METHYLATION OF NUCLEIC ACIDS IN HAMSTER KIDNEY CELLS INFECTED WITH HERPES SIMPLEX VIRUS

by

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ABBREVIATIONS

In addition to the accepted standard abbreviations, the following were used:

DNase 1	=	pancreatic deoxyribonuclease
RNase	=	pancreatic ribonuclease
S. D. S.	=	sodium dodecyl sulphate
MAK	=	methylated albumin Kieselguhr
P. I.	=	post infection
P. F. U.	=	plaque forming units
P. P. O.	=	2, 5-diphenyloxazole
P. O. P. O. P.	=	1, 4 di-2-(5-phenyl oxazolyl)-benzene
5-mC	=	5-methylcytosine
6-mA	=	6-methyladenosine (6-methylaminopurine)
EC2F [x% met]	=	Eagle's medium containing 2% (v/v) calf serum, 20mM-sodium formate and x% normal methionine concentration
HSV	=	Herpes simplex virus
FMDV	=	foot-and-mouth-disease virus
SV40	=	simian virus 40
NmpM	=	dinucleotide of 2'-o-methyl nucleotide linked to a base-methylated nucleotide
NmpN	=	2'-o-methyl nucleotide linked to a nucleotide

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I. INTRODUCTION

1. VIRUSES

1.1. Introduction

Viruses are incapable of independent replication, relying on host-cell systems, such as protein-synthesizing ribosomal complexes, for many of the processes involved in self-duplication. One important property of viruses which differentiates them from other organisms is that they contain only one nucleic acid, in some cases, RNA, and in others, DNA. In every case, this nucleic acid is enclosed by a protein shell.

1.2. Viral Structure

Viruses vary in complexity from infective particles (virions) consisting only of nucleic acid contained in a protein shell (capsid) to structures in which further chemical, morphological and antigenic components can be distinguished. Thus the nucleic acid-protein complex, the viral nucleocapsid, may be naked or enclosed in an outer envelope.

1.3. Viral Nucleic Acid

Whether DNA or RNA, the nucleic acid of all viruses contains genetic information necessary for the formation of complete virus particles in infected host cells. Some particles, for instance,

can be stripped of their protein coats yet still remain infective, although with reduced virulence, e.g. polio-virus, tobacco mosaic virus. The amount of information carried by the nucleic acid is probably related to its size.

Viral RNA may be single-stranded, of molecular weight around 1 - 2 x 10^6 daltons, as in the bacteriophages f2, MS2 and R17, the plant viruses, tobacco mosaic virus and turnip yellow mosaic virus, and the animal viruses, polio, encephalomyocarditis and influenza; or of molecular weight around 10 x 10^6 daltons, as in the leukosis and murine leukemia viruses; or, again, it may be double-stranded of molecular weight about 2 x 10^6 daltons, as in reovirus.

Single-stranded DNA exists in viruses, such as bacteriophages ϕ x 174, S13, and animal viruses, Kilham rat virus and mouse minute virus where it has a molecular weight of around 1.7 - 1.8 x 10^6 daltons. However, double-stranded DNA is much more common, ranging in molecular weight from 3 to 200 x 10^6 daltons. Among the small viruses which contain double-stranded DNA there are several whose genome exists as a covalently-closed circle, often with extensive "supercoiling" (e.g. polyoma virus, the papova virus SV40 and the papilloma viruses, of molecular weight 3 - 5.3 x 10^6 daltons). So far as is known the double-stranded DNA in viruses of

intermediate size (the bacteriophages T3, T7, λ and the human adenoviruses), and of large size (the Escherichia coli T-even phages, Bacillus subtilis phage SP8, the herpesviruses, the poxviruses and several insect viruses) is present as a linear double helix.

1.4. Viral Capsid

The capsid is composed of apparently identical protein subunits packed in a regular fashion around the nucleic acid. This results in either rod-shaped viruses (e.g. tobacco mosaic virus) with helical symmetry, or isometric or nearly spherical viruses with icosahedral symmetry (Horne & Wildy, 1961; Casper & Klug, 1962). Different planes of symmetry exist in the latter type. Groups of protein subunits may cluster together to form electron-microscopically visible units. Some isometric viruses, e.g. reovirus, poxviruses, herpesviruses and turnip crinkle virus, have outer and inner protein coats, but this capsid type has not been detected in the helical viruses. Viral coat protein may have enzymic functions as in myxoviruses, reovirus and vaccinia virus (Hirst, 1943; Shatkin & Sipe, 1968; Pogo & Dales, 1969).

1.5. Viral Envelope

The coat protein may be surrounded by an envelope, which in some cases is an essential part of the infective virus, e.g. in influenza virus. Although the main components of the outer envelope are considered to be of viral origin, normal host components may

also be incorporated into the structure (Wildy & Watson, 1962). The envelope may therefore contain lipid and carbohydrate as well as protein. Some proteins of the envelope are virus-coded and these frequently form "spikes", e.g. in influenza virus, whose "spikes" have neuraminidase or haemaglutinin activity. Viral envelopes may either be in close association with the nucleocapsid as in influenza viruses, or in the form of a loose cover as in the herpesviruses (Figure 1).

2. HERPES SIMPLEX VIRUS (HSV)

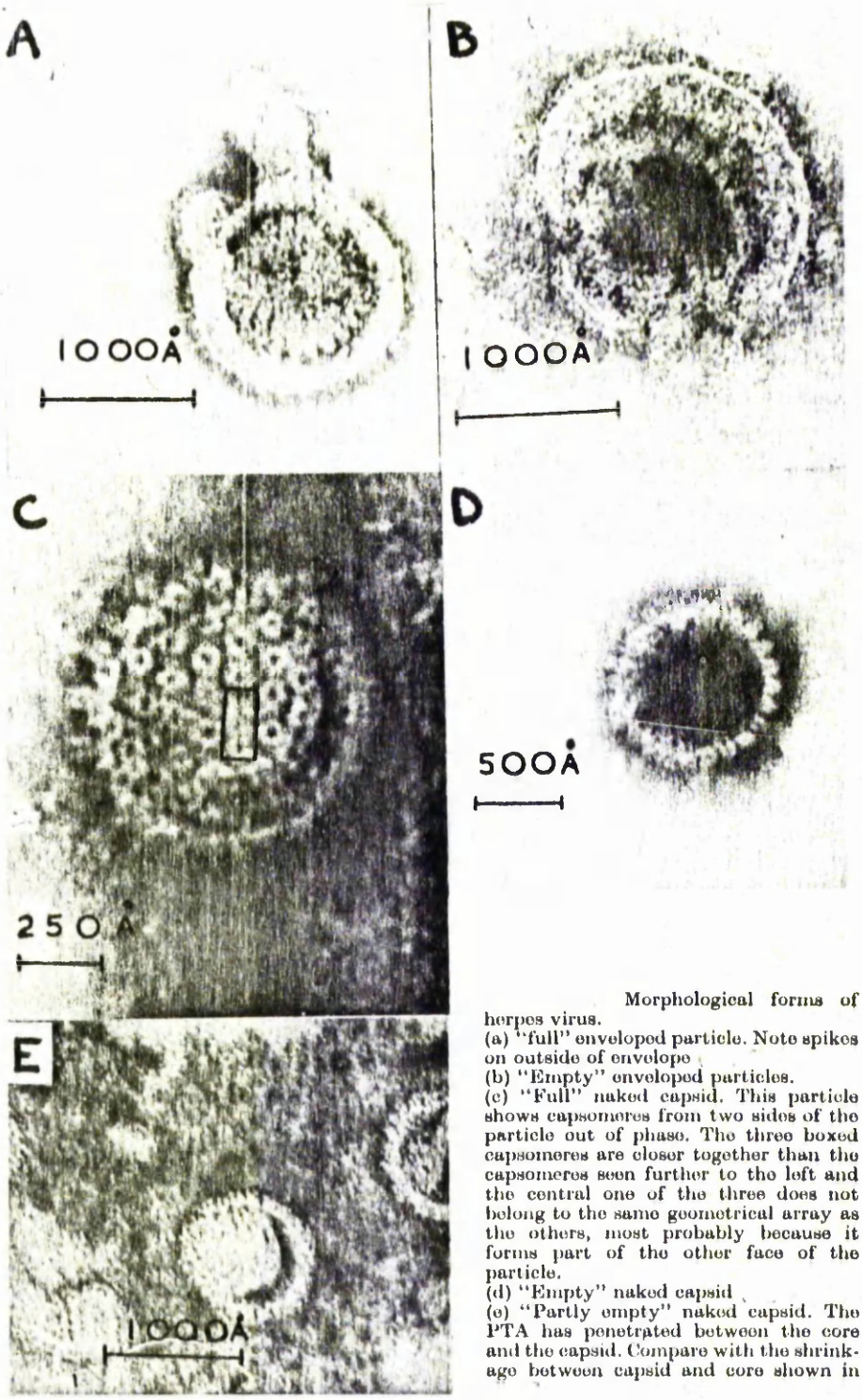
2.1. Herpes Viruses

HSV is one of a group of herpesviruses which include B virus, marmoset virus, pseudorabies virus, and equine, canine, avian, rabbit and feline herpesviruses. Viruses which may belong to the same group are Burkitt's human lymphoma virus (Yamaguchi, Hinuma & Grace, 1967), Marek's disease virus (Epstein, Achong Churchill & Biggs, 1968) and Lucké tumour (frog adenocarcenoma) virus (Stackpole & Mizell, 1968).

2.2. HSV Structure

HSV has been shown, in common with all the viruses of this group, to contain DNA (Epstein, 1962). The nucleic acid forms the inner core of the virion, which is probably in close association

Figure 1



Morphological forms of herpes virus.
(a) "full" enveloped particle. Note spikes on outside of envelope
(b) "Empty" enveloped particles.
(c) "Full" naked capsid. This particle shows capsomeres from two sides of the particle out of phase. The three boxed capsomeres are closer together than the capsomeres seen further to the left and the central one of the three does not belong to the same geometrical array as the others, most probably because it forms part of the other face of the particle.
(d) "Empty" naked capsid
(e) "Partly empty" naked capsid. The PTA has penetrated between the core and the capsid. Compare with the shrinkage between capsid and core shown in

Reprinted from Watson and Wildy, Cold Spring Harbor Symp. Quant. Biol., 27, 40.

with protein. Surrounding this core there is a series of three capsids; the innermost is an electron opaque shell with bead-like projections; the middle is an electron translucent shell; and the outermost is an icosahedral structure composed of 162 capsomere units (Fig. 1). External to the capsomeres are, first, an inner, lipid-containing envelope, and, second, an outer envelope which is probably derived largely from the inner lamellae of the nuclear membrane of the host cell (Morgan, Ellison, Rose & Moore, 1954; Nii, Morgan & Rose, 1968).

2.3. HSV Replication

Replication of HSV takes place in the infected cell as four distinct phases - infection, eclipse, maturation and release.

From electron microscopic examination, it has been shown that the five steps involved in the infection phase of HSV replication are: (a) attachment; (b) digestion of the viral envelope; (c) digestion of the cell wall; (d) passage of the capsid directly into the cytoplasm; and (e) digestion of the capsid with release of the core (Morgan, Rose & Mednis, 1968). Viral DNA is probably transported into the nucleus of the host cell at this time (Roizman, 1969).

The eclipse phase spans the events occurring between the disappearance of the infecting virus particle and the appearance of the

progeny virus. In HEp-2 cells infected with HSV, the length of the eclipse phase is affected by temperature, multiplicity of infection and prior infection of the cells with another mutant (Kaplan & Vatter, 1959; Roizman, 1963).

Also during the eclipse phase there is a gradual, irreversible inhibition of synthesis of host protein, DNA and RNA, the rate of inhibition accelerating as infection progresses. In the early stages of eclipse, this reduced metabolism may be, at least partly, counter-balanced by the synthesis of nonstructural and, later, structural components specified by the virus.

Roizman (1969) speculates that when the viral coat protein synthesized on polyribosomes in the cytoplasm has migrated to the nucleus, a DNA-protein core is first assembled and the final assembly of the capsid is then initiated at some point on this core. This phase, maturation, is followed by the last phase, release of virus.

The nucleocapsid of the progeny virus is now enveloped by the inner lamellae of the nuclear membrane as it exits from the nucleus (Morgan, Ellison, Rose & Moore, 1954; Stoker & Ross, 1958; Morgan, Rose, Holden & Jones, 1959; Siegert & Falke, 1966; Spring & Roizman, 1968). Enveloped virions are either contained in a vacuole and are released from the cell by "reverse phagocytosis"

(Morgan, Rose & Mednis, 1968) or are released along tubules continuous with the outer lamellae of the nuclear membrane and with the cytoplasmic membrane (Schwartz & Roizman, 1969).

This release of virus particles completes the HSV growth cycle and results in cell death.

2.4. Protein Metabolism in HSV Infection

Much of the study of the biochemistry of viral replication has involved events taking place during the eclipse phase of growth since, during this period, the replication, transcription and translation events central to new virus production are initiated and developed.

Work on protein synthesis during eclipse of HSV, other than that involving specific enzyme synthesis, has been carried out using HEp-2 cells infected with HSV (Roizman, Borman & Rousta, 1965; Sydiskis & Roizman, 1966). In this system proteins synthesized in the cytoplasm are slowly and selectively transferred to the nucleus (Spear & Roizman, 1968), although less than half the protein synthesized undergoes this transfer. Some of the proteins formed at an early stage of eclipse are enzymes involved in nucleic acid and protein synthesis required for viral replication.

2.5. Enzymes Involved in HSV Nucleic Acid Synthesis

Infection of BHK21/C13 cells with HSV produces an increased DNA polymerase (DNA nucleotidyl transferase, EC 2.7.7.8) activity at this early eclipse stage. Evidence infers the enzyme to be virus-coded (Keir, 1965; Keir, Hay, Morrison & Subak-Sharpe, 1966; Keir, Subak-Sharpe, Sheddon, Watson & Wildy, 1966; Keir, 1968). HEp-2 cells infected with HSV exhibit similarly elevated levels of DNA-polymerase activity. Interestingly, pseudorabies virus is related to HSV, yet Hamada, Kamiya & Kaplan (1966) do not differentiate in neutralization tests between DNA polymerase extracted from pseudorabies-infected and uninfected rabbit kidney cells, although enzyme activity is substantially increased.

Studies on the DNase (deoxyribonuclease, EC 3.1.4.5) enzymes in HSV-infected cells support the hypothesis that at least one of them is virus-coded (Newton, 1964; McAuslan, Herde, Pett & Ross, 1965; Sauer, Orth & Munk, 1966; Morrison & Keir, 1967; Keir, 1968). Pseudorabies virus also induces an endonuclease, which may be virus-coded.

Other enzymes involved in nucleic acid synthesis which have been shown to have increased activity in HSV-infected cells and are probably relevant to virus growth are deoxycytidine monophosphate deaminase (Keir, 1968), deoxythymidine kinase (Kit & Dubbs, 1963;

Newton, 1964; Borman & Roizman, 1965; Prusoff, Bakhle & Sekely, 1965; Klemperer, Haynes, Sheddon & Watson, 1967), thymidine monophosphate kinase in L-cells (Newton, 1964), although there is no detectable difference in its level in monkey kidney cells (Prusoff et al, 1965), deoxycytidine kinase (Perera & Morrison, 1970) and a requirement for DNA-dependent RNA polymerase (EC 2.7.7.6) activity may be inferred from the inhibitory effect of Actinomycin D on HSV multiplication (Roizman, 1963; Sauer, Orth & Munk, 1966; Sauer & Munk, 1966).

2.6. DNA Metabolism in HSV Infection

During the early stages of the eclipse phase HSV causes a disaggregation of the host cell nucleolus and an aggregation of chromosomes at the nuclear membrane (Sydiskis & Roizman, 1966, 1967). Synthesis of HSV DNA has been shown histochemically (Newton & Stoker, 1958; Munk & Sauer, 1964) and biochemically (Sydiskis & Roizman, 1966) to take place in the nucleus of the host cell, and is associated with an electron translucent matrix. After an initial lag, viral DNA synthesis increases to a maximum prior to maximum release of virus.

HSV DNA is double-stranded (Russell, 1962; Darlington & Randall, 1963) and has a molecular weight in the range 6 - 10 x 10^7

daltons (Russell & Crawford, 1963; Becker, Dyn & Sarov, 1968). The DNA content per virus particle is 1.14×10^{-10} μg (Darlington & Randall, 1963), as compared to the approximate content of the host cell of 8×10^{-6} μg. HSV DNA has a base content such that the G + C content is approximately 70% of the total base content (Ben-Porat & Kaplan, 1962; Russell & Crawford, 1964; Subak-Sharpe, Shepherd & Hay, 1966), thus being quite different from mammalian DNA, which has a G + C content of about 40% (Wyatt, 1951; Swartz, Trautner & Kornberg, 1962). Most animal DNA viruses studied have a G + C content more similar to that of their hosts (Subak-Sharpe et al, 1966; Morrison, Keir, Subak-Sharpe & Crawford, 1967).

2.7. RNA Metabolism in HSV Infection

Infection of host cells with HSV causes a rapid decline in total RNA synthesis (Roizman, Borman & Rousta, 1965; Aurelian & Roizman, 1965; Hay, Köteles, Keir & Subak-Sharpe, 1966; Flanagan, 1967; Wagner & Roizman, 1969). Later in the cycle of infection there is a less rapid decline in synthesis, the exact timing of which is dependent on the cell system used, and on the conditions of infection and incubation. The degree of inhibition observed is dependent on the multiplicity of infection used (Wagner & Roizman, 1969). Both Hay et al and Flanagan used a multiplicity of infection of about 16 P. F. U. /

cell, the former in BHK21/C13 cells and the latter in KB cells. Wagner & Roizman used a multiplicity of infection of between 80 and 200 P.F.U./cell in HEp-2 cells. The last named workers fractionated their cells before extraction of the RNA, mainly after long pulse-labelling times, while the other workers extracted total RNA after labelling times of 2 hours or 30 minutes and 15 minutes, respectively. Wagner and Roizman claimed that only by their method is it possible to indicate any alteration in RNA metabolism.

2.8. Ribosomal RNA Metabolism in HSV Infection

Hay et al reported a rapid inhibition in the synthesis of 45S and 35S RNA, which was borne out by the sucrose density gradient results of Flanagan (1967). From studies of nuclear RNA, Wagner and Roizman (1969) found that synthesis and methylation of 45S continued at a reduced rate for some time after infection, even when it was not being converted to 18S and 28S RNA. They deduced that processing as well as synthesis of 45S RNA is inhibited by HSV. Thus HSV is like the animal RNA viruses in this respect. The significance of these findings will be discussed later.

Wagner and Roizman (1969 (b)) found, in the nucleus of infected cells, a significant amount of viral-specified RNA (i.e. RNA which will anneal to HSV DNA) of 50S or greater sedimentation coefficient.

Neither Flanagan (1967) nor Hay et al examined the high molecular weight RNA fraction, but both reported RNA of a lower sedimentation coefficient which annealed to HSV DNA. Hay et al reported this as "20S" RNA and Flanagan, who analyzed RNA later in the viral growth cycle, also found the majority of virus-specified material to be in the range 12 to 24S. The base composition of this material was similar to that of viral DNA (Flanagan, 1967). Wagner & Roizman (1969 (b)) found that the majority of cytoplasmic RNA was in the range of 14S - 20S and that at least some of the polysomal RNA resulted from cleavage of high molecular weight virus-coded nuclear RNA.

2.9. HSV Transfer RNA

Gross inhibition of RNA synthesis after HSV infection has already been discussed (Section 2, 7). Synthesis of RNA of approximately 4S declines more slowly than does rRNA synthesis, and remains at a significant level late into the infective cycle (Hay, Kötteles, Keir & Subak-Sharpe, 1966; Wagner & Roizman, 1969). Wagner & Roizman (1969) report that some of the apparent loss of 4S RNA is caused, at least partly, by leakage from the cells. Flanagan (1967) could show virtually no annealment of small molecular weight RNA to HSV DNA, whereas Hay Kötteles, Keir & Subak-Sharpe (1966) have shown that at least part of the 4S RNA fraction is virus-specified. In addition, an

arginyl-tRNA with properties different from host arginyl-tRNA appears to be formed after infection.

3. DEOXYRIBONUCLEIC ACID

3.1. General Structure of DNA

In the Watson-Crick double-helical DNA structure (Watson & Crick, 1953) all the constituent bases are linked to deoxyribose phosphate. All parts of a DNA molecule are potentially susceptible to change by substitution, but specific substitution in the deoxyribose phosphate backbone of naturally-occurring DNA has not yet been detected. This portion of the nucleic acid cannot be considered at present to donate to the structure any bonding properties useful for distinguishing one part of the polydeoxynucleotide from another, unless interactions between bases produce changes which are transmitted to the backbone structure. Thus only information in the bases themselves seems capable of producing distinguishing structural features. The pyrimidine bases (Cyt and Thy) share a common ring structure as do the purines (Ade and Gua). Thus, intermolecular forces which differentiate among the purines and the pyrimidines must involve extraannular atoms.

3.2. Bonding of Bases

The regular helical configuration of the polymer is stabilized by internal and external secondary bonds (Watson & Crick, 1953).

The bases have side groups which are at least potentially hydrophobic, but these are mainly involved in internucleotide bonds within the DNA structure. Interactions involving London and Van der Waals forces, and perhaps some hydrogen bonding, exist among the purines and pyrimidines, and virtually all the surface atoms in the sugar and phosphate groups form bonds to water molecules (Michelson, Massoulié & Guschlbauer, 1967). Calculation of total electronic interaction energy, obtained by adding the in-planar energy between bonded bases (Ade & Thy and Cyt & Gua) in opposing strands of DNA, and the vertical energy involved in "stacking" between near-neighbour base-pairs, shows that the different combinations of base-pairs, may be divided into three groups (Pullman & Pullman, 1969). First, the most stable combinations are those formed by (Gua, Cyt) pairs only; second and less stable are the mixed combinations of (Ade, Thy) and (Cyt, Gua); and third, the least stable, are those containing only (Ade, Thy) pairs (Pullman & Pullman, 1969). This finding is supported by the greater thermal stability of nucleic acids rich in Gua and Cyt relative to those rich in Ade and Thy (Marmur & Doty, 1964). Clearly, base content is an important factor in producing structural change.

3.3. Effect of Base Substituents

Synthetic polynucleotide complexes have been studied,

and several major factors governing their structural stability can be defined. The direct interplanar interactions between bases (London, Van der Waals forces (Michelson, 1962)) play an important role, and can be modified by substitution of the bases. "Hydrophobic" forces, defined as organization of solvent molecules in the vicinity of the helix (Kauzmann, 1959) exert a considerable control, and can also be affected by base substitution. Interaction of bases, especially the inplanar interactions between Ade & Thy and Cyt & Gua are specific, and contribute to the structural stability of the polymer. One substituent group which may affect the interaction specificity of the bases is the methyl group. This group is hydrophobic and bulky, and so may significantly alter interactions within the structure of a polynucleotide complex. A wide variety of polynucleotides containing analogues of the naturally-occurring purines and pyrimidines has been examined, and it has been confirmed that certain methyl groups, e.g. at C6 of a purine, give lower structural stability to the homo- and hetero-polymer (Michelson & Pochon, 1966), whereas methylation at C5 in pyrimidine nucleotides increases stability in both the homo-polynucleotide and in the heteropolynucleotide complex (Massoulié, Michelson & Pochon, 1966; Szer & Shugar, 1966). Methylation at the 7 position of Gua residues in model compounds and in DNA results in lability of the C-8 proton, giving rise to behaviour of the substituted

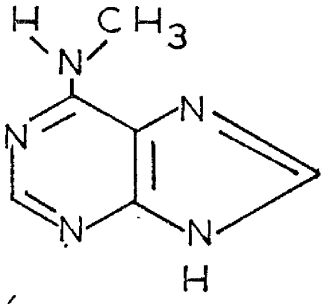
molecule similar to that of molecules related to dThy (Tomasz, 1970). Thus the introduction of a methyl group into a polynucleotide complex in vitro has a profound effect on its secondary structure.

3.4. Methylation of DNA

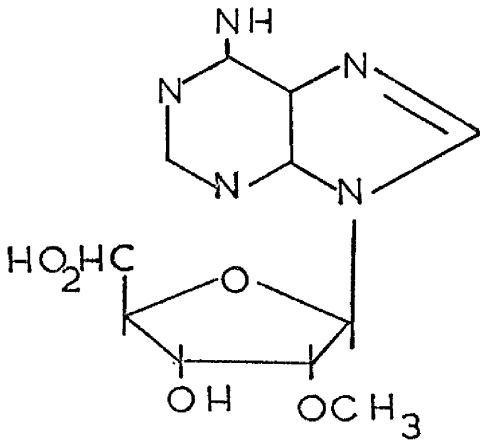
Methylation of certain DNA bases has been found to occur in vivo. Biochemically, there are two classes of methylated deoxyribonucleotide, the first of which is derived directly from TTP during DNA synthesis, and is present in almost all DNAs. The quantitatively less important class of methylated deoxyribonucleotide which arises when 5-methylcytosine (5-mC) and 6-methyladenine (6-mA) (see Figure 2) are formed in DNA at the polynucleotide level is perhaps more involved in distinguishing one part of the polynucleotide from another. From the time that Hotchkiss detected the presence of a trace amount of 5-mC in calf thymus DNA in 1948 (Hotchkiss, 1948) there have been reports that DNA from many organisms contains methylated bases. The presence of 6-mA appears to be restricted to the DNA from lower organisms, such as the blue-green alga, Plectonema boryanum (Kaye, Salomon & Fridlender, 1967), and bacteria, where it may be the sole methylated nucleotide, as in Esch. coli 15 T^- where it is present to the extent of 1 mole % of the DNA nucleotides (Dunn & Smith, 1958; Remy, 1961; Theil & Zamenhof, 1963; Doskočil & Šormová, 1965). The occurrence of 5-mC is more

Figure 2.

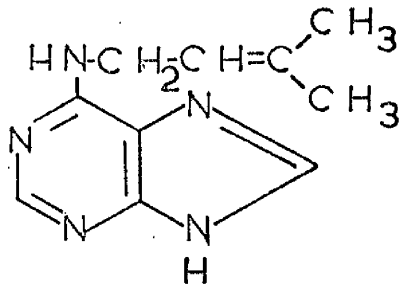
Methylated Bases and Nucleosides.



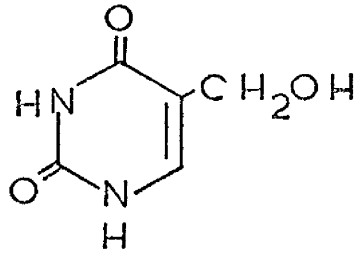
N^6-methyladenine



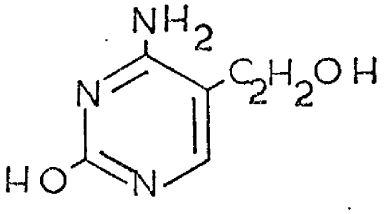
2'-0-methyladenosine



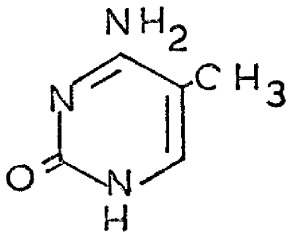
N^6-Δ^2-isopentenyladenine



5-hydroxymethyluracil



5-hydroxymethylcytosine



5-methylcytosine

widespread, being present to different extents, e.g. to 2.3 mole % in the algal flagellate, Euglena gracilis (Brawerman, Hufnagel & Chargaff, 1962; Ray & Hanawalt, 1964), to 5.6 mole % in bracken fern (Thomas & Sherratt, 1956), in strains of Esch. coli (Gold, Hurwitz & Anders, 1963b) and Bac. subtilis var. aterrimus (Doskočil & Šormová, 1965), to 5.5, 5.8 and 4.3 mole % in the angiospermae rye grass, wheat germ and tobacco leaves (Wyatt, 1951; Brawerman & Chargaff, 1951; Tewari & Wildman, 1966), to 1.7 mole % in the Annelida nephthys ciliata (Antonov, Favorova & Belozerskii, 1962), to an almost negligible extent in insects (Wyatt, 1951; Wyatt & Linzen, 1965), to 1.8 mole % in echinoderms (Wyatt, 1951; Grippo, Iaccarino, Parisi & Scarano, 1968), to almost 2 mole % in herring sperm and the frog (Wyatt, 1951; Dawid, 1965), and in many mammalian tissue DNAs (Wyatt, 1951; Laland, Overend & Webb, 1952; Hurst, Marko & Butler, 1953; Antonov, Favorova & Belozerskii, 1962; Spencer & Chargaff, 1963; Burdon, 1966; Salomon, Kaye & Herzberg, 1969). The extent of methylation of mammalian DNA is reasonably uniform, the variation between 1.0 & 1.9 mole % perhaps being partly due to its measurement by different techniques. Not only is the methylation of Cyt therefore widespread, but the extent to which it is present varies, even within different organelles in one cell. For example, in

euglena gracilis the main band DNA is methylated but satellite DNA is not (Ray & Hanawalt, 1964); in tobacco leaves nuclear DNA is methylated, but not chloroplast DNA (Tewari & Wildman, 1966) and the level of methylation of mouse nuclear satellite DNA is twice that of the main band nuclear DNA (Salomon, Kaye & Herzberg, 1969).

3.5. Distribution of Methylated Bases

That the occurrence of methylated bases is not related to the base composition of the DNA only suggests that there is a non-random distribution of methyl groups, and also that neighbouring bases may determine the sites of methylation. The frequency of occurrence of particular nucleotides next to methylated nucleotides in DNA has been examined by several groups. Sinsheimer (1954, 1955) degraded calf thymus DNA enzymically and measured the relative 5-mdC content of the resulting dinucleotides. More than 30% of the 5-mdC was found to occur in the dinucleotide 5-mdCpdG while the majority of dC occurs in dGpdC. Deoxycytidine and 5-mdC are not found in similar environments within calf thymus DNA, but in wheat germ and rye grass DNA 5-mdC and dT do seem to be positioned similarly (Shapiro & Chargaff, 1960; Spencer & Chargaff, 1963). Doskočil & Šorm (1962) degraded DNA with diphenylamine and DNAse I, and examination of these dinucleotide products confirmed that the highest degree of replacement of dC by 5-mdC in mammalian DNA was observed in the sequence dCpdG. This was also observed in wheat germ, but not in B. subtilis DNA. A small

quantity of the 5-mC in mammalian DNA was found in the sequence dCpdA, but none in dCpdC or dCpdT. Grippo, Iaccarino, Parisi & Scarano (1968) using an approach similar to that of Doskočil & Šorm hydrolyzed DNA from developing sea urchin embryos. Analysis of dideoxynucleotides showed that 90% of the 5-mdC, the only methylated nucleotide detected in sea urchin embryo DNA, again occurs in the dideoxynucleotide (dC, dG). A very little occurs as (dC, dC), and trace amounts as (dC, dT) or (dC, dA). Thus the dideoxynucleotide (5-mdC, dG) seems to be the major naturally occuring methylated doublet in DNA from higher organism.

Non-exhaustive enzymic degradation of DNA yields a series of oligodeoxynucleotides which can be separated according to size and base composition. The environment and frequency of occurrence of a methylated base can then be estimated. On this basis it has been shown that in Esch. coli C the most frequent dideoxynucleotide is dCp 5-mdC, while there is little specificity for the nucleotide linked to the 3'-phosphorus of 5-mdC (Doskočil, 1966). In contrast, dAp 5-mdC, dGp 5-mdC and 5-mdC pdT occur most frequently in B. subtilis aterrimus (Doskočil, 1966). As discussed above, in calf thymus DNA there is a high specificity for dG linked to the 3'-phosphorus of 5-mdC, but dA, dG, dC, dT are all found linked to the 5'-phosphorus (Sinsheimer, 1955). Thus the position of a methylated nucleotide may be determined by the identity of both adjacent nucleotides (5' & 3') as in B. subtilis, or by either one as in Esch. coli or higher organisms. It is perhaps

significant that in no case so far investigated is the identity of both nearest neighbour nucleotides totally unrestricted.

3.6. Methylation of Viral DNA

The number of different base substitutions, and the degree to which they occur in bacteriophage DNA has led to hypotheses regarding the role of these modified deoxynucleotides in the replication of viruses (Arber & Linn, 1969). The following modifications have been found to occur in bacteriophage: replacement of thymine by uracil in PBS phage for B. subtilis (Takahashi & Marmur, 1963): replacement of cytosine by 5-hydroxymethylcytosine in the coliphage T2, T4, T6 and Klebsiella 2-phage (Wyatt & Cohen, 1953; Lehman & Pratt, 1960; Gold, Hausmann, Maitra & Hurwitz, 1964; Anisymova, Gabrilovich, Soshina & Cherenkevich, 1969), and by 5-hydroxymethyl-uracil in B. subtilis phage SP8 (Kallen, Simon & Marmur, 1962). The methylated bases, 5-mC and 6-mA also occur in bacteriophage, e.g. 5-mC in Esch. coli λ phage and in a phage for Xanthomonas oryzae (Ledinko, 1964; Kuo, Huang & Teng, 1968) and 6-mA in the coliphages T2, T4, T7 & P1b (Hausmann & Gold, 1966; Hudnik-Plevnik & Melechen, 1967). In contrast, the coliphages T3 & T5 have a complete lack of methylated bases other than Thy (Gefter, Hausmann, Gold & Hurwitz, 1966).

