. The time course of incorporation of (^{3}H)thymidine

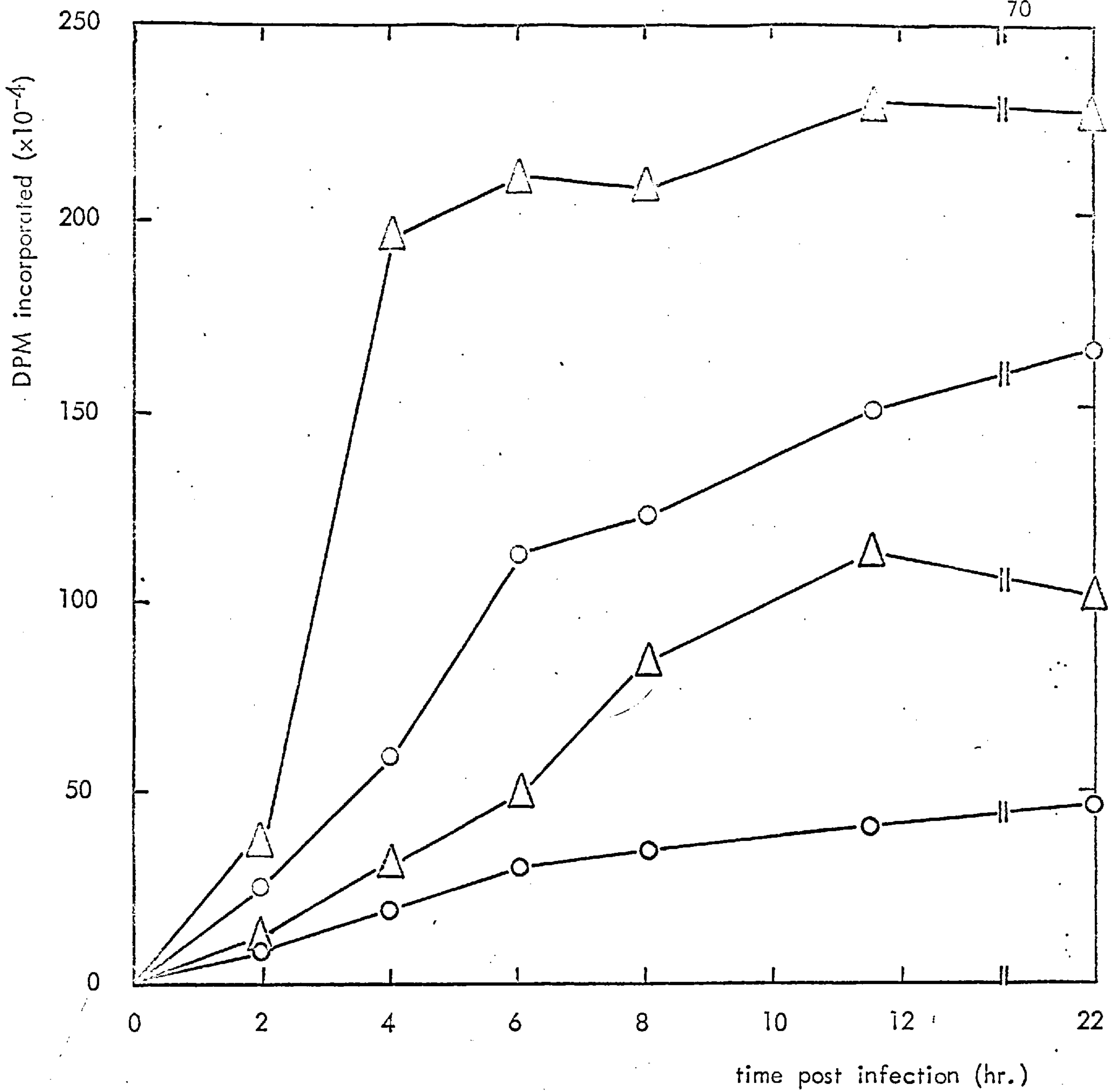

Fig. (15). The effect of arginine deprivation on the synthesis of DNA.

○—○ control cultures maintained in the presence of arginine;

○—○ control cultures maintained in the absence of arginine;

△—△ infected cultures maintained in the presence of arginine;

△—△ infected cultures maintained in the absence of arginine.

into infected and control cultures maintained in the presence of nucleosides with and without arginine is shown in fig. 16

The time course of incorporation into uninfected control cultures maintained in the presence of arginine was approximately linear. In infected cultures under these conditions, there was a marked stimulation of incorporation between 2 and 4 hr. post infection compared with controls. In the absence of arginine incorporation of (^{3}H)thymidine was greatly reduced in both, although incorporation into infected cultures was still greater than into controls. The amount of incorporation ocurring in infected cells between 2 and 4 hr. post infection was reduced by 93% on deprivation of arginine.

It has already been demonstrated that radioactivity supplied as (^{3}H)thymidine can be recovered almost exclusively in the nucleic acid fraction. It is highly probable that the stimulation of incorporation into infected cells maintained in the presence of arginine represents the synthesis of viral DNA. Joklik and Becker (1964) have shown that in a similar system 90% of the viral DNA is synthesised between 1.5 and 4.5 hr. post infection, and at a maximum rate several times that of DNA replication in uninfected cells. Deprivation of arginine inhibited DNA synthesis in both infected and control cultures, although some degree of stimulation due to infection was still apparent. The fact that this effect was obtained in the presence of an exogenous source of nucleosides suggests that the inhibition is not related directly to a role of arginine in the synthesis of these compounds.

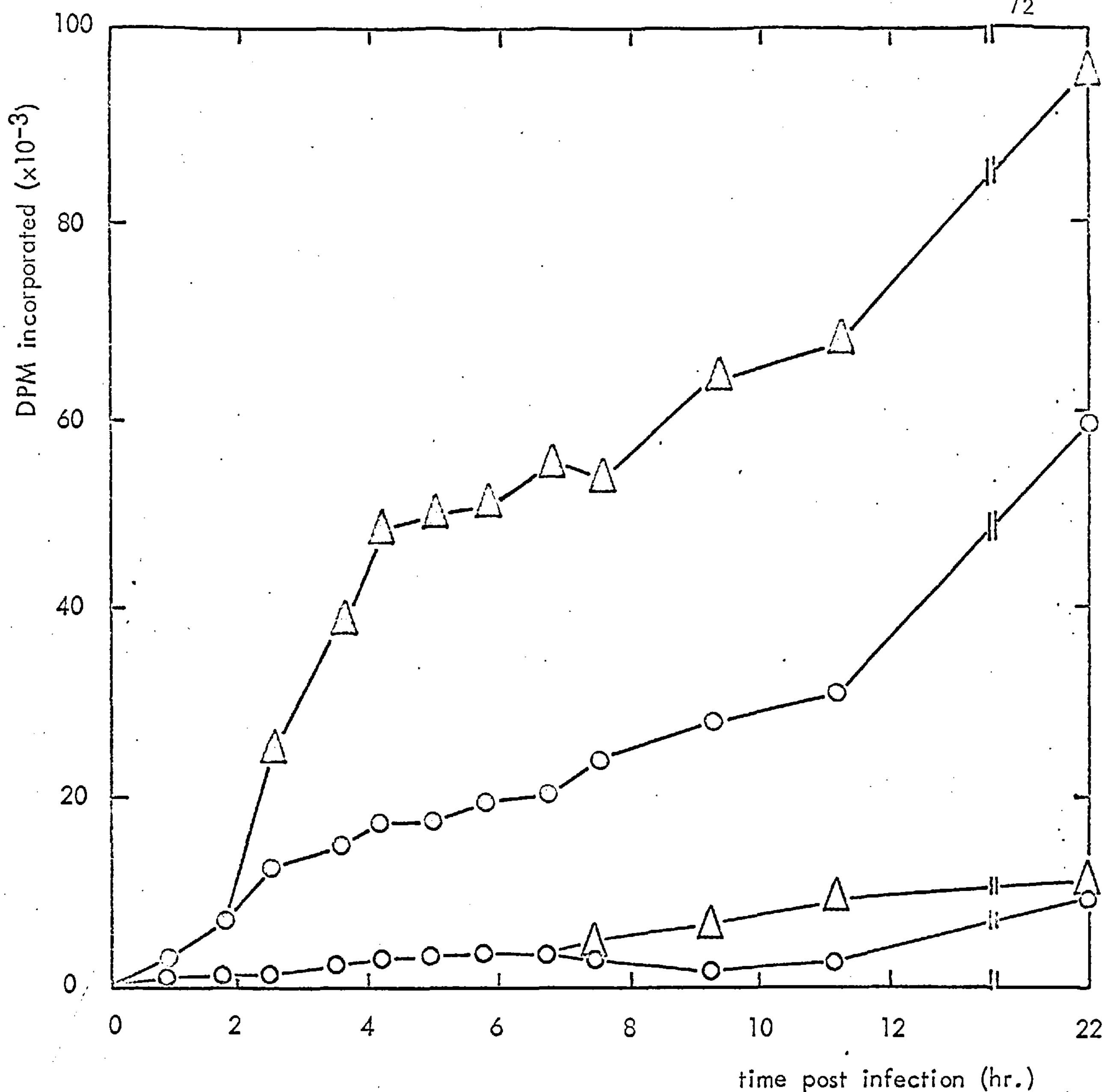

Fig. (16). The effect of arginine deprivation on the synthesis of DNA in the presence of nucleosides.

○—○ control cultures maintained in the presence of arginine;

○—○ control cultures maintained in the absence of arginine;

△—△ infected cultures maintained in the presence of arginine;

△—△ infected cultures maintained in the absence of arginine.

5.2 Synthesis of RNA

HeLa cell cultures were equilibrated in serum free experimental medium which either lacked arginine or contained 1 mM. arginine. After virus infection of some cultures from each set, all cultures were reincubated with media similar to those used for equilibration but containing 0.05 μCi. per ml. (^{14}C)uridine. Samples were taken from each series of cultures at various times after the addition of media containing the labelled precursor and assayed for incorporated radioactivity. The time course of incorporation of (^{14}C)uridine into infected and control cultures maintained in the presence and absence of arginine is shown in fig.17.

As before, the rate of incorporation into uninfected control cultures maintained in the presence of arginine declined after 6 hr. In infected cultures under these conditions, incorporation was greatly reduced after 4 hr. post infection compared with controls. Deprivation of arginine produced further reductions of incorporation in both, although incorporation into controls was still greater than into infected cultures.