In addition, the T-even coliphages have sugars bound to some of their 5-hydroxymethylcytosine, in the mono-or di-sugar form,

and with varying frequency (Lehman & Pratt, 1960; Lunt, Siebke & Burton, 1964).

The only DNA-containing animal virus whose methylation pattern has been established is polyoma virus. Kaye & Winocour (1967) found that virus grown in the presence of radioactively-labelled methionine yielded viral DNA which contained no radioactivity. They concluded from this that the viral DNA contained no methyl groups other than that of Thy.

3.7. Bacterial DNA Methylase

As discussed above, methylation of DNA takes place at the polynucleotide level (Fleissner & Borek, 1962), apparently at specific sites. The DNA methylase reactions are catalyzed by specific enzymes (Gold, Hurwitz & Anders, 1963), the DNA methylases (methyl-transferases) as predicted by Kornberg, Zimmerman, Kornberg & Josse (1959). The source of methyl groups for the transfer reaction is the methyl group of methionine, (Remy & Smith, 1957), in the form of an intermediate with a high free energy of hydrolysis, S-adenosyl-L-methionine (S-AM). The mechanism of the reaction is depicted in Figure 3. S-AM was first used as methyl donor in vitro by Gold, Hurwitz & Anders (1963).

DNA methylase from Esch. coli W may be purified four hundred-

Figure 3

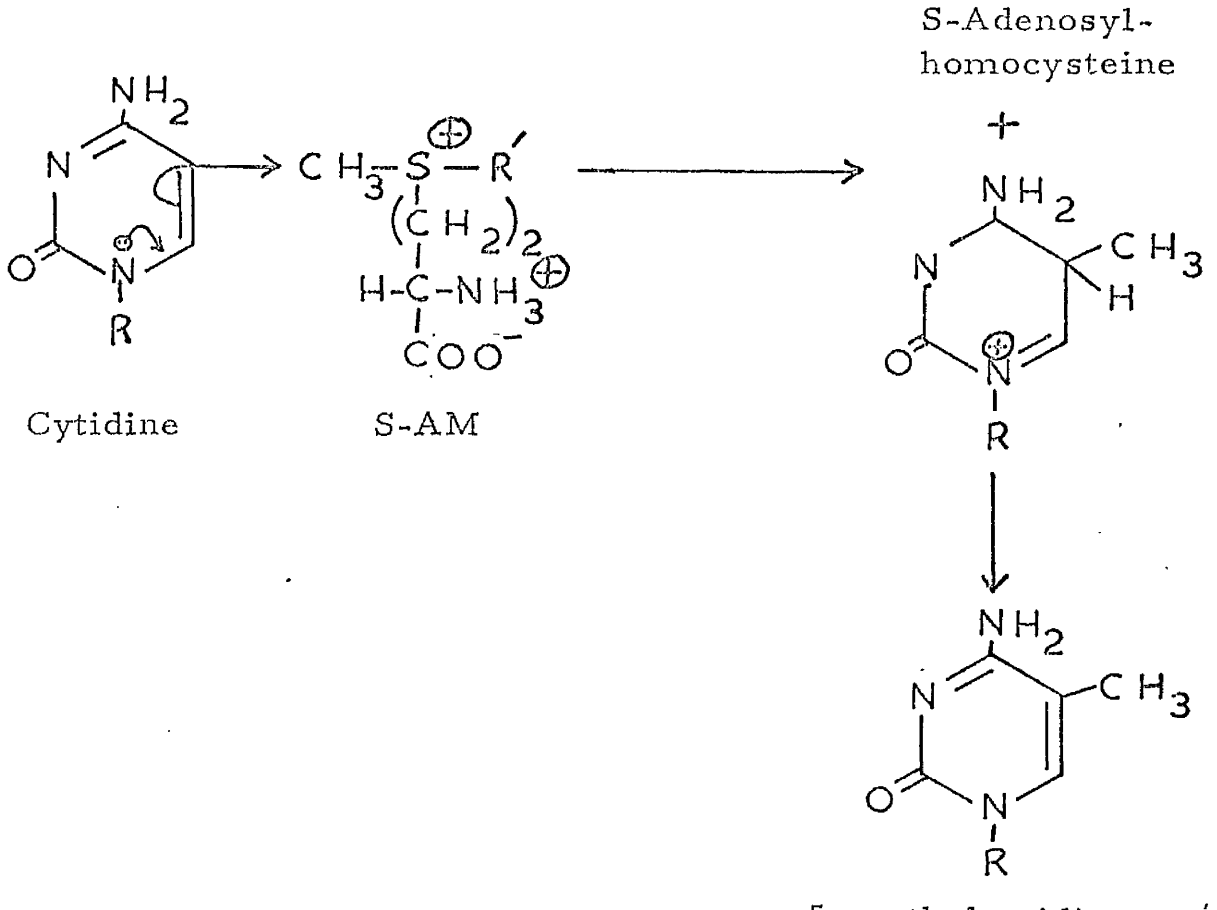
Mechanism of Nucleic Acid Methylase Reaction

S-Adenosylmethionine + nucleotide (in DNA or RNA)

DNA or RNA

Methylase

S-Adenosylhomocysteine + methylated nucleotide (in DNA or RNA)



fold without the ratio of the extents of *in vitro* methylation of A & C being altered (Gold & Hurwitz, 1964). Using one DNA methylase extract, but varying the source of the substrate DNA (heterologous), the comparison of incorporation into 6-mA and 5-mC can be used to compare primary structures of the substrate DNAs (Fujimoto, Srinivasan & Borek, 1965; Kaye, Fridlender, Salomon & Bar-Meir, 1967). On the other hand, if one DNA substrate is used with heterologous DNA methylases from varying sources, varying ratios of 6-mA to 5-mC are obtained which reflect the natural methylated base composition of DNA homologous with the enzyme (Kay, Fridlender, Salomon & Bar-Meir, 1967).

In this latter system, methylase extracts from *Esch. coli* B methylate only dA, those from *Esch. coli* K12 methylate dA and dC, and those from *B. subtilis* methylate only dC (Borek & Srinivasan, 1966). These results suggest that separate methylase enzymes for the formation of 5-mC and 6-mA exist *in vivo*.

3.8. Bacteriophage DNA Methylases

Certain bacteriophages have been shown to induce DNA methylase activities which are distinct from those of their host cells and which therefore probably play a major role in the infective process. Infection of *Esch. coli* B and *Esch. coli* K12 with bacteriophage T2,

which contains 6-mA as its sole methylated nucleotide, induces a greatly increased capacity to methylate dA specifically *in vitro*, while the capacity to methylate dC is unaltered (Gold, Hausmann, Maitra & Hurwitz, 1964; Fujimoto, Srinivasan & Borek, 1965). *De novo* protein synthesis is required for the induction of this DNA methylase activity, and the kinetics of its appearance support the hypothesis that it is a new, phage-directed enzyme (Hausmann & Gold, 1966; Sellin, Srinivasan & Borek, 1966). A smaller elevation of DNA methylase activity occurs after T1 and T4 infection of *Esch. coli*, but there is no such change after T6 or T7 infection. Phage T3 infection results in marked inhibition of the DNA methylase activity of *Esch. coli* extracts (Gold, Hausmann, Maitra & Hurwitz, 1964).

A bacteriophage 15-coded DNA methylase activity has been found after treatment of *Esch coli* 15T⁻ cultures with the bacteriostatic agents 5-aminouracil and 2-thiothymine, or with lysogen-inducing agents, u. v.-light and mitomycin C (Yudelevich & Gold, 1969). This new enzyme activity is specific for dA, in contrast to the host-produced enzyme.

3.9. Mammalian DNA Methylases

Methylation of DNA *in vitro* by extracts of mammalian cells has proved difficult to define biochemically, although it has been reported to occur in Krebs II ascites tumour cells, rat spleen and

rat liver with/without Reuber minimal hepatomas (Burdon, Martin, & Lal, 1967; Sheid, Srinivasan & Borek, 1968; Sheid & Bilik, 1969; Kalousek & Morris, 1969). Mammalian DNA methylase activity is normally contained mainly in the chromatin fraction of the cell (Burdon, Martin & Lal, 1967; Sheid, Srinivasan & Borek, 1967; Kalousek & Morris, 1969), and it seems surprising that DNA methylase activity reported in hamsters bearing tumours induced by adenovirus-12 was detected in the cell supernatant fraction (McFarlane & Shaw, 1968).

No reports have appeared of the induction of DNA methylase activity associated with animal virus infection.

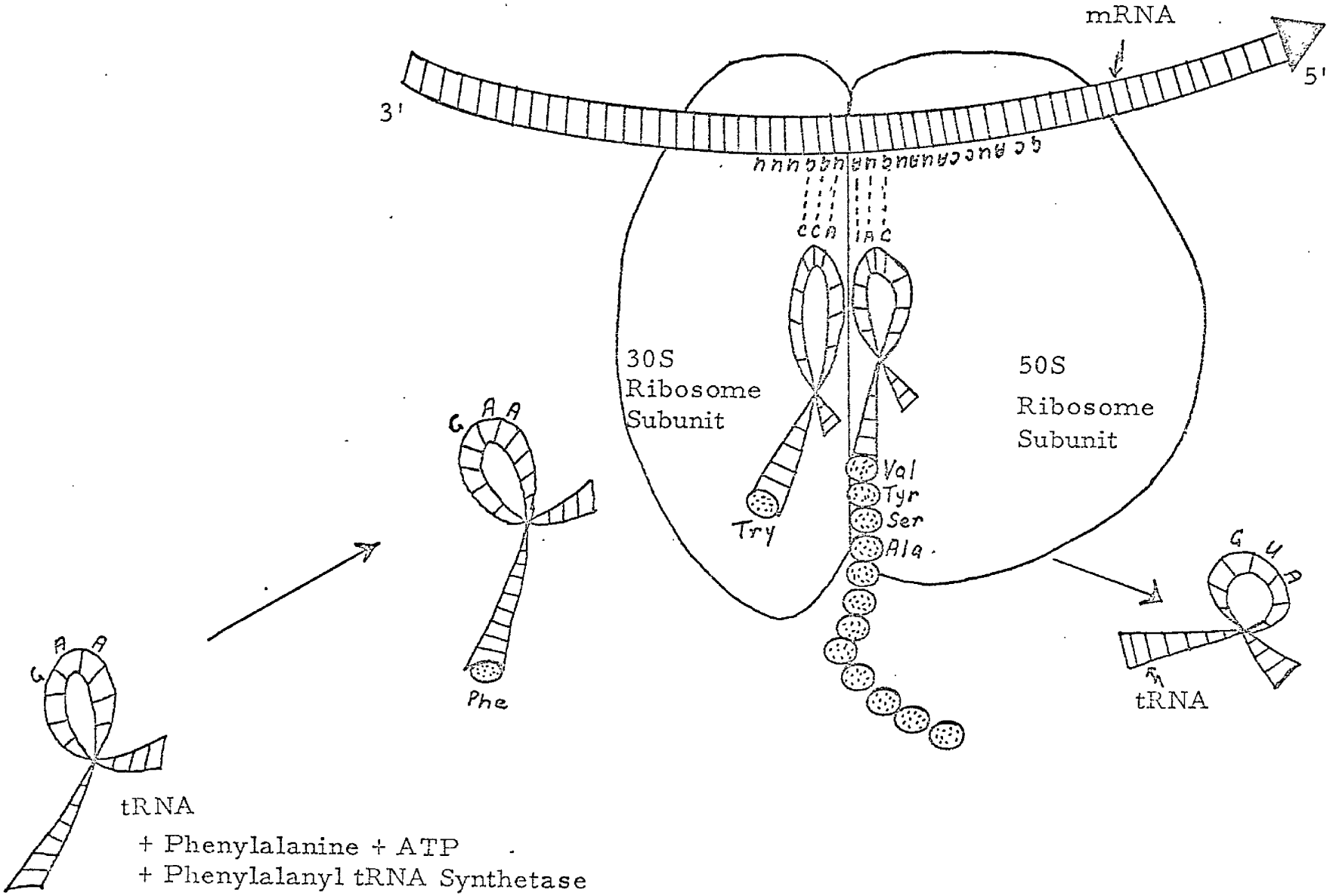
4. CELLULAR RIBONUCLEIC ACID

RNA molecules, including the main species, tRNA, rRNA and mRNA, are transcribed in the cell from DNA by DNA-dependent RNA polymerase (EC2.7.7.6). In mammalian cells RNA synthesis is mainly nuclear while the synthesis of rRNA and tRNA takes place in the nucleolus. This RNA is then transported to the cytoplasm, where rRNA is in conjugation with protein to form ribosomes, which may be linked together by mRNA molecules to give polysomes, the sites of protein synthesis.

The RNA species and their interactions and involvement in

Figure 4

Depiction of Protein Synthesis

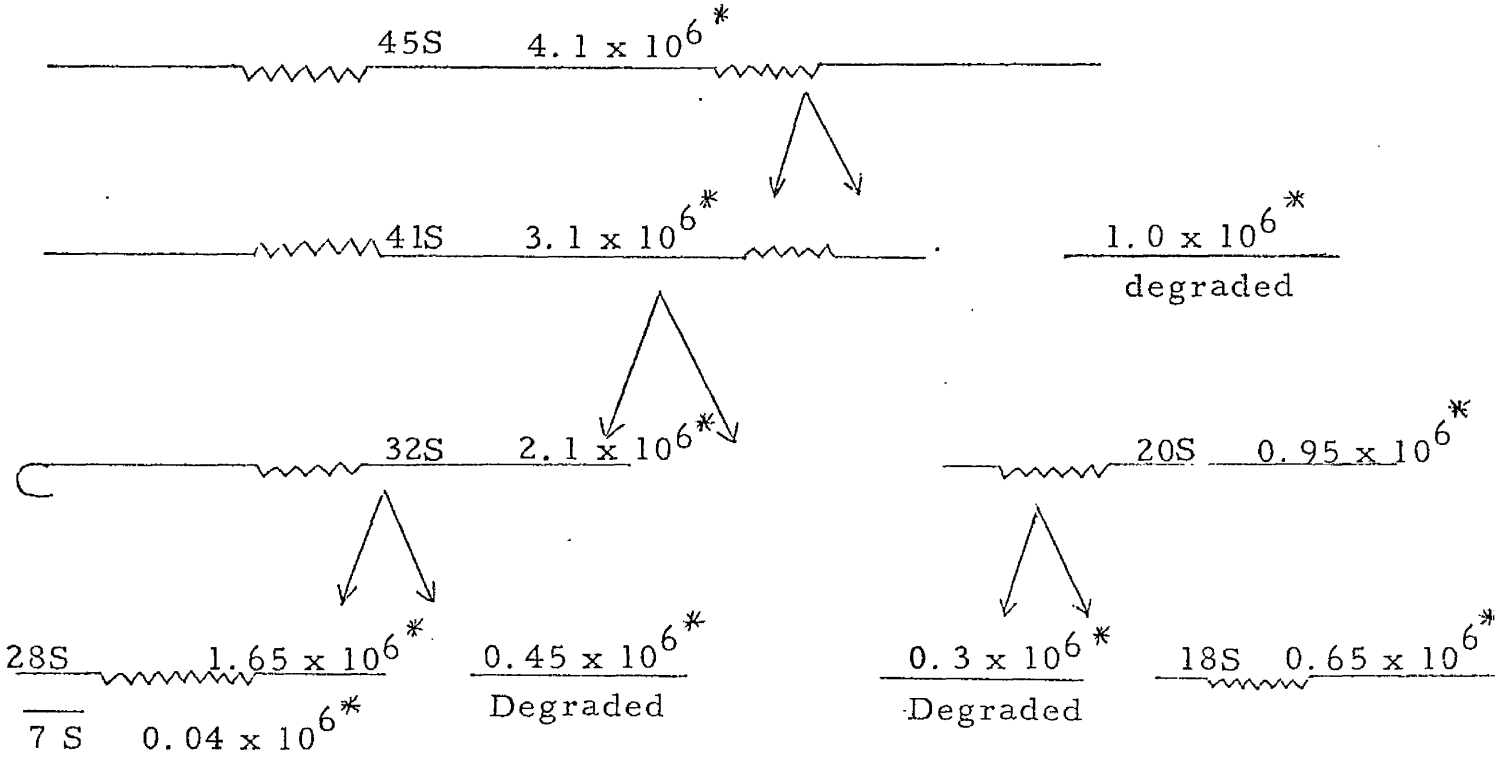


protein synthesis are dealt with in Sections 5 to 8. An outline of the steps of protein synthesis is illustrated in Figure 4.

5. RIBOSOMAL RIBONUCLEIC ACID

5.1. Synthesis and Processing

Synthesis of rRNA takes place in the nucleolus of mammalian cells in the form of precursor molecules with a sedimentation coefficient of 45S (Hiatt, 1962; Rake & Graham, 1964; Harbers & Müller, 1962; Perry, 1964). Much of the work concerned with the processing of this molecule into the rRNA species found in the ribosome (i.e. 18S in the 30S ribosomal subunit and 28S in the 50S subunit) has been carried out by Penman and coworkers, using HeLa cells. The sequence of events which they suggest occurs in the nucleolus is as follows:



* denotes Molecular Weight in daltons

A 36S species which can also be extracted from nuclei could arise from 45S splitting to give 28S + 36S, or from 41S breaking up to give 18S + 36S (Penman, Smith & Holtzman, 1966; Wagner, Penman & Ingram, 1967; Weinberg, Loening, Willems & Penman, 1967). The RNA not found in the 18S and 28S RNA species may form some of the species of low molecular weight RNA also found in the nucleus; but several other species of nuclear RNA have been shown to arise in a way quite distinct from rRNA (Knight & Darnell, 1967; Dingman & Peacock, 1968; Rein & Penman, 1969), and these exhibit size heterogeneity and some species variation (Weinberg & Penman, 1969). The relationship between rRNA and its precursors has been confirmed using high-resolution RNA-DNA hybridization (Jeanteur & Attardi, 1969).

Although some of the characterization of these events was carried out using inhibitors of RNA synthesis following the location of radioactively-labelled RNA precursors (Perry, 1962 ; Sherrer, Latham & Darnell, 1963), a comparison of methylation patterns of the RNA species (Penman, Smith & Holtzman, 1966; Wagner, Penman & Ingram, 1967; Weinberg, Loening, Willems & Penman, 1967; Zimmerman, 1968), or a combination of the two techniques has also been used (Burdon, 1967).

5.2. Ribosomal RNA Methylation

It has been established that the heavy (28S) and light (18S) rRNAs have different base sequences (Aronson, 1963; Delihas, 1964; Munro, 1964) but it is not yet clear whether each type of rRNA is made up of one or more sequences of nucleotides. Lane and Tamaoki (1969), for example, suggest that the slower-sedimenting rRNA is heterogeneous in L-cells. Whether or not the rRNA is heterogeneous within each species, it has been clearly shown experimentally that the different species have substantial variation in methylation patterns.

Esch. coli rRNA was shown to contain methylated bases (Srinivasan, Nofal & Sussman, 1964; Starr & Fefferman, 1964), the lighter species containing approximately 20% more methylation than the heavier species. Brown and Attardi (1965) showed that HeLa cell rRNA was also methylated. Ribosomal RNA from many sources has, since then, been shown to be methylated at the polynucleotide level. The methylated bases and nucleosides of rRNA are included in Table I.

In rRNA, nucleoside methylation occurs both in the base and in the sugar group (Figure 2), the amounts varying with the origin and species of RNA. For example, in bacterial rRNA, methylation of the bases is about four times that of the sugar residues (Starr &

Table I

Unusual Bases and Nucleosides in RNA

Dihydrouracil

5-hydroxyuracil

2-thiouracil

4-thiouracil

1, 5-diribosyluracil

5-ribosyluracil

2-thiocytosine

N^6-acetylcytosine

N^6-aminoacyladenine

N^2-ribosylguanine

N^3-ribosylguanine

Orotic Acid

Hypoxanthine

Xanthine

Pseudouridine

2'(-3')-O-ribosyl-adenine

2'-O-methyladenosine

2'-O-methylcytidine

2'-O-methylguanosine

2'-O-methyluridine

2'-O-methylpseudouridine

7-methylxanthine

1-methylhypoxanthine

1-methyluracil

3-methyluracil

5-methyluracil (thymine)

5-hydroxymethyluracil

5-methylaminomethyl-2-thiouracil

3-methylcytosine

N^4-methylcytosine

5-methylcytosine

5-hydroxymethylcytosine

6-amino-N^5-methylformamido-isocytosine

N^1-methylcytosine

1-methyladenine

2-methyladenine

7-methyladenine

6-methyladenine

6-dimethyladenine

N^6, N^6-dimethyladenine

1-methyl-N^6-methyladenine

1-Δ^2-isopentenyladenine

N^6-Δ^2-isopentenyladenine

2-methylthio-N^6-isopentenyladenine

1-methylguanine

7-methylguanine

N^2-methylguanine

N^2, N^2-dimethylguanine

Fefferman, 1964; Hayashi, Osawa & Miura, 1966; Dubin & Günulp, 1967), whereas in plants many more sugar molecules than bases are methylated (Lane, 1965; Isaksson & Phillips, 1968); this is also true for mammalian rRNA (Wagner, Penman & Ingram, 1967; Lane & Tamaoki, 1969).

Sugar methylation (see Figure 2), in the 2'-OH position in all four methylated nucleosides, occurs naturally in rRNA, as do all the sixteen possible NmpM dinucleotides. The latter are readily obtained, as methylation of the sugar moiety in the 2'-OH group renders the 3'- phosphate bond resistant to alkali and to most nuclease digestion. Comparison of the frequencies of occurrence of the various 2'-OH methylated nucleotides with the base composition of rRNA indicates that this methylation is non-random.

The rRNAs differ quantitatively and qualitatively in their methylated nucleoside content. For example, in HeLa cells, 1-1.2% and 1.5-1.7% of the nucleosides are methylated in 28S and 18S rRNA respectively (Brown & Attardi, 1965; Vaughan, Soeiro, Warner, & Darnell, 1967). In L-cells the methylated bases N^6-methyladenine and N^6-dimethyladenine are present in the slower sedimenting rRNA, while both N^6-methyladenine and 5-methyladenine occur in the heavier material (Lane & Tamaoki, 1969). In general, the occurrence

of N^6-dimethyladenine distinguishes 18S from 28S RNA in mammalian cells.

Methylation of 45S ribosome-precursor RNA occurs at the site of its synthesis in the nucleolus (Greenberg & Penman, 1966; Zimmerman & Holler, 1967; Muramatsu & Fujisawa, 1968). About half of the 45S RNA is lost during maturation (see Section 5.1.); but very little methylated nucleotide is lost (Weinberg, Loening, Willems & Penman, 1967), the discarded RNA being of higher G+C content than 18S and 28S rRNA. An equimolar mixture of 18S and 28S contains the methylation pattern of 45S rRNA while patterns of methylation are the same in 32S as in 28S and in 20S as in 18S. Some secondary methylation, to N^6-dimethyladenine, occurs while 18S is still in the nucleolus (Zimmerman, 1968). Kinetic studies show that a similar rRNA product/precursor relationship exists in yeast (Retèl, Van Den Bos & Planta, 1969).

HeLa cells deprived of methionine synthesize undermethylated RNA, and do not form complete ribosomes (Vaughan, Soeiro, Warner & Darnell, 1967). This undermethylated RNA can be methylated when methionine is returned to the system, thus confirming that methylation occurs at the polynucleotide level.

5.3. Ribosomal RNA Methylases

Ribosomal RNA methylases recognize specific sites on the RNA molecule independent of its length or state of processing. These enzymes, which, like DNA methylase (Figure 3), use S-adenosylmethionine as the source of methyl groups, were first shown in Esch. coli (Gordon & Borman, 1964; Srinivasan, Nofal & Sussman, 1964; Hurwitz, Anders, Gold & Smith, 1965), and have since been described for other systems. Both sugar and base moieties can be methylated in vitro (Nichols & Lane, 1968).

5.4. Effect of Virus Infection on Ribosomal RNA

RNA viruses differ in the degree of inhibition they invoke on host rRNA synthesis. For example, the coliphages F2 and Qβ give rise to considerably less inhibition than do R17 and R23, which, in common with the animal RNA viruses polio-, mengo- and FMDV have a very marked inhibitory effect (Franklin & Baltimore, 1962; Holland & Peterson, 1964; Darnell, Girard, Baltimore, Summers & Maizel, 1967; Hudson & Paranchych, 1967; Grado, Friedlender, Ihl & Contreras, 1968; Ascione & Vande Woude, 1969). In at least some cases, RNA virus infection causes a reduction in rRNA methylation concomitant with the reduction in synthesis (Grado, Friedlender, Ihl & Contreras, 1968; Ascione & Vande Woude, 1969),

and this has been suggested to result from nuclear and nucleolar disintegration (Bernhard & Granboulan, 1968). DNA-containing viruses may also cause a reduction in RNA synthesis, but new species of RNA may be synthesized which are virus-coded, as in Esch. coli infected with phage T4 (Weiss, Hsu, Foft & Scherberg, 1968), vaccinia virus (Becker & Joklik, 1964; Salzman, Shatkin & Sebring, 1964; Kates & McAuslan, 1967) and the adenoviruses (Fujinaga & Green, 1968; Fujinaga, Piña & Green, 1969; Warren, 1969).

6. SMALL RIBONUCLEIC ACID SPECIES

6.1. General

Viral infection may stimulate the accumulation of small molecular weight RNA in infected cells. Some of this may result from breakdown of larger RNA molecules but it seems clear that some may also represent a genuine synthesis of intact small RNA molecules (Rose, Reich & Weissman, 1965; Reich, Forget, Weissman & Rose, 1966; Baguley, Bergquist & Ralph, 1967). In uninfected cells themselves there are both stable and labile small molecular weight RNA species, at least 9 of which occur in the nucleus (Weinberg & Penman, 1969). The origins and functions of the majority of small molecular weight RNA molecules are not known.

6.2. "5S" RNA

One small molecular weight RNA which has been characterized is the ribosomal "5S" RNA. "5S" RNA is 120 nucleotides long, and contains no unusual bases (Brownlee, Sanger & Barrell, 1967; Forget & Weissman, 1967). There is one "5S" molecule per ribosome, associated with the 50S subunit (Siddiqui & Hosokawi, 1968; Morell & Marmur, 1968), but whether 5S RNA is synthesized as a separate entity or as part of the rRNA complex is not unequivocally determined, although evidence is accumulating that it is of separate origin (Knight & Darnell, 1967; Brown & Weber, 1968; Brown & David 1968; Hatlen, Amaldi & Attardi, 1969).

6.3. 7S RNA

Another small rRNA molecule is 7S RNA, which originates from the breakdown of the 32S ribosome precursor RNA. This species remains associated with 28S RNA, also with an unknown function (Pene, Knight & Darnell, 1968), in common with 8S RNA (Prestayko, Tonato & Busch, 1970).

The only small molecular weight RNA species whose functions are known is tRNA.

7. TRANSFER RNA

7.1. Introduction

Transfer RNA molecules are transcribed from cellular DNA

genes (Giacomoni & Spiegelman, 1962; Goodman & Rich, 1962) which may map in the same region as rRNA, as in Bac. subtilis (Dubnau, Smith & Marmur, 1965; Oishi, Oishi & Sueoka, 1966) or bear no relationship to ribosomal cistrons, as in Drosophila melanogaster (Ritossa, Atwood & Spiegelman, 1966). Although others have been suggested, there are several known functions of tRNA: it forms an ester bond between its terminal adenosine and an amino acid with a clear specificity towards one amino acid. It then transfers this activated amino acid to the ribosomes, recognizing a specific triplet sequence in mRNA (Crick, 1958; Chapeville, Lipman, von Ehrenstein, Weisblum, Ray & Benzer, 1962) prior to the formation of a peptide bond during polypeptide synthesis.

7.2. Transfer RNA Structure

At least 31 tRNAs are required to translate all the codons of mRNA in terms of the Wobble Hypothesis (Crick, 1966). The primary structure of the 75 - 85 nucleotides of at least 14 of these are now known, and these sequences have been used to suggest several secondary and tertiary structures for tRNA (Lake & Beeman, 1968; Cramer, Doepner, Van de Haar, Schlimme & Seidel, 1968; Doctor, Fuller & Webb, 1969; Melcher, 1969; Levitt, 1969; Ninio, Favre & Yaniv, 1969). These are all variations on the clover-leaf structure, proposed originally by Holley et al (1965) (see Figure 5), which has a probability

Figure 5.

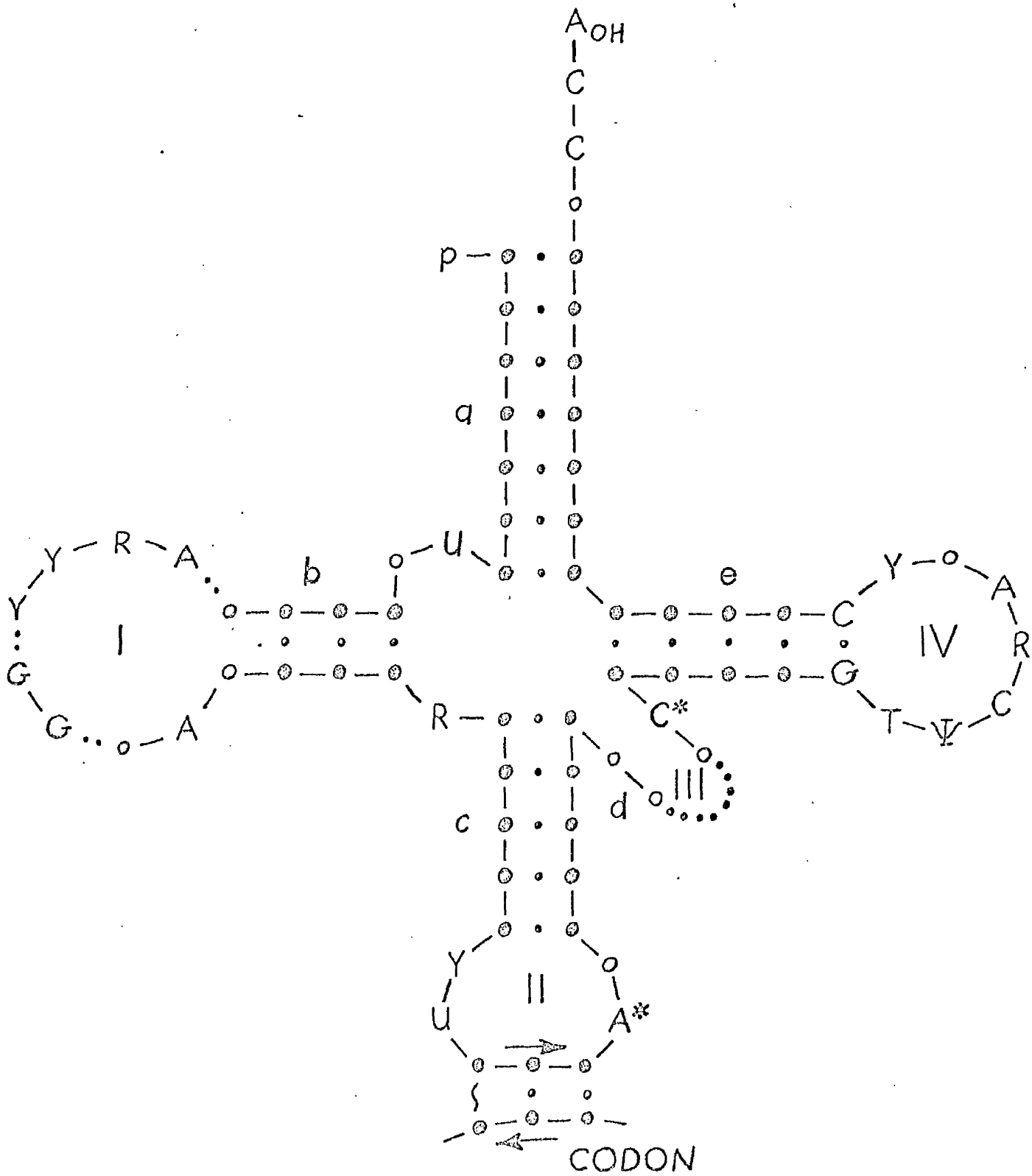
Generalized Representation of a Transfer RNA Molecule in the "Cloverleaf" Form.

(As elaborated by Clark & Jukes).

I, II, III, IV

I, II, III, IV:	"loops" (unpaired regions).
a, b, c, d, e:	helical (base-paired) regions, or "arms".
Solid circles:	bases in helical regions, usually paired by H-bond (indicated by centred dot).
Open circles:	bases usually unpaired.
R; Y:	purine; pyrimidine (nucleosides).
T; ψ:	ribothymidine; pseudouridine.
*:	modified base.
→ :	3' p 5' direction (upper arrow indicates anticodon).
~ :	"wobble" pairing.
	loop III and arm d are sometimes absent. The numbering and lettering are not influenced thereby.

Figure 5.



of less than one in ten of being an artifact of random sequencing (Dube, Marcker, Clark & Cory, 1968). All tRNA molecules whose sequences are known can be portrayed in terms of the clover-leaf model, but this is not absolutely identical for all tRNAs (Blake, Fresco & Langridge, 1970). In this model complementary regions form α-helices and non-bonded regions "loop" out as "stacked" single-stranded stretches (Figure 5). Theoretical predictions from the clover-leaf structure are supported experimentally by measurements of physico-chemical parameters (Lake & Beeman, 1968) and by the accessability of parts of the sequence to chemical or enzymic modification (Zamir, Holley & Marquisee, 1965; Armstrong, Hagopian, Ingram & Wagner, 1966; Nelson, Ristow & Holley, 1967). It has been suggested that the formation of such a clover-leaf tertiary structure is largely a function of the preferred orientation and interaction of non-bonded looped regions rather than interaction between helical segments (Henley, Lindahl & Fresco, 1966; Lake & Beeman, 1968; Levitt, 1969; Doctor, Fuller & Webb, 1969). Little is known of quaternary structure of tRNAs but Loehr & Keller (1968) have proposed that clover-leaf "loops" of tRNA molecules may again be able to interact intermolecularly and be involved in forming structure between different tRNAs.

7.3. Aminoacyl-tRNA Synthetase Reaction

The functions of tRNA are probably all mediated by enzyme action, and certainly require recognition of specific sites on the tRNA. The aminoacyl-tRNA synthetase enzyme has single binding sites for ATP, the specific amino acid and tRNA (Norris & Berg, 1964; Allende, Allende, Gatica, Celis, Mora & Matamala, 1966; Baldwin & Berg, 1966; Yarus & Berg, 1967), and forms an enzyme-aminoacyladenylate intermediate complex (Norris & Berg, 1964; Lagerkvist, Rymo & Waldenstrom, 1966). In mammalian cells there appears to be more than one aminoacyl-tRNA synthetase for certain amino acids, as mitochondrial systems have different enzymes from homologous cytoplasmic systems (Nass, 1969), while there appears to be a singular relationship in bacterial cells (Niyomporn, Dahl & Strominger, 1968). During the reaction, the synthetase must be able to interact with a specific region of the tRNA molecule. Attempts have been made to locate this area by limited enzymic or chemical modification of the tRNA alone (Harriman & Zachau, 1966; Lindahl, 1967; Smith, 1969), although such an approach is likely to be inconclusive. For example, modification which disrupts base-pairing, and thereby inactivates the codon recognition function of the tRNA

may leave the molecule still competent to interact with the synthetase enzyme. Although this approach, reviewed by Miura (1967) and Madison (1968), has given rise to disparity in results, comparison of these with results derived from different approaches have proved extremely valuable. In a more definitive experiment utilizing u. v.-irradiation followed by enzymic digestion of aminoacyl acceptor and non-acceptor alanine-specific tRNA molecules, where chain cleavage was not involved in the inactivation reaction, Schulman & Chambers (1968) indicated that the base-pairs involving the 5th, 6th and 7th residues from the 3'-end are involved in the synthetase recognition, and that the ordered structure of the stem region and possibly other parts of the molecule are required for recognition. Gefter & Russell (1969), by characterization of suppressor tyrosine tRNAs produced on infection of Esch. coli with the defective transducing phage 80d su^{+}_{III}, have shown that "loop" II (Figure 5) is not involved in synthetase recognition in that particular tRNA. Other possible locations of the enzyme attachment site are in the loop nearest the 5'-end (Loop I) of the tRNA molecule or in loop III (Doctor, Loebel, Sodd & Winter, 1969). Conflicting results have meant that so far no definite location of the attachment site has been ascertained for any tRNA, although there seems to be no inherent reason why the same region of different tRNA molecules should be

involved.

7.4. Coding in Messenger RNA

After the formation of the aminoacyl-tRNA, the tRNA molecule becomes involved in binding to the ribosomes, in recognition of a unique sequence of nucleotides in the mRNA and, finally, in formation of the peptide bond. Messenger RNA, bearing the base sequence complementary to one strand of DNA, is active in association with the ribosomes and aminoacyl-tRNA molecules. The reading of the information in the mRNA required for protein synthesis is contained in a degenerate triplet nucleotide code, and commences, at least in bacterial systems, at the 5'-end of the mRNA, with the sequence AUG (and perhaps GUG) corresponding to N-formylmethionine (Clark & Marcker, 1966; Sundararajan & Thach, 1966). Reading is terminated by a sequence containing UGA, UAG or UAA (Last, Stanley, Salas, Hille, Wahba & Ochoa, 1967; Brenner, Stretton & Kaplan, 1965). Synonym codons are probably recognized by different (iso-accepting) tRNA molecules specific for the same amino acid (von Ehrenstein & Dias, 1963; Galizzi, 1967; Weisblum, Cherayil, Bock & Söll, 1967). In vitro, obvious differences in the coding response of several aminoacyl-tRNAs have been observed, ranging from those in which a tRNA can recognize only one codon to those in which one tRNA can recognize up to 3

codons (Söll, Cherayil & Bock, 1967). This multiple recognition involves only the third (5'-) base of the mRNA codon and seems likely to occur only under the conditions predicted by Crick in his Wobble Hypothesis (Crick, 1966). More tRNAs than are required to balance the degenerate nature of the genetic code have been isolated and these "extra" tRNAs have been termed "redundant" (Söll & Raj-Bhandary, 1967; Goodman, Abelson, Landy, Brenner & Smith, 1968).

7.5. Anticodon Site

The position of the anticodon site in the tRNA has been shown to occur in loop II of the clover-leaf model (Levitt, 1969). As mentioned previously, the 5'- nucleotide of the anticodon triplet is free to "wobble", and both the 3'- and 5'- nucleotides adjacent to the triplet are, in all species examined, respectively, an unmodified or modified A and an unmodified U.

7.6. Base and Nucleoside Modification

The structural integrity of the tRNA molecule is likely to be important for ribosome-binding, for codon-anticodon interaction, and for manoeuvring the aminoacyl molecule into the correct position for its transfer on to the growing peptide chain. Interactions similar to those previously discussed in sections 3.2. and 3.3 for DNA exist between nucleotides in tRNA and will also be dependent on the

presence of substituent groups on the base.

The several types of unusual base in tRNA are introduced at different points in its synthesis. Thiolation, which occurs in a tRNA precursor molecule at the polynucleotide level, produces the minor components 4-thiouracil (Lipsett, 1965), 2-thiocytosine, 5 methylaminomethyl-2-thiouracil (Carbon, David & Studier, 1968), or 2-methylthio-N^6-isopentenyladenosine (Burrows, Armstrong, Skoog, Hecht, Boyle, Leonard & Occolowitz, 1968). Other compounds introduced at the polynucleotide level are pseudouridine (Cohn, 1960) and N^6-acetylcytosine (Madison, 1968), while dihydrouracil (Madison & Holley, 1965) and hypoxanthine (Holley, Everett, Madison & Zamir, 1965) are utilized as nucleoside triphosphates at the time of transcription. As listed in Table I, a wide variety of unusual bases and nucleosides of this kind have been reported, and it seems likely that more labile modifications remain to be identified. However, of all the base modifications present in tRNA, quantitatively the most important are the methylated bases and nucleosides. These are inserted at the polynucleotide level, and naturally-occurring examples are listed in Table I, and the structures of some depicted in Figure 2.

7.7. Distribution of Modifications

Several modified bases are positioned near the 5'- terminus

of the tRNA molecule, but the majority (about 80%) is present in single-stranded "looped" regions. Those in a double-stranded part of the molecule are generally in juxtaposition to a loop, although 5-mC in phenylalanine tRNA is not (Madison, 1968). This is consistent with the finding that methylation in many positions in bases reduces their base-pairing ability, whereas in the formation of 5-methylcytosine from cytosine that ability is enhanced.

Not only do tRNA molecules share the general property of carrying the modified bases in non-H-bonded regions, but the bases are also often present in common nucleotide sequences. For example, N^6-isopentenyladenosine and 2-methylthio-N^6-isopentenyladenosine are located next to the 3'-end of the anticodon site (Hall, Robins, Stasiuk & Thedford, 1966; Burrows, Armstrong, Skoog, Hecht, Boyle, Leonard & Occolowitz, 1968; Harada, Gross, Kimura, Chang, Nishimura & Raj Bhandary, 1968; Gefter & Russell, 1969), and this specific base modification is almost certainly concerned with the binding of the tRNA to the ribosome mRNA complex (Gefter & Russell, 1969). Heterologous tRNA from rat liver and yeast have the same 6 oligonucleotides surrounding 1-methyladenine A (Baguley & Staehelin, 1969), and 3 homologous yeast tRNAs have the sequence - GpCpGpCp - in the same position, in each case the second G being present as N^2-dimethylguanosine. A base modification close to the 3'- OH end

of tRNA has been identified both in Esch. coli and rat liver tRNA (Lagerkvist & Berg, 1962; Herbert & Wilson, 1962), and it seems likely that other such similarities among tRNAs will emerge.

The distribution of modified bases reported above suggests that, in addition to the primary sequence surrounding a base, the accessibility of that base to the modifying enzyme(s) is an important factor in determining its modification. It is likely that methylated bases will be found in similar positions in tRNAs where a common function is required: however, where one tRNA molecule is involved in a specific function, a methylated base will probably be found to play an important distinguishing ro

7.8. Transfer RNA Methylases

Transfer RNA methylase enzymes catalyze the transfer of an intact methyl group from an activated methionine molecule (S-adenosylmethionine) to a C-, or N- atom of a purine or pyrimidine base, or an O-atom of ribose in a precursor tRNA molecule (cf. Figure 3). Enzyme activity is located mainly in the supernatant fraction of cells (Hurwitz, Gold & Anders, 1964; Tsutsui, Srinivasan & Borek, 1966; Burdon, Martin & Lal, 1967; Baguley & Staehelin, 1968; Rodeh, Feldman & Littauer, 1967) although tRNA is apparently synthesized in the nucleus.

Several tRNA methylase activities are present in any one system. For example, Hurwitz, Gold & Anders (1964) separated

6 enzyme fractions from Esch. coli; Nichols & Lane (1968) measured 6 base methylase activities and 3 sugar methylase activities in vitro with Esch. coli extracts; and Svensson, Björk & Lundahl (1969) separated 8 enzyme fractions from Sacchanomyces cerevisiae.

The substrate tRNA generally used in vitro is extracted in an undermethylated form from Esch. coli M12 (58-161), which exhibits relaxed control over RNA synthesis in the absence of methionine (Mandel & Borek, 1963). Transfer RNA methylase will methylate homologous substrate only if it is methyl-deficient (Gold, Hurwitz & Anders, 1963; Srinivasan & Borek, 1963; Svensson, Bowman, Eriksson & Kjellin, 1963); thus methyl-deficient tRNA can first be methylated by homologous enzymes and then supermethylated by heterologous enzymes in vitro. Extracts from tumours have higher tRNA methylase activity than extracts from similar "normal" tissues, and can supermethylate tRNA from these tissues, as described for Krebs II ascites tumour cells, SV40-induced tumours, adenovirus-12-induced tumours, murine leukemia virus-induced tumours, Novikoff hepatoma tumours, and in tissues of rat with Dunning leukemia (Burdon, 1966; Tsutsui, Srinivasan & Borek, 1966; Mittelman, Hall, Yohn & Grace, 1967; Silber, Goldstein, Berman, Decter & Friend, 1967; McFarlane & Shaw, 1968, Baguley & Staehelin, 1968; Hacker &

Mandel, 1969). This elevated enzyme activity in tumour tissue extracts may be related to the appearance of new species of tRNA. For example, in Novikoff hepatoma cells, 8 new species of tRNA can be separated, perhaps because of altered physical properties owing to additional methylation of pre-existing molecules, or owing to the production of tRNA molecules with a new primary structure (Baliga, Borek, Weinstein & Srinivasan, 1969). However, Kaye & Leboy (1968) find that, in vitro, the activity of the tRNA methylases is ion-dependent, and that, if "optimal" ionic conditions are employed, extracts of "normal" and tumour-bearing tissue methylate tRNA at the same rate, to the same extent and with similar specificity. Clearly further characterization of these enzyme systems is required to differentiate meaningfully between "normal" and tumour cells in this respect.

7.9. Effect of Virus on Transfer RNA Methylation

Bacteriophage-infection has widespread effects on tRNA methylation. The coliphage T1 causes no alteration in extent or type of methylation, whereas coliphage T2 produces a different base methylation pattern in tRNA after infection, caused by changes in the relative levels of the base-specific methylases (Wainfan, Srinivasan & Borek, 1965). Bacteriophage T4-infection of Esch. coli

also alters the base-methylation patterns, perhaps to cope with the phage T4-specified tRNA (Boezi, Armstrong & De Backer, 1967; Daniel, Sarid & Littauer, 1968; Weiss, Hsu, Foft & Scherberg, 1968). On the other hand, reduced methylase activity is the result of induction in a lysogenic strain of Esch. coli K12 (λ^+), apparently owing to inhibition of host methylase activity by a dialyzable product of the infection (Wainfan, Srinivasan & Borek, 1966).

Carcinogenic viruses induce tumours which exhibit high levels of tRNA methylase activity. In contrast, the rate of tRNA methylation in polio-infected HEp-2 cells is one third that of control cells (Grado, Friedlender, Ihl & Contreras, 1968), but base analysis reveals formation of 6-methyladenosine which is absent in host cells. In the case of FMDV, 4S RNA methylation is inhibited by 57% after one hour of infection, although no tRNA methylase inhibitor can be detected (Ascione & Vande Woude, 1969). There are no reports on the effects of a DNA-containing animal virus on tRNA methylation in cell culture systems.

8. ONCOGENIC VIRUSES

The sequence of events described in Sections 2, 3 and 2, 5 for the eclipse phase of HSV growth is not necessarily followed when an oncogenic DNA virus infects a cell in vitro. These viruses, the

papova viruses (polyoma virus, SV40, the papilloma viruses) and the adenoviruses produce either a cytopathic effect or cause transformation depending to a large extent on the type of cell used as host. Transformation is defined as "a heritable change in the properties of a cell subsequent to virus infection which is manifested by the loss of the regulatory restraints of its growth potential". Proteins which are synthesized late in the growth cycle of a productively growing oncogenic virus do not appear in transformed cells, but some "early" proteins do. Included amongst these are some enzymes related to nucleic acid synthesis (Kit, Dubbs, de Terres & Melnick, 1965; Hartwell, Vogt & Dulbecco, 1965; Kit, Dubbs, Frearson & Melnick, 1966). Either the viral genome or a viral mRNA induces host enzymes of the DNA biosynthetic pathway and host DNA synthesis is stimulated, but there does not seem to be any synthesis of viral DNA (Gershon, Hausen, Sachs & Winocour, 1965; Gershon, Sachs & Winocour, 1966; Sheinin, 1966). It is thought that the viral genome is integrated into the host cell genome (Todaro & Green, 1966; Sauer & Defendi, 1966). It is not known whether the viral DNA is linked to the cellular DNA by alkali-stable covalent linkages as one large piece at a single site, or connected at multiple sites after many individual insertions (Sambrook, Westphal, Srinivasan & Dulbecco, 1968).

When cells are productively infected with oncogenic viruses, the normal processes required for the production of progeny virus must take place. Included in these is the synthesis of enzymes involved in nucleic acid synthesis, e.g. in polyoma-infected cells, DNA polymerase, whose activity is increased (Hartwell, Vogt & Dulbecco, 1965), is, at least partly, a virus-induced enzyme (Smart, Fried & Pitts, 1967), and thymidine kinase activity is increased (Sheinin, 1966). However, in contrast to the situation in most virus-infected cells, DNase activity is not significantly altered (Fried & Pitts, 1968). Control of whether events lead to transformation or to cytopathic effect in oncogenic virus infection is unknown.

MATERIALS AND METHODS

1. MATERIALS

1.1. Chemicals and Analytical Materials

All chemicals were, where possible, "AnaLar" grade, or its equivalent, and were purchased mainly from B. D. H., Poole, Dorset; Hopkins & Williams, Ltd., London, or Sigma Chemical Co., London: CsCl was purchased from Harshaw Chemical Co., Cleveland, Ohio; materials for liquid scintillation counting from Nuclear Enterprises, Ltd., Edinburgh; L-amino acids, nucleosides and bases; DNase I, phospholipase; Esch. coli B tRNA, and DNA from different species from Sigma Chemical Co., London, Calbiochem Inc., Los Angeles, or Schwartz BioResearch Inc., Orangeburg, New York; agarose powder from L'Industrie Biologie Francaise, S. A., Gennevilliers, France; Whatman 1 MM and 3 MM chromatography paper, Dowex 1-X8 and Amberlite CG-50 from H. Reeve Angel & Co. Ltd., London; cellulose acetate membrane filters (0.45 μ pore size) from Millipore Filter Corporation, Bedford, Mass.; and Industre X-ray film, type D from Kodak, Ltd.

1.2. Radioactive Material

Radioactive compounds were purchased from the Radiochemica Centre, Amersham, Bucks.

1.3. Virus and Tissue Culture Cells

Viruses used in this study were Herpes Simplex Virus (HSV) strain α of HFEM (Russell, Gold, Keir, Omura, Watson & Wildy, 1964), and pseudorabies virus (Kaplan & Vatter, 1959), a gift from W. Shepherd.

Tissue culture cells were a continuous line of hamster fibroblasts, BHK21/C13, as described by Macpherson & Stoker (1962), or human epidermoid carcinoma cells (HEp-2) as described by Moore, Sabachewsky & Toolan, (1955) (cultured by W. Shepherd).

2. COMPOSITION OF MEDIUM

2.1. Eagle's Medium

A modification of Eagle's medium (Busby, House & Macdonald, 1964) containing 100 units/ml Penicillin, 100 µg/ml Streptomycin, 0.2 µg/ml of the antimycotic agent n-butyl, p-hydroxylbenzoate and 0.002% w/v phenol red was used.

2.2. Media for Cell and Virus Growth

2.2.1. Tryptose Phosphate Broth consisted of a 2.95% w/v solution of tryptose phosphate broth (Difco Bacto) in distilled water.

2.2.2. EC10 (2) contained 10% v/v (or 2% v/v) Calf Serum in Eagle's Medium.

2.2.3. EC2F [x%met] contained 2% v/v calf serum, 20 mM sodium formate and x% normal concentration of methionine in Eagle's medium. 100% methionine was 100µM.

2.2.4. EHu2 contained 2% v/v pooled human serum in Eagle's medium.

2.3. Solutions

2.3.1. Phosphate buffered saline (A) (PBS(A)) was a solution of 0.17 M-NaCl, 3.4 mM-KCl, 10 mM-Na_2HPO_4 and 2 mM-K H_2PO_4, pH 7.4 (Dulbecco & Vogt, 1954).

2.3.2. Tris-saline was a solution of 25 mM-tris-HCl buffer, pH 7.4, 0.14 M-NaCl, 5 mM-KCl, 0.7 mM-Na_2HPO_4, and 5 mM-dextrose containing 0.002 % w/v phenol red, 100 units/ml Penicillin and 100 μg/ml Streptomycin.

2.3.3. Versene solution consisted of EDTA dissolved to 0.6 mM in PBS(A), to which 0.002% w/v phenol red was added.

2.3.4. Trypsin-versene solution consisted of 1 volume of 0.25% w/v trypsin solution (Difco trypsin in Tris-saline) in 4 volumes of 0.6 mM-EDTA.

2.3.5. Formol saline consisted of 4% v/v formaldehyde in 85 mM-NaCl and 0.1 M-Na_2SO_4, diluted one in ten.

2.3.6. Giemsa stain was a 1.5% w/v suspension of Giemsa in glycerol, heated at 56° from 90 to 120 min. It was used diluted with an equal volume of methanol (Dacie, 1956).

2.3.7. Toluidine blue was used as a 0.05% w/v solution in distilled water.

2.3.8. Standard saline citrate (S.S.C.) was a solution of 0.15 M-NaCl and 15 mM-trisodium citrate, pH 7.0.

2.3.9. R.S.B. was an isotonic buffer solution containing 0.01 M-tris-HCl buffer, pH 7.4, 0.01 M-KCl and 0.0015 M-$MgCl_2$ (Becker & Joklik, 1964).

2.3.10. Chloroform/iso-amylalcohol mixture consisted of 24 parts chloroform to 1 part iso-amylalcohol.

2.3.11. Phenol solutions were prepared by saturating freshly distilled phenol with the buffer present in the cell or virus preparation. In some cases 8-hydroxyquinoline was added as a preservative to a final concentration of 0.1% w/v.

2.4. Suspensions

2.4.1. Bentonite was used as an approximately 2.5% w/v sterile suspension prepared by a modification of the method of Singer & Fraenkel-Conrat (1961) as follows: bentonite powder, washed with water, was left at room temperature as a suspension in 0.1 M-EDTA, pH 7.0. The top part of the sediment formed after 25,000g for 30 min was resuspended in 0.02 M-tris-HCl buffer, pH 7.5, autoclaved, and stored at 4°.

2.4.2. "Macaloid" (from National Lead Co., Houston, Texas) was

a 2.5% w/v sterile suspension in water, diluted 1000-fold for use.

2.4.3. Kieselguhr (from Koch Light Laboratories, Ltd., Colnbrook Bucks) was purified by extensively washing the crude preparation with 1M-NaOH, distilled water, 1M-HCl and finally to neutrality with distilled water. It was then dried and crushed to a powder. Ten g of this was suspended in 0.1M-NaCl, 50 mM-Na H_2PO_4-Na_2HPO_4 buffer, pH 6.3 and boiled for 1 min. After cooling, 25 mg freshly dissolved N-methylated albumin (Mandell & Hershey, 1969), was mixed with the 50 ml suspension of Kieselguhr to give methylated albumin Kieselguhr (MAK).

3. CELL AND VIRUS GROWTH

3.1. BHK21/C13 Cell Culture

3.1.1. Cell growth. The BHK21/C13 cell line was maintained by subculturing confluent monolayers of cells. Monolayers in 80 oz. roller bottles (or 40 oz. Roux bottles) were washed with 20 ml (8 ml) warmed versene and the cells removed by shaking after a few minutes of contact with 20 ml (8 ml) trypsin-versene. After inhibition of the trypsin activity with 2 ml calf serum, the cells were resuspended, after centrifugation (400 g, 5 min), in EC10. The cells were then dispensed into roller bottles or Roux bottles in 180 ml (or 60 ml) EC10 at a concentration of approximately 10^5 cells/ml. After gassing to

give an atmosphere of 5% CO_2, 95% air, the vessels were incubated at 37° for 2 - 3 days, by which time the cells had divided into confluent monolayers. Cells were, from time to time, grown on 50 mm or 90 mm plastic Petri dishes in 5 ml or 15 ml EC10, seeded once more at 10^5 cells/ml. In this case incubation was carried out at 37° in a humidified incubator flushed continuously with 5% CO_2, 95% air.

3.1.2. "Serum-depleted cell cultures." For the production of "serum-depleted" cells, confluent monolayers in roller bottles were washed with 20 ml ice-cold versene, and the cells removed with 20 ml cold trypsin/versene. The cell suspension was mixed with 80 ml ice-cold Eagle's medium and harvested by centrifugation (15 min, 200g). After pipetting to form a single-cell suspension in EC0.5, this was dispensed into roller bottles (180 ml), Roux bottles (60 ml) or 90 mm Petri dishes (15 ml), and incubated at 37° in an atmosphere of 5% CO_2, 95% air for 4 or 5 days.

3.1.3. Stock BHK21/C13 cells. Cells were prepared for stock by harvesting as detailed above (Section 3.1.1.). They were then suspended in 70% v/v Eagle's medium, 25% v/v calf serum and 5% v/v sterile glycerol at a concentration of not more than 10^7 cells/ml, and, after freezing slowly, were stored at -70°.

Cells brought out of storage were collected by centrifugation (400g for 5 min) before setting up in Roux bottles as described above. Such cells were passaged at least twice before being used for experimental purposes.

3.2. HSV Growth

For the production of HSV stocks, HSV (1 P.F.U. to 300 cells) in 20 ml EC2 was allowed to adsorb for 60 min to confluent monolayers of BHK21/C13 cells in roller bottles revolving slowly at 37°. The medium was then replaced with 50 ml EC2 and the cultures rolled at 37° for a further 42 hours. After approximately 24 hours, sodium bicarbonate (0.9% w/v) was used to readjust the pH to neutral. The infected cells were then removed either by shaking or by incubating for 3 min with versene. After collection by centrifugation at 4° (200g for 15 min) the cells were disrupted either by sonication in a small volume of the supernatant, or by freezing and thawing four times in solid CO_2, ethanol, and the debris removed by centrifugation. This treatment released the cell-associated virus which was concentrated by centrifugation at 30,000g for 60 min at 4°. The resulting pellets were suspended by sonication in a small volume of PBS(A) or 0.1 M-tris-HCl buffer, pH 8.0. The virus was stored at -70°. For experimental purposes, elapsed time after infection was calculated from the time of inoculation of the cells with virus.

3.3. Plaque Assay

Infectivity of HSV was measured by a plaque assay technique. Confluent monolayers of BHK21/C13 cells on 50 mm Petri dishes were washed in EC2, and incubated for 60 min at 37° to allow adsorption of serial dilutions of the virus from 0.2 ml EC2. The medium was then replaced by 4 ml EHu2 , and incubation continued for 2 days. The cell sheets were washed with PBS(A), fixed with formol saline (30 min at room temperature) and stained with Giemsa stain (30 min at room temperature). Plaques were counted using a low-powered microscope after excess stain had been removed with PBS(A).

4. FRACTIONATION METHODS

4.1. Cell Fractionation

Cells suspended in R.S.B. were left at 0° for 10 min before being disrupted by homogenization. Differential centrifugation was then carried out at 600g for 4 min to remove nuclei and cell debris, and at 105,000g for 60 min to sediment the ribosomes (Becker & Joklik, 1964).

4.2. Fractionation of Nucleic Acid

4.2.1. CsCl equilibrium density gradients were made by raising the density of a DNA solution in S.S.C. to 1.70g/ml with solid CsCl. Refractive indices of such solutions were measured in an Abbé refractometer. Liquid paraffin was layered over the DNA solution

(to fill the centrifuge tubes) before centrifugation at 86,000g (swing-out rotor) or 70,000g (fixed angle rotor) for 66 hour at 18°C (Flamm, Bond & Burr, 1966). Fractions were collected dropwise from the bottom of the tube, and the CsCl density gradient estimated in the refractometer. After dilution of the fractions to 0.5 ml with 0.01 M-tris-HCl buffer, pH 8.2, their absorbancy at 260 nm was measured and their radioactivity estimated by precipitating the nucleic acid with carrier DNA onto millipore filters with 5% w/v ice-cold trichloroacetic acid. After three washes with 5% w/v trichloroacetic acid, and drying, 10 ml of a toluene-based scintillant was added. A 60 min incubation at 37° in 0.5 M-KOH was occasionally included before precipitation to digest contaminating RNA.

4.2.2. MAK column chromatography. The MAK, prepared as described above, was packed on top of a layer of washed sand over a scintered glass plate in a glass tube of uniform bore. A suspension of Kieselguhr ($^{1}/10$ volume of MAK used) was layered on top of the MAK. The column was washed with 10 volumes 0.2 M-NaCl in 0.05 M-NaH_2PO_4-Na_2HPO_4 buffer, pH 6.3 under pressure from a peristaltic pump. The flow rate was maintained at 2 - 3 ml/min. The nucleic acid sample was applied at 100 μg/ml and at not more than 1 ml/min in a solution of S.S.C. After washing with 2 volumes of the same buffer,

the adsorbed nucleic acid was eluted with a linear gradient (0.2 M to 1.6 M-NaCl) in buffer at room temperature. The flow rate was maintained at about 2 ml per min, and 2 ml samples were collected. Fractions were estimated directly for absorbancy and radioactivity as described for CsCl gradients (Section 4.2.1.).

4.2.3. Agarose gel electrophoresis. Two variations of this method, based on the method of McIndoe & Munro (1967), were employed. For the first, 2% agarose gels were formed in small diameter glass tubes, using 0.02 M-tris-citrate buffer, pH 7.9 as the aqueous phase, as described by the above authors. Approximately 50 μg RNA sample was applied in a sucrose-containing 0.01 M-tris buffer, pH 7.4. Electrophoresis was carried out using 0.2 M-tris-citrate, pH 7.9 in the electrode compartments, by applying 4 mA across each gel for 30 min. The gels were removed from the tubes, stained for 60 min in toluidine blue which forms an insoluble salt with RNA, and destained with 7% w/v acetic acid for 18 hours. Dye uptake along the gel was recorded in a "Vitatron" spectrophotometer. Gels were then cut into 1 mm fractions. These were treated with hydrogen peroxide or water, hyamine and 2-methoxyethanol, as adapted from Tishler & Epstein (1968) before radioactivity was measured in 8 ml toluene scintillant.

In the second method, 2% gels were formed in 1 ml disposable,

calibrated pipettes using 0.04 M-tris-0.02 M-sodium acetate-acetic acid, pH 7.8, and containing 0.002 M-EDTA as aqueous phase. The same buffer system was present in the electrode compartments as in the gels during electrophoresis, and 3 mA per gel was applied for 65 min at room temperature. Gels were either removed and stained for tracing as described above, or equal volumes of gel were fragmented and expelled into scintillation vials by forcing the gel through the narrow orifice of the syringe. These fractions were mixed with 2 ml 2-methoxyethanol for 2 hours before measuring radioactivity with 8 ml toluene-based scintillant.

4.3. Nucleic Acid Hydrolysate Analysis

4.3.1. DNA base analysis. Purified DNA was evaporated to dryness under reduced pressure in a pyrex tube. It was then heated to 170 - 200° for 60 min with 0.25 ml 90% v/v formic acid in the sealed tube wrapped in glass fibre in a hydrolysis "bomb". The tube was frozen before opening and the residual formic acid evaporated off under reduced pressure. The residue was dissolved in a small volume of 12M-HCl and applied to Whatman 1 MM or 3 MM chromatography paper together with marker samples of 5-mC and 6-mA. The chromatograms were developed either in the descending direction using n-butanol: water (86: 14 v/v) with 5% ammonia in the atmosphere for

22 hours, or in this sytem followed by descending elution for 22 hours at 90° to the first direction in iso-propanol: conc. HCl: water (68: 16.4: 15.6 by volume). Spots were located by u. v.-absorption.

4.3.2. RNA nucleotide analysis. Purified RNA (approximately 10 mg/ml) was hydrolyzed with 0.3M-KOH, for 18 hours at 37°. The solution was neutralized with perchloric acid and after 10 min at 0° the $KClO_4$ was removed by centrifugation. From time to time the hydrolysis was carried out in 72% v/v perchloric acid at 100° for 60 min, followed by neutralization with KOH.

Chromatographic analysis of the hydrolysates was carried out as follows: Aliquots of the supernate were spotted on Whatman 1 MM chromatography paper. Separation of the nucleoside monophosphate was obtained by two-dimensional descending elution in the following solvents: first, iso-butyric acid: 0.5 M-NH_4OH (50: 30 v/v) for 22 hours, and second, iso-propanol: conc HCl: water (53.5: 19.3: 15.6 by weight) (Leech, Dyer, Poole & Smith, 1968) for 20 hours. Spots were again located on the dried chromatograms by their u. v.-absorption. This method was based on that of Hayashi, Osawa & Miura (1966).

4.3.3. Autoradiography of chromatograms. Autoradiograms were obtained after contact of X-ray films with the chromatograms for 3 - 11 weeks. Where material being analyzed had been dual-

labelled, (^{14}C & ^{3}H) a sheet of cellophane was inserted between the chromatogram and the X-ray film to cut out the weak β-emissions from tritium.

4.3.4. Estimation of radioactivity of chromatograms. Sections of chromatograms were cut out and counted in 10 ml dioxan-based scintillant, or incubated in 0.5 ml hyamine (60°, 10 min or 37°, 60 min) before counting in 10 ml toluene-based scintillant, or counted dry in a gas-flow counter with a thin window.