For the reasons already discussed, similar experiments were performed in which HeLa cell cultures were equilibrated in media containing 0.02 mM. concentrations of nucleosides. After virus infection of some cultures from each set, all cultures were reincubated with media similar to those used for equilibration but containing 0.02 μCi. per ml. (^{14}C)uridine. Each series of cultures was sampled and assayed for incorporated radioactivity as before. The time course of incorporation of (^{14}C)uridine into infected and control cultures maintained in the presence of nucleosides with and without arginine is shown in fig. 18

Again, the time course of incorporation into uninfected control cultures maintained in the presence of arginine was

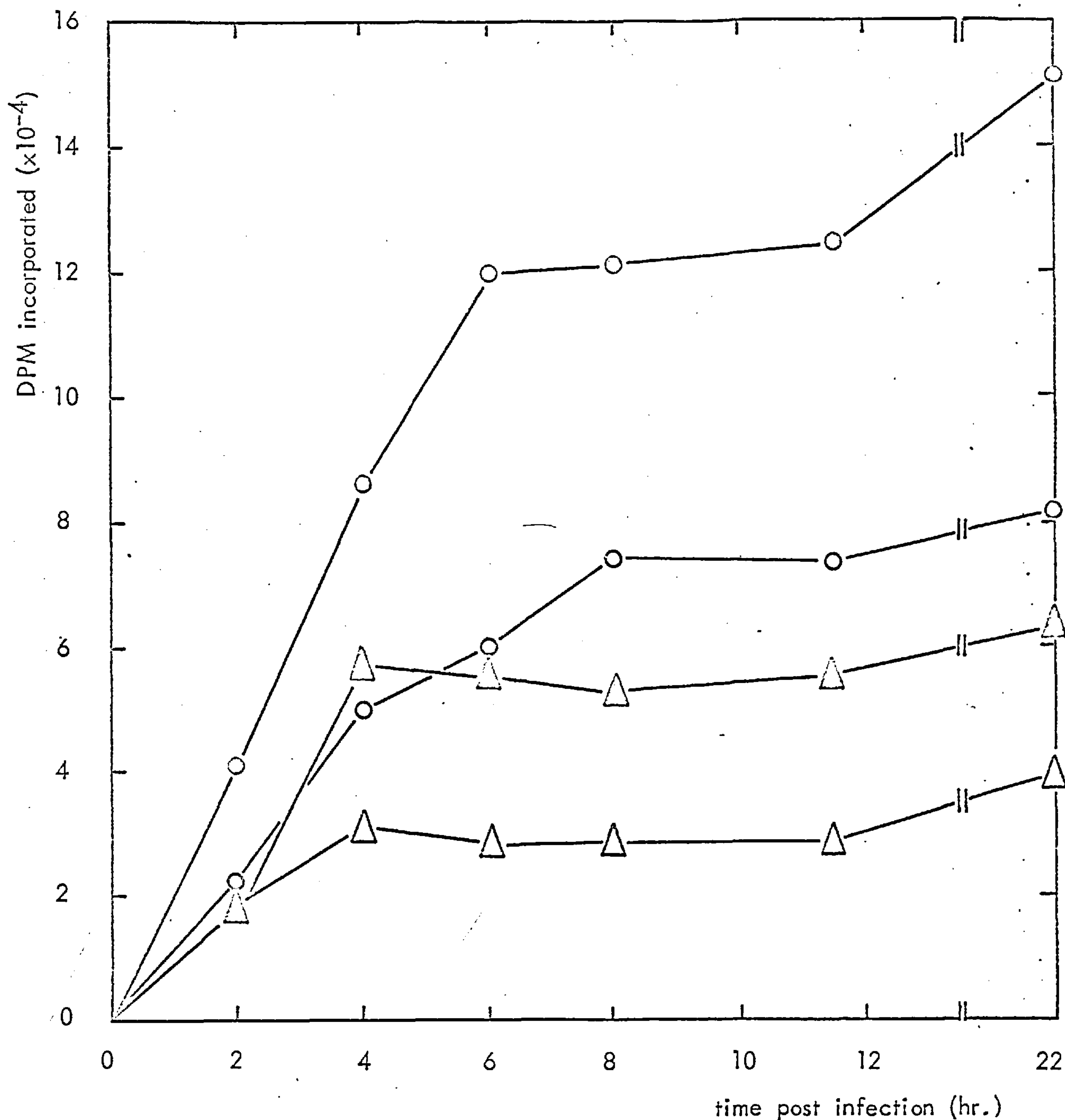

Fig. (17). The effect of arginine deprivation on the synthesis of RNA.

○——○ control cultures maintained in the presence of arginine;

○——○ control cultures maintained in the absence of arginine;

△——△ infected cultures maintained in the presence of arginine;

△——△ infected cultures maintained in the absence of arginine.

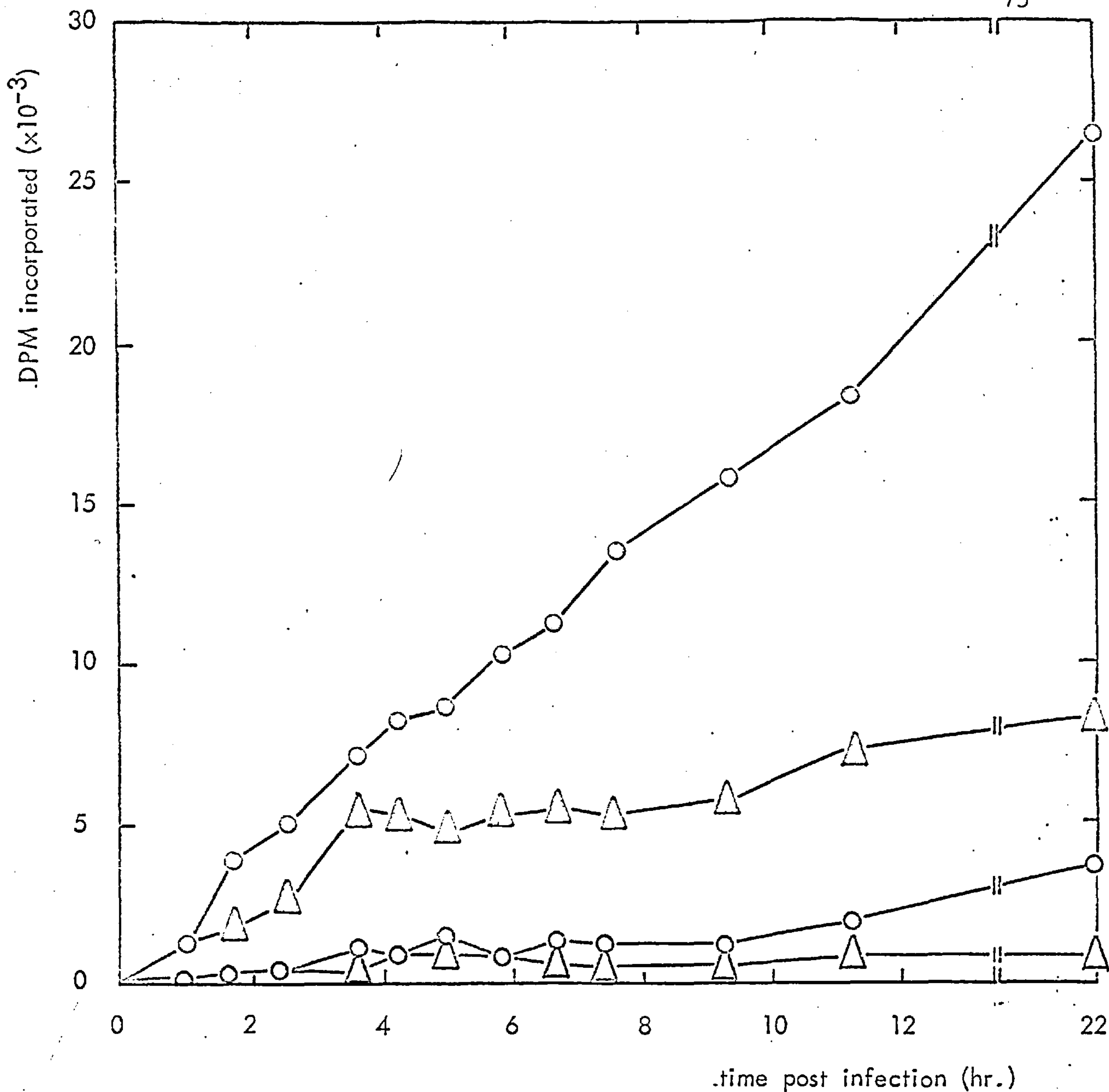

Fig. (18). The effect of arginine deprivation on the synthesis of RNA in the presence of nucleosides.

○—○ control cultures maintained in the presence of arginine;

○—○ control cultures maintained in the absence of arginine;

△—△ infected cultures maintained in the presence of arginine;

△—△ infected cultures maintained in the absence of arginine.

approximately linear. In infected cultures under these conditions, incorporation was greatly reduced after 3.5 hr. post infection compared with controls. Deprivation of arginine produced further reductions of incorporation in both, although incorporation into controls was still greater than into infected cultures. The amount of incorporation ocurring in infected cells during the first 4 hr. post infection was reduced by 84% on deprivation of arginine. After 3 hr. post infection there was negligible incorporation into arginine starved, infected cells.

In the presence of nucleosides it is unlikely that the labelled uridine supplied is metabolised to cytidine or thymidine and incorporated into DNA to any significant extent. This is supported by the pattern of (^{14}C)uridine incorporation which differs totally from that of (^{3}H)thymidine incorporation and suggests that uridine incorporation is a true measure of RNA synthesis. Becker and Joklik (1964) have shown that in a similar system the rates of both host and viral RNA synthesis decrease rapidly after 4 hr. post infection.

Deprivation of arginine inhibited RNA synthesis in both infected and control cultures although some further degree of repression due to infection was still apparent. Again, the inhibition by arginine deprivation is probably not related to a role of arginine in the synthesis of nucleosides. Becker and Joklik (1964) have also shown that the maximum rate of viral RNA synthesis amounts to only about 20% of the initial rate of host RNA synthesis in infected cells. Consequently, it is not possible to estimate the extent to which virus specific RNA synthesis is inhibited by deprivation of arginine from the results presented above.

5.3 Synthesis of protein

HeLa cell cultures were equilibrated in serum free experimental medium which either lacked arginine or contained 1 mM. arginine. After virus infection of some cultures from each set, all cultures were reincubated with media similar to those used for equilibration but containing 0.5 μCi. per ml. (^{3}H)leucine and 0.2 μCi. per ml. (^{14}C)phenylalanine. Samples were taken from each series of cultures at various times after the addition of media containing labelled precursors and assayed for incorporated radioactivity. The time courses of incorporation of (^{3}H)leucine and (^{14}C)phenylalanine into infected and control cultures maintained in the presence and absence of arginine are shown in fig. 19 and 20 and are essentially similar.

Infection in the presence of arginine slightly reduced the incorporation of protein precursors compared with control cultures. There was negligible incorporation into cells deprived of arginine although under these conditions incorporation into controls was still greater than into infected cultures. Salzman and Sebring (1967) have shown that in a similar system, cellular protein synthesis is inhibited by 80-90% at 4 hr. post infection. It follows that virus specific protein synthesis in the presence of arginine is only slightly less extensive than host protein synthesis in uninfected cells. The amount of protein synthesis ocurring in infected cells during the first 12 hr. post infection was reduced by 93% on deprivation of arginine, measured by incorporation of either leucine or phenylalanine.

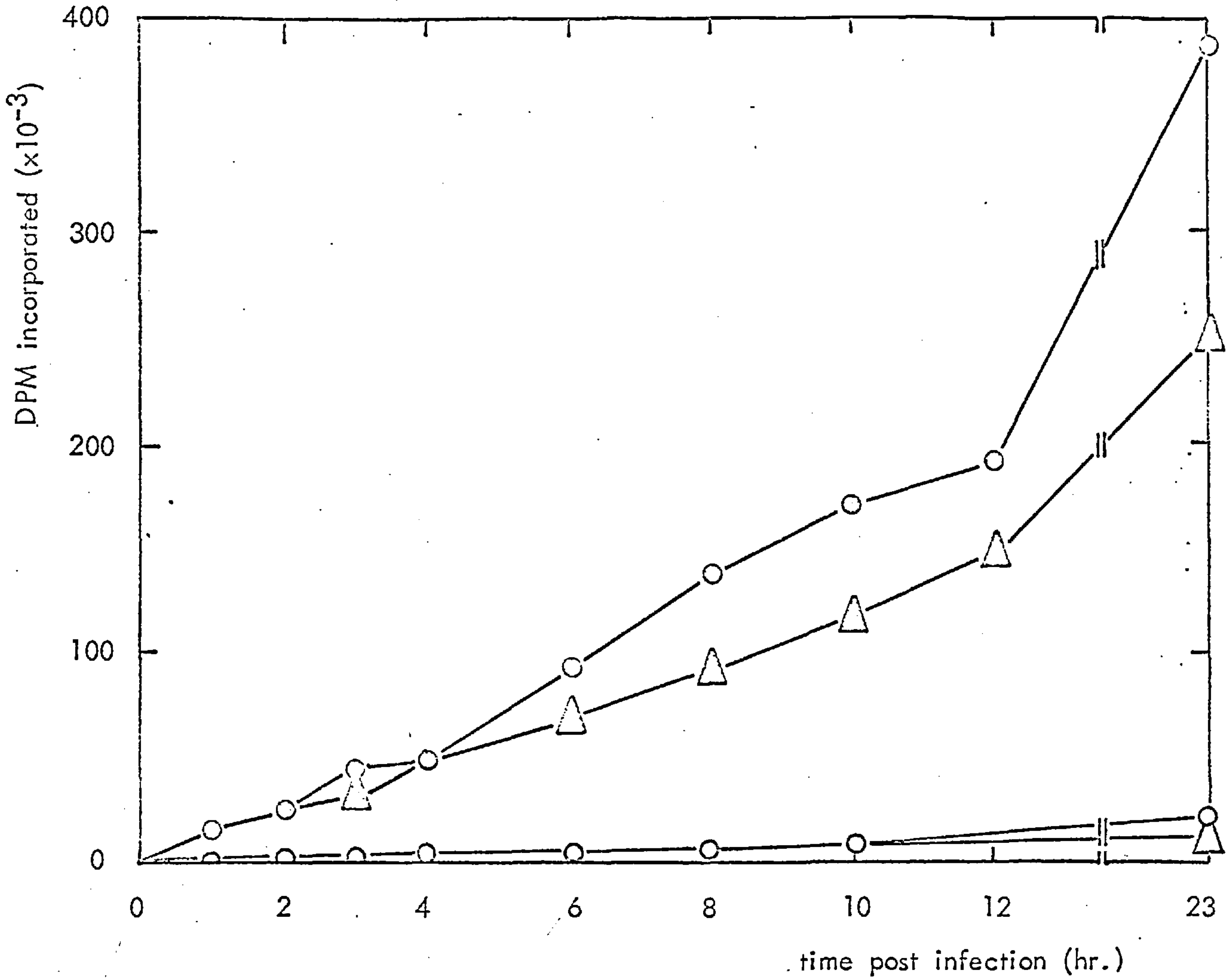

Fig. (19). The effect of arginine deprivation on the synthesis of protein measured by the incorporation of leucine.