5. ESTIMATION PROCEDURES

5.1. Methods for Estimating Radioactivity. In some cases ^{14}C-labelled samples were counted by drying onto aluminium planchettes in a low-background Nuclear-Chicago gas-flow counter. The gas routinely used was 98% helium and 1 - 3% butane and the efficiency of counting was about 20%.

^{14}C and ^{3}H-labelled material was counted in a Packard Series 4,000, a Nuclear Chicago Series 725, or a Philips Liquid Scintillation spectrometer, in toluene-based liquid scintillator (0.5% w/v P.P.O. ± 0.03% w/v P.O.P.O.P.) or dioxan-based scintillator (10% w/v naphthalene, 0.7% w/v P.P.O. ± 0.03% w/v P.O.P.O.P.). Efficiencies were determined by the channels ratio method, or the external standard

method, depending on the homogeneity of the sample. Where it was necessary to solubilise polar samples in toluene scintillant 1.0 M-hyamine hydroxide (0.5 ml/10 ml scintillant) or 2-methoxy-ethanol (2 ml/8 ml scintillant) was used.

5.2. NaCl and CsCl Estimation

A graphic relationship can be obtained between salt concentration and refractive index (see Figure 6). The refractive indices of solutions of either salt were measured using an Abbé refractometer.

5.3. Estimation of Protein and Nucleic Acid

5.3.1. Protein estimation was carried out using the method of Lowry, Rosebrough, Farr & Randall (1951), with bovine serum albumin as standard.

5.3.2. DNA was estimated either by the method of Burton (1956) or Ceriotti (1955), using calf thymus DNA as standard, or by its absorbancy at 260 nm.

5.3.3. RNA Estimation was carried out by the Orcinolmethod (Kerr & Seraidarian, 1945) or by its absorbancy at 258 nm.

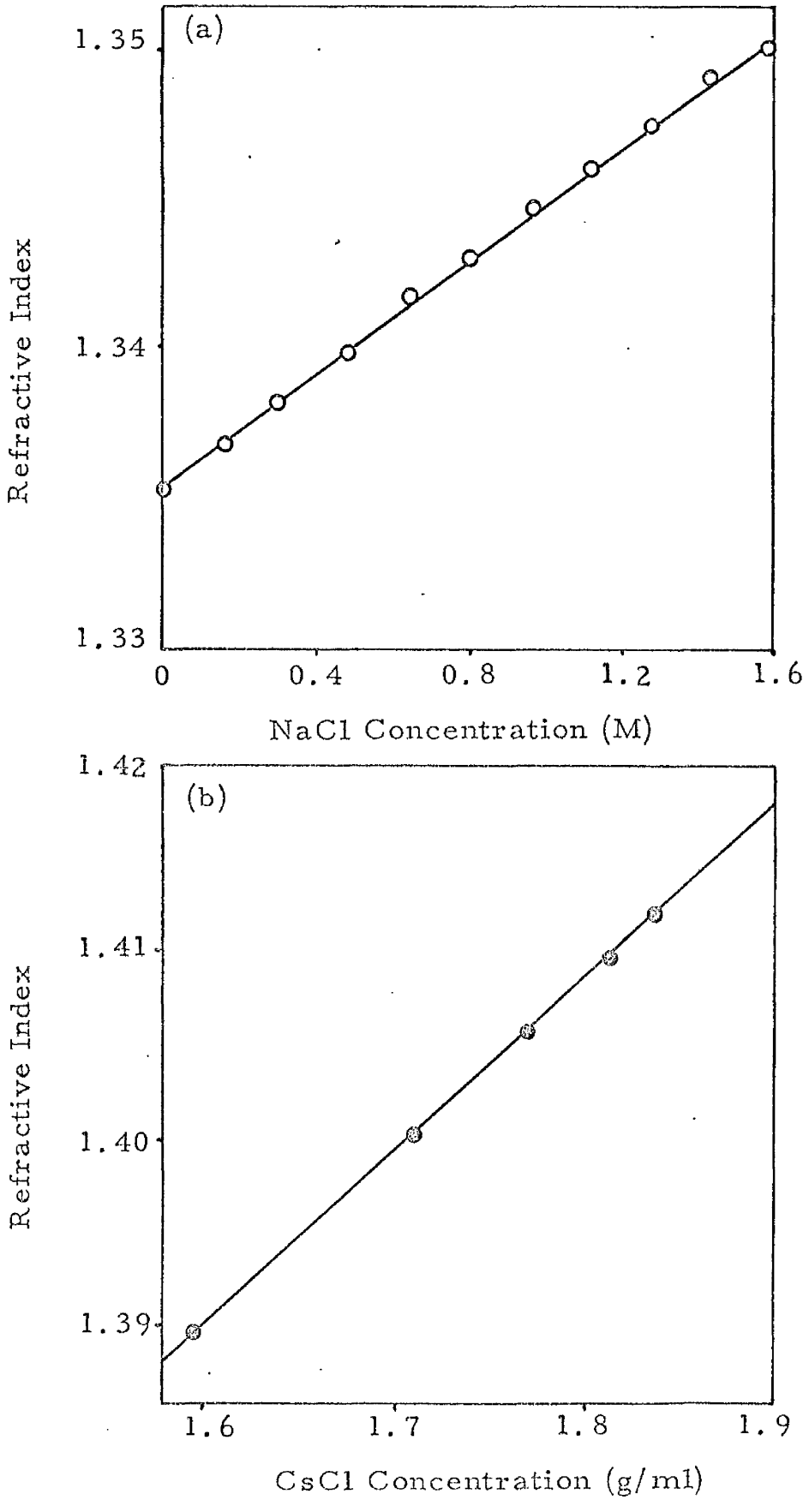
6. EXTRACTION OF NUCLEIC ACID

6.1. Extraction of Total Nucleic Acid

Total nucleic acid was extracted by a modification of the

Figure 6

Calibration of (a) NaCl and (b) CsCl Concentration by Refractometry



method of Saito & Miura (1963).

Cells were suspended in 0.1 M-tris-HCl buffer, pH 8.0, 0.1 M-NaCl and 1% w/v S.D.S. and frozen rapidly. After thawing, the suspension was stirred at 0° for 20 min with an equal volume of buffer-saturated phenol. Phases were separated by centrifugation and the aqueous phase re-extracted with phenol. The aqueous phase was twice extracted with ether, excess ether blown off with nitrogen and nucleic acids precipitated from 67% v/v ethanol, 18 hours at -10°. The nucleic acid, harvested by centrifugation, was dissolved in 0.1 x S.S.C. containing bentonite and made to S.S.C. with 10 x S.S.C.

6.2. Extraction of Nucleic Acid by the Phenol Method.

A suspension or solution containing nucleic acid in buffer was made 1 or 2% w/v with S.D.S. and shaken 10 minutes with an equal volume of water- or buffer-saturated phenol containing 0.1% w/v 8-hydroxyquinoline at room temperature. Phases were separated by centrifugation, and residual phenol extracted from the aqueous phase by ether. When excess ether had been blown off with nitrogen, the nucleic acid was precipitated from 67% v/v ethanol or 67% v/v ethanol-2% w/v potassium acetate at 0°, and harvested by "spooling" or centrifugation.

6.3. Extraction of Cytoplasmic RNA

The cytoplasmic fraction of cells (the supernatant from the

600g centrifugation described for cell fractionation (Section 4.1.)) was extracted twice with phenol-0.1% w/v 8-hydroxyquinoline. The aqueous phase was then extracted twice with ether, excess ether blown off with nitrogen, and the solution incubated overnight with 100 μg/ml pronase. The phenol extraction procedure was repeated, and the resultant solution precipitated at -10° overnight from 67% v/v ethanol - 2% w/v potassium acetate.

6.4. Extraction of Ribosomal RNA

(Modified from the method of Steele & Busch, (1967)).

Ribosomes suspended in 0.1 M-sodium acetate, pH 5.0 containing 0.1 M-NaCl and 0.5% w/v S.D.S. were homogenized with a loose-fitting teflon pestle, then very briefly with an equal volume of water-saturated phenol before being shaken for 20 min at room temperature. The aqueous phase after centrifugation was re-extracted by shaking with phenol for 10 min. The RNA was precipitated overnight from 67% v/v ethanol-2% w/v potassium acetate. The RNA, dissolved in 0.02 M-tris-HCl buffer, pH 7.5, 0.002 M-$MgCl_2$ and made 2% w/v w.r.t. S.D.S. was incubated overnight at 37° with 100 μg/ml pronase, extracted with buffer-saturated phenol, and the aqueous phase extracted twice with ether and precipitated overnight from 67% v/v ethanol - 2% w/v potassium acetate. The nucleic acid was incubated at 37° for

10 min and then 25° for 20 min with 50 μg/ml DNase I in 0.05 M-acetate buffer, pH 5.0 and 0.005 M-$MgCl_2$ containing macaloid. RNA was precipitated from 2 M-NaCl, dissolved in 0.05 M-acetate buffer, pH 5 containing macaloid, and reprecipitated from 67% v/v ethanol - 2% w/v potassium acetate.

6.5. Extraction of Soluble RNA

The 105,000g supernatant fraction from cells was precipitated overnight from 67% ethanol v/v - 2% w/v potassium acetate. After centrifugation, the precipitate was dissolved in 0.05 M-tris-HCl buffer, pH 7.4, 0.001 M-$MgCl_2$ containing macaloid and extracted 3 times with buffer-saturated phenol at room temperature. The final aqueous phase was extracted twice with ether, and precipitated overnight from 67% v/v ethanol - 2% w/v potassium acetate. The RNA was incubated for 60 min at 37° in 1 M-tris-HCl, pH 9.0 and reprecipitated from 67% v/v ethanol - 2% w/v potassium acetate. The final precipitates were dissolved in 1 M-NaCl, and the resultant solutions removed and reprecipitated from 67% v/v ethanol - 2% w/v potassium acetate.

6.6. Extraction of DNA (a) Modified Marmur Procedure

The starting material, made to 2% w/v with S.D.S. and 1 M with $NaClO_4$ was shaken gently at 37° for 1 - 2 min. It was then twice

deproteinized with chloroform-iso-amylalcohol and the DNA "spooled" or centrifuged out from 67% v/v ethanol. The DNA was dissolved in 0.01 M-tris-HCl buffer, pH 8.2, and incubated overnight with 150μg/ml pronase. Protein was removed by a phenol - 0.1% w/v 8-hydroxyquinoline extraction, followed by a chloroform-iso-amylalcohol extraction. The DNA was then either precipitated from 67% v/v ethanol or used directly for CsCl density gradient analysis.

(b) Pronase Extraction

Supernatant virus which had been treated with RNase and DNase, or a cell extract, was suspended in 0.01 M-tris-HCl buffer, pH 8.2, made 2% w/v w.r.t. S.D.S., and incubated 60 min at 37° with 150μg/ml pronase. This material was used for CsCl density gradient analysis.

6.7. Pretreatments.

6.7.1. Pronase (from Calbiochem Inc., Los Angeles) solutions were heated for 2 min at 100° and then self-digested for 30 min at 37° to remove hydrolytic enzymes.

6.7.2. RNase (bovine pancreatic, from B.D.H., London) solutions were heated 10 min at 100° to destroy any DNase activity.

7. ENZYME ASSAY

7.1. DNA Methylase

7.1.1. Mammalian. The methods of Burdon, Martin & Lal (1967) Sheid, Srinivasan & Borek (1968), Kalousek & Morris (1968) and

Kalousek & Morris (1969) were used for enzyme preparation and assay. These methods will be compared later.

7.1.2. Esch. coli DNA methylase. Enzyme extracts were prepared as described by Fujimoto, Srinivasan & Borek (1965). DNA methylase assay was based on the method of Fujimoto, Srinivasan & Borek (1965) or of Hausmann & Gold (1966). In some reaction mixtures BHK21/C13 homogenates from infected and control cultures were included to test for inhibition of activity by the virus. The reaction product was deproteinized by the Marmur method, hydrolyzed in 0.3M-KOH for 60 min at 37°, precipitated in trichloracetic acid and washed thoroughly on millipore filters with 5% w/v trichloroacetic acid before drying and counting in toluene scintillant.

7.2. Transfer RNA Methylase.

Cells were harvested either with versene or by scraping, washed with Eagle's medium and twice with 0.01M-tris-HCl buffer, pH 8.0, 0.01M-$MgCl_2$, 0.005M-2-mercaptoethanol and 0.15M-KCl. After contact at 0° for 10 min with hypotonic buffer, cells were homogenized and fractionated. The method of assay was that of Wainfan & Borek (1967) unless otherwise stated. Approximately 1 mg protein was used in a 0.5 ml assay volume, containing 200 µg Esch. coli B. tRNA, 10.0 nmole S-AM (0.05 µC-[^{14}C-Methyl]), 0.01M-tris-HCl buffer,

pH 8.0, 0.01 M-$MgCl_2$, 0.005 M-2-mercaptoethanol. The reaction mixture was incubated 30 min at 37°, and the incubation continued for 10 min with 2 ml 1.5 M-hydroxylamine, pH 7.0. When cooled, 0.6 ml 6M-HCl and 0.3 ml 50% w/v trichloroacetic acid were added and the mixture left at 0° for 15 min before being centrifuged. The precipitate was washed 5 times with 5% w/v trichloroacetic acid, taken up in NH_4OH, transferred onto steel planchettes and dried. Incorporation of ^{14}C was measured using the gas flow counter. Alternatively, the trichloroacetic acid precipitates were transferred onto millipore filters, dried and counted in 10 ml toluene-based scintillant.

RESULTS

1. CONDITIONS FOR OPTIMAL INCORPORATION OF METHYL GROUPS FROM METHIONINE

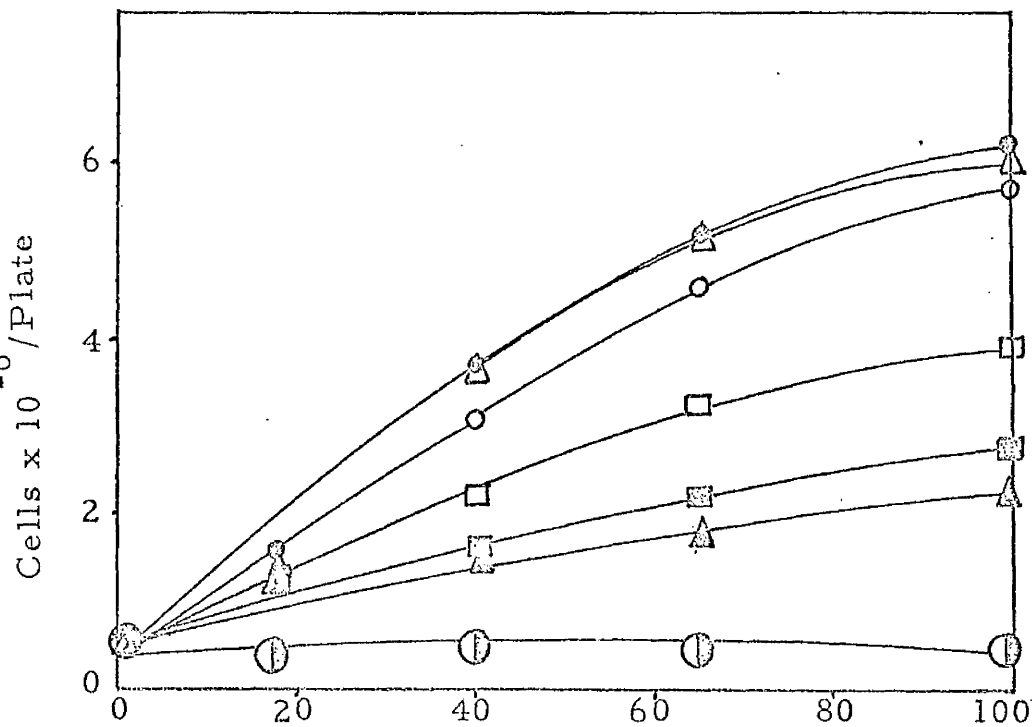
1.1. The Effect of Sodium Formate Concentration on Cell Growth

Methionine contributes to purine and thymidylate biosynthesis via the "one carbon pool" in mammalian cells (Hermann, Fairley & Byerrum, 1955, Kit, Beck, Graham & Gross, 1958). Hence experiments involving in vivo incorporation of radioactively-labelled methyl groups from methionine into nucleic acid may be compromised by the incorporation into the carbon skeleton of bases. However, unlabelled sodium formate can be used to dilute out the "pool" of 1-carbon units derived from radioactively-labelled methionine and so reduce, to a negligible extent, entry of radioactively-labelled methyl groups of methionine into the base structures of nucleotides. To establish the tolerance of BHK21/C13 cells to added sodium formate in growth medium, cells were dispensed into 50 mm Petri dishes (Methods, Section 3.1) in EC10 containing sodium formate (0 - 100 mM). Cells were harvested at intervals using trypsin and resuspended in PBS(A) for an estimation of cell number in the haemocytometer.

It was concluded from the results depicted in Figure 7 that sodium formate was non-toxic to BHK21/C13 cells up to a final concentration of 20 mM, and this concentration was adopted for routine use in experiments involving radioactively-labelled methionine.

Figure 7

Effect of Sodium Formate on Cell Growth



○—○ , 0 mM-sodium formate ;

◎—◎ , 10 mM-sodium formate ;

△—△ , 20 mM-sodium formate ;

□—□ , 30 mM-sodium formate ;

▨—▨ , 40 mM-sodium formate ;

▲—▲ , 50 mM-sodium formate ;

◐—◐ , 100 mM-sodium formate.

BHK21/C13 cells grown in the sodium formate concentration shown were harvested at intervals and counted in a haemocytometer.

1.2. The Effect of Methionine Concentration on the Growth of BHK21/C13 Cells

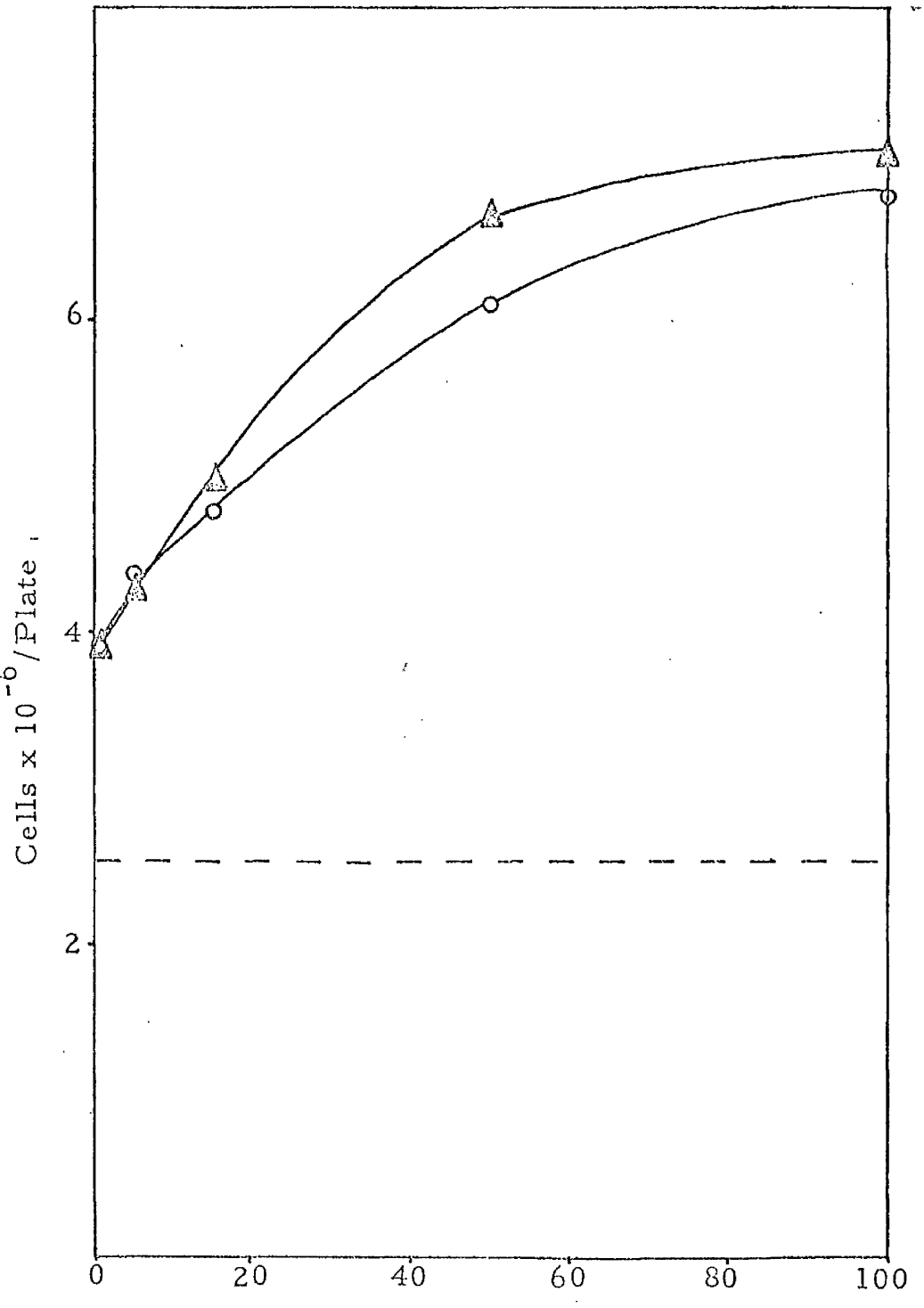
In order to obtain maximum incorporation of methyl groups into nucleic acids from added radioactively-methyl-labelled methionine, it was necessary to add, in the medium, the smallest concentration of methione which will still support normal metabolism. The effect of methionine concentration on gross cellular metabolism, reflected in cell growth, was therefore studied.

BHK21/C13 cells in 50 mm Petri dishes were grown normally for 18 hours in EC10 before replacement of the medium by EC5 or 10 containing 20 mM-sodium formate, and with varying levels of methionine. The cells were harvested and counted in the haemocytometer after 48 hours' further incubation. As can be seen from Figure 8, cell division is methionine-dependent, but a reasonable level of cell growth is supported by only 5% of the normal methionine concentration. Similar results were obtained using EC2 growth medium.

Thus a cell growth-requirement was shown for methionine. Since the study to be undertaken involved investigation of nucleic acid metabolism in HSV-infected cells, it was then necessary to determine the effect of methionine concentration on viral growth and nucleic acid metabolism in BHK21/C13 cells.

Figure 8

Effect of Methionine Concentration on
BHK21/C13 Cell Growth



o——o , 5% Calf Serum;

▲——▲ , 10% Calf Serum;

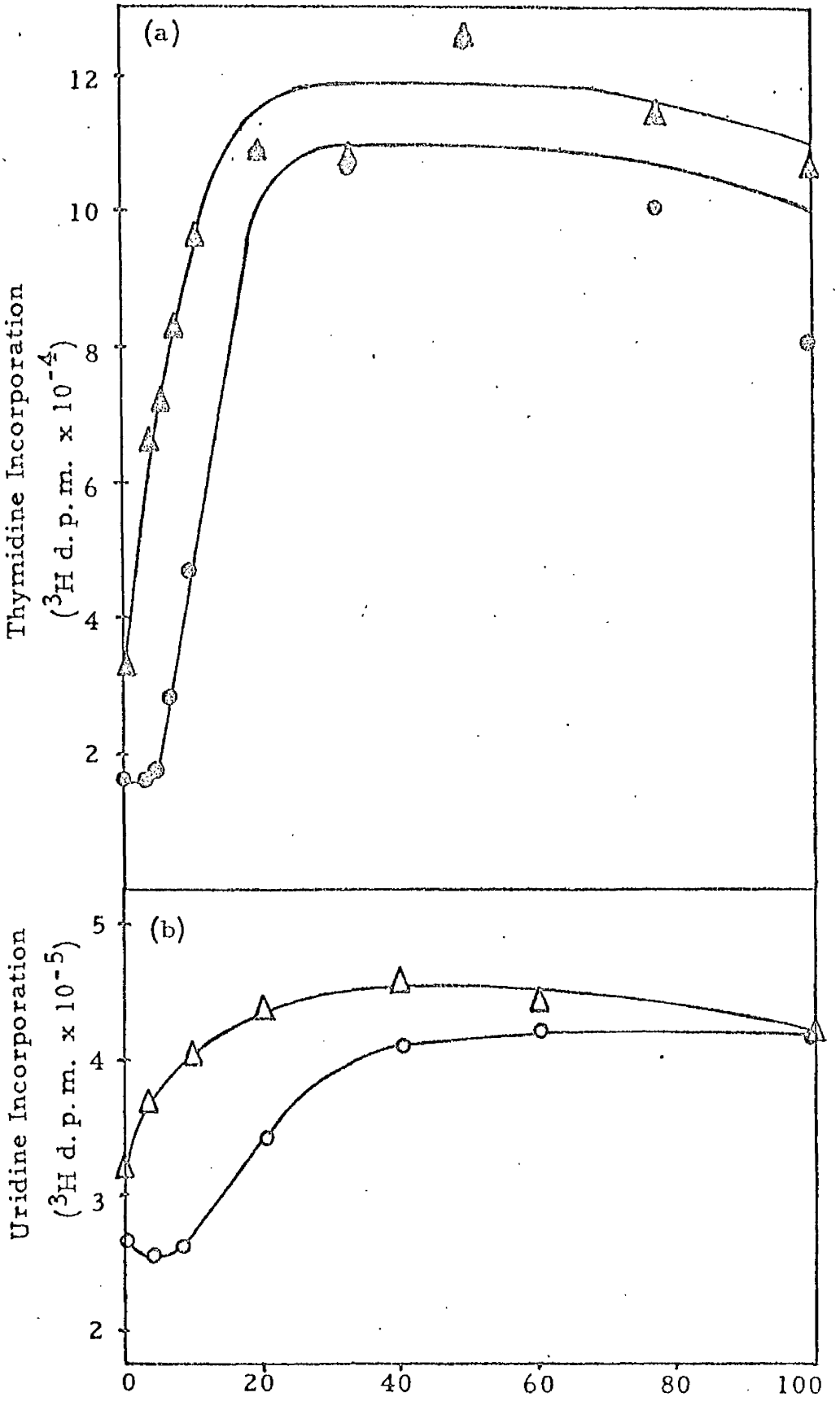
— — — , probable cell number before the medium was changed to [x% met] .

1.3. The Effect of Methionine Concentration on DNA and RNA Synthesis in BHK21/C13 Cells

Cultures of BHK21/C13 cells, grown almost to a monolayer in 50 mm Petri dishes, were preincubated for 4 hours in EC2F (i.e. EC2 made 20 mM w.r.t. sodium formate) containing different levels of methionine. Five - or 4-hour pulse-labelling, in EC2F (1.5 ml/plate) containing these different levels of methionine, was then carried out using [6 - ^{3}H]-thymidine (0.5 μC and 0.075 μmol per plate) or [5 - ^{3}H]-uridine (0.5 μC and 0.0015 μmol per plate), with the addition of unlabelled cytidine (to 5×10^{-6} M or 2×10^{-5} M respectively). After the pulse period the cells were washed with ice-cold Eagle's medium, scraped from the plates, extracted four times with 5% w/v trichloroacetic acid and washed finally with ethanol and then ether before being dissolved in 0.5 ml hyamine hydroxide for measurement of radioactivity in toluene scintillant. The results obtained for DNA and RNA synthesis are depicted in Figures 9 (a) and (b) respectively. These suggest that down to 10% normal methionine concentration the growth medium is not restrictive for DNA and RNA synthesis up to 8 hours, while up to 14 hours RNA synthesis and DNA synthesis continue normally down to concentrations of 40% and 20% normal methionine respectively. The limiting factor may thus be rate of methylation of the nucleic acid, or rate or extent of synthesis of protein. Later studies suggest that the former probably does not apply.

Figure 9.

Effect of Methionine Concentration on (a) DNA and (b) RNA Synthesis in BHK21/C13 Cells.



(a) ——— , 10 - 15 hours; (b) ——— , 9 - 13 hours;
——— , 5 - 10 hours. ——— , 5 - 9 hours.

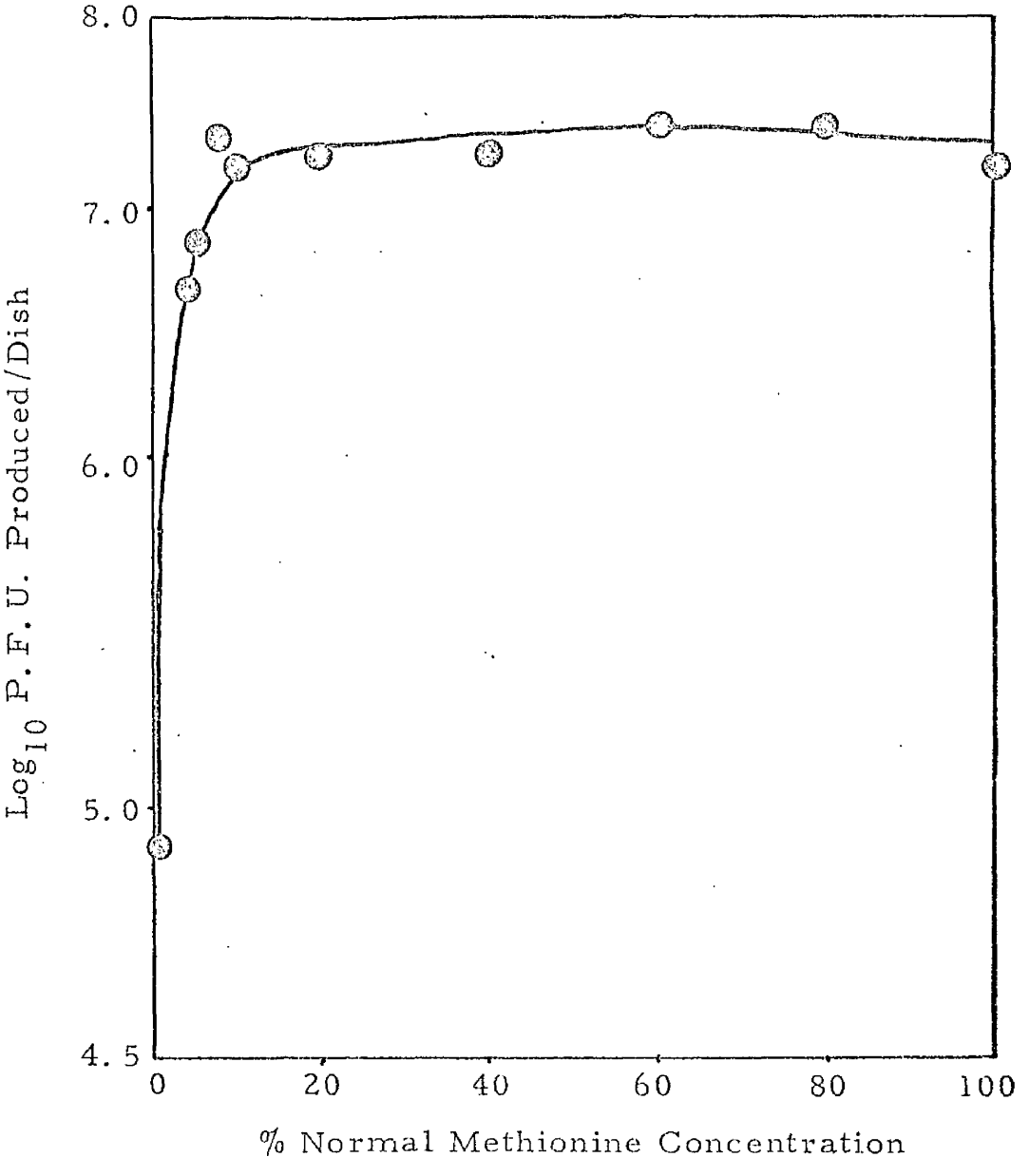
1.4. The Effect of Methionine Concentration on Herpes Simplex Virus Replication

Virus was grown as described in Methods Section 3.2. in confluent BHK21/C13 cell cultures on 50 mm Petri dishes. The cells were preincubated for 4 hours in EC2F containing different levels of methionine and then incubated for a further 42 hours after infection in 2 ml replacement medium (EC2F). Cells were broken by sonication in the incubation medium, and the resultant suspension plaque-assayed for HSV (see Methods, Section 3.3). These results (Figure 10) suggested that, as for arginine (Tankersley, 1964; Becker, Olshevsky & Levitt, 1967; Inglis, 1968), some extracellular methionine was required for the maximum formation of HSV virions. However, it also revealed that HSV was replicated normally in levels of methionine much lower than those required for normal levels of host nucleic acid synthesis (Figure 9 (a) and (b)). At 7% normal methionine concentration, virus growth was normal, while host DNA and RNA synthesis had been reduced to approximately 30% and 40% normal after 9 - 14 hours and 8 - 12 hours respectively. The use of low levels of methionine in subsistence medium provides a possible system for the study of HSV synthesis in a system with reduced host metabolism.