○——○ control cultures maintained in the presence of arginine;

O——O control cultures maintained in the absence of arginine;

△——△ infected cultures maintained in the presence of arginine;

Δ——Δ infected cultures maintained in the absence of arginine.

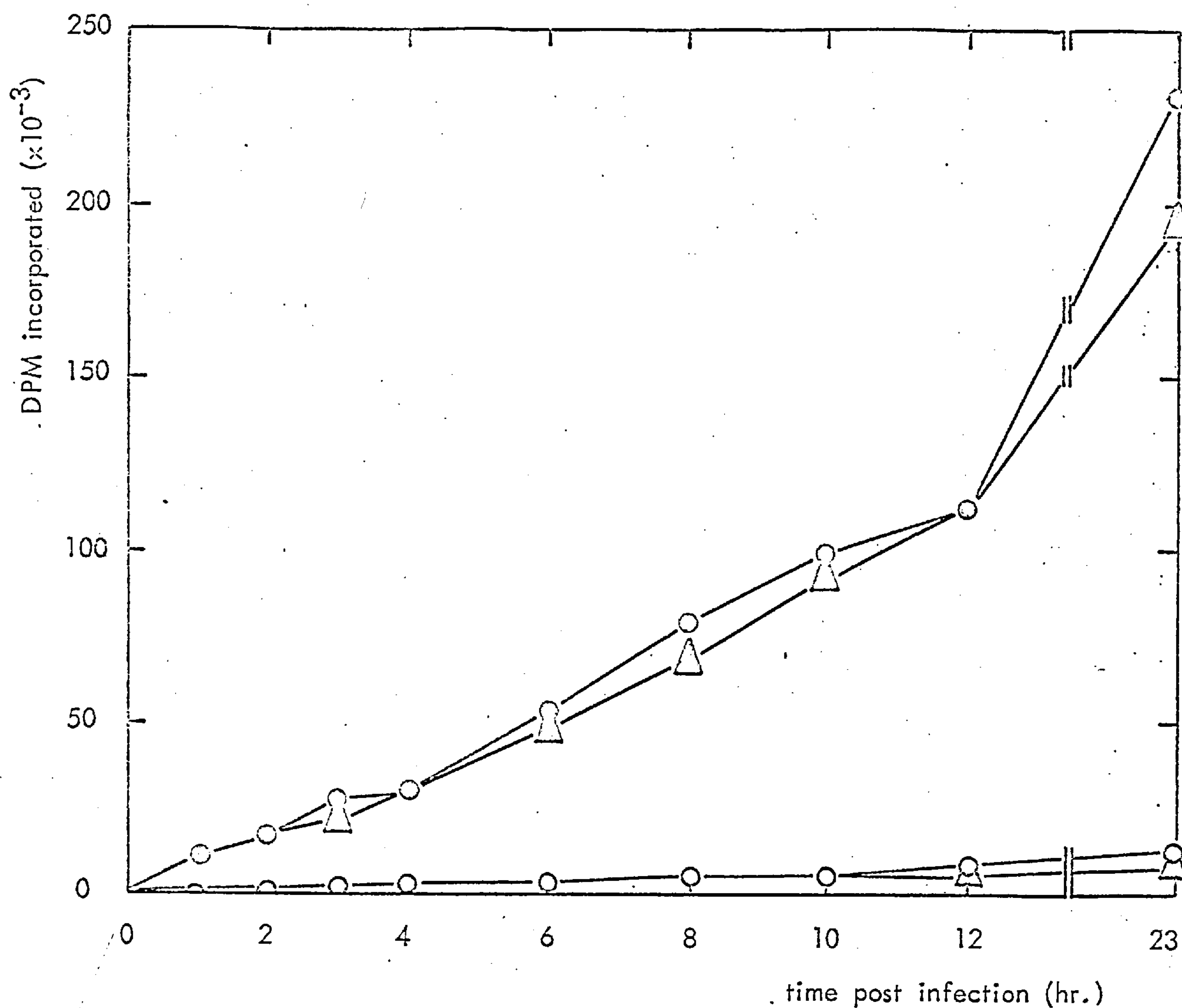

Fig. (20). The effect of arginine deprivation on the synthesis of protein measured by the incorporation of phenylalanine.

○—○ control cultures maintained in the presence of arginine;

○—○ control cultures maintained in the absence of arginine;

△—△ infected cultures maintained in the presence of arginine;

△—△ infected cultures maintained in the absence of arginine.

5.4 <u>Synthesis of virus specific antigens in arginine deprived, infected cells.</u>

Previously described results demonstrate an overall reduction in RNA synthesis in infected cells maintained in the absence of arginine but do not indicate the extent to which virus specific RNA synthesis is inhibited under these conditions. Consequently, the production of virus specific antigens in arginine deprived, infected cells was studied, using diffusion in gel tests, as a qualitative examination of the extent of virus specific RNA synthesis.

HeLa cell cultures were equilibrated in serum free experimental medium which either lacked arginine or contained 1 mM. arginine. After virus infection, the cultures were reincubated with similar media. At 24 hr. post infection cell extracts containing soluble antigens were prepared as described and employed in diffusion in gel tests against the hyperimune rabbit antivaccinia serum. The diffusion plates were allowed to develop for 2 days after which time no further development of line precipitation patterns took place.

Reactions of complete identity between both of the antigen preparations were obtained. Extracts prepared from infected cultures maintained in the presence of arginine gave no line of precipitation not obtained with extracts of arginine deprived, infected cultures. This indicates that in the absence of arginine, at least synthesis of virus RNA species coding for antigens detectable by the antiserum takes place. However, the marked reduction of protein synthesis resulting from arginine deprivation of infected cultures suggests either that overall synthesis of antigens is greatly reduced or that synthesis of some antigens constituting the greater part of virus specific protein synthesis is selectively inhibited. Complete identification of the virus

specific antigens produced in the presence of arginine with those produced in the absence of arginine suggests that the serum contains antibodies raised only against early antigens, as deprivation of arginine was previously shown to result in almost total inhibition of virus specific DNA synthesis.

As Salzman and Sebring (1967) have demonstrated that both early and late virus proteins are capable of participipating in precipitation reactions in diffusion in gel tests, the specificity of the antiserum was examined by a comparison of the line precipitation patterns obtained against extracts of HeLa cell cultures infected in complete medium in the presence and absence of 5-bromodeoxyuridine (BUDR). This compound is a structural analogue of thymidine and appears to result in a non-competitive inhibition of DNA synthesis. The effectiveness of BUDR as an inhibitor of DNA synthesis was demonstrated in the following way. HeLa cell cultures were equilibrated in serum free experimental medium containing 1 mM. arginine and 0.02 mM. concentrations of nucleosides. After virus infection of some cultures, all were reincubated with medium similar to that used for equilibration but containing 0.5 μCi. per ml. (^{3}H)thymidine. Additionally, the medium with which some cultures from each set were reincubated contained 0.02 mM. BUDR which had previously been shown to inhibit completely the formation of infective progeny virus. Samples were taken from each series of cultures at various times after the addition of media containing the labelled precursor and assayed for incorporated radioactivity. The time course of incorporation of (^{3}H)thymidine into infected and control cultures maintained in the presence and absence of BUDR is shown in fig. 21

As previously demonstrated, infection of cultures in complete medium resulted in a stimulation of incorporation between 2 and 4 hr. post infection. In the presence of BUDR however, incorporation

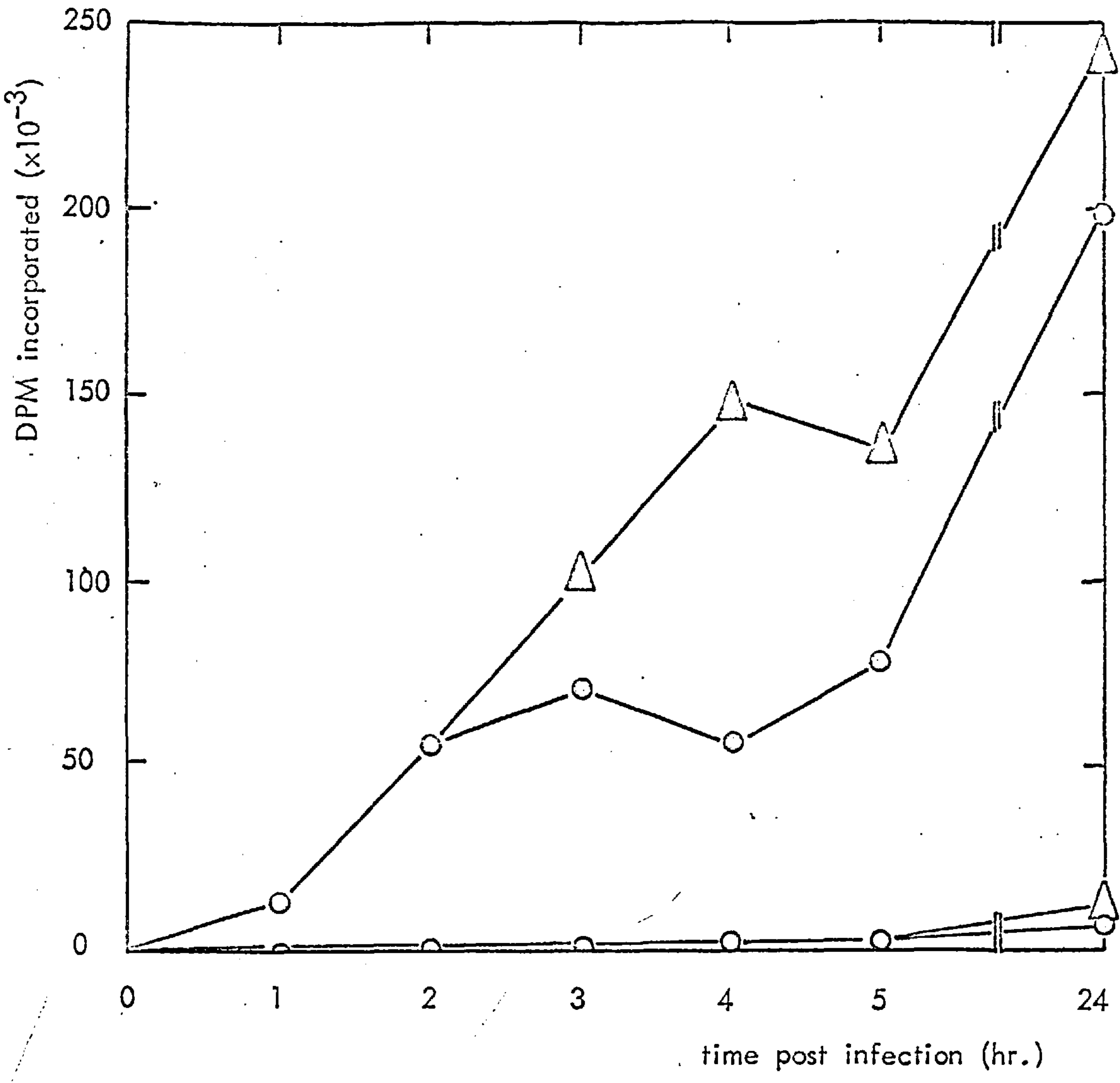

Fig. (21). The effect of BUDR on the synthesis of DNA.

○—○ control cultures;

○—○ control cultures maintained in the presence of BUDR;

△—△ infected cultures;

△—△ infected cultures maintained in the presence of BUDR.

into both infected and control cultures was greatly reduced and the stimulation of incorporation associated with virus DNA replication was completely inhibited. A reduction of incorporation of 94% was observed at 24 hr. post infection.

In diffusion in gel tests, extracts of cultures infected in the presence of arginine gave no line of precipitation not obtained with extracts of similar cultures maintained in the additional presence of BUDR (plate 4). As the presence of BUDR completely inhibits the formation of functional virus DNA, it follows that the serum contains antibodies only against early antigens whose synthesis is directed by RNA species transcribed from the infecting viral genome rather than from replicated DNA molecules. Production of some virus antigens in vaccinia infected cultures in which DNA synthesis was inhibited has previously been demonstrated (Shatkin, 1963 ; Baxby and Rondle 1968).

Thus, deprivation of arginine does not prevent the synthesis of at least some virus messenger RNA species and the consequent production of the early antigens for which these code.

Plate (4). Diffusion in gel line precipitation patterns.

S - rabbit hyperimmune antivaccinia serum;

A - soluble extract of HeLa cells infected and maintained in the presence of arginine;

B - soluble extract of HeLa cells infected and maintained in the presence of arginine and BUDR;

C - soluble extract of HeLa cells infected and maintained in the absence of arginine.

CHAPTER 6

THE INCORPORATION OF ARGININE INTO INFECTED AND CONTROL CULTURES AND INTO MATURE VIRUS PARTICLES

6.1 Time course of incorporation of arginine into infected and control cultures

HeLa cell cultures were equilibrated in serum free experimental medium containing 0.5 mM. arginine. After virus infection of some cultures, all were reincubated with medium similar to that used for equilibration but containing 0.2μCi. per ml. uniformly labelled (^{14}C)arginine. Samples were taken from each series of cultures at various times after the addition of medium containing the labelled precursor and assayed for incorporated radioactivity. The time course of incorporation of arginine into infected and control cultures is shown in fig. 22. Infection of cultures reduced incorporation after 2 hr. compared with controls and at 12 hr. post infection a reduction of 57% was observed.

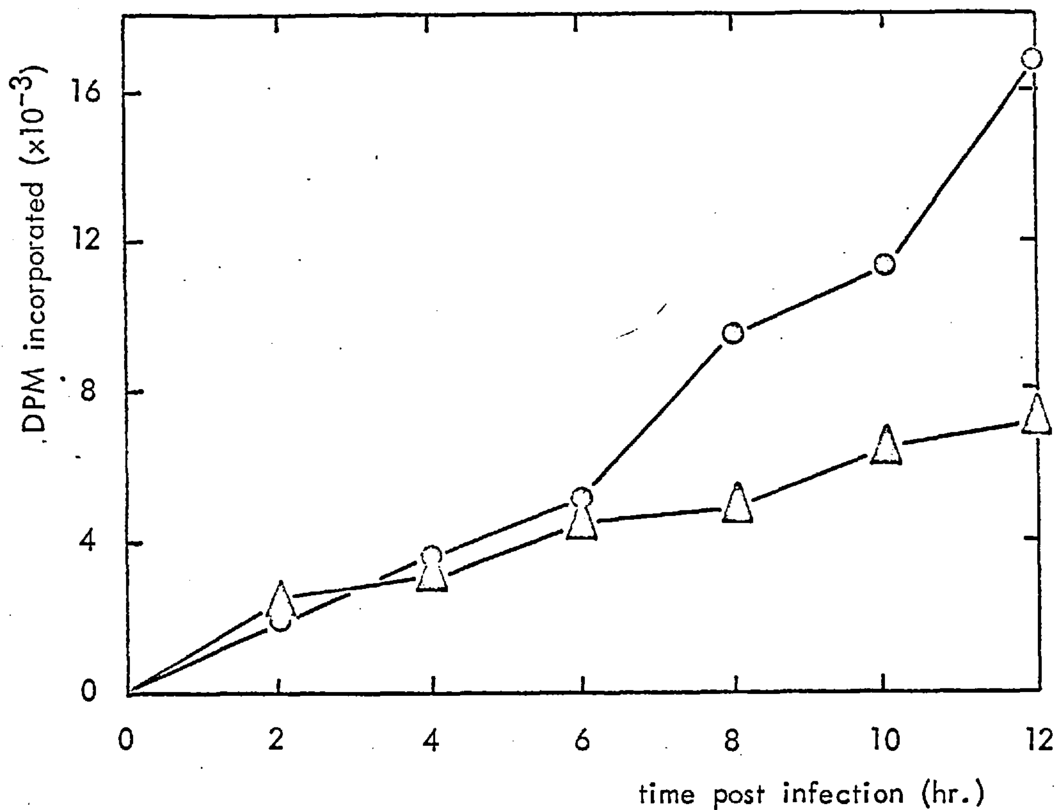

Fig. (22). The incorporation of arginine into control and infected cultures.

○——○ control cultures;

△——△ infected cultures.

These results are similar to those obtained measuring the incorporation of the amino acids leucine and phenylalanine. However, the greater reduction in incorporation of arginine, compared with leucine or phenylalanine, on infection of cells in complete medium may indicate that overall virus specific protein synthesis is less dependent on the presence of arginine than is host specific protein synthesis.

6.2 Rate of incorporation of arginine following infection.

The kinetics of the incorporation of arginine into infected and control cultures were further investigated by following changes in the rates of incorporation. A change in rate is not readily demonstrable by following the time course of incorporation in which the significance of increments is related to preceeding values. Such a change may however, be demonstrated by determination of the amount of radioactivity incorporated during short periods of incubation with medium containing the labelled precursor at various times in the replication cycle. The shorter the period of exposure of cultures to the labelled precursor, the more accurate a measure of the rate of incorporation at the median time is obtained from determination of incorporated radioactivity. The application of this hypothesis is limited in practice by the amount of radioactivity which can be supplied in the culture medium without causing cell damage and the necessity for incorporation of sufficient activity for accurate determination. Preliminary experiments indicated that using 0.2 μCi. per ml. (^{14}C)arginine in the presence of a background concentration of unlabelled arginine sufficiently high to disregard the possibility of depletion during the experiments, a pulse time of about 20 min. was desirable.

HeLa cell cultures were equilibrated in serum free experimental medium containing 0.25 mM. arginine. After virus infection of some cultures, all were reincubated with medium similar to that used for equilibration. A shortened virus adsorption period of 30 min. was used in order to examine early events in the replication cycle. At various times post infection beginning at the end of the virus adsorption period, samples from each series of cultures were incubated for 20 min. with medium containing 0.2 μCi per ml. guanido(^{14}C)arginine and assayed for radioactivity incorporated

within this period (fig. 23).

The results obtained showed a 50% increase in the rate of incorporation of arginine by infected cells compared to control cells within 50 min. of first exposing cultures to virus. Following this very early stimulation, the rate of incorporation rapidly declined while incorporation into control cultures remained relatively constant throughout. A further, reproducible stimulation of incorporation into infected cultures was observed at 3 hr. post infection. An essentially similar pattern of results was obtained using uniformly labelled (^{14}C)arginine although the extent of incorporation appeared to be greater (fig. 24). This is almost certainly an artifact resulting from metabolism of arginine and incorporation into high molecular weight material of some radioactively labelled carbon atoms in the form of arginine derivatives.

However, these experiments do indicate a stimulation of the incorporation of arginine into infected cells at two distinct times in the virus replication cycle. The first certainly precedes virus DNA replication while the second is concurrent with this.

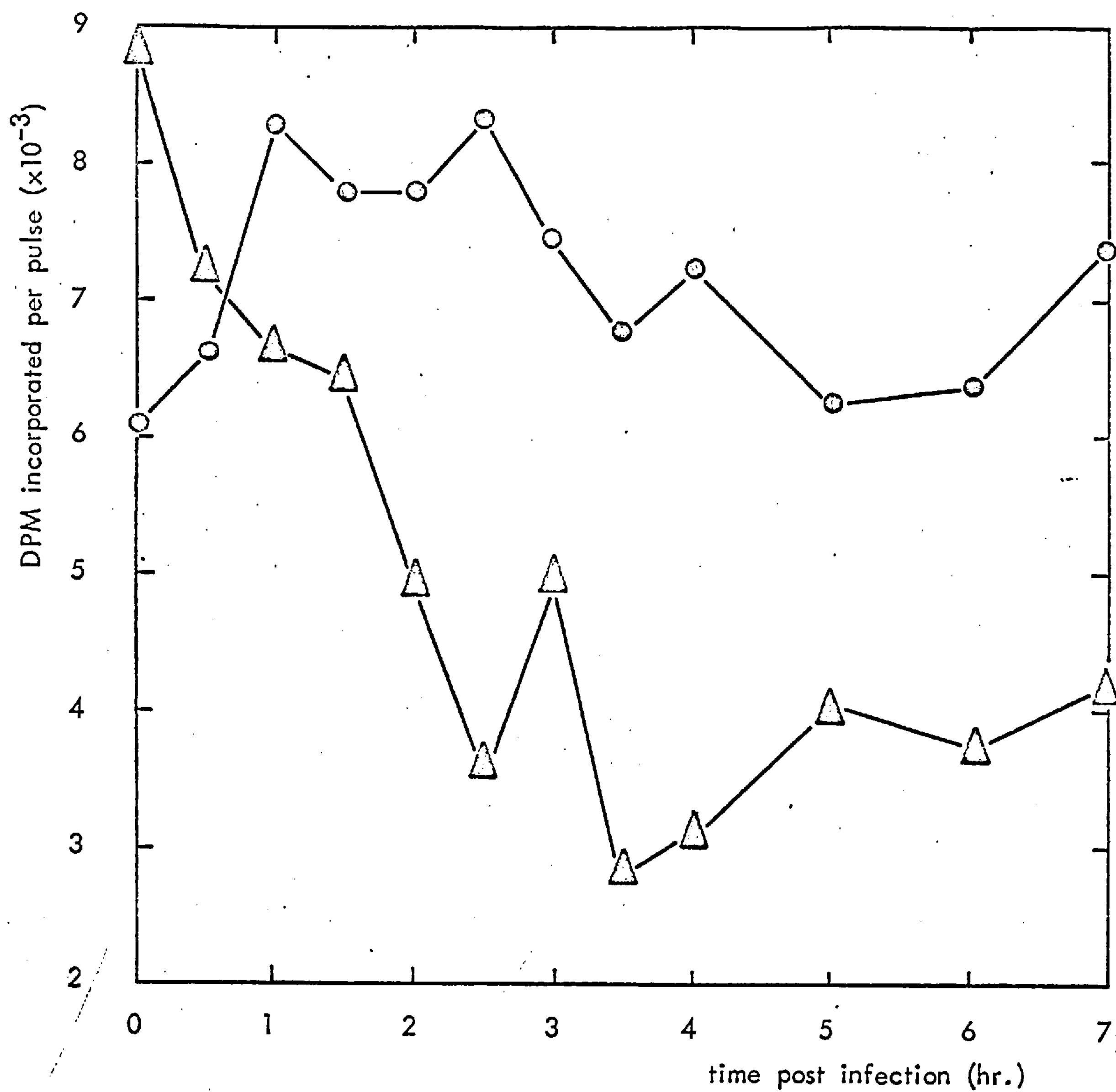

Fig. (23). The rate of incorporation of guanido(^{14}C)arginine into control and infected cultures.