From these studies it was concluded that the optimum concentration of methionine to be present in the incubation medium for studies on the methylation of nucleic acid was 20%, but that this level could be reduced

Figure 10.

Effect of Methionine Concentration on HSV Growth



to 7% if necessary for studies on the formation of progeny virus.

1.5. Equilibration of Methionine between Medium and Cells

Confluent monolayers of BHK21/C13 cells on 50 mm Petri dishes were preincubated for 5 hours in EC2F [20% met] and 0.1 μC [methyl - ^{14}C] L-methionine (53.6 μC/μmol). After washing the cell sheets with the low-methionine EC2, the medium was then replaced by 1.5 ml non-radioactive EC2F [20% met], and incubation continued. The radioactivity in the medium and in the trichloroacetic acid-soluble fraction of the cells was then estimated at intervals (see Figure 11).

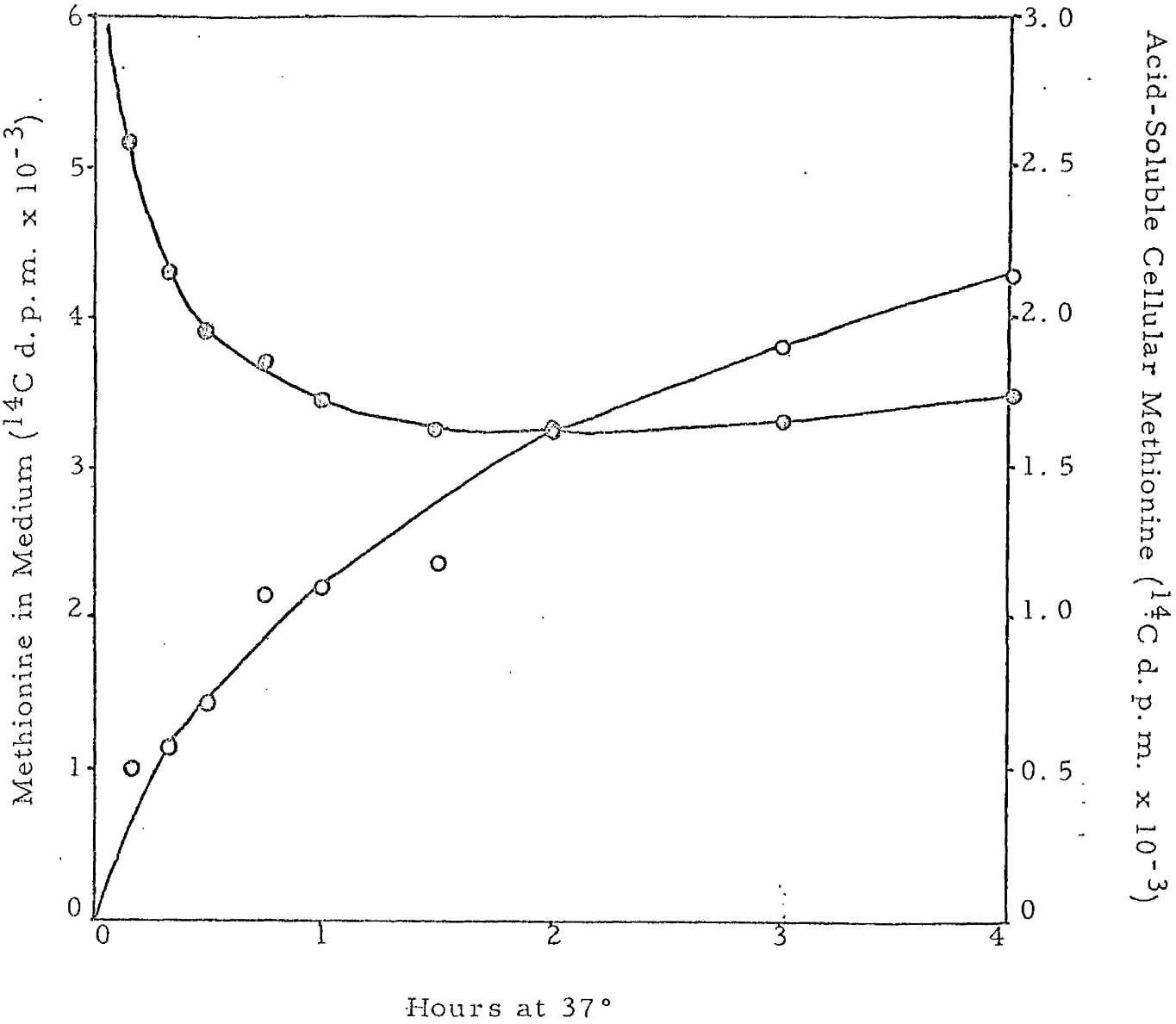
There was an initial rapid loss of radioactively-labelled methionine from the cells, which suggests a rapid exchange between cells and medium. Also, an apparent complete equilibration of methionine in the cell was attained after about $1\frac{1}{2}$ hours incubation. On the other hand the extracellular methionine took much longer to equilibrate. For this reason, although the more important factor in subsequent work was the effective equilibration of the intracellular methionine, a preincubation period of 4 hours was routinely used in experiments involving low-methionine medium.

Using values from the equilibrated system, an estimation was obtained of the "pool" size of approximately 1.5×10^{-15} mol "free" methionine per BHK21/C13 cell incubated in EC2F [20% met].

When radioactively-labelled methionine is included in incubation

Figure 11.

Equilibration of Methionine between Medium and Cells



o——o , d.p.m. in medium;

⊙——⊙ , d.p.m. in acid-soluble cellular material.

medium, it is likely that this amino acid will be diluted out by intracellular unlabelled material. The above result suggested a dilution factor of approximately 1.25 when 20% normal methionine was used in EC2. In Section 1.6 further estimations of the dilution factor are described which are consistent with this value.

1.6. Estimation of a Methionine Dilution Factor for BHK21/C13 Cells

1.6.1. Amino acid analysis

A confluent monolayer of BHK21/C13 cells was incubated in an 80 oz roller bottle for 8 hours in EC2F [7% met] at a final specific activity of 15 μC/μmol [methyl - ^{14}C] - L-methionine. Cells were scraped off and suspended briefly in ice-cold 0.1M-tris-HCl buffer, pH 7.7 and 0.15M-NaCl, and then in 0.5M-perchloric acid. After homogenization, cell debris was washed with perchloric acid, the supernatants pooled, neutralized with KOH, and freeze-dried. A sample of the medium used was made 0.5N with respect to perchloric acid, neutralized with KOH and also freeze-dried. Samples of cell extract and medium were dissolved in water and the methionine content measured by automatic amino acid analysis (by courtesy of Drs. G. Leaf and W. Wunner). Radioactivity was measured in dioxan scintillant. Specific activities calculated for the cell extract and medium were 11.6 μC/μmol and 14.0 μC/μmol methionine respectively, indicating a dilution factor of 1.2.

1.6.2. Comparison of Incorporation of [^{3}H] - and [^{14}C] - methyl-labelled methionine

A confluent monolayer of BHK21/C13 cells in an 80 oz roller bottle was preincubated for 4 hours in EC2F [7% met]. This medium was then replaced by the same mixture containing 3 μC [methyl - ^{14}C] - L-methionine (53.7μC/μmol) to give a final specific activity of 0.77 μC/μmol, and 1 μC [methyl - ^{3}H] -L-methionine (5.4 mC/μmol) to give a final specific activity of 3 μC/μmol. The medium was sampled after intervals at 37° and its radioactive content measured. It was found that both isotopes were taken up by the cells at an equal rate. Finally the cells were washed 5 times with ice-cold Eagle's medium, removed from the plate with versene, precipitated and washed 5 times with 5% w/v trichloroacetic acid at 0°. After drying, the precipitates were dissolved in 0.5 ml hyamine hydroxide for counting in toluene scintillant. Summation of relative specific activities of methionine originally present in the medium was equated with those of the acid-precipitable cell material after 24 hours. It was assumed that total incorporation of isotope into acid-insoluble material (mainly protein) over a relatively long time interval reflected the specific activity of intracellular methionine "pools", and that the rate of protein synthesis was not dependent on the level of methionine in the medium under the conditions employed. The dilution factor thus obtained was 1.3. A similar study employing labelling of BHK21/C13

cells infected with HSV, or mock-infected gave results indicative of dilution factors of 1.0 and 1.5 respectively.

1.6.3. Methylation of newly-synthesized BHK21/C13 cell DNA

(a) Newly confluent cultures of BHK21/C13 cells in 80 oz roller bottles were preincubated in 50 ml EC2F [100% met] or [20% met] and incubation continued with 50 ml of fresh medium for a further 5 hours. [6 - ^{3}H]-thymidine and [methyl - ^{14}C]-L-methionine were then added to give final specific activities of 2.8 μC/μmol (at 5 x 10^{-5}M) and 7.6 μC/μmol respectively, and deoxycytidine to give a final concentration of 5 x 10^{-6}M. After a further 5 hours' incubation, cultures were extensively washed with medium, the cells removed with trypsin and further washed with ice-cold R.S.B. before DNA was extracted by the method of Marmur (1961). The DNA was purified by CsCl equilibrium centrifugation (Methods, Section 4.2.1) and fractions from this analysis which contained DNA were pooled and estimated for radioactivity in toluene scintillant after precipitation on millipore filters.

In cells incubated in medium containing the normal concentration of methionine, one mole of nucleotide was methylated per 71 moles of newly synthesized nucleotide, assuming the base content of this DNA was 30% thymine and that the rates of synthesis and methylation were equal, after a 30 min lag in the case of the latter, as in HeLa cells (Burdon & Adams, 1969). In cells incubated in medium containg 20%

normal methionine concentration 0.75 mole% methylation of newly synthesized DNA nucleotide was indicated. But analysis of DNA suggested approximately $^{1}/_{3}$ of the methionine incorporated appeared as thymine (see later). Thus, on this basis, the frequency of occurrence of 5-methylcytosine was calculated for the two exogenous methionine levels to be 1.07% and 1.13% DNA nucleotide respectively. Similarly, a level of 1.05 mole % methylated nucleotide was indicated in newly-synthesized DNA from cells incubated in EC2F [7% met]. Comparison of these values with those obtained for 5-methylcytosine by chemical estimation (Wyatt, 1951; Antonov, Favorova & Belozerskii, 1962; Vanyushin, Tkachera & Belozersky, 1970) (0.9 to 1.3 mole % methylation of nucleotide) indicated very small or negligible dilution factors.

Calculation of "free" methionine "pool" sizes suggested values of 2×10^{-15} mol/cell and 10^{-15} mol/cell from data from Sections 1.6.2 and 1.6.3 respectively. These values are in agreement with that of 1.5×10^{-15} mol/cell obtained from methionine equilibration studies (Section 1.5).

A summary of methods used and values obtained for methionine dilution factor estimation is given in Table II.

These estimations, with one exception, were made for dilution of methionine by uninfected BHK21/C13 cells. It was still considered possible that infection of these cells by HSV causes increased

Table II

Effect of Dilution of Medium Methionine by Intracellular Methionine Pool

Section Number	Method	Methionine Concentration	Dilution Factor
1.6.1.	Amino Acid Analysis	7% Normal	1.2
1.6.2	^{3}H-and ^{14}C-Incorporation	7% Normal	1.3
1.5.	Equilibration Study	20% Normal	1.25
1.6.3.	Newly Synthesized DNA	7% Normal	1.05
1.6.3.	Newly Synthesized DNA	20% Normal	1.13
1.6.3.	Newly Synthesized DNA	7% Normal	1.07

degradation of protein, and that this augments the cellular methionine "pools". A study of the involvement of methionine in protein metabolism during HSV-infection of BHK21/C13 cells was therefore carried out.

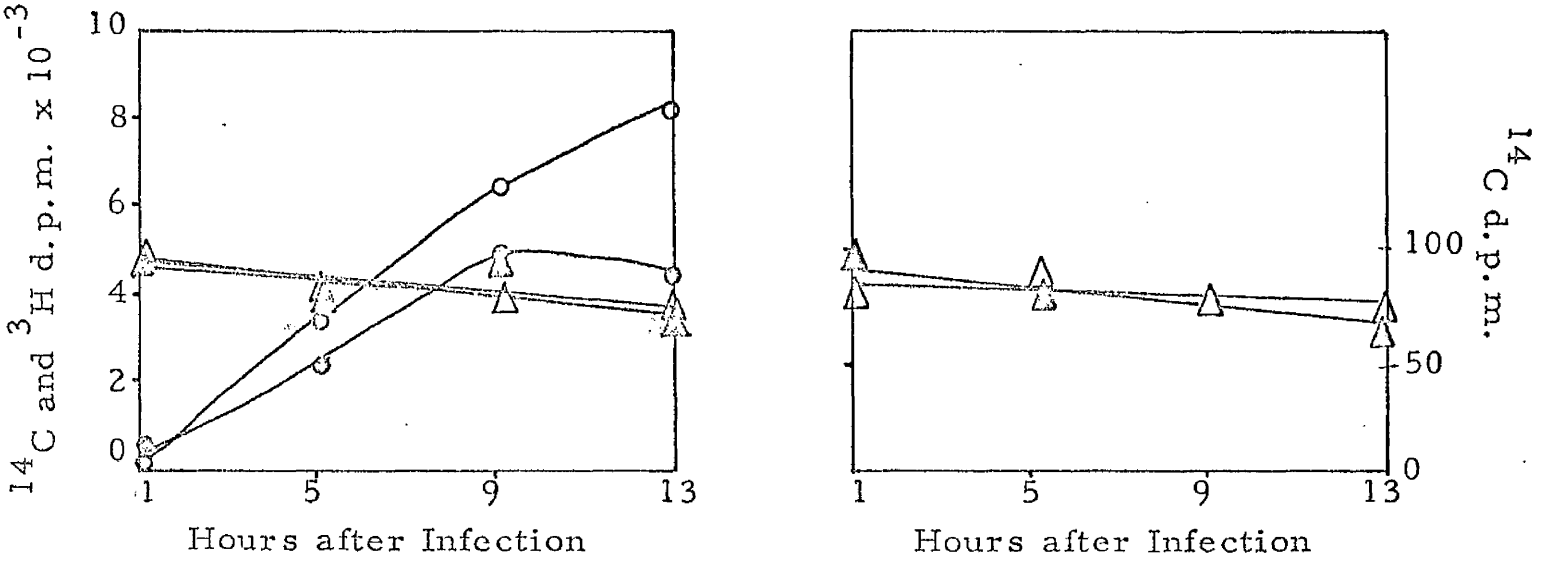
1.7. Protein Metabolism in HSV-Infected BHK21/C13 Cells

BHK21/C13 cells were grown to confluence on 90 mm Petri dishes in EC2F in the presence of [methyl - ^{14}C] -L-methionine at a final specific activity of 6μC/μmol, washed extensively with EC2, and incubated for 24 hours at 37° in 10 ml EC2. Cultures were infected with HSV (10 P.F.U./cell) or mock-infected, and incubated in EC2F containing [methyl - ^{3}H] -L-methionine at a final specific activity of 1.5 μC/μmol after removal of excess virus at the end of 1 hour's adsorption. At intervals, the medium was sampled and the cells removed and homogenized in 0.5 ml R.S.B. at 0°.

Figure 12 (a) depicts total protein synthesis and breakdown and dilution measured by continual monitoring of the methionine label (^{3}H and ^{14}C respectively) during the course of the experiment. The overall rate of protein synthesis (as measured by methionine incorporation) is seen to have been slightly lower in virus-infected cells than in uninfected cells. Over a 9 hour period, protein synthesis in the infected cells, measured by methionine uptake, amounted to 80%

Figure 12

Methionine Metabolism in Control and HSV-infected BHK21/C13 Cells



BHK21/C13 cells grown in the presence of [methyl - ^{14}C] - L-methionine were washed, mock-infected, or infected with HSV (10 P.F.U./cell) and incubated in EC2F containing [methyl - ^{3}H] -L-methionine. (a) Acid-insoluble and (b) acid-soluble cell material.

△——△ , Control BHK21/C13 cell ^{14}C d.p.m.;

▲——▲ , HSV-infected BHK21/C13 cell ^{14}C d.p.m.;

o——o , Control BHK21/C13 cell ^{3}H d.p.m.;

⊙——⊙ , HSV-infected BHK21/C13 cell ^{3}H d.p.m.

of control protein synthesis. This result is in agreement with the findings of Kaplan & Ben-Porat (1959), Roizman, Borman & Rousta (1965), Hamada & Kaplan (1965) and Sydiskis & Roizman (1966) who showed that protein synthesis continues in HSV-infected cells (as measured by arginine and leucine incorporation) at a level slightly below that of the host cell.

It is interesting to note, however, that at around 13 hours P.I. very little apparent synthesis takes place (Figure 12 (a)) and this is perhaps due to virus release balancing the uptake of precursor. Fractionation of the cells (Methods, Section 4.1) showed that reduction in protein synthesis occurred in each fraction (soluble, ribosomal and nuclear) after infection in a manner similar to that found for the whole cell (Figure 12 (a)).

The decrease in specific activity of infected and uninfected cell protein labelled during the preincubation period prior to infection (represented by ^{14}C content) (Figure 12 (a)) can be equated with the turnover of protein in these cells.

The levels of radioactivity from [methyl - ^{14}C] -L-methionine (with which the cultures had been preincubated) in the acid-soluble fraction of infected and uninfected cells (Figure 12 (b)) showed no significant differenceover the time period studied, while the ^{3}H levels, from [methyl - ^{3}H] -L-methionine present in the medium during

incubation, in this fraction were identical throughout the experiment. Thus the results of this experiment indicate that there is no appreciable difference between infected and uninfected cells in terms of methionine uptake and release, and therefore it was assumed in subsequent experiments that the dilution effects in HSV-infected cells were similar to those in uninfected cells.

2. SYNTHESIS AND METHYLATION OF TOTAL NUCLEIC ACID FROM HSV-INFECTED CELLS

2.1. MAK Column Fractionation

In an initial study of nucleic acid methylation, BHK21/C13 cell cultures, in 80 oz roller bottles, were incubated at 37° from 1 to 5 or 1 to 9 hours after infection with HSV (80 P.F.U./cell) in 50 ml EC2F containing 20 μC [methyl - ^{14}C] -L-methionine (56.8 mC/mmol) at a final specific activity of 28 μC μmol. The cells were harvested using versene, and thoroughly washed with cold medium before extraction of the nucleic acid by a modification of the method of Saito & Miura (1963) (Methods, Section 6.1).

Fractionation of the nucleic acids on MAK columns was carried out as described in Methods, Section 4.2.2. The NaCl eluting gradient was measured by refractometry (Methods, Section 5.2.). This technique was employed in an attempt to obtain fractionation of DNA from the major species of RNA. In this, it was successful.

Figure 13.

Fractionation of Nucleic Acids by MAK Column Chromatography

Total nucleic acid was extracted, from uninfected and HSV-infected BHK21/C13 cells incubated in EC2F [7% met] and labelled for the times shown below with [methyl-^{14}C] -L-methionine, by a cold phenol method. The samples were applied to the column in S.S.C., and eluted with a NaCl gradient in 0.2 M-phosphate buffer, pH 6.3 at 2 ml/min. Samples were precipitated on millipore filters with trichloroacetic acid for counting in toluene-based scintillant.

o——o, u.v.-absorbance.

●——●, ^{14}C d.p.m. from [methyl-^{14}C] -L-methionine.

Δ——Δ, NaCl gradient.

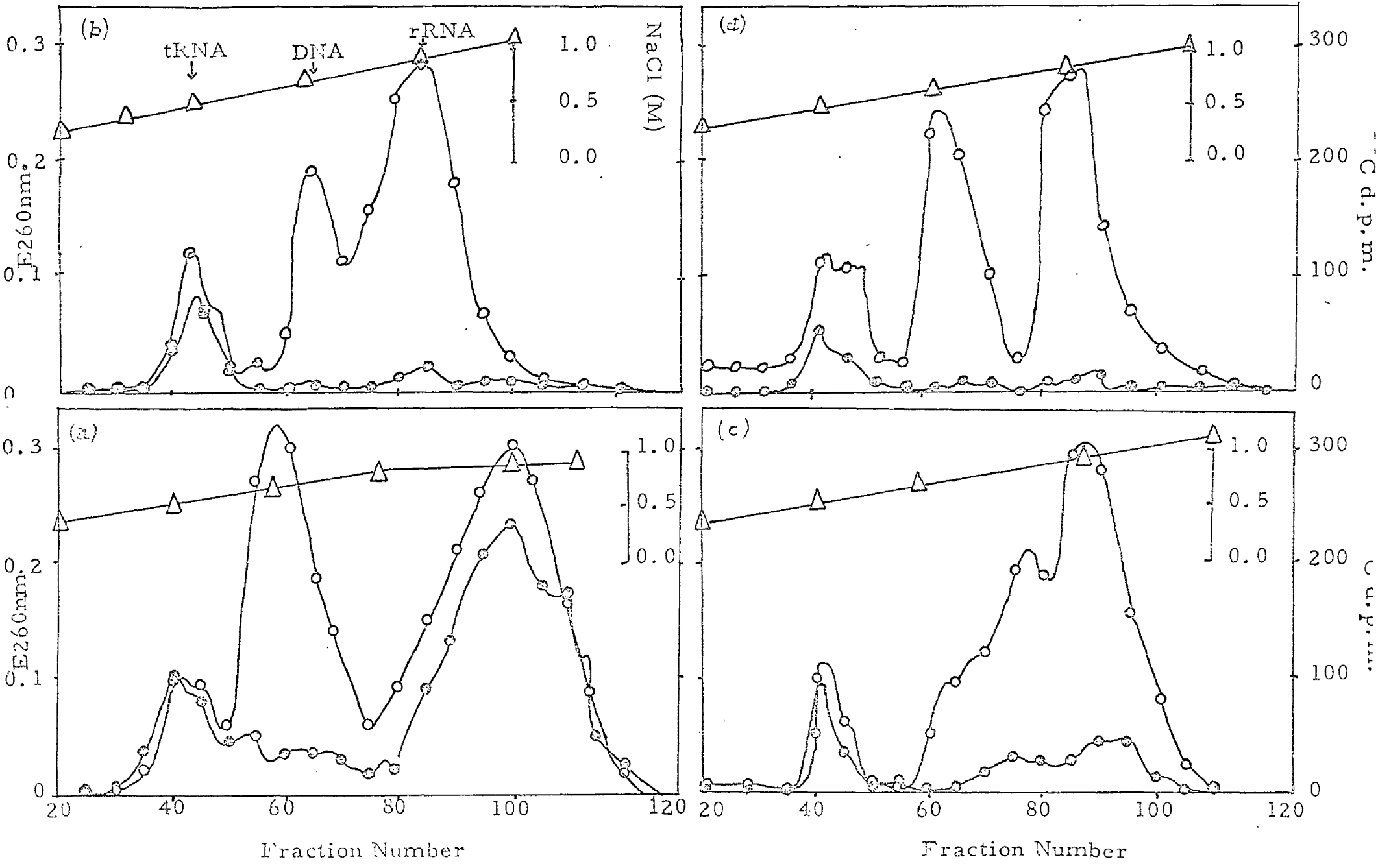
(a) uninfected BHK21/C13 cells pulsed 8 hours.

(b) HSV-infected BHK21/C13 cells pulsed 1 - 9 hours P.I.

(c) uninfected BHK21/C13 cells pulsed 4 hours.

(d) HSV-infected BHK21/C13 cells pulsed 1 - 5 hours P.I.

Figure 13


(b)
tRNA
DNA
rRNA
1.0
0.5
0.0
NaCl (M)
0.3
0.2
0.1
0
E260nm.
(d)
300
200
100
0
c.p.m.
(a)
(c)
20
40
60
80
100
120
Fraction Number

The results (Figure 13) showed that the total extent of methylation of each of the species fractionated, tRNA, DNA and rRNA, eluting from the column with 0.52M, 0.68M and 0.88M NaCl respectively was lower in infected cells than in control cells. There was a marked fall in rRNA methylation, especially over the 9 hour labelling period, there being apparently negligible further incorporation of methyl groups after the 1 - 5 hour pulse, while tRNA methylation was much less affected. A fall in DNA methylation proved difficult to quantitate owing to low levels of incorporation even in the control cells. These results probably reflect a decrease in synthesis of nucleic acid after HSV infection, and, therefore, attempts were made to investigate this using the above technique. However, this type of fractionation proved to be unreliable for the separation required, and was therefore abandoned in favour of a more accurate fractionation procedure.

2.2. Fractionation of Nucleic Acids on the Basis of Cellular Location

In place of a technique which separated DNA, tRNA and rRNA, the following procedures were routinely used primarily to fractionate DNA from cytoplasmic RNA, which then could easily be further fractionated.

After preincubation in EC5F [7% met], 80 oz roller bottle cultures of BHK21/C13 cells were infected with HSV (10 P.F.U./cell)

or were mock-infected. Following one hour's adsorption at 37°, the cultures were incubated for 4 hours in EC2F [7% met] before the addition of [G - ^{3}H] -uridine and [methyl - ^{14}C] -L-methionine at final specific activities of 12.5 μC/μmol and 39 μC/μmol, respectively, and cytidine (to 3 x 10^{-5}M) for a further 4 hours' incubation at 37°, i.e. 5 to 9 hours P.I. The cytidine was added to prevent the incorporation of label from labelled cytidine via labelled uridine into the rapidly-turned-over CCA-terminus of tRNA. The concentration used was found to have no inhibitory effect on cellular RNA metabolism.

After the cells had been extensively washed, they were fractionated by osmotic disruption using the method of Becker & Joklik (Methods, Section 4.1.). The cytoplasmic soluble fraction and the ribosomes were separated centrifugally as described in Methods, Section 4.1. and the RNA isolated from them by the phenol method (Methods, Section 6.2). These RNA fractions were further purified by treatment with DNase (50 μg/ml) to remove contaminating DNA followed by pronase (100 μg/ml) to remove any residual protein and to deacylate any radioactively-labelled methionyl-tRNA, and finally two further extractions with phenol. The RNA was then precipitated from ethanol, washed with ethanol, ethanol-ether (3:1 v/v) and ether. Additionally, the rRNA fractions were purified by MAK column

chromatography (Methods, Section 4.2.3). DNA was extracted from the nuclear fraction by the modified method of Marmur (Methods, Section 6.6.(a)). After precipitation from ethanol, the DNA was washed twice with ethanol, and once each with ethanol-ether (3:1 v/v) and with ether. Such DNA samples were further purified by CsCl density gradient centrifugation (Methods, Section 4.2.1). Fractions containing DNA were pooled, diluted 1:5 with 0.01M-tris-HCl buffer, pH 8.2 and pelleted by centrifugation (100,000 g, 4°, 18 hour). The specific activity of each nucleic acid sample was measured and the results are summarized in Table III.

In Table III, the ratio of incorporation of ^{14}C from [methyl - ^{14}C] - L-methionine to ^{3}H from [G - ^{3}H] -uridine reflects the specific activity of newly synthesized RNA. In this experiment treatment with pronase was carried out to remove methionine acylated to tRNA, and to digest any protein which had survived the phenol extraction, and it is evident that in BHK21/C13 cells methylation of newly synthesized tRNA is apparently inhibited by 45% 5 - 9 hours after infection with HSV. Further, from these results it would appear that methylation of newly-synthesized rRNA was increased on HSV infection of BHK21/C13 cells, but this may well have been caused by inaccuracy of measurement of ^{3}H in the relative ^{14}C and ^{3}H levels present in the infected cell rRNA preparation. It is clear, however, that rRNA synthesis was inhibited

(93%) much more than the cytoplasmic soluble RNA (25%), as has previously been shown (Hay, Kötteles, Keir & Subak-Sharpe, 1966; Wagner & Roizman, 1969), and as found with MAK (Section 2.1).

As was evident from results involving MAK fractionation, this present study showed that gross DNA methylation decreased in BHK21/C13 cells after infection with HSV. However, this is likely to be related, at least partly, to inhibition of host DNA synthesis by the virus.

Similar studies have subsequently been carried out to compare RNA synthesis and methylation in two cell types after infection with HSV, examining particularly the RNA labelled from 1 to 7 hours P.I. These cell lines were BHK21/C13 and HEp-2 in which similar patterns of RNA metabolism were observed. Methylation of newly-synthesized tRNA was inhibited in both systems, and, in contrast to the results shown in Table III, methylation of newly-synthesized rRNA was lower in infected than in uninfected cells. It was clear that the pattern emerging was one of inhibition of synthesis of tRNA and rRNA and also inhibition of methylation of these species. It was not,however, apparent from these quantitative studies whether the latter inhibition was dependent only on the former, or whether some additional factor was involved. The following qualitative study was therefore carried out.

Table III

Methylation of Nucleic Acids Fractionated on the Basis of Cellular Location

Sample	µg used	^{14}C d.p.m./µg	^{3}H d.p.m./µg	$^{14}C/^{3}H$
BHK21/C13 DNA	23	14	-	-
BHK21/C13/HSV DNA	25	6.8	-	-
BHK21/C13 rRNA	377	128	257	0.5
BHK21/C13/HSV rRNA	115	32	18	2.1
BHK21/C13 soluble RNA	46	366	156	2.3
BHK21/C13/HSV soluble RNA	45	197	134	1.3

^{14}C d.p.m. derived from [methyl - ^{14}C] -L-methionine; ^{3}H d.p.m. derived from [5 - ^{3}H] -uridine. Samples were prepared and estimated as described in Section 2.2.

2.3. Determination of the Methylated Base Pattern in Soluble RNA After Infection of BHK21/C13 Cells with HSV

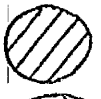
Purified cytoplasmic soluble RNA was obtained from BHK21/C13 cells, infected with HSV (10 P.F.U./cell), which had been incubated in EC2F containing [methyl - ^{14}C] - L-methionine at a final specific activity 3.4 μC/μmol from 1 to 7 hours P.I. Samples of the RNAs were hydrolyzed by incubating for 18 hours at 37° with 0.3M-KOH followed by neutralization with perchloric acid. The hydrolysates were chromatographed on Whatman 1MM paper (Methods, Section 5.3.2) and autoradiograms made on X-ray film over a period of 3 months' exposure. A comparison of the results from this study of the base content of soluble RNA from BHK21/C13 cells (control and infected with HSV) is shown in Figure 14. Eight, and possibly nine, methylated species were found, and their locations on the two chromatograms allowed superimposition of the autoradiograms. The autoradiograms of control and infected samples did not differ to any significant qualitative extent except in one case, spot No. 4, this being present on the control film but not on the film from HSV-infected BHK21/C13 soluble RNA hydrolysate. The areas of the chromatograms corresponding to each spot on the X-ray films were cut out and counted using a low-background gas flow counter. These results are given in Table IV. Minor differences have probably arisen because of the

Figure 14

Soluble RNA extracted from BHK21/C13 Cells infected with HSV or mock-infected as detailed in Section 2.3 was hydrolyzed and subjected to chromatography on Whatman 1MM paper. Solvent for 1st direction was iso-butyric acid: 0.5M-NH_4OH (50:30 v/v); solvent for 2nd direction was iso-propanol: conc HCl: H_2O (53.5: 19.3: 15.6 by weight).

 , u-v absorbing spots.

Autoradiograms obtained by exposing X-ray films to the chromatograms for 3 months were compared.

 , Spot present on Control Film;

 , Spot present on Infected Film.

Table IV

Spots arbitrarily numbered as shown in Figure 14 were excised from the chromatograms and measured for radioactivity using a Low Background Gas Flow Counter. Average c.p.m. and the % contributions from each spot are listed.