○—○ control cultures;

△—△ infected cultures.

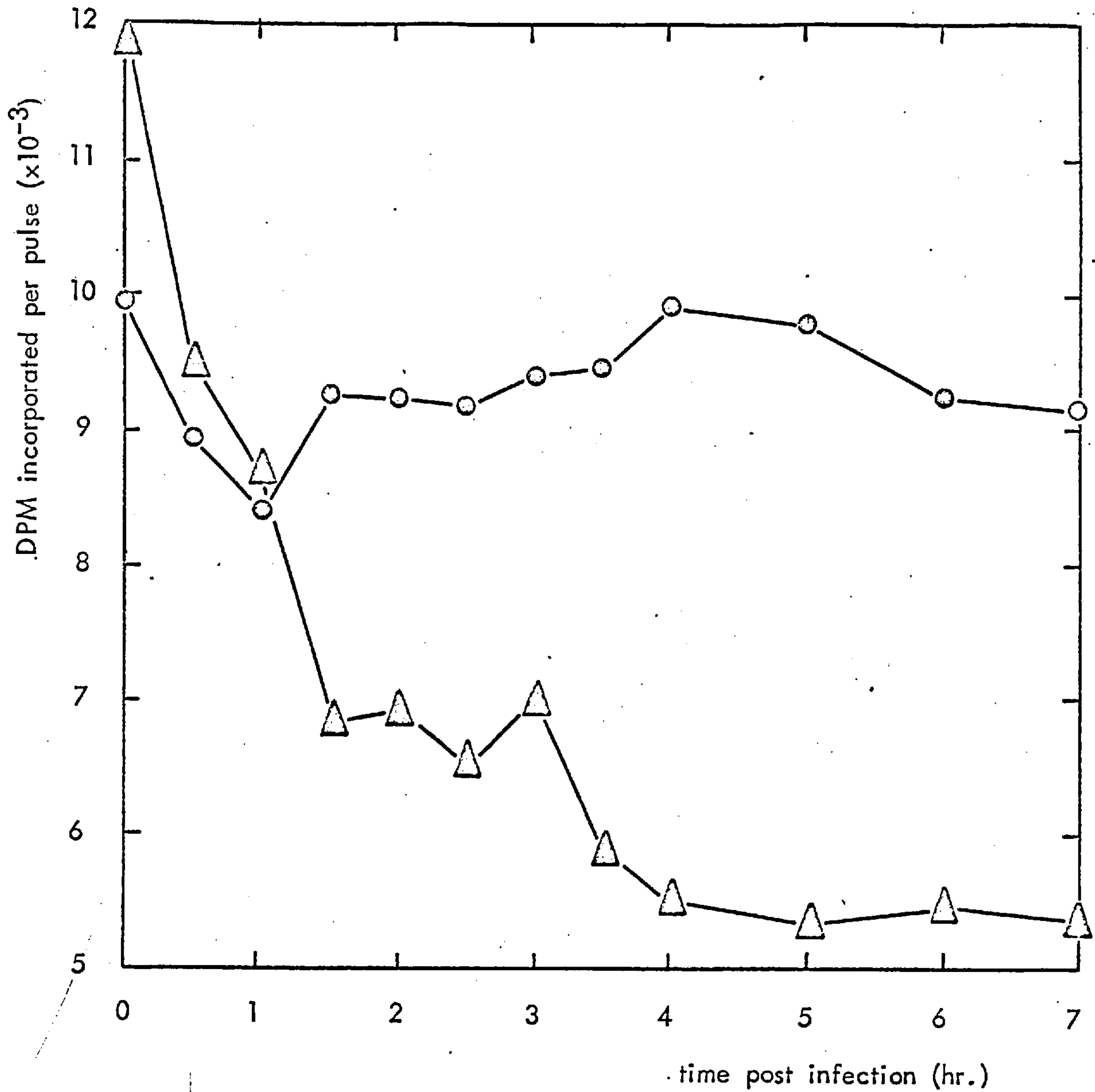

Fig. (24). The rate of incorporation of uniform(^{14}C)arginine into control and infected cultures.

○—○ control cultures;

△—△ infected cultures.

6.3 Incorporation of arginine into mature virus particles.

As virus infection of cells was shown to stimulate the incorporation of arginine at specific times in the virus replication cycle, purified progeny virus grown in the presence of radioactively labelled arginine was examined for incorporated radioactivity.

HeLa cell cultures in 40 oz. bottles were infected at a multiplicity of 5 pfu per cell. The inocula were replaced with 50 ml. aliquots of serum free experimental medium containing 0.5 mM. arginine and either 40 μCi. total of guanido(^{14}C)arginine or 40 μCi. total of uniformly labelled (^{14}C)arginine. At 24 hr. post infection, progeny virus was extracted and purified as described. After centrifugation of the purified virus preparations in potassium tartrate density gradients, a thin white band was visible in each gradient at a position corresponding to about 45% potassium tartrate (specific gravity 1.25). No pellets were observed. Fractions from the gradients were assayed for infectivity and for incorporated radioactivity (fig. 25). In both case the single peaks of infectivity and radioactivity were coincident. However, the specific amount of radioactivity incorporated into the uniformly labelled virus preparation was greater than that incorporated into the guanido labelled preparation.

The apparent molar ratio of arginine incorporated into the two differently labelled virus preparations was 1.56 As previously discussed, this is almost certainly due to metabolism of arginine and the incorporation of resulting derivatives. The value of the apparent molar ratio of incorporation of uniformly labelled arginine to guanido labelled arginine gives an indication as to which part of the arginine molecule is incorporated after metabolism. Consideration of the specific activity and molecular weight of the uniformly labelled arginine preparation indicates that labelled molecules have an isotopic abundance of 83%, that is statistically,

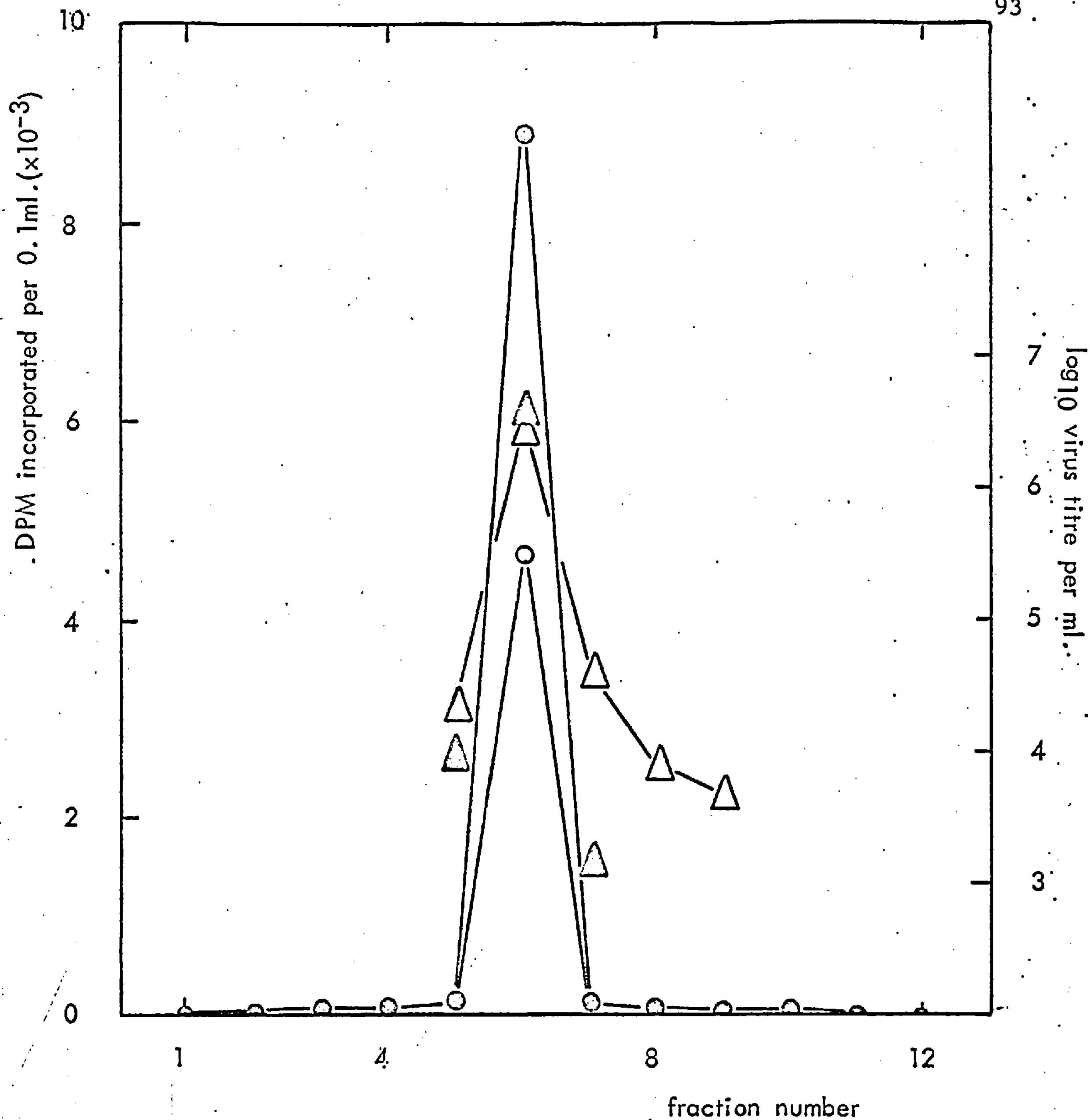

Fig. (25). The incorporation of arginine into mature virus particles.

O——O uniform(^{14}C) activity incorporated;

O——O guanido(^{14}C) activity incorporated;

△——△ infective virus, uniformly labelled;

△——△ infective virus, guanido labelled.

any one of the six carbon atoms has an 83% probability of being an isotopic carbon atom and consequently, 83% of labelled molecules are labelled in the guanido position. Disregarding the small porportion of uniformly labelled molecules not labelled in this position, 20% of the radioactivity is located in the guanido group and the remaining 80% is distributed over the five remaining carbon atoms. In the guanido labelled arginine preparation however, labelled molecules have an isotopic abundance of 100% in the guanido position and consequently no radioactivity is present in the remainder of the molecule. Principle pathways of arginine metabolism have been indicated previously and show that in general, the guanido carbon and the remaining carbon chain are mutually exclusive in their participation in metabolism. It follows therefore, that if no incorporation other than that of the arginine molecule took place, an apparent molar ratio of unity would be observed. If metabolism of arginine resulted in the incorporation of derivatives containing carbon atoms other than that of the guanido group an apparent molar ratio of between unity and infinity would be observed, the value depending on the extent of incorporation of derivatives compared with that of arginine itself. Finally, if metabolism of arginine resulted in the incorporation of derivatives containing the carbon atom of the guanido group an apparent molar ratio of between unity and 0.2 would be observed, the value again depending on the extent of incorporation of derivatives. The experimentally obtained value indicates that metabolism of arginine predominantly results in the incorporation of derivatives containing carbon atoms other than that of the guanido group, perhaps as proline, aspartate or a polyamine.

CHAPTER 7

QUANTITATIVE EFFECTS OF THE DELAYED ADDITION OF ARGININE ON THE PRODUCTION OF INFECTIVE VIRUS

Previously described experiments demonstrated both the requirement for arginine associated with the synthesis of DNA, RNA and protein in infected cells and the incorporation of arginine into mature virus particles. The following experiments were designed to investigate a possible requirement for arginine at a late stage in the replication cycle such as the formation of mature virus particles.