Figure 14

Autoradiographs of Hydrolysis Products of RNA from BHK21/C13 Cells (Control and Infected)

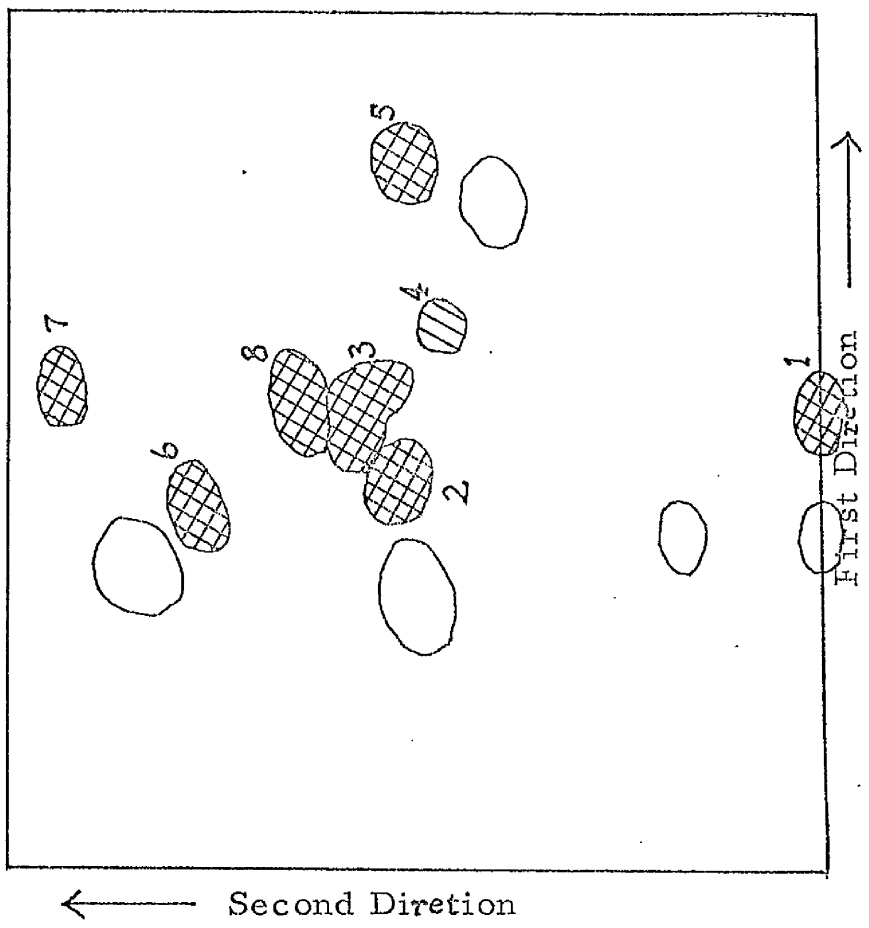


Table IV

Spot No.	^{14}C c.p.m.	% Total	Spot No.	^{14}C c.p.m.	% Total
Infected 1	17	4.3	Control 1	16	2.6
Infected 2	49	11.6	Control 2	76	12.6
Infected 3	82	19.5	Control 3	136	22.6
Infected 4	4	0.9	Control 4	21	3.5
Infected 5	51	12.1	Control 5	64	10.7
Infected 6	79	18.7	Control 6	89	14.8
Infected 7	46	10.9	Control 7	98	16.7
Infected 8	93	22.1	Control 8	130	21.7

difficulty of excising areas from the two chromatograms in an identical fashion. Again, however, the only major quantitative difference occurred in spot No. 4, which gave approximately background activity in the case of the HSV-infected material, but gave a significant count in the case of the control material. A possible second quantitative difference, in spot No. 7, was also apparent, again showing a loss of activity in the infected cell relative to the control cell. It is possible that the presence of some activity in spots 4 and 7 in the X-ray films of HSV-infected material arose from host RNA synthesis in uninfected (or infected) cells during the period that the cultures were labelled.

2.4. Comparison of HSV with Pseudorabies Virus

Since pseudorabies virus and HSV are closely related, it seemed proable that the former virus could be used as a comparison for the effect of Herpes Viruses on RNA synthesis and methylation.

Studies were carried out in BHK21/C13 cells and RK cells infected with pseudorabies virus (10 P.F.U./cell). In this case incubation with isotope was from 1 to 5 hours of virus infection, otherwise the procedure was as already defined (Section 2.2.). The results in the two cell systems were similar, and those from BHK21/C13 cells are summarized in Table V. It is clear that, after pseudorabies

Table V

Methylation of Newly-synthesized RNA in Control and Pseudorabies Virus (PRV)-infected BHK21/C13 Cells

Sample	14C d.p.m.	^{3}H d.p.m.	$^{14}C/^{3}H$
Control BHK21/C13 soluble RNA	895	359	2.5
PRV-infected BHK21/C13 soluble RNA	1727	1079	1.4
Control BHK21/C13 rRNA	1228	1104	1.1
PRV-infected BHK21/C13 rRNA	587	1214	0.5

^{14}C d.p.m. from [methyl - ^{14}C]-L-methionine

^{3}H d.p.m. from [5 - ^{3}H]-uridine

Multiplicity of infection was 11 P.F.U./cell, and the pulse-labelling period was 1 - 5 hours P.I.

virus infection methylation of newly synthesized RNA of both species (tRNA and rRNA) was inhibited, and that the soluble RNA was inhibited slightly less than the rRNA. Although not to exactly the same extent as in HSV infection, the synthesis of these species was also inhibited. Thus the methylation of newly synthesized soluble RNA and rRNA have been shown to be similarly inhibited in different cell systems after infection by the related viruses, pseudorabies and HSV.

It seemed probable, therefore, that the inhibition of methylation was caused by the virus, or by something induced by the virus, and that a knowledge of the time of inhibition might be indicative of its source.

3. TIME COURSE OF RNA METHYLATION IN HSV-INFECTED CELLS

3.1. In Exponentially-Growing BHK21/C13 Cells

3.1.1. Infected at a multiplicity of infection of 10 P.F.U./cell

Confluent monolayers of BHK21/C13 cells on 90 mm Petri dishes were treated as previously described. Cultures incubated in EC2F [20% met] were pulsed for 90 minutes with [methyl - ^{14}C] - L-methionine and [5 - ^{3}H] -uridine at final specific activities of 56 μC/μmol and 1.1 μC/nmol respectively, and cytidine (to 3.2×10^{-6}M). Cells were washed extensively before and after removal by scraping, and the nucleic acid from the cytoplasm purified by double phenol extraction. An overnight incubation with pronase (100 μg/ml) was then included to digest any remaining protein, and to remove amino acids acylated to the

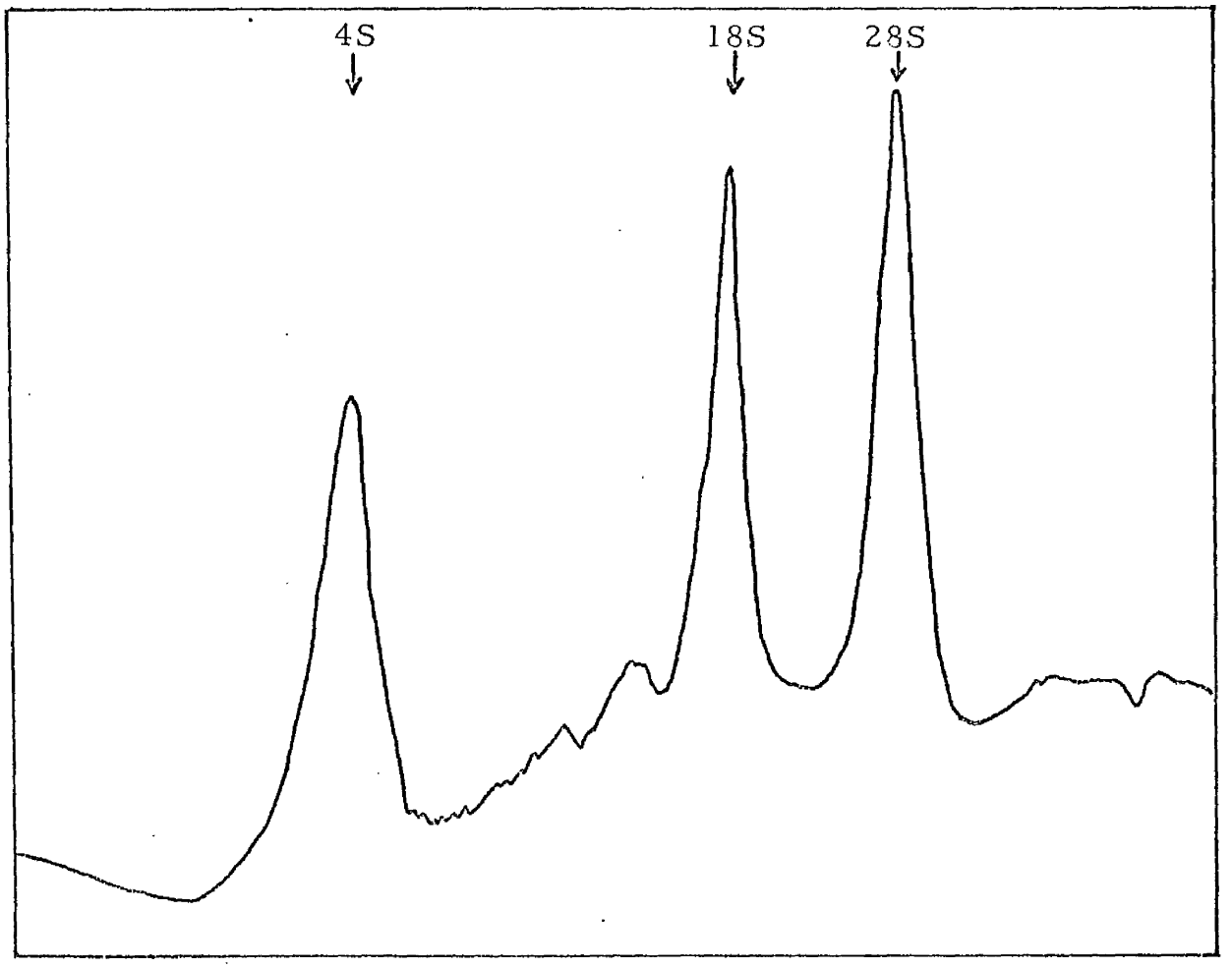
tRNA. The nucleic acid was further purified by phenol extraction before precipitation from ethanol - 2% w/v potassium acetate. "Macaloid" (0.0025 μg/ml) was present throughout the preparation, and in the 0.02M-tris-acetate buffer, pH 7.9, containing 0.005M-EDTA used to dissolve the nucleic acid for its analysis by agarose gel electrophoresis (Methods, Section 4.2.3.). A "Vitatron" tracing of the stained 2% w/v agarose gel after electrophoresis (Methods, Section 4.2.3.) of a sample of RNA from control cells together with a sample from infected cells (a total of 15 μg RNA) is shown in Figure 15.

Results for the main species of RNA are summarized in Table VI. These are shown graphically in Figure 16. From these results it appears, from the $^{14}C/^{3}H$ ratios, that there is probably no significant change in the extent of methylation of newly-synthesized rRNA (cf. Section 2.2). Again, however, a decrease in the extent of methylation of newly-synthesized "4S" material was indicated, as previously shown (Table III). No values could be obtained for the relative levels of synthesis of the different species of RNA, as quantitative estimation could not be obtained from the gels.

At least 4 species of RNA were detected in the agarose gels, corresponding in "S" value to approximately 45, 28, 18 and 4. The first-mentioned band disappeared early in infection, there being no

Figure 15.

"Vitatron" Tracing of Toluidine-blue Stained RNA Species Separated by Electrophoresis on a 2% Agarose Gel.



← Direction of Running

Figure 16

Methylation of Newly-synthesized RNA in Exponentially-growing BHK21/C13 Cells Infected with HSV (10 P.F.U./Cell)

Purified cytoplasmic RNA from (a) BHK21/C13 cells, pulsed 90 min in EC2F [20% met] with [methyl - ^{14}C]-L-methionine and [^{3}H - 5]-uridine, and HSV-infected BHK21/C13 cells pulsed (b) 1 - 2½ hours P.I., (c) 2½ - 4 hours P.I., (d) 4 - 5½ hours P.I., (e) 5½ - 6 hours P.I. and (f) 6 - 7½ hours P.I. was fractionated by electrophoresis on 2% agarose gels.

o—o, ^{14}C d.p.m. from [methyl - ^{14}C]-L-methionine;

o—o, ^{3}H d.p.m. from [5 - ^{3}H]-uridine.

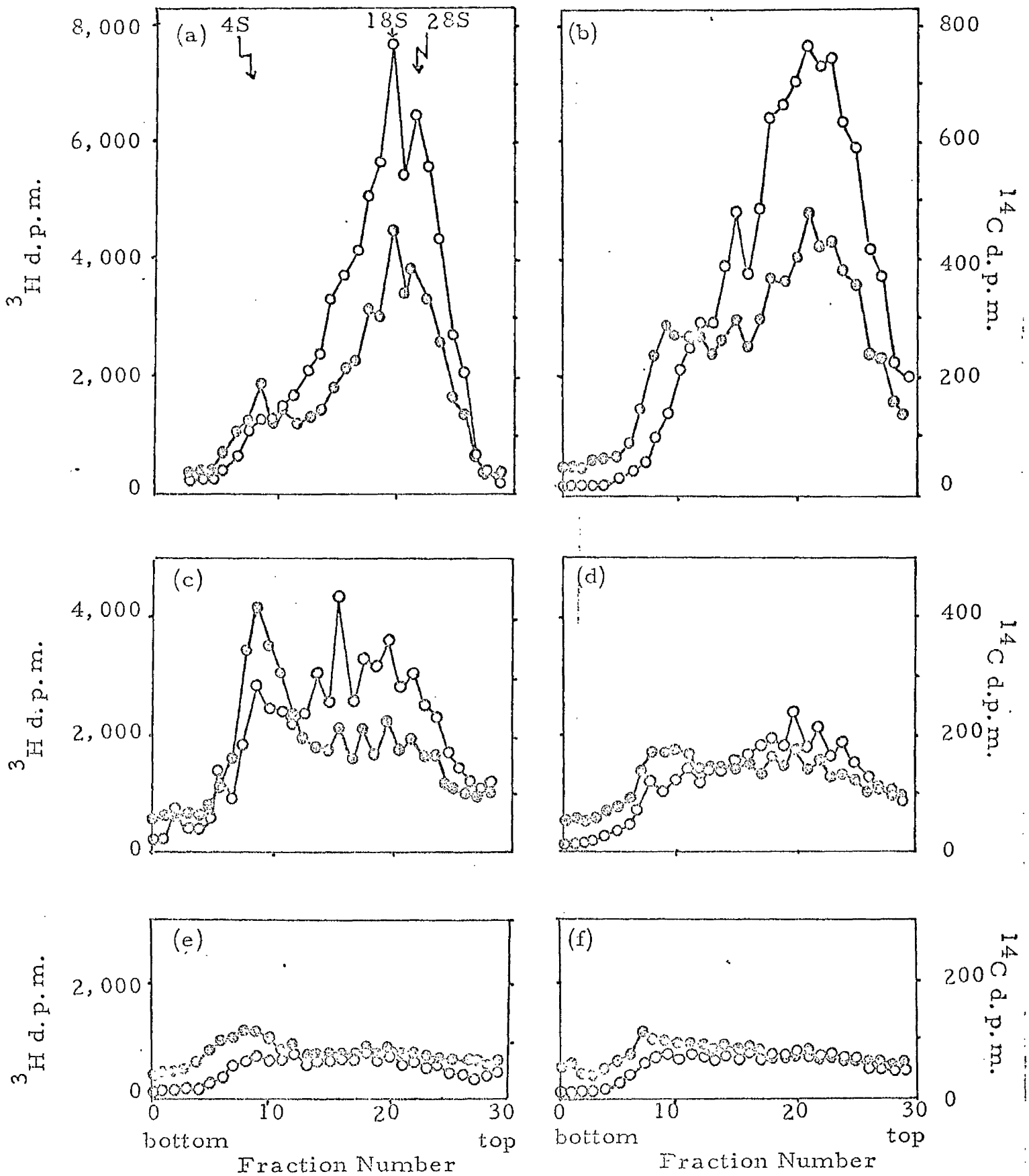

(a)
4S
18S
28S
8,000
6,000
4,000
2,000
0
^{3}H d.p.m.
(b)
800
600
400
200
0
^{14}C d.p.m.
(c)
4,000
2,000
0
^{3}H d.p.m.
(d)
400
200
0
^{14}C d.p.m.
(e)
2,000
0
^{3}H d.p.m.
(f)
200
0
^{14}C d.p.m.
0
10
20
30
bottom
top
Fraction Number
0
10
20
30
bottom
top
Fraction Number

Table VI

Time-Course of Methylation of Newly-Synthesized RNA in Exponentially-growing BHK21/C13 Cells Infected with HSV (10 P.F.U./Cell)

Species	"45S"			"28S"			"18S"			"4S"		
Source and Labelling Time	^{14}C d.p.m.	^{3}H d.p.m.	$\frac{^{14}C}{^{3}H}$	^{14}C d.p.m.	^{3}H d.p.m.	$\frac{^{14}C}{^{3}H}$	^{14}C d.p.m.	^{3}H d.p.m.	$\frac{^{14}C}{^{3}H}$	^{14}C d.p.m.	^{3}H d.p.m.	$\frac{^{14}C}{^{3}H}$
Uninfected BHK21/C13	272	4,194	0.065	659	11,102	0.065	240	15 272	0.076	387	1,925	0.193
BHK21/C13/HSV 1 - $2\frac{1}{2}$ hours P.I.	245	4,205	0.058	433	7,464	0.058	482	7,651	0.063	288	2,011	0.143
BHK21/C13/HSV $2\frac{1}{2}$ - 4 hours P.I.	-	-	-	194	3,071	0.063	225	3,631	0.062	412	2,835	0.145
BHK21/C13/HSV 4 - $5\frac{1}{2}$ hours P.I.	-	-	-	153	2,140	0.071	185	2,433	0.076	168	925	0.181
BHK21/C13/HSV $5\frac{1}{2}$ - 7 hours P.I.	-	-	-	-	-	-	-	-	-	113	738	0.151
BHK21/C13/HSV 7 - $8\frac{1}{2}$ hours P.I.	-	-	-	-	-	-	-	-	-	96	767	0.125

^{14}C d.p.m. from [methyl - ^{14}C]-L-methionine; ^{3}H d.p.m. derived from [5 - ^{3}H]-uridine after agarose gel fractionation of cytoplasmic RNA

alteration in the extent of methylation of this species. In each sample where there was significant incorporation of ^{14}C and ^{3}H into the species, 18S rRNA was found to be slightly more methylated than 28S rRNA. However, synthesis as well as methylation of both of these species was seen to decline quite rapidly after infection of BHK21/C13 cells with HSV. Synthesis of "4S" RNA was inhibited more slowly, although methylation appeared to be affected early in infection. There appeared to be a slight increase in methylation of this species of RNA 4 - $5\frac{1}{2}$ hours P.I. over that occurring $2\frac{1}{2}$ - 4 hours P.I. By 7 - $8\frac{1}{2}$ hours P.I., however, methylation of "4S" RNA had been inhibited 35% when compared to the uninfected species.

It seemed possible that the effect of HSV on the methylation of RNA species was less pronounced because of residual host synthesis and methylation in uninfected cells, and therefore it was decided to compare these results with those from cultures infected with virus at 5 times the input multiplicity.

3.1.2. Infected at a High Multiplicity of Infection (50 P.F.U./Cell)

BHK21/C13 cells were infected in suspension by shaking with 50 P.F.U./cell of HSV for 20 minutes, and then plated out on 90 mm Petri dishes. Two-hour pulse-labelling in the presence of cytidine (3.2×10^{-6}M) was carried out with [5 - ^{3}H] -uridine and [methyl - ^{14}C] -L-methionine at final specific activities of 1.2 μC/nmol

Figure 17

Methylation of Newly-synthesized RNA in Exponentially-growing BHK21/C13 Cells Infected with HSV (10 P.F.U./Cell)

Purified cytoplasmic RNA from (a) BHK21/C13 cells, pulsed 2 hours in EC2F with [methyl - ^{14}C]-L-methionine and [5 - ^{3}H]-uridine, and HSV-infected cells pulsed (b) 0 - 2 hours P.I., (c) 2 - 4 hours P.I., (d) 4 - 6 hours P.I., (e) 6 - 8 hours P.I. and (f) 8 - 10 hours P.I. was fractionated by electrophoresis on 2% agarose gels.

o—o, ^{14}C d.p.m. from [methyl - ^{14}C] -L-methionine;

o—o, ^{3}H d.p.m. from [5 - ^{3}H] -uridine.

Figure 17.

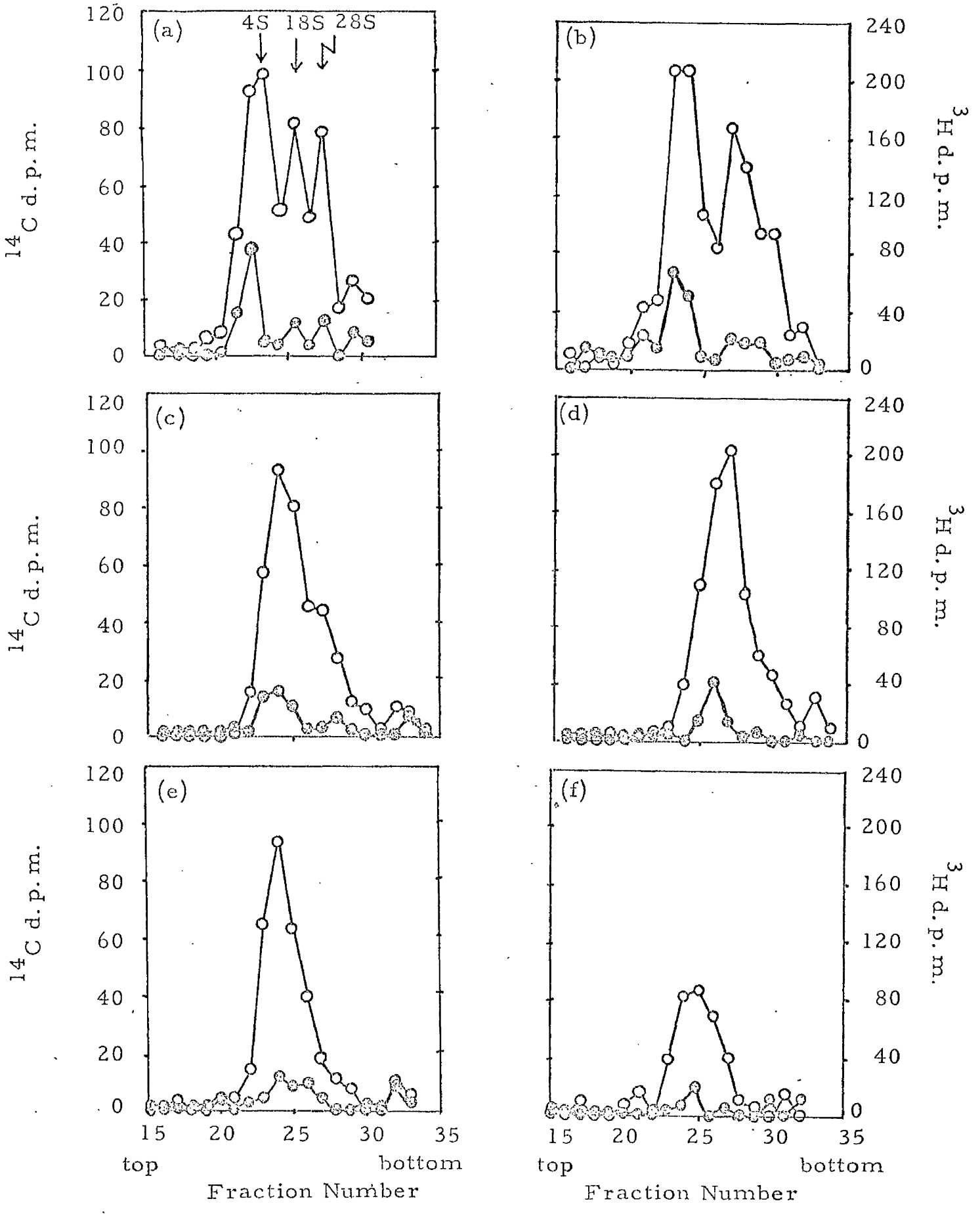

(a)
4S 18S 28S
(b)
(c)
(d)
(e)
(f)
^{14}C d.p.m.
^{3}H d.p.m.
120
100
80
60
40
20
0
240
200
160
120
80
40
0
15 20 25 30 35
top
bottom
Fraction Number

Table VII

Time-Course of Methylation of Newly-synthesized RNA in Exponentially-growing BHK21/C13 Cells Infected with HSV (50 P.F.U./Cell)

Species of RNA	"28S" + "18S"			"4S"		
Source and Labelling Time	^{14}C d.p.m.	^{3}H d.p.m.	$\frac{^{14}C}{^{3}H}$	^{14}C d.p.m.	^{3}H d.p.m.	$\frac{^{14}C}{^{3}H}$
BHK21/C13, 2 hour pulse	16	340	0.047	57	570	0.10
BHK21/C13 HSV, 0-2 hour P.I.	31	483	0.066	70	566	0.124
BHK21/C13 HSV, 2-4 hour P.I.	14	174	0.080	43	500	0.086
BHK21/C13 HSV, 4-6 hour P.I.	6	342	0.018	35	450	0.078
BHK21/C13 HSV, 6-8 hour P.I.	-	-	-	39	511	0.076
BHK21/C13 HSV, 8-10 hour P.I.	-	-	-	16	280	0.057

^{14}C d.p.m. derived from [methyl - ^{14}C] -L-methionine; ^{3}H derived from [5 - ^{3}H] - uridine, after agarose gel fractionation of cytoplasmic RNA

and 35 μC/μmol respectively. RNA was prepared as described in Section 3.1. The incorporation of both isotopes into the different RNA fractions after agarose gel electrophoresis is shown in Figure 17, and these results are summarized in Table VII.

The decrease in synthesis and in methylation was shown to be more rapid in cells infected at high multiplicity of infection than when a multiplicity of infection of 10 P.F.U./cell was used (Section 3.1.1.). After an early slight increase, the extent of methylation of "4S" RNA was greatly inhibited (as shown previously (Section 3.1.1.)). It seemed possible that an early increase in the level of methylation of newly synthesized RNA was being masked by BHK21/C13 metabolism which was probably not completely inhibited at this stage. The study was therefore repeated using "serum-depleted" BHK21/C13 cell cultures (which have low levels of RNA synthesis and methylation) to emphasize virus effect.

3.2. In "Serum-depleted" BHK21/C13 Cells Infected with HSV at a High Multiplicity of Infection (50 P.F.U./cell)

"Serum-depleted" cultures were prepared on 90 mm Petri dishes as described in Methods, Section 3.1.2. Just prior to infection the medium was removed from each plate. Five ml was made to 20 mM with respect to sodium formate and replaced on each culture at

Figure 18.

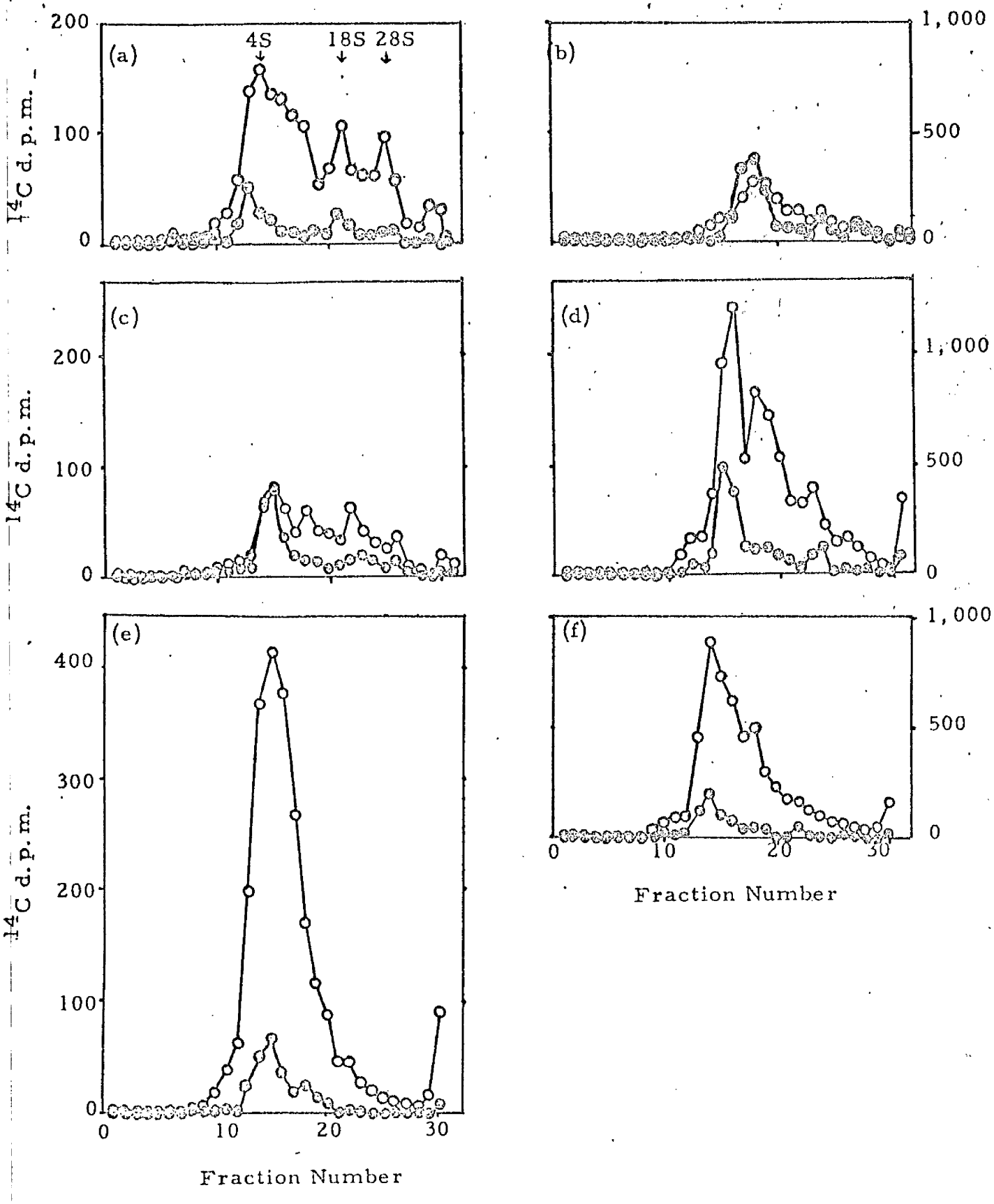

(a)
4S
18S
28S
(b)
(c)
(d)
(e)
(f)
200
100
0
1,000
500
400
300
10
20
30
^{14}C d.p.m.
Fraction Number

Figure 18

Methylation of Newly-synthesized RNA in Serum-depleted BHK21/C13 Cells Infected with HSV (50 P.F.U./cell)

Purified cytoplasmic RNA from (a) BHK21/C13 cells, pulsed 2 hours in EC0.5F with [methyl - ^{14}C]-L-methionine and [5 - ^{3}H]-uridine, and HSV-infected BHK21/C13 cells pulsed (b) 1 - 3 hours P.I., (c) 3 - 5 hours P.I., (d) 5 - 7 hours P.I., (e) 7 - 9 hours P.I. and (f) 9 - 11 hours P.I. was fractionated by electrophoresis on 2% agarose gels.

●—●, ^{14}C d.p.m. from [methyl - ^{14}C]-L-methionine;

○—○, ^{3}H d.p.m. from [5 - ^{3}H]-uridine.

Table VIII

Time-Course of Methylation of Newly-synthesized RNA in "Serum-depleted BHK21/C13 Cells Infected with HSV (50 P.F.U./Cell)

Species of RNA	"18S" + "28S"			"4S"		
Sample and Labelling Time	^{14}C d.p.m.	^{3}H d.p.m.	$\frac{^{14}C}{^{3}H}$	^{14}C d.p.m.	^{3}H d.p.m.	$\frac{^{14}C}{^{3}H}$
Uninfected BHK21/C13 Cells	40	453	0.11	176	843	0.21
Uninfected BHK21/C13 (EC0.5—EC10) 8-10 hour	223	2,760	0.081	407	1,420	0.21
BHK21/C13/HSV, 1-3 hours P.I.	39	318	0.12	221	850	0.26
BHK21/C13/HSV, 3-5 hours P.I.	37	700	0.05	187	1,083	0.17
BHK21/C13/HSV, 5-7 hours P.I.	23	186	0.12	208	2,540	0.08
BHK21/C13/HSV, 7-9 hours P.I.	-	-	-	200	7,350	0.03
BHK21/C13/HSV, 9-11 hours P.I.	-	-	-	81	2,340	0.03

^{14}C d.p.m. derived from [methyl - ^{14}C] -L-methionine; ^{3}H d.p.m. derived from [5 - ^{3}H] -uridine after fractionation by agarose gel electrophoresis of cytoplasmic RNA.

the end of the 1 hour's adsorption of HSV (50 P.F.U./cell). The cultures were incubated for 2 hour periods with [5 - ^{3}H] -uridine and [methyl - ^{14}C] -L-methione (final specific activities of 1.2 μC/nmol and 35 μC/μmol respectively) and cytidine (to 3.2 x 10^{-6}M). RNA was prepared and fractionated as described in Section 3.1. Results are depicted graphically in Figure 18 and in tabular form in Table VIII.