HeLa cell cultures were equilibrated in serum free experimental medium which contained the virus growth limiting concentration 0.015, 0.03 or 0.06 mM. arginine. After virus infection, the cultures were reincubated with media similar to those used for equilibration. At 6 hr. post infection the arginine concentration in some cultures maintained in media initially containing 0.015 or 0.03 mM. arginine was increased by the addition of arginine equivalent to 0.06 mM. Samples were taken from each series of cultures at various times post infection and titrated for infective virus (fig. 26).

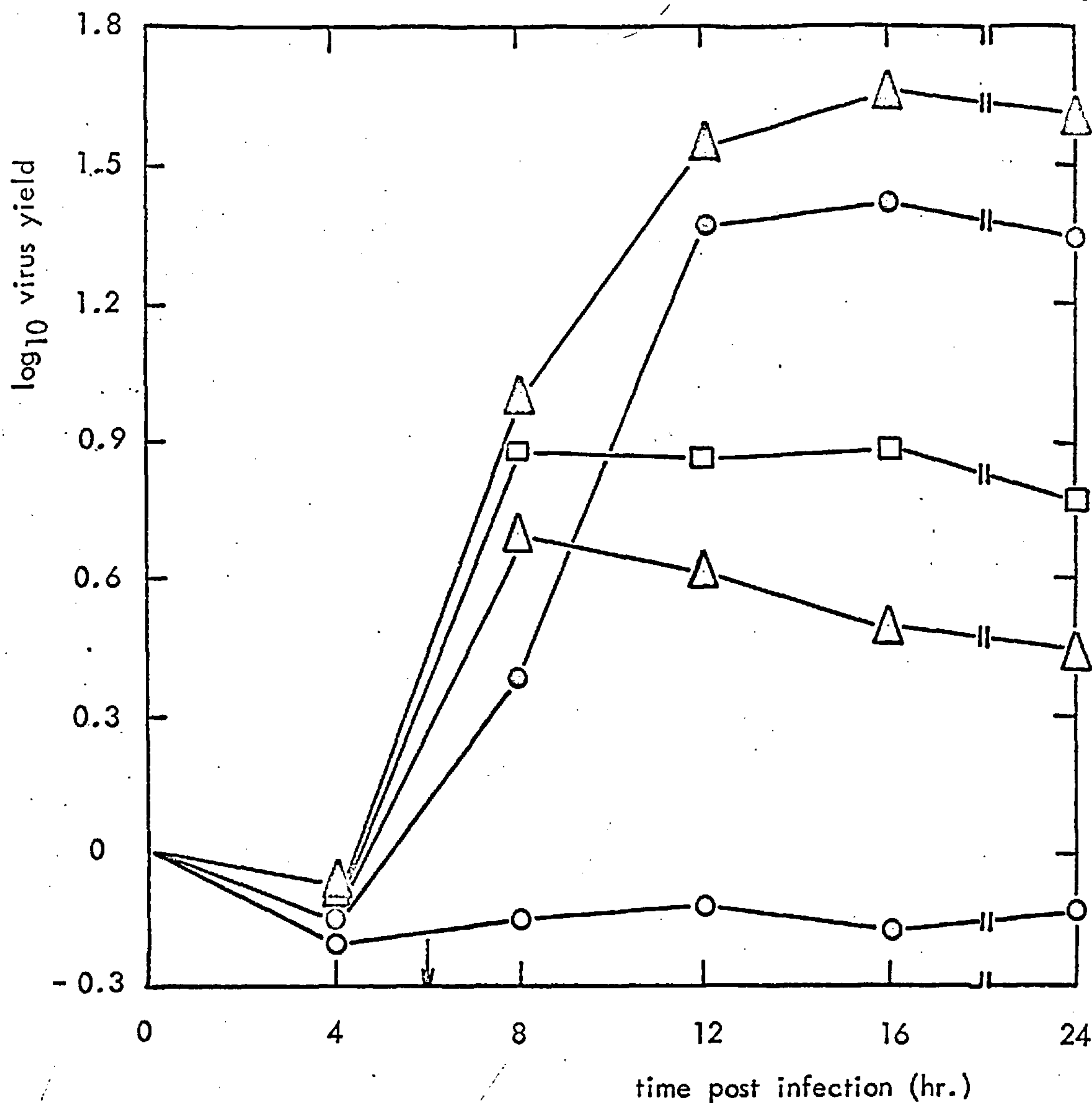

Fig. (26). Quantitative effects of the delayed addition of arginine on the production of infective virus.

○—○ cultures infected in the presence of 0.015mM. arginine;

○—○ similar cultures supplied with 0.06mM. arginine at 6hr. p.i.;

△—△ cultures infected in the presence of 0.03mM. arginine;

△—△ similar cultures supplied with 0.06mM. arginine at 6hr. p.i.;

□—□ cultures infected in the presence of 0.06mM. arginine.

In the presence of 0.015 mM. arginine no progeny virus was produced, but addition of 0.06 mM. arginine at 6 hr. post infection permitted virus growth to titres greater than those in cultures maintained in medium initially containing this higher concentration of arginine. Infective progeny virus was first detected within 2 hr. after supplying additional arginine to the cultures. Similarly, the addition of 0.06 mM. arginine at 6 hr. post infection to cultures previously maintained in medium initially containing 0.03 mM. arginine resulted in an increased yield of virus compared to that from cultures maintained in medium initially containing the higher concentration. This additional yield was produced without delay. In further experiments, in which 0.06 mM. arginine was added at 6 hr. post infection to cultures equilibrated, infected and maintained in its absence, production of infective progeny virus was not detected until 5 hr. after the addition of arginine (fig. 27).

It was previously shown that in cultures infected in the presence of complete medium, infective progeny virus was first detectable at 5 hr. post infection. A similar lag was observed between the addition of arginine at 6 hr. post infection to arginine deprived, infected cultures and the first detection of infective progeny virus. This period must represent the time required under normal circumstances for the virus specific events preceeding the formation of infective virus to take place. The detection of progeny virus within 2 hr. after the addition of 0.06 mM. arginine to infected cultures previously maintained in medium initially containing 0.015 mM. arginine indicates that the latter concentration, although insufficient to allow the formation of infective virus, permitted earlier, virus specific events to take place. The immediate production of an increased yield of infective virus following the addition of 0.06 mM. arginine to cultures previously maintained in medium initially containing the virus

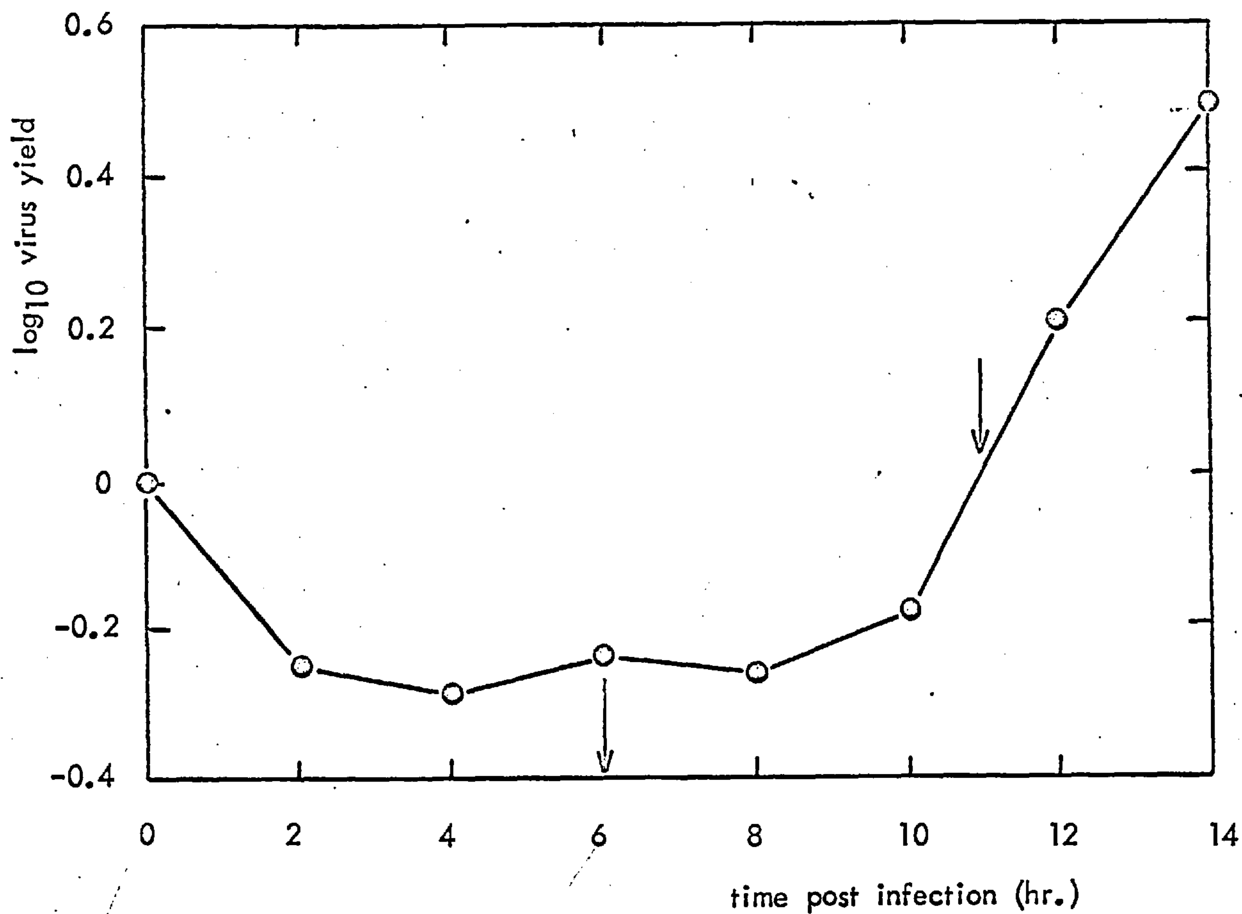

Fig. (27). The quantitative effect of the delayed addition of arginine to cultures previously maintained in its absence on the production of infective virus.

growth limiting concentration 0.03 mM. arginine demonstrates that events in the virus replication cycle following the synthesis of viral DNA are quantitatively dependent on the availability of arginine. A concentration of 0.015 to 0.03 mM. arginine suffices for completion of the virus specific events preceeding the formation of infective progeny virus whereas the production of maximum yield of virus requires 0.09 mM. arginine.

CHAPTER 8

DISCUSSION

The results obtained in this study confirm and extend the previous observations of Holtermann (1969) and of Singer et al., (1970) that arginine is essential for the growth of vaccinia virus. Arginine is shown to be required at both an early and a late stage in the virus replication cycle.

As the synthesis of virus specific products is accomplished mainly by the existing metabolic capacity of the host cell under the redirection of the infecting virus nucleic acid, the nutritional requirements for virus replication must be considered in relation to those of the host. Animal cells in culture require a number of amino acids to be supplied in the culture medium in order to continue growth. Such requirements may be relative in that a nutrient is normally synthesised only at a growth limiting rate or absolute in that no endogenous synthesis takes place. If host cells are deprived of an essential amino acid, the synthesis of proteins containing that amino acid will be inhibited whether these are host or virus specified and this inhibition of protein

synthesis may have indirect effects on other metabolic functions. However, under conditions of starvation, cellular proteins may be turned over and an endogenous supply of an essential amino acid may thus become available. A virus nutritional requirement which is not also a cellular requirement cannot be demonstrated by omission of that nutrient from the culture medium as the virus requirement is fulfilled endogenously. Alternatively, virus induced modification of host metabolic function can negate a cellular nutritional requirement which otherwise would also be required for virus replication. This is demonstrated by the restoration of the thymidine phosphorylating ability of thymidine kinase deficient cells following infection by vaccinia virus. Similarly, it is possible that virus infection enhances the turnover of cellular proteins by the induction of either a host or virus specified protease. A final factor to be considered in the host virus relationship is the superinfection of cultures with microorganisms which may have nutritional requirements or metabolic capacities in addition to those of the cells. Thus, the depletion of arginine in PPLO contaminated cultures has been shown to affect the growth of a number of viruses.

In the complete absence of arginine, vaccinia infected HeLa cell cultures produced no infective progeny virus even when incubation was continued for a total of 48 hr. It should be emphasised however, that the depletion of arginine by starvation did not irreversibly affect the ability of either the cells to support virus replication or the virus to direct its replication, distinguishing this inhibition from that resulting from cell damage or the action of an antimetabolite. Restoration of arginine at the time of infection resulted in a production of progeny virus which followed normal kinetics. Restoration of arginine to deprived cultures at 6 hr. post infection totally reversed the inhibition of virus replication. The contribution of serum to the

otherwise defined culture medium should be considered in relation to arginine starvation. Although there may be some variation between batches of serum in this respect, it was found that the addition of 2% unheated calf serum had an arginine sparing effect equivalent to 0.03 mM. arginine and consequently, serum was omitted from all experimental media.

The production of infective virus in adenovirus infected cells (Rouse et al., 1963) and in herpesvirus infected cells (Tankersley, 1964) has also been reported to depend on the presence of arginine. Jeney et al., (1967) reported that arginine was required for the growth of herpesvirus in cultures of the continuous cell lines HeLa, HEp_2 and KB but not in cultures of human or chick embryonic fibroblasts or of primary monkey kidney cells. The ability of these arginine deprived, primary cell cultures to support virus replication suggests the availability of endogenous arginine. However, of 31 types of primary cell cultures examined by Tytell and Neuman (1960), all exhibited a marked growth response to the presence of exogenous arginine but no significant growth response to ornithine in substitution for arginine. As in the majority of these cultures citrulline can substitute for arginine, an inability to convert ornithine to citrulline and consequently, a lack of the enzyme carbamoyl phosphate:L-ornithine carbamoyl transferase can be inferred. This effect may well be relative as the growth response as measured by Tytell and Neuman takes little account of the normal growth rates and final population densities achieved by different cell cultures. Thus, the primary cell cultures may synthesise arginine at a growth limiting rate and may themselves be less dependent on the presence of exogenous arginine, permitting the growth of an arginine requiring virus under conditions of experimental arginine deprivation.

The results obtained in the present study show that HeLa cell cultures deprived of arginine have greatly reduced ability to synthesise DNA, RNA and protein. Although cultures appeared cytologically normal after the depletion of the intracellular pool of arginine by starvation, the amount of DNA synthesis occurring in a 22 hr. period was reduced by 84%, RNA synthesis by 86% and protein synthesis by 95% compared with control cultures maintained in the presence of arginine. Gonczol et al., (1967) reported a 61% inhibition of DNA synthesis in similar cells deprived of arginine although cultures were not depleted of arginine prior to use. These inhibitions of macromolecular synthesis may be interrelated but the primary effect is likely to be the inhibition of protein synthesis. Continued DNA replication probably requires the synthesis of both enzymatic protein associated with its formation and basic, structural protein such as histones associated with its stability and function. While the enzyme activity may continue to some degree in the absence of de novo protein synthesis, newly synthesised DNA not protected by association with concurrently synthesised protein may be degraded by cellular nucleases. In addition to a probable arginine requirement for the synthesis of ribosomal protein, polyamine derivatives of arginine may have a role in the synthesis and stabilisation of RNA. Hare (1969) found that the presence of the arginine structural analogue canavanine inhibited the synthesis of DNA and RNA in addition to that of protein in mouse embryo cells but ascribed these effects to the formation of abnormal canavanyl protein. The possibility of a direct role of arginine in the elaboration of nucleic acids should be considered at this point. A contribution of the guanido carbon of arginine rather than carbon dioxide to the synthesis of pyrimidines and to some extent of purines in Lactobacillus leichmannii has been reported (Hutson and Downing, 1968). Such a pathway is unlikely to be significant in the system described here as experiments investigating

patterns of nucleic acid synthesis were performed in the presence of media which contained nucleosides.

A quantitative relationship was demonstrated between the concentration of arginine supplied in the medium and the yield of infective vaccinia virus, maximum yield being obtained in the presence of medium containing 0.09 mM. arginine. This dependence of virus yield on arginine concentration was shown to be a direct dose response phenomenon in which the total production of progeny virus is quantitatively related to an arginine requiring event such as the synthesis of a macromolecular product. This concept could represent a requirement for continued synthesis of a virus induced protein on which the formation of mature virus particles depends. The relationship is not a dose delay phenomenon in which the rate of production of progeny virus is related to an arginine requiring event such as the synthesis of a specific enzyme nor is it a threshold phenomenon in which the completion of an arginine requiring event is necessary for subsequent events to take place. Becker et al., (1967) have shown that in herpesvirus infected BSC_1 cells, at least 0.105 mM. arginine is required to obtain maximum yield of virus, a value very similar to that presented here for vaccinia infected HeLa cells.

Deprivation of arginine significantly altered patterns of macromolecular synthesis in vaccinia virus infected HeLa cells. Virus DNA replication was normally observed as a marked stimulation of DNA synthesis occurring between 2 and 4 hr. post infection. Joklik and Becker (1964) showed that in a similar system, virus DNA was synthesised between 1.5 and 4.5 hr. post infection. The results presented previously demonstrate that this is almost completely abolished in cells infected in the absence of arginine, a situation similar to that observed by Gonczol et al., (1967) in herpesvirus infected HeLa cells. Overall RNA synthesis

in vaccinia virus infected HeLa cells was characteristically reduced after 3.5 hr. post infection in agreement with the results of Becker and Joklik (1964) who observed that the rates of both host and virus RNA synthesis decrease rapidly after 4 hr. post infection. Deprivation of arginine resulted in a marked inhibition of overall RNA synthesis which was apparent from the time of infection. As Becker and Joklik (1964) have shown that the initial rate of host RNA synthesis in infected cells greatly exceeds the maximum rate of virus RNA synthesis, it is difficult to estimate the extent to which virus specific RNA synthesis is inhibited on deprivation of arginine. However, the detection of virus specific proteins by a serological technique indicates that synthesis of at least some early,virus specific messenger RNA species takes place in arginine deprived, infected cells. Overall protein synthesis in cultures infected in complete medium was only slightly reduced in comparison to uninfected control cultures when measured by the incorporation of leucine. Overall protein synthesis measured by the incorporation of phenylalanine was almost unchanged. As cellular protein synthesis has been shown to be inhibited by 80 to 90% at 4 hr. post infection (Salzman and Sebring, 1967), it follows that the extent of virus specific protein synthesis is normally almost as great as that of host specific synthesis. This is not unexpected in that the same authors reported that only about half of the virus specific protein synthesised during the replication cycle eventually appears in mature virus particles. Deprivation of arginine from infected cells resulted in an almost total inhibition of protein synthesis measured by the incorporation of either leucine or phenylalanine.

The results presented show that during the first 2 hr. post infection after which time virus specific DNA synthesis would normally commence, RNA synthesis was reduced by 82% and

protein synthesis by 86% on deprivation of arginine. It is highly probable that failure to synthesise virus DNA resulted from prior reductions in the synthesis of early messenger RNA species transcribed from input virus genomes and of proteins normally translated from these messengers. Requirements for the synthesis of proteins involved in the initiation of virus nucleic acid replication are well known in DNA viruses including vaccinia virus (Joklik and Becker, 1964), herpes simplex virus (Roizman et al., 1963) and adenovirus type 2 (Polasa and Green, 1965). In many cases these have been shown to represent the synthesis of virus induced enzymes associated with DNA replication. Such are the thymidine kinase and DNA polymerase induced on infection by vaccinia virus (Green, 1962) and by pseudorabies virus (Hamada et al., 1966). The synthesis of vaccinia early messenger RNA species very shortly after uncoating in the cytoplasm of HeLa cells infected in complete medium has been demonstrated (Becker and Joklik, 1964) and these almost certainly direct the synthesis of the early enzymes (Jungwirth and Joklik, 1965). Becker et al., (1967) and Rouse and Schlesinger (1967) reported that in herpesvirus infected BSC_1 cells and adenovirus infected KB cells respectively, the synthesis of virus specific DNA proceeded normally in the absence of arginine. In neither of these cases however, were cultures starved of arginine prior to infection. It may be that without depletion of the intracellular pool, sufficient arginine is available to permit the synthesis of early proteins required for DNA replication.

It is no doubt significant that the earliest events in the vaccinia replication cycle, preceding even the formation of early messenger RNA, are dependent on protein synthesis, the metabolic function most likely to be inhibited by deprivation of an essential amino acid. Joklik (1964a ; 1964b) has described the uncoating of phagocytised poxvirus particles as a two stage process. The first stage, which results in the formation of DNase resistant cores, is

thought to be mediated by existing lysosomal enzymes and the second, which results in the release of functional virus DNA available for the transcription of early messenger RNA, is thought to be mediated by a virus induced enzyme. While it is likely that the activity of the existing cellular enzymes is relatively unaffected by arginine deprivation, it is certain that the capacity for de novo protein synthesis is greatly reduced. Thus, it may be that one of the earliest effects of arginine deprivation in infected cells is to inhibit the second stage of the uncoating of virus particles. The demonstration of the synthesis in arginine deprived, infected cells of some virus specific early messenger RNA species and the proteins for which they code has already been mentioned. This appears to be contradictory but, as the vaccinia virus genome could code for as many as 150 proteins only a few of which are detected here, may result from a selective inhibition. A limited synthesis of virus specific RNA would be possible even in the absence of complete uncoating. Kates and McAuslan(1967b) presented evidence that some transcription of early messenger RNA takes place in the absence of protein synthesis, that is before poxvirus cores are fully uncoated and raised the question of the source of the DNA- dependent RNA polymerase responsible for the transcription. Further investigation has demonstrated the presence of this enzyme activity within purified virus particles (Kates and McAuslan, 1967c ; Munyon et al., 1967). Whether messengers synthesised by cores have access to the sites of protein synthesis is open to speculation. In any case, the ability of messenger RNA species to direct protein synthesis in arginine deprived cells would depend on the amino acid composition of the proteins for which they code. The early virus proteins detected in this system are presumably not rich in arginine. The actual extent of virus specific RNA formation in the absence of arginine is unresolved but there seems to be no basis for a selective inhibition of host rather than virus specific synthesis. It may

be that the formation of early virus messengers whose synthesis depends on prior protein synthesis is inhibited: failure of late messenger RNA synthesis would result from failure of virus DNA replication.

The requirement for arginine in the early events of the vaccinia replication cycle is further emphasised by the increased rate of arginine incorporation immediately following virus infection but preceding virus DNA replication. This very marked stimulation presumably reflects a requirement for arginine in the synthesis of early protein. It is of great interest that the synthesis of at least three vaccinia virus structural proteins commences early in the infection cycle and continues in the presence of an inhibitor of DNA synthesis (Holowczak and Joklik, 1967). Two of these proteins are constituents of the viral core and by analogy with other systems are those which might be expected to be arginine rich. The presence of such arginine rich, basic proteins associated with the virus core rather than with the capsid has been demonstrated in adenoviruses (Prage et al., 1968 ; Russell and Becker, 1968) and in herpes simplex virus (Olshevsky and Becker, 1970a ; 1970b). The increased rate of arginine incorporation at 3 hr. post infection may represent the synthesis of an arginine rich protein produced concurrently with replicated virus DNA. The synthesis of protein in addition to that associated enzymatically with DNA replication is known to be required for the formation of functional poxvirus DNA. Kates and McAuslan (1967a) have reported a requirement in rabbitpox virus infected HeLa cells for a protein synthesised concurrently and associated stoichiometrically rather than catalytically with DNA replication. This is an early virus function in that such protein can accumulate in the absence of DNA synthesis yet is unusual in that its synthesis is directed by a relatively unstable messenger RNA: the synthesis of poxvirus early enzymes has been shown to be directed by extremely stable messenger RNA species (Jungwirth and Joklik, 1965). Evidence of requirements for protein synthesis

concurrent with adenovirus DNA replication (Polasa and Green, 1965) and with polyoma virus DNA replication (Shimono and Kaplan, 1969) has also been reported.