From the experiments carried out in exponentially-growing cell cultures, infected with HSV at high multiplicity of infection (Section 3.2.) it was clear that both synthesis and methylation of rRNA were rapidly inhibited (see also Section 3.1). This is also true of the residual rRNA synthesis and methylation in "serum-depleted" cells. Also, as observed previously, RNA appearing in the 4S region in electrophoresis continued to be synthesized, albeit at a rate lower than that of the uninfected cells, for some time after infection. From infection of "serum-depleted" cells it appeared that this synthesis might be virus-induced or virus-coded, as the rate of "4S" RNA synthesis in these cells reached 5 times the low level of synthesis found in the uninfected cells during a 7 - 9 hour P.I. pulse. The band of "4S" RNA appearing on gel electrophoresis (Figure 15) became broader during the course of infection, spreading into the region of slightly larger RNA.

Figure 19

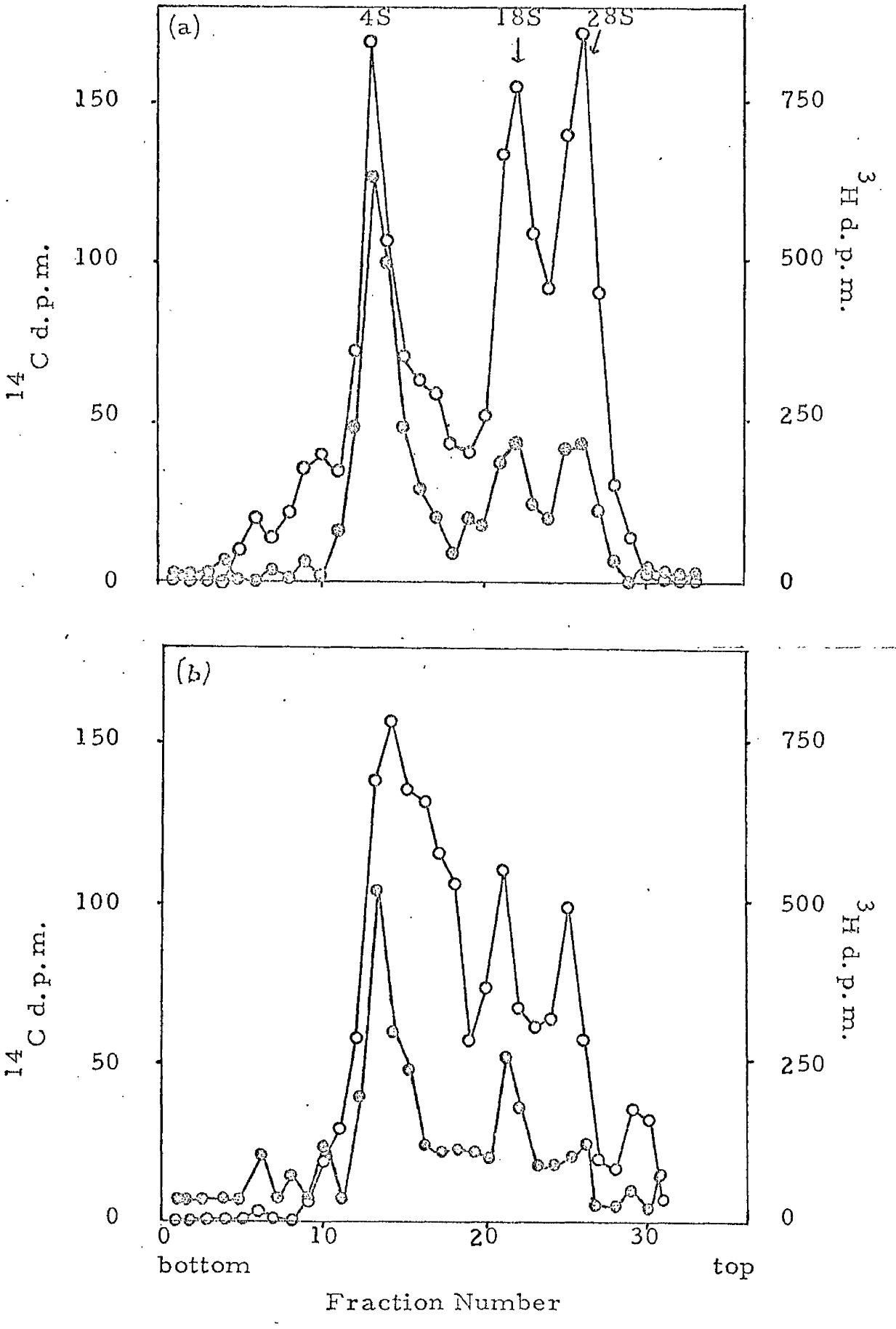
Methylation of Newly-synthesized RNA in Exponentially-growing and Serum-depleted BHK21/C13 Cells

Purified cytoplasmic RNA from (a) Serum-depleted BHK21/C13 cells, pulsed 2 hours in EC0.5F with [methyl - ^{14}C]-L-methionine and [5 - ^{3}H]-uridine, and (b) BHK21/C13 cells incubated 5 days in EC0.5, and pulsed 8 - 10 hours after the start of incubation in EC10F.

o——o, ^{14}C d.p.m. from [methyl - ^{14}C]-L-methionine;

o——o, ^{3}H d.p.m. from [5 - ^{3}H]-uridine.

Figure 19


(a)
4S
18S
28S
150
100
50
0
750
500
250
0
^{14}C d.p.m.
^{3}H d.p.m.
(b)
150
100
50
0
750
500
250
0
^{14}C d.p.m.
^{3}H d.p.m.
0
10
20
30
bottom
top
Fraction Number

The radioactivity of this band was unchanged after DNase treatment, but was completely dispelled by KOH digestion at all times P.I. After a slight initial increase in the level of methylation of newly-synthesized soluble RNA, this was rapidly inhibited. This effect was clearer from the results from "serum-depleted" cells (Figure 18), as, in these, effects of the infection were not masked by residual host metabolism. Comparison of results from the different systems suggests that a slight stimulation of methylation and synthesis may occur about 1 - 2 hours P.I.

That cell metabolism is at a low level in "serum-depleted" cells is made clear by comparison of RNA metabolism in these to that in cells when incubation medium was changed from EC0.5 to EC10. "Serum-depleted" cells resuspended in high-serum-containing medium resume DNA synthesis and cell metabolism after a delay (Burk, 1966). From Figure 19 it can be seen that RNA synthesis was greatly stimulated in resting cells 8 - 10 hours after contact with serum, but the extent of methylation of the 4S RNA was unchanged. The level of methylation of rRNA was slightly lower in exponentially-growing than in "serum-depleted" cells, and this may be caused by a slower stimulation or synthesis of rRNA methylases relative to RNA polymerase.

In the HSV-infected "serum-depleted" cells, peaks of incorporation of [5 - ^{3}H] -uridine occurred in positions intermediate between those

of rRNA and 4S RNA in gel electrophoresis (Figure 18), and one peak appeared to migrate between 18S and 28S. These RNA molecules could be those detected by Hay et al (1966), Flanagan (1967) and Wagner & Roizman (1969) and tentatively classified by them as mRNA molecules.

4. TRANSFER RNA METHYLASES IN HSV-INFECTED CELLS

Any changes in methylation in vivo may be reflected in the relevant enzymes, or their activity in vitro. This possibility was now tested in the case of tRNA methylases after HSV infection.

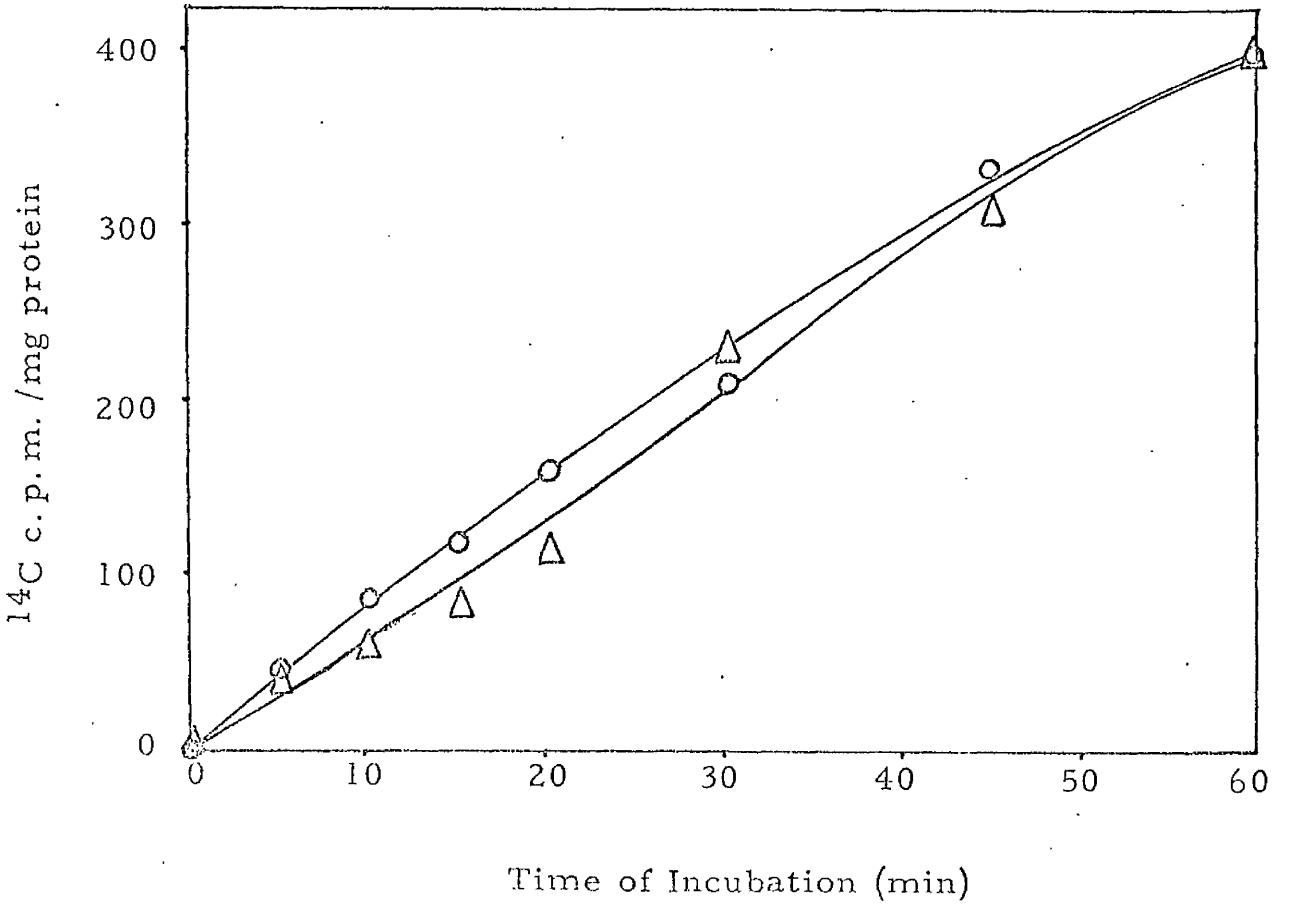
4.1. From Exponentially-growing Cells Infected at a Low Multiplicity of Infection (10 P.F.U./Cell).

4.1.1. From BHK21/C13 cell cultures

BHK21/C13 cell cultures in 80 oz roller bottles were treated as for the preparation of RNA (Section 3) except that no isotope was present during incubation. Enzyme preparations were prepared as detailed previously (Methods, Section 7.2) from control and infected cells. Preliminary assays carried out using the standard technique (Methods, Section 7.2) and Esch. coli tRNA as substrate revealed that extracts of control and infected cells behaved similarly with respect to activity as a function of protein concentration but differed only in their initial rate of reaction and then to a very small extent (Figure 20). Additionally, these enzyme preparations did not differ significantly in the final levels (saturation levels) of methylation which they were able

Figure 20.

Time-Course of Transfer RNA Methylase from Exponentially-growing BHK21/C13 Cells, Uninfected and Infected 6 hours with HSV (10 P. F. U. /Cell)



o——o , Uninfected BHK21/C13 cell extract;

Δ——Δ , HSV-infected BHK21/C13 cell extract.

to introduce into substrate tRNA. Several experiments of this nature were carried out using BHK21/C13 cells infected at a low multiplicity of infection and, while some inhibition of tRNA methylase activity was generally revealed after infection this varied from 10 - 40% relative to control. This could reflect the extent of infection of the cells.

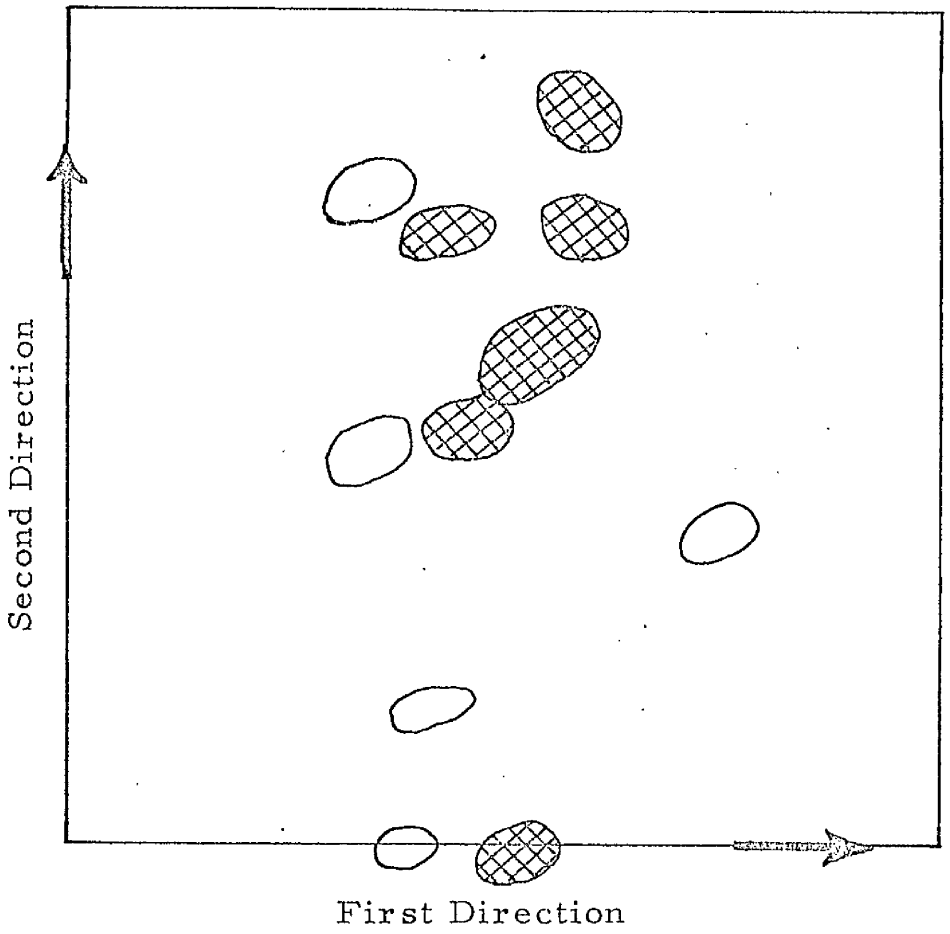
Purification of the assay products (labelled with methyl - ^{14}C groups from [^{14}C]-S-adenosylmethionine) from 6 hour HSV-infected and control BHK21/C13 cell methylase-catalyzed reactions, followed by KOH hydrolysis of the RNA, two-dimensional chromatography of the hydrolysates on Whatman 1MM paper (Methods, Section 4.3.2.) and autoradiography of the chromatograms (Methods, Section 4.3.3.) revealed no significant differences in the nucleotides methylated by the two enzyme preparations (Figure 21). However these autoradiograms of heterologous (Esch. coli B) tRNA methylated by the BHK21/C13 enzyme did differ substantially from those obtained similarly from homologous RNA methylated in vivo (Figure 22).

4.1.2. From HEp-2 cells infected with HSV at a low multiplicity of infection (11 P.F.U./cell)

HSV grown in HEp-2 cells had caused inhibition of 4S RNA methylation (Section 2.4.) similar to that produced when the virus was grown in BHK21/C13 cells. It therefore seemed reasonable to test whether the two systems were also parallel in the activity of their tRNA methylases.

Figure 21

Autoradiography of Products of Assay for Transfer RNA Methylase Activity in Control and HSV-Infected BHK21/C13 Cells



Chromatograms were obtained by eluting assay products (Esch. coli tRNA) on Whatman 1MM in the first direction with iso-butyric acid: 0.5M-NH_4OH (50: 30 (v/v) and in the second direction with iso-propanol: conc. HCl: H_2O (53.5: 19.3: 15.6 by weight).

Autoradiograms were obtained after contact of X-ray films with the chromatograms for 9 weeks.

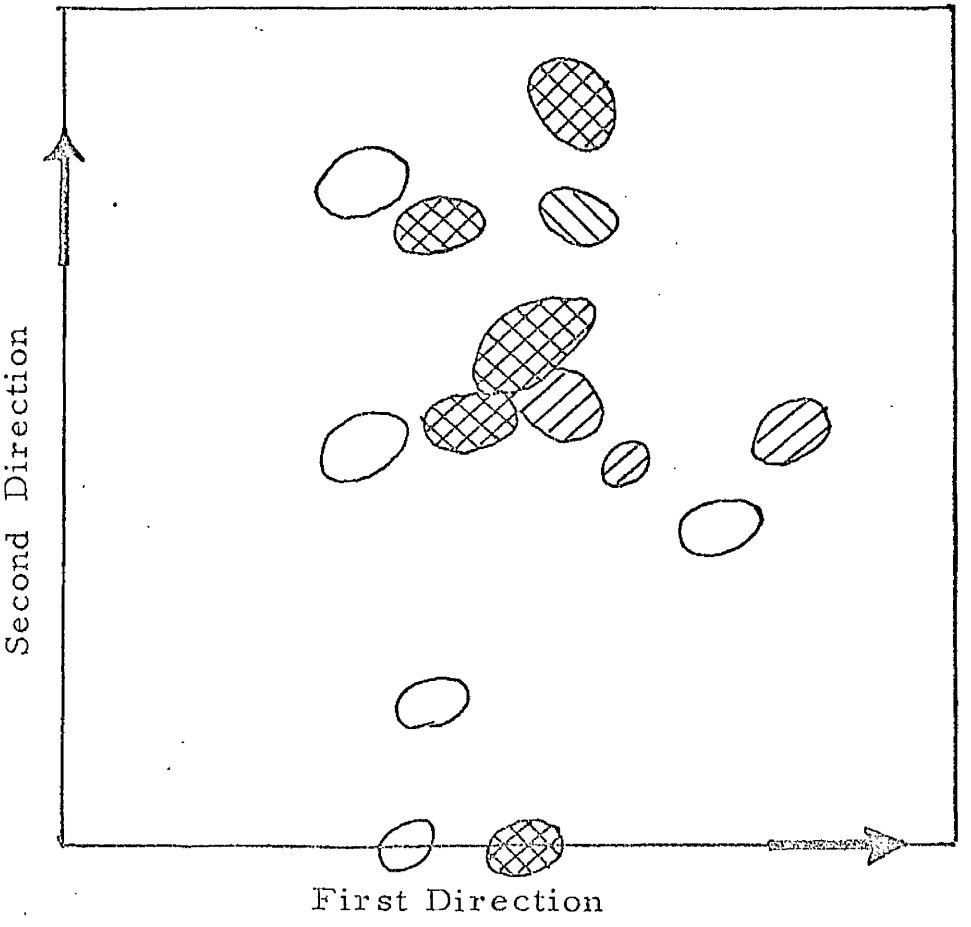
◯, u.v.-absorbing spots on chromatograms ;

▨, spots on autoradiogram from control assay product ;

▨, spots on autoradiogram from HSV-infected assay product.

Figure 22

Comparison of Hydrolysis Products of RNA Methylated in vivo and in vitro



Chromatograms were obtained by running alkaline hydrolysis products of (a) uninfected BHK21/C13 cells 4S RNA labelled in vivo with [methyl - ^{14}C] -L-methionine and (b) tRNA methylase assay products (Esch. coli tRNA) on Whatman 1MM in the first direction with iso-butyric acid: 0.5M-NH_4OH (50: 30 (v/v)) and in the second direction with iso-propanol: conc HCl: H_2O (53.5: 19.3: 15.6: by weight). Autoradiograms were obtained after contact of X-ray films with the chromatograms for 9 weeks.

◯; u.v.-absorbing spots on chromatograms;

◯; spots on autoradiograms from in vivo methylation;

◯; spots on autoradiograms from in vitro methylation.

An enzyme preparation from HEp-2 cells infected for 6 hours with HSV (at a multiplicity of infection of 11 P.F.U./cell) catalyzed the incorporation of methyl groups from S-adenosylmethionine as shown in Table IX. These results revealed a slightly decreased activity in the ribosomal fraction and in the 105,000 g supernatant fraction, and essentially no alteration in the nuclear fraction as compared to the control enzyme preparation. Transfer RNA methylase activity was seen to be present in both the 105,000 g supernatant fraction and the nuclear fraction to about the same extent. Differences between control and infected cell enzyme preparations might have been caused by a viral-induced enzyme or inhibitor, or might have been caused by leakage of tRNA methylase from the cytoplasm, as some leakage of material does take place from the cytoplasm of cells after HSV infection (Wagner & Roizman, 1969).

As discussed previously for the in vivo studies, it was possible in this sytem that changes in tRNA methylase activity after infection were being masked by residual host activity. Any inhibition of activity would presumably be augmented by infection of exponentially-growing cells with a high multiplicity of infection of HSV, so this system was now used for assay of tRNA methylases.

4.2. From Exponentially-growing Cells Infected at a High Multiplicity of Infection (50 P.F.U./Cell).

These 105,000 g supernatant fractions from infected and

Table IX

Transfer RNA Methylase from HEp-2 Cells Infected with HSV (10 P.F.U./Cell)

Enzyme Preparation	Activity
HEp-2, Crude Extract	22.0
HEp-2/HSV, Crude Extract	20.7
HEp-2, ribosomal fraction	25.3
HEp-2/HSV, ribosomal fraction	18.8
HEp-2, nuclear fraction	20.0
HEp-2/HSV, nuclear fraction	20.8
HEp-2, 105,000 g supernatant	33.3
HEp-2/HSV, 105,000 g supernatant	30.4

Activity is expressed as pmol methyl group incorporated into tRNA from [methyl - ^{14}C]-S-adenosylmethionine/mg protein, using the standard tRNA methylase assay procedure. Enzyme fractions were obtained 6 hours after infection.

Table X

Transfer RNA Methylase from Exponentially-growing BHK21/C13, Uninfected and HSV-infected (50 P.F.U./Cell)

Source of Enzyme Extract	Activity
BHK21/C13 Cells	10.6
BHK21/C13/HSV, 0 time P.I.	11.6
BHK21/C13/HSV, 3 hours P.I.	10.6
BHK21/C13/HSV, 6 hours P.I.	10.0
BHK21/C13/HSV, 9 hours P.I.	9.8

Activity is expressed as pmol methyl group incorporated from [methyl - ^{14}C]-S-adenosylmethionine/mg protein, using the standard tRNA methylase assay procedure.

The enzyme extracts were 105,000 g supernatant fractions.

uninfected BHK21/C13 cells at several times P.I. were prepared in parallel with the in vivo RNA methylation experiment discussed previously (Section 3.1.2). The fractions were assayed as before (Methods, Section 7.2.). The results show that there appeared to be a slight and possibly insignificant decrease in methylase activity with time after infection (Table X).

In in vivo studies of 4S RNA methylation (Section 3) a slight initial rise in methylation early in infection had been routinely observed and this had been visible especially when cells were infected at high multiplicity of infection. This might have been caused by tRNA methylase activity induced by HSV and masked in exponentially-growing cells. If so, it should be possible to demonstrate this activity in "serum-depleted" cultures of BHK21/C13 cells infected at high multiplicity of infection.

4.3. From "Serum-depleted" BHK21/C13 Cells Infected at a High Multiplicity of Infection (50 P.F.U./Cell).

This study was carried out in parallel with the in vivo RNA methylation experiment discussed previously (Section 3.2). The assay of the 105,000 g supernatant fractions from the cell cultures was by the method previously described (Methods, Section 7.2). The results shown in Table XI indicated that when BHK21/C13 cells were grown in EC 0.5, tRNA methylase activity fell to about one fifth of the level present in

Table XI

Transfer RNA Methylase from "Serum-depleted" BHK21/C13, Uninfected and HSV-infected (50 P.F.U./Cell)

Source of Enzyme Extract	Activity
BHK21/C13, exponentially growing	23.5
BHK21/C13 (EC 0.5)	4.5
BHK21/C13/HSV (EC 0.5), 3 hours P.I.	9.1
BHK21/C13/HSV (EC 0.5), 6 hours P.I.	4.5

Activity is expressed as pmol methyl group incorporated into tRNA from [methyl - ^{14}C]-S-adenosylmethionine/mg protein, using the standard tRNA methylase assay procedure. The enzyme extracts were 105,000 g supernatant fractions.

Figure 23.

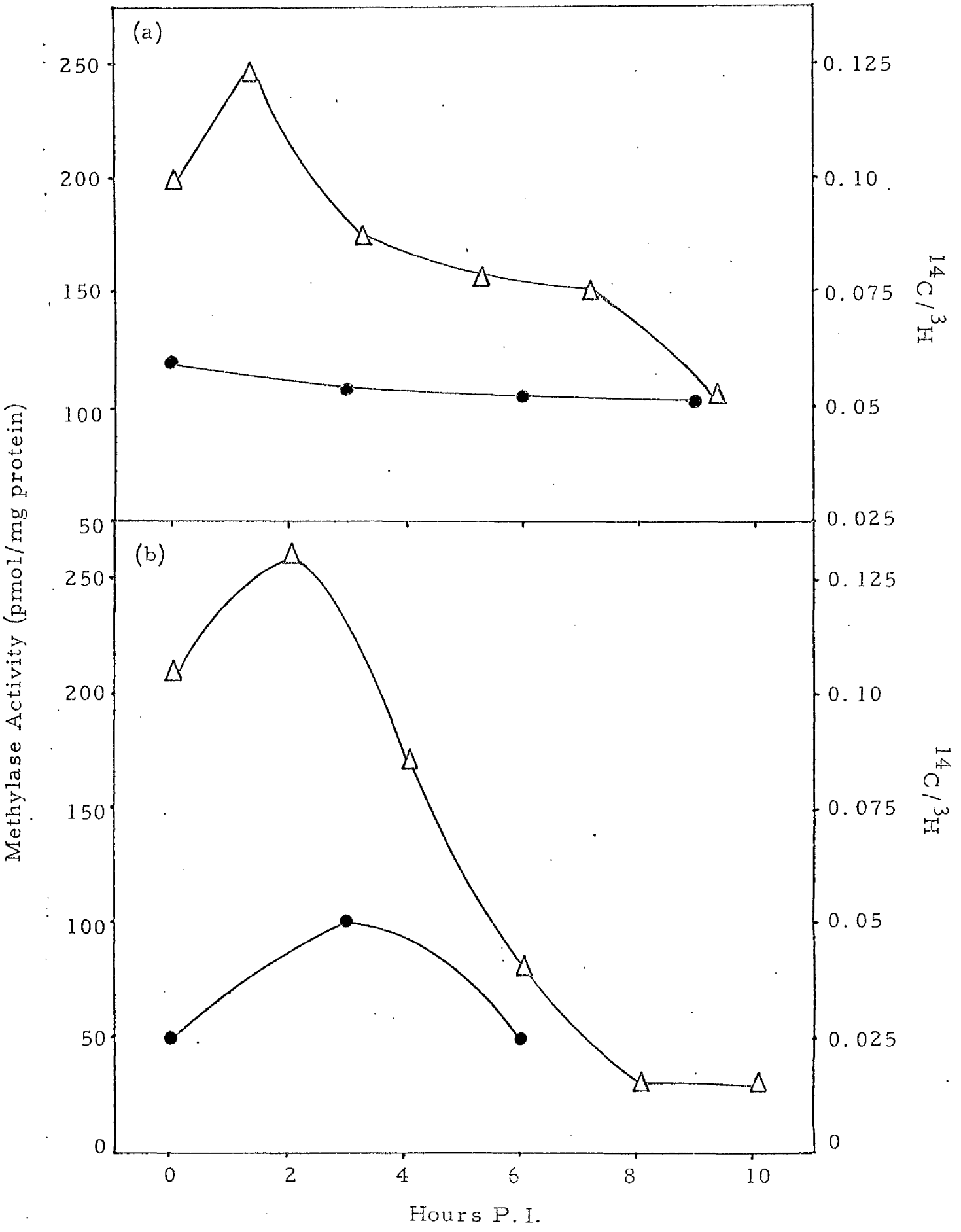
Comparison of Transfer RNA Methylation *in vitro* and *in vivo* in (a) Exponentially-growing and (b) "Serum-depleted" BHK21/C13 Cells infected with HSV (50 P. F. U. /Cell).

In vivo activity is depicted as methylation of newly-synthesized "4S" RNA, measured by incorporation of ^{14}C and ^{3}H from [*methyl*-^{14}C]-L-methionine and [5-^{3}H]-uridine (Sections 3. 1(ii) and 3. 2). *In vitro* activity was measured by incorporation into *Esch. coli* tRNA of ^{14}C from [*methyl*-^{14}C]-S-adenosylmethionine, in the presence of enzyme extracts (Sections 4. 2 and 4. 3).

Δ——Δ , $^{14}C/^{3}H$ (*in vivo*);

o——o , pmol/mg proteins (*in vitro*).

Figure 23.


(a)
(b)
250
200
150
100
50
0
0.125
0.10
0.075
0.05
0.025
0
2
4
6
8
10
Methylase Activity (pmol/mg protein)
$^{14}C/^{3}H$
Hours P.I.

exponentially-growing cells. From these results, and from a plot of the measurements of methylation in vivo and in vitro tRNA methylase activity after infection (Figure 23) it appeared possible that the initial increase in the level of methylation of 4S RNA under both growth conditions was produced by an early increase in tRNA methylase activity in HSV-infected cells. This activity was visible only when cells with a low endogenous methylase activity were infected. After this early rise, both levels of methylation relative to control and tRNA methylase then decreased.

The depression of methylation could be produced by the presence of an inhibitor in infected cells, but if this were so a mixture of enzyme preparations from exponentially-growing control cells and HSV-infected cells would be expected to catalyze methylation to an extent less than the sum of the activities of each enzyme preparation. Such "mixing" experiments were carried out, with the results shown in Table XII. It was noted that when enzyme preparations from cells infected with HSV for various times P.I. were mixed with a preparation from uninfected cells, a combined methylase activity of about 120% of the expected summed activities was obtained. Thus no inhibition of tRNA methylase activity is apparent. The augmented activity might be produced by each preparation containing RNA available for methylation by a heterologous

Table XII

Transfer RNA Methylase "Mixing" Experiments

Source of Enzyme Extract		Activity (pmol)	
Uninfected	Infected	Expected	Measured
Exponentially-Growing BHK21/C13	"Serum-depleted", 3 hours P.I.	73	84
Exponentially-Growing BHK21/C13	"Serum-depleted", 6 hours P.I.	68	83
Exponentially-Growing BHK21/C13	Exponentially-growing, 3 hours P.I.	30	46
Exponentially-Growing BHK21/C13	Exponentially-growing, 9 hours P.I.	36	46
"Serum-depleted" BHK21/C13	"Serum-depleted", 9 hours P.I.	23	33

Enzyme preparations were those described in Section 4.1 - 4.3. Activity is expressed as pmol methyl group incorporated into tRNA from [methyl - ^{14}C]-S-adenosylmethionine/mg protein using the standard tRNA methylase assay procedure.

enzyme, e.g. host RNA may be methylated by any HSV-induced enzyme and vice versa. It might also be produced by a loosening of the substrate RNA structure by partial RNase digestion, thus making more sites accessible to the methylase.