The incorporation of arginine into vaccinia infected HeLa cultures was reduced soon after infection compared to that into uninfected control cultures. This reduction was greater than that observed when protein synthesis was measured by the incorporation of either leucine or phenylalanine and may indicate that overall virus specific protein synthesis is less dependent on the presence of arginine than is host specific protein synthesis. This is in contrast with the results of Kaplan et al., (1970) who showed that the proteins synthesised in pseudorabies virus infected rabbit kidney cells contained more arginine but less phenylalanine relative to leucine than did the proteins synthesised in uninfected cells. This does not however, preclude the synthesis of specific, arginine rich proteins in the system studied here and the possibility of their selective incorporation into mature virus particles.

The utilisation of arginine in the formation of complete virus particles was demonstrated by preparing purified virus from infected cultures maintained in the presence of guanido(^{14}C)arginine or uniformly labelled (^{14}C)arginine. In both instances radioactivity was associated with mature virus particles of a characteristic density after centrifugation in density gradients. This shows that in addition to the requirement for DNA replication, arginine is also incorporated into virus structural proteins. The presence of arginine in vaccinia virus has been directly demonstrated by chemical analysis of purified virus particles (Turner and Kaplan, 1968). Comparison of the amounts of radioactivity incorporated from the two differently labelled arginine preparations shows that some arginine is metabolised before incorporation of the label. Consideration of the positions of

isotopic caron atoms in the differently labelled arginine molecules suggests that the incorporation of arginine derivatives containing atoms of the carbon chain predominates over that of derivatives containing the carbon atom of the guanido group. Such derivatives are likely to include proline and aspartate and possibly polyamines. The polyamines spermidine and spermine have been shown to be minor constituents of the RNA containing turnip yellow mosaic virus (Beer and Kosuge, 1970) although this may be related to the nature of the nucleic acid.

It is interesting to compare the amount of arginine which would be expected to be found in virus particles with that actually incorporated into vaccinia infected cultures and into preparations of purified virus:

(1) The amount of arginine present in vaccinia virus particles estimated from the known chemical composition of the virus.

The data of Turner and Kaplan (1968) show that the arginine content of vaccinia virus protein is about 5% by mass. As protein constitutes about 91% of the mass of the virus particle (Joklik, 1966) which has been given as 5.5×10^{-15}g. (Smadel et al., 1939), the mass of protein per 10^6 virus particles is about 5.0×10^{-9}g. and the mass of arginine is about 2.5×10^{-10}g.

This is equivalent to about 1.4×10^{-6} µmoles arginine per 10^6 particles.

(2) The amount of arginine incorporated into vaccinia infected cultures.

From results presented, 5×10^6 HeLa cells infected under one step growth conditions incorporated 8.2×10^{-3} µmoles of arginine measured by the incorporation of uniformly labelled (^{14}C)arginine in the 12 hr. following infection. By comparison with growth curves, these cells would be expected to produce about 50 pfu

per cell or, taking a value of 10 for the particle to infectious unit ratio (Joklik, 1962), a total of 2.5×10^9 particles. The data of Salzman and Sebring (1967) suggest that, of the total protein synthesised during this period, about 30% appears in mature virus particles.

This is equivalent to about <u>1×10^{-6} µmoles arginine per 10^6 particles</u>.

(3) The amount of arginine incorporated into preparations of purified virus.

The crude virus suspension contained not more than about 2×10^9 pfu or, again taking a value of 10 for the particle to infectious unit ratio, about 2×10^{10} particles. Neglecting quantitative losses in the purification procedure which are likely to be small, this number of particles contained about 2.9×10^{-2} µmoles of arginine measured by the incorporation of guanido(^{14}C)arginine.

This is equivalent to about <u>1.5×10^{-6} µmoles arginine per 10^6 particles</u>.

No direct measurement of the particle to infectious unit ratio in purified virus preparations was made but this value was expected to increase greatly as a result of aggregation of virus particles during centrifugation through potassium tartrate density gradients. This behavior in the presence of high concentrations of salts was noted by Craigie and Wishart (1934). Very little infective virus was present in gradient fractions other than the fraction containing the visible band of purified virus. In this case a recovery of 6.8×10^6 pfu was obtained, indicating a value of about 2,900 for the particle to infectious unit ratio. This is not inconsistant with the purification procedure.

The values presented in (2) and (3) for the amount of arginine found by radioactivity studies to be incorporated into virus particles are in excellent agreement with the value derived by consideration of the composition of the virus particle and indicate

that a substantial amount of the arginine incorporated into infected cultures eventually appears in the structual proteins of complete virus particles. However, the value obtained by consideration of the incorporation of uniformly labelled (^{14}C)arginine into infected cultures (2) is probably an overestimate as not all the label represents arginine, but even so is lower than that expected from the composition of the virus particle (1). As this estimate was obtained on the basis of a random incorporation of radioactively labelled proteins into virus particles and guanido labelled purified virus particles contained as much arginine as was expected (3), it may be that arginine rich proteins synthesised in infected cells are selectively incorporated.

Changes in protein synthesis in vaccinia infected cultures deprived of arginine should be compared with those reported in cultures infected with other DNA viruses in the absence of arginine. The expression of virus functions under these conditions in cells infected with adenovirus, herpesvirus and papovaviruses has already been discussed. Although quantitative differences were shown, Rouse and Schlesinger (1967) found that the full complement of adenovirus capsid proteins was produced. Similarly, in arginine deprived cultures infected with herpesvirus Courtney et al., (1970) demonstrated the synthesis of some virus specific proteins although these were atypically confined to the cytoplasm and Becker et al., (1967) showed that the rate of protein synthesis in such cells was reduced. Normal amounts of these antigens were detected in cell nuclei after the restoration of arginine suggesting the possibility of a role of arginine in the transport of materials across the nuclear membranes. Such transport mechanisms are obviously important in the replication of the intranuclear viruses where virus specific components synthesised in the cytoplasm are assembled in the nuclei of infected cells. It is possible however, that nuclear transport

mechanisms are also involved in the cytoplasmic replication of vaccinia virus as the presence of a functional nucleolus is thought to be required for the expression of cellular genetic information and messenger and structural RNA species pass from the nucleus to the cytoplasm together (Sidebottom and Harris, 1969).

Again, polyoma virus specific protein synthesis was shown to continue in the absence of arginine (Winters and Consigli, 1969). In all these cases virus specific DNA synthesis was uninhibited yet virus DNA remained uncoated suggesting that arginine is involved in a synthetic step required for the maturation of virus particles. The presence of arginine rich internal proteins in adenovirus and in herpes simplex virus has been established. In vaccinia infected cultures deprived of arginine the expression of late virus functions is presumably inhibited as a result of the inhibition of virus DNA synthesis.

Further evidence of the dual nature of the arginine requirement for the replication of vaccinia virus and a degree of quantification of each stage of the requirement is provided by the effects of the delayed addition of arginine on the production of infective virus. When arginine was added to cultures previously infected and maintained in its absence, a lag of 5 hr. was observed before the production of infective virus. This is identical to the lag observed in cultures infected in complete medium and presumably represents the time required for early events, the synthesis of virus DNA and some synthesis of late proteins to take place. An arginine concentration of 0.015 mM. is sufficient to permit virus DNA synthesis and those early events on which this depends as the addition of 0.06 mM. arginine to such cultures at 6 hr. post infection results in a prompt appearance of infective virus although a slightly reduced final yield is obtained. An arginine concentration of 0.03 mM is sufficient for the formation of a

limited yield of infective virus but the addition of 0.06 mM. arginine to these cultures after DNA synthesis has taken place results in the immediately continued production of a full yield of virus. Thus, the synthesis of protein(s) required in the assembly of virions is quantitatively dependant on the presence of arginine and while 0.015 to 0.03 mM. arginine suffices for the completion of virus specific events preceding the formation of infective particles the further addition of 0.06 mM. arginine is required to complete virus maturation. However, these results do not indicate that such arginine containing structural protein is necessarily the product of late virus expression but merely that it is functional at a late stage in the virus replication cycle. The marked stimulation of the rate of incorporation of arginine into cultures very soon after infection may indicate that the synthesis of such protein is an early virus function which in cells infected in complete medium normally begins before virus DNA replication. Thus, it is possible that this protein synthesis corresponds to the formation of the early, internal structural proteins of Holowczak and Joklik,(1967) which accumulate until virus maturation begins in the presence of complete medium. These early core proteins are normally subject to switch off and only small amounts continue to be synthesised throughout the replication cycle. It is possible however, that under conditions of arginine deprivation the switch off mechanism, which in the case of at least some early proteins requires both DNA synthesis (Salzman and Sebring, 1967) and protein synthesis (McAuslan, 1963), is non functional and that stable messenger RNA species remain competent to direct the synthesis of those proteins until arginine becomes available. A striking analogy is now apparent with the replication of the large, intranuclear DNA viruses in which deprivation of arginine inhibits the synthesis of arginine rich, internal structural proteins required for the maturation of virus particles. In arginine deprived, herpesvirus infected cells the messenger RNA species directing the synthesis

of such proteins are present and competent in the cytoplasm and function on restoration of arginine (Becker et al., 1967). In adenovirus infected cells the protein involved is related to a virus specific antigen normally synthesised early in the replication cycle (Russell et al., 1967b ; Russell and Becker, 1968). If a similar situation obtains in the vaccinia virus replication cycle, the effect of the delayed addition of arginine is to separate chronologically on the basis of their function two distinct arginine requirements which occur at about the same time in normally infected cells. Thus, one requirement is for the synthesis of early proteins on which virus DNA replication depends and another is for the synthesis of structural protein on which virus maturation will eventually depend. Whether such protein is in fact an internal component of the virus or a constituent of the capsid is at this stage unresolved. The presence of an arginine rich internal component as in adenovirus and herpesvirus has not yet been demonstrated in vaccinia virus particles. However, several of the proteins synthesised in response to vaccinia infection of HeLa cells have been shown to be arginine rich and to have isoelectric pH values indicative of a basic nature (J.D. Williamson, personal communication). It is possible that a requirement for arginine in a synthetic step resulting in the formation of internal basic protein(s), perhaps associated specifically with the nucleic acid, is a feature common to the replication of DNA viruses.

In this context, it is interesting to speculate on the redundancy observed in the synthesis of vaccinia virus products. Salzman and Sebring, (1967) have shown that in HeLa cells infected in complete medium, the quantity of early protein not incorporated into mature virus is almost as large as the quantity of protein which does appear in virus particles. Only 15% of the structural protein of virions is synthesised early and the quantity of early protein remaining appears to be far greater than that which is

required to function enzymatically although the vaccinia virus genome is capable of coding for many more enzyme functions than are recognised at present. Similarly, late proteins are produced in excess and contribute to the pool of soluble antigen. Westwood et al., (1965) found that 7 immunologically detectable components of the virus were also present in soluble antigen preparations in addition to a further 10 antigens which may not be structural proteins. Virus specific DNA synthesis appears to be similarly uneconomic. Joklik and Becker, (1964) have shown that only about one third of the DNA is actually coated although some of that remaining presumably directs late virus functions. However, a virus specific product is known which does not appear to be produced in excess. This is the nucleoprotein (NP) antigen which is not found in soluble antigen preparations, comprises 50% of the mass of the mass of the virus and accounts for all the nucleic acid (Smadel et al., 1942). The major component of this multiple antigen is not present on the surface of intact virus particles, is thus internal and may be related to an internal protein associated with the virus DNA (Woodroofe and Fenner, 1962). Thus, it is possible that assembly of virus particles is normally limited by the availability of such a protein and that partial deprivation of arginine increases this effect.

Finally, some indication of future work seems appropriate at this point. The isolation of vaccinia virus specific, arginine rich proteins, the determination of their time of appearance in the replication cycle and their possible identification with known virus antigens is a logical development. The nature and extent of the inhibitions of virus specific RNA synthesis in arginine deprived cells remains an open question but could be resolved by the use of an inhibitor of transcription combined with a technique for the identification of virus specific proteins. It would also

be interesting to examine the extent of early enzyme induction in arginine deprived, infected cells in order to clarify the cause of the inhibition of DNA synthesis which has so far been observed only in this system.

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