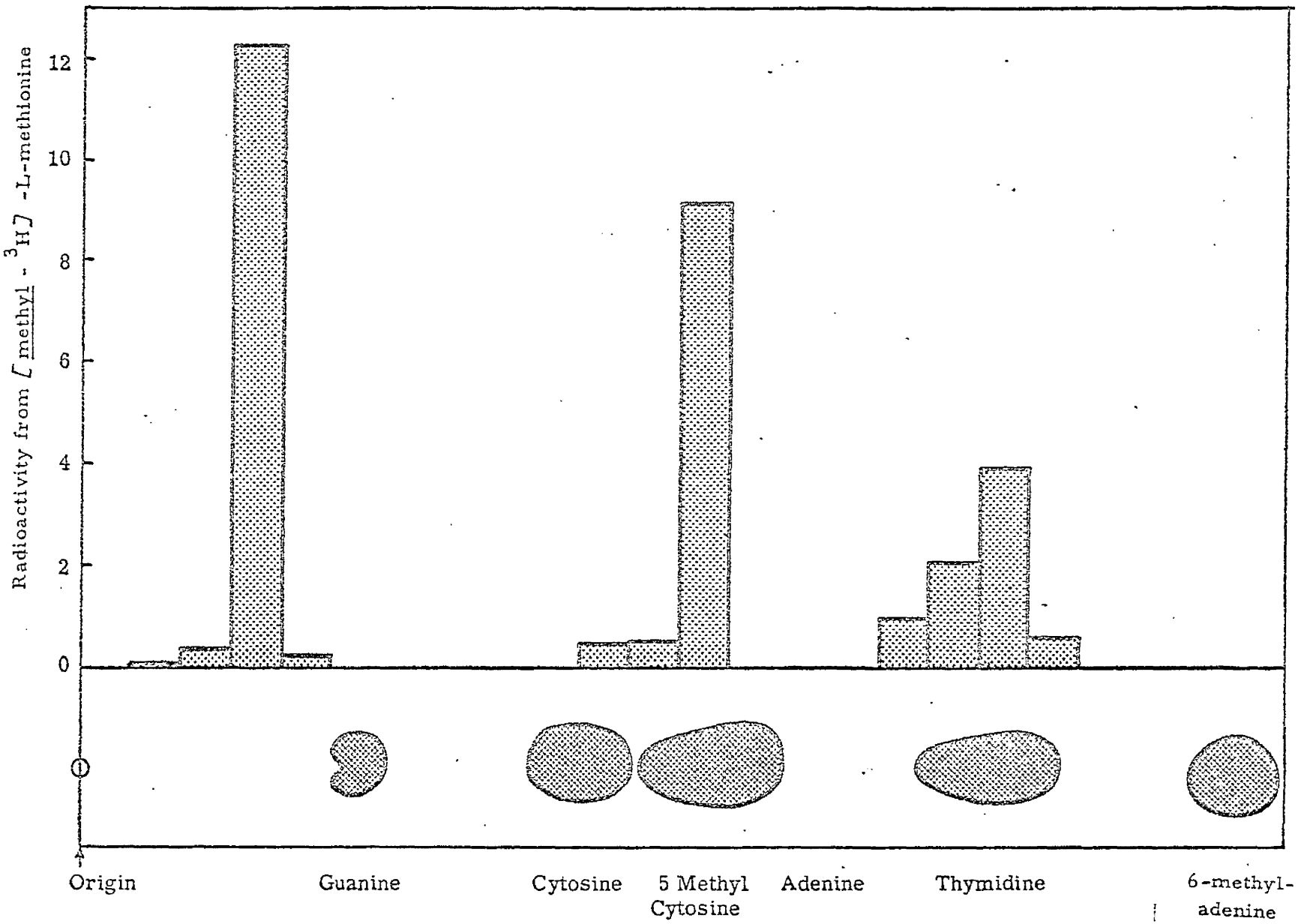
5. DNA METHYLATION IN VIVO

5.1. BHK21/C13 DNA Methylation

As already described in Section 1.6. BHK21/C13 DNA is methylated to a level of approximately one mole % nucleotides. Purified DNA synthesized in BHK21/C13 cells in the presence of radioactively-methyl-labelled methionine was hydrolyzed and analyzed chromatographically as described in Methods Section 4.3.1. The experimental details were as follows: BHK21/C13 were dispersed and incubated with [methyl - ^{3}H] -L-methionine (at a final specific activity of 640 μC/μmol) in EC2F [7% met] for 4 days, as at this level of methionine cell growth was very low (Section 1.2.). The cells were thoroughly washed before purification of the DNA by phenol extraction and CsCl density gradient centrifugation. The pooled material was used for base analysis. The results depicted in Figure 24 show that 5-methylcytosine was methylated in this system, but 6-methyladenine was not. The source of the radioactivity near the origin of the chromatogram was not determined. It was, however, shown to be neither methionine nor a breakdown product of 5-methylcytosine.

Figure 24

Chromatogram of [methyl - ^{3}H] -L-methionine-labelled BHK21/C13 DNA hydrolysate



DNA hydrolysed in formic acid, 190°, 60 mins. Run on Whatmann No. 1 paper, in butanol. H_2O(86: 14 v/v); 5% NH_3 in atmosphere, 24 hours. Radioactivity measured by liquid scintillation.

About 40% of the total incorporation of methyl groups into DNA bases was found to occur in thymine. That thymine incorporates methyl groups from methionine has been shown in HeLa cells, although to a lesser extent (20%) (Burdon & Adams, 1969). There was negligible inclusion of one-carbon units into adenine, cytosine and guanosine, as expected (Section 1.1.).

5.2. Effect of HSV Infection on BHK21/C13 DNA Methylation

5.2.1. Study of pulse-labelled DNA

From earlier fractionation studies using MAK columns (Section 2.1), it appeared that the methylation of total DNA in BHK21/C13 cells decreased after infection with HSV.

CsCl gradient fractionation of the same material as was subsequently fractionated on MAK columns (Section 2.1) after treatment with RNase gave results which also indicated decreased methylation of total DNA in infected cells to approximately 20% of the uninfected cell DNA level after 1 - 5 hours' labelling with methionine. However, this could not be related to newly synthesized DNA or to viral or host DNA. The indicated reduction in methylation could be due to (a), decrease in host methylation owing to inhibition of host DNA synthesis alone, or (b), (a) with synthesis of viral DNA containing a low level of methylation, or (c) reduced synthesis and therefore methylation of all species of DNA synthesized. Therefore, experiments were carried out to measure DNA synthesis as well as methylation, and also to fractionate viral from host DNA.

5.2.2. Synthesis and methylation of DNA in BHK21/C13 infected with HSV

Nearly-confluent BHK21/C13 cell cultures preincubated for 4 hours in EC2F [7% met] were infected with HSV (10 P.F.U./cell) before incubation with 50 ml EC2F [7% met]. Pulse-labelling from 1 to 6 hours or 6 to 11 hours P.I. was carried out with [methyl - ^{14}C]-L-methionine and [6 - ^{3}H]-thymidine to final specific activities of 16.7 μC/μmol and 2.4 μC/μmol respectively, and deoxycytidine, to a final concentration of 5 x 10^{-6}M. The addition of the latter was required as the concentration of thymidine used produces feedback-inhibition of pyrimidine biosynthesis, thereby curtailing cytidine production. DNA was extracted by the pronase method (Methods, Section 6.6.(b)) prior to purification of the total DNA on CsCl density gradients (Methods, Section 4.2.1.). Total incorporation of ^{14}C from methionine and ^{3}H from thymidine was estimated for each sample, and from this the level of methylation of newly synthesized DNA (viral plus host) was calculated. From Table XIII it can be seen that the degree of methylation of total DNA synthesized in BHK21/C13 cells decreased after infection with HSV. For the calculation the dilution factors estimated in Section 1.6. were employed. HSV DNA and probably host DNA will have been synthesized during both pulse periods used, the former predominantly in the later time period and vice versa. The viral DNA has a G + C content of 70% (Russell & Crawford, 1964),

so that the calculated synthesis of DNA, espcially from 6 to 11 hours P.I., will be an underestimate, inferring that deviation from "normal" methylation of BHK21/C13 DNA after HSV infection is greater than shown.

As all cells are not infected when a multiplicity of infection of 10 P.F.U. of HSV/cell is used (Wagner & Roizman, 1969) it is probable that part of the methylation of newly synthesized DNA occurred in uninfected cells. This was confirmed experimentally by separating viral and host DNA on CsCl gradients. Examination of the radioactivity in the fractions obtained from the DNA of BHK21/C13 cells infected with HSV, and labelled 6 - 11 hours P.I. (Figure 25) indicated methylation was associated with the fractions of DNA of lower buoyant density. Also, the two peaks of incorporation of ^{3}H from thymidine represent the newly-synthesized DNA of HSV (buoyant density 1.727 g/ml) and BHK21/C13 (of buoyant density 1.709 g/ml). Figure 26 depicts the incorporation of ^{3}H and ^{14}C (from thymidine and methionine respectively) into DNA of uninfected BHK21/C13 cultures labelled in parallel with the infected cultures. As seen, plots of E 260 nm, ^{14}C incorporation and ^{3}H incorporation are all coincident. As shown in Table XIII, this represent 0.96 mole % methylated DNA nucleotide. Calculations based on the results depicted in Figure 25 indicated that the extent of methylation of host cell DNA 6 - 11 hours P.I. is 1.5 mole %, thus inferring an inhibition of BHK21/C13 DNA methylation after infection of these cells

Figure 25

DNA from BHK21/C13 infected with Herpes Simplex Virus, labelled 6 -11 hours; fractionated at pH 8.2

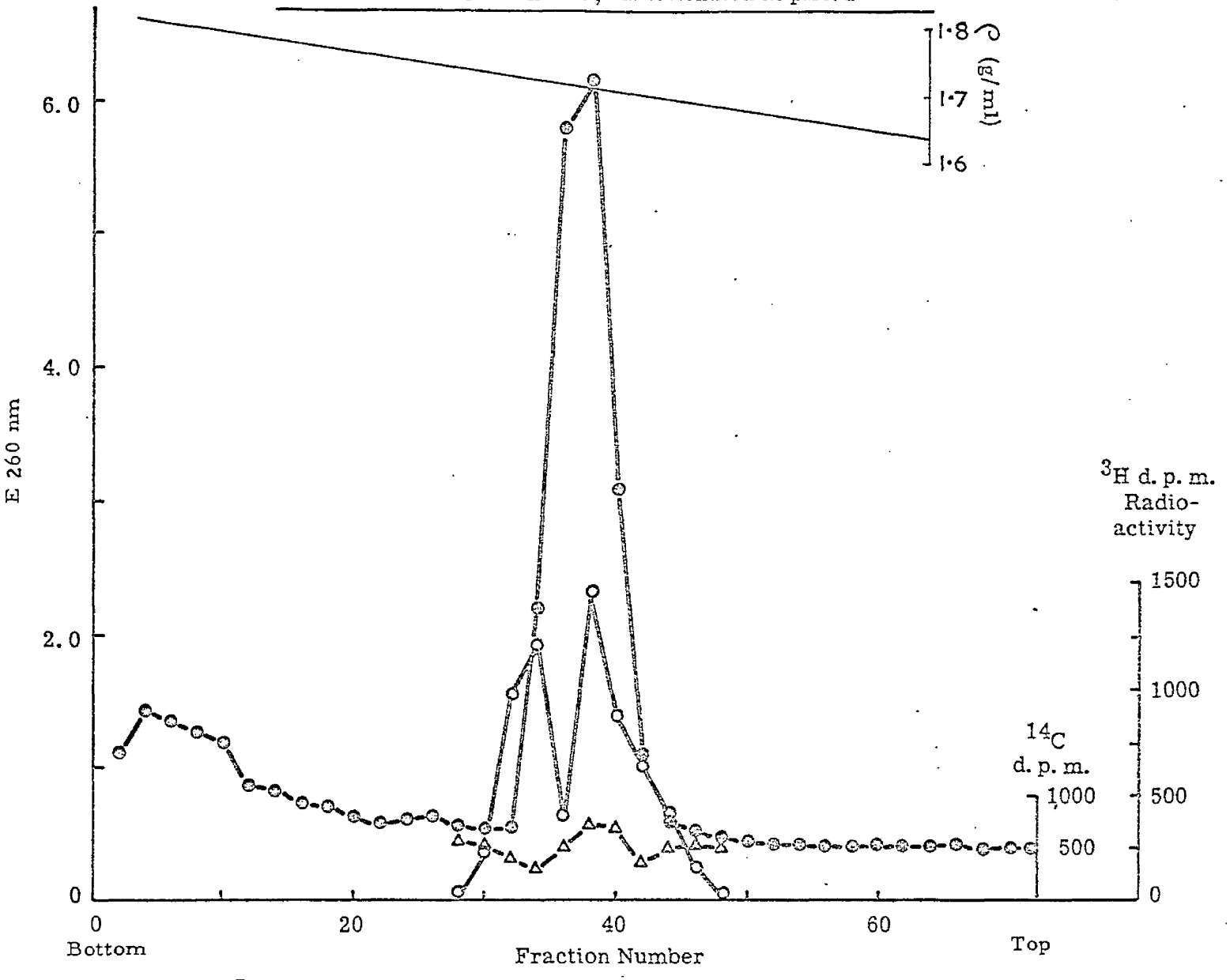


Figure 26

BHK21/C13 DNA fractionated at pH8.2

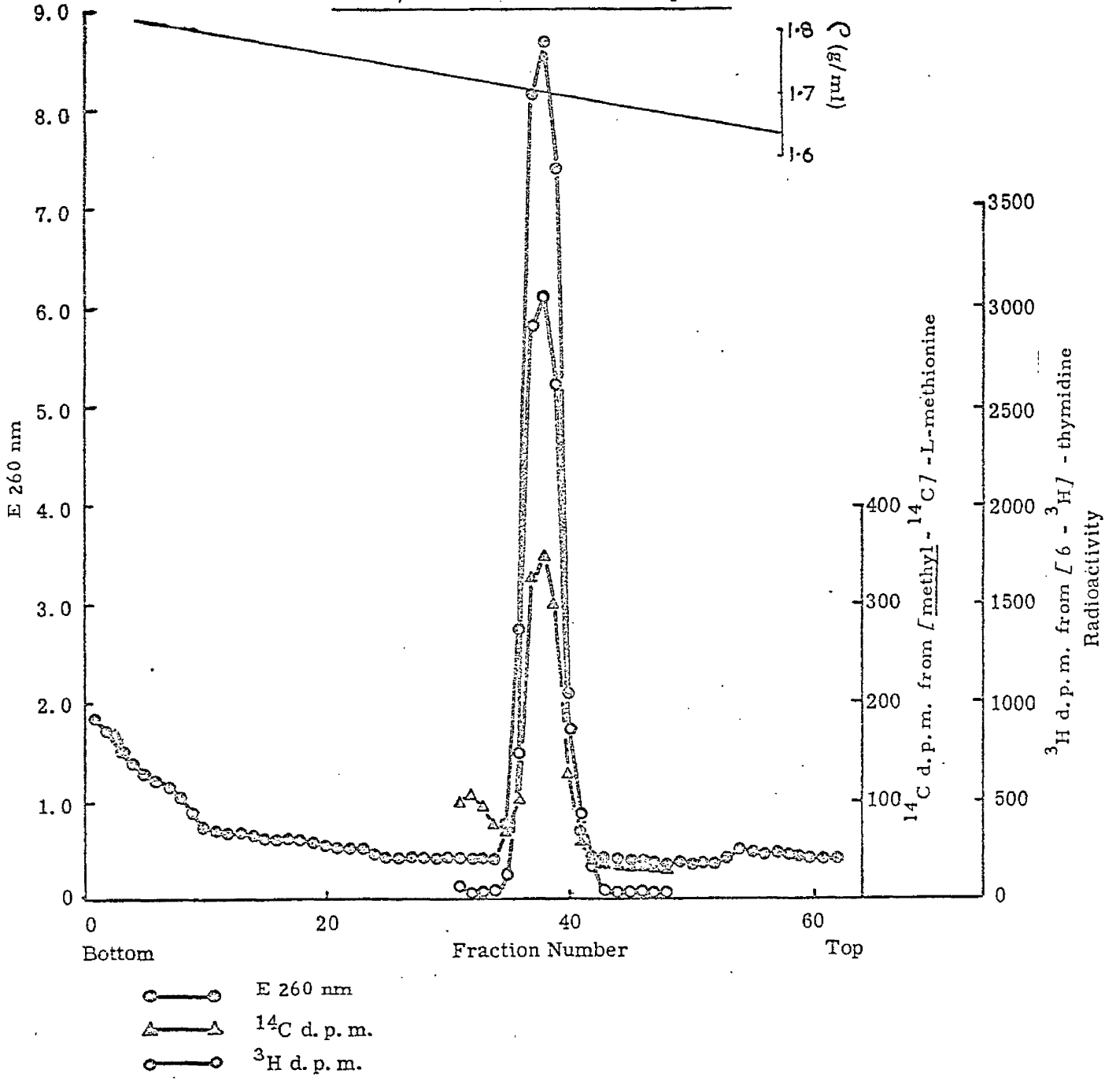


Table XIII

Effect of HSV Infection of BHK21/C13 Cells on Methylation of Newly-synthesized DNA

Source of DNA	^{3}H d.p.m.	^{14}C d.p.m.	moles % methylated nucleotide
BHK21/C13, pulsed 5 hours	4.74×10^{3}	1,554	1.04
BHK21/C13/HSV, 1 - 6 hours P.I.	4.86×10^{3}	855	0.56
BHK21/C13/HSV, 6 - 11 hours P.I.	3.2×10^{3}	350	0.37

The source of the purified DNA was BHK21/C13 cells pulsed in EC2F [7% met] for 5 hour periods with [6 - ^{3}H] thymidine and [methyl - ^{14}C]-L-methionine after HSV infection (10 P.F.U./cell).

by HSV.

The above results suggest that the DNA of HSV has a much lower methylated base content than its host DNA, but an estimation of the level of methylation in HSV DNA required isolation of this nucleic acid from virus, and the use of larger amounts of material.

6. METHYLATION OF DNA FROM HSV

6.1. Method of Production of Methionine-labelled HSV

Newly confluent monolayers of BHK21/C13 cells in 80 oz roller bottles were preincubated for 4 hours with 50 ml EC2F containing the level of methionine to be used for viral growth. Cultures were infected at a multiplicity of infection of approximately 1 P.F.U./cell in 20 ml of this medium, allowing one hour for adsorption before replacement by 50 ml of the same medium containing isotopically-labelled methionine. Virus was grown and harvested as for stock (Methods, Section 3.2.). This growth schedule was employed using several methionine concentrations as follows:

6.2. Virus Growth in 7% Normal Methionine Concentration

HSV was grown in the presence of 300 μC [methyl - ^{3}H] -L-methionine, at a final specific activity of 291 μC/μmol. The virus was washed twice with 0.005M-tris-HCl buffer, pH 7.4 and 0.14mM-NaCl, then incubated for 30 min at 37° with 50 μg/ml DNase and 100 μg/ml RNase in 0.05M-tris-HCl buffer, pH 7.4 and 10^{-3}M-$MgCl_2$. The harvested virus was mixed with twelve times the quantity of unlabelled

virus and extracted by the modified Marmur procedure (Methods, Section 6.6.(a)) together with carrier DNA (salmon sperm).

Sixty μg of this DNA was centrifuged for 24 hours at 44, 770 r.p.m. and 20° in a Spinco Model E Ultracentrifuge. A densitometer tracing of a 60 sec exposure is shown in Figure 27, and this showed that 15% of the total DNA was viral.

The DNA was then centrifuged to equilibrium on a preparative CsCl gradient as described in Methods, Section 4.2.1., and fractions collected as described. Fractions 19 - 22 (Figure 28), containing viral DNA, were pooled and precipitated with carrier DNA onto millipore filters for scintillation counting. Eighty-five d.p.m. were obtained from 2.6 μg HSV DNA, corresponding to 1 methylated nucleotide per 5×10^4 nucleotides. A "blank" sample of carrier, salmon sperm, DNA from the "light" side of the DNA, gave an activity of 63 d.p.m.

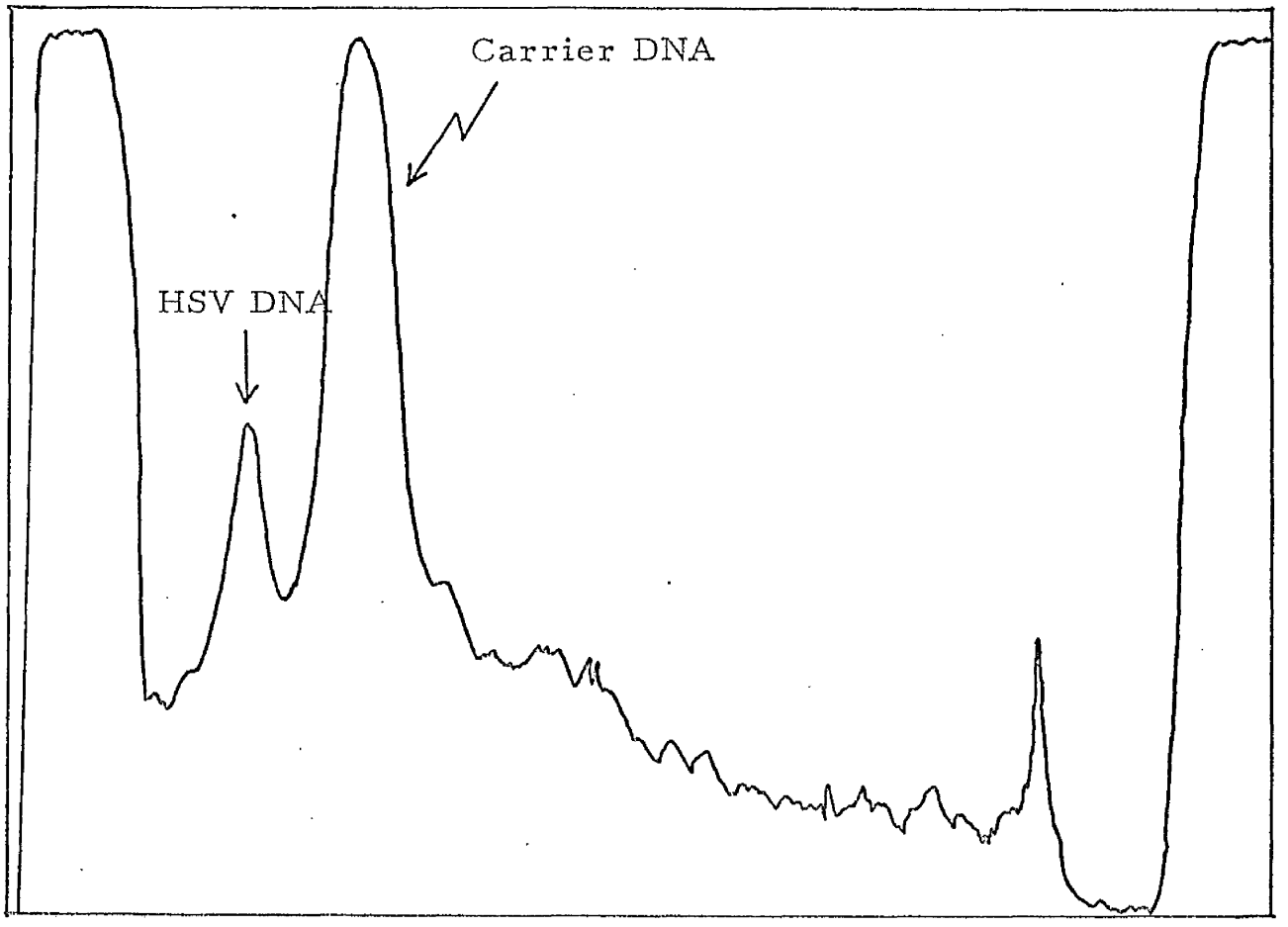
These results suggest a negligible level of methylation in HSV DNA, but this estimation was based on a very small amount of DNA, giving near-background radioactivity. A more decisive result, it appeared, required more radioactively-labelled virus and so the following experiment was undertaken.

6.3. . Virus grown in 7% normal methionine concentration

HSV was grown in the presence of 200 μC [methyl - ^{3}H] -L-methionine per 80 oz. roller bottle, at a final specific activity of 527 μC/

Figure 27

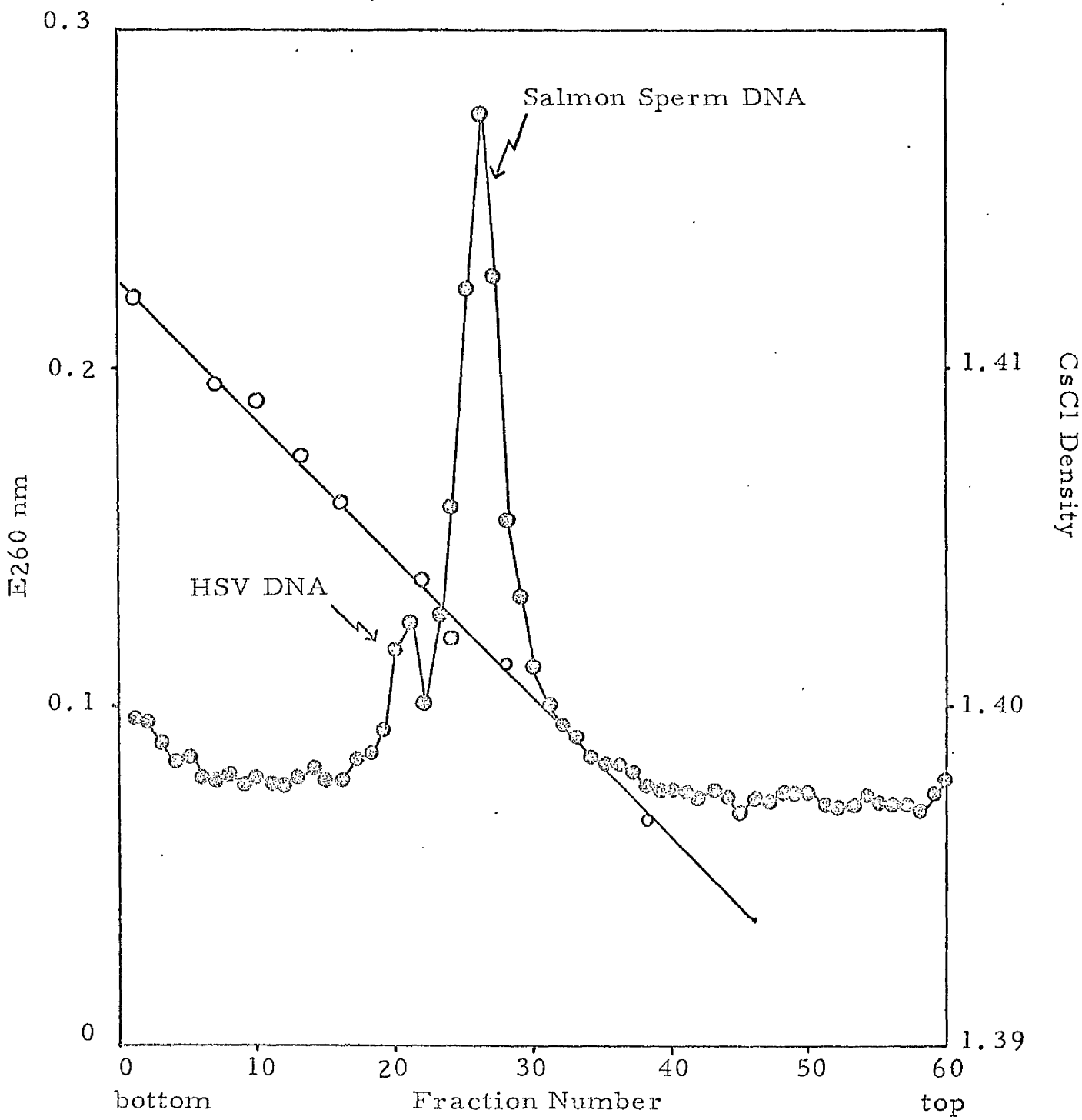
Densitometer Tracing of HSV DNA and Carrier DNA



HSV DNA was extracted with carrier salmon sperm DNA and made to 1.705 g/ml, then centrifuged 24 hours, 44,770 r.p.m., 20° in Spinco Model E; 60s exposure.

Figure 28

HSV DNA and Salmon Sperm (Carrier) DNA Separated by Equilibrium Centrifugation in CsCl Density Gradients



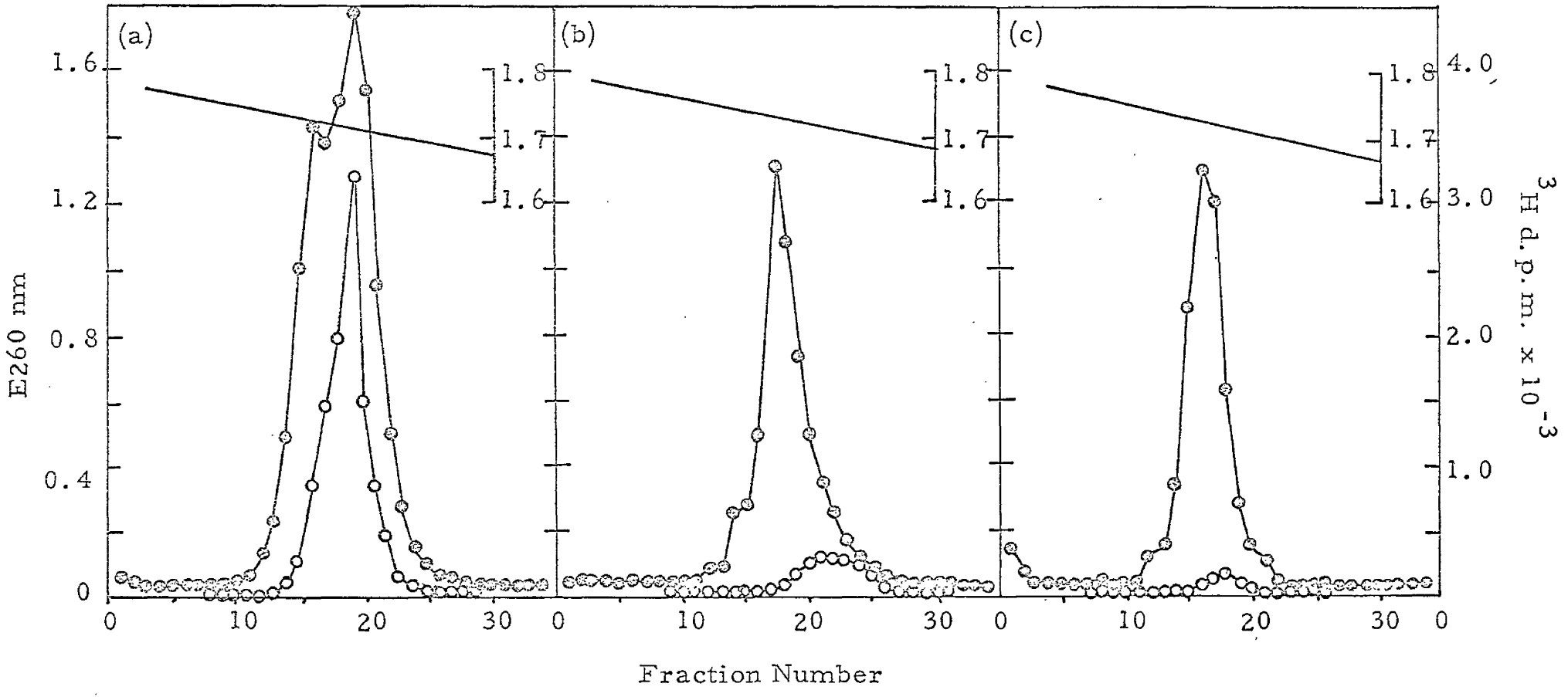
o—o , E260 nm ;

o—o , CsCl Gradient.

μmol. The extracted virus was mixed with an equal amount of unlabelled virus for DNA extraction as before, except that the DNase treatment was omitted, in case sonication had disrupted the viral coat, leaving viral DNA exposed. BHK21/C13 DNA, some of which was labelled, acted as carrier during DNA extraction. Viral DNA was successively purified from contaminating BHK21/C13 DNA by CsCl buoyant density gradient fractionation (Methods, Section 4.2.1.). Figure 29 (à) shows 2 peaks of u.v.-absorbing material, the buoyant densities of which correspond to those of HSV and BHK21/C13 DNA. The radioactivity appeared to be associated with BHK21/C13 DNA. Fractions 14 - 17 were combined and recentrifuged, giving the results shown in Figure 29 (b). Ninety % of the radioactivity in (a) was lost, as was 90% of BHK21/C13 u.v.-absorbing material. Combination and recentrifugation of fractions 12 - 17 gave the results shown in Figure 29 (c). Then only 2 - 3% of the radioactivity remained, with no u.v.-absorbance visible for BHK21/C13 DNA. The peak of radioactivity occurred in samples with a density of 1.709 g/ml, which is within the density range of mammalian DNA. Herpes DNA is relatively homogeneous, banding at 1.727 g/ml. Calculations using the quantities obtained in Figure 29 (c) for methylation and absorbancy indicated that there are 3×10^6 moles of nucleotide per mole of methylated nucleotide in the DNA of HSV.

Figure 29

Separation of HSV DNA from BHK21/C13 DNA



Equilibrium centrifugation in CsCl density gradients of DNA from HSV grown in BHK21/C13 cells in the presence of [methyl -^{3}H] -L-methionine. A starting density of 1.720 g/ml was used for centrifugation at 20°C, 33,000 r.p.m., 62 hours in SW50 rotor of Spinco L2 ultracentrifuge. 0.1 ml samples, diluted to 0.5 ml were measured for absorbance at 260 nm, and acid-insoluble material counted for radioactivity.

(a) Initial DNA preparation; (b) recentrifugation of fractions 14 - 17 from (a); (c) recentrifugation of fractions 12 - 17 from (b); o——o, Absorbance at 260 nm; o——o, ^{3}H radioactivity; ———, buoyant density.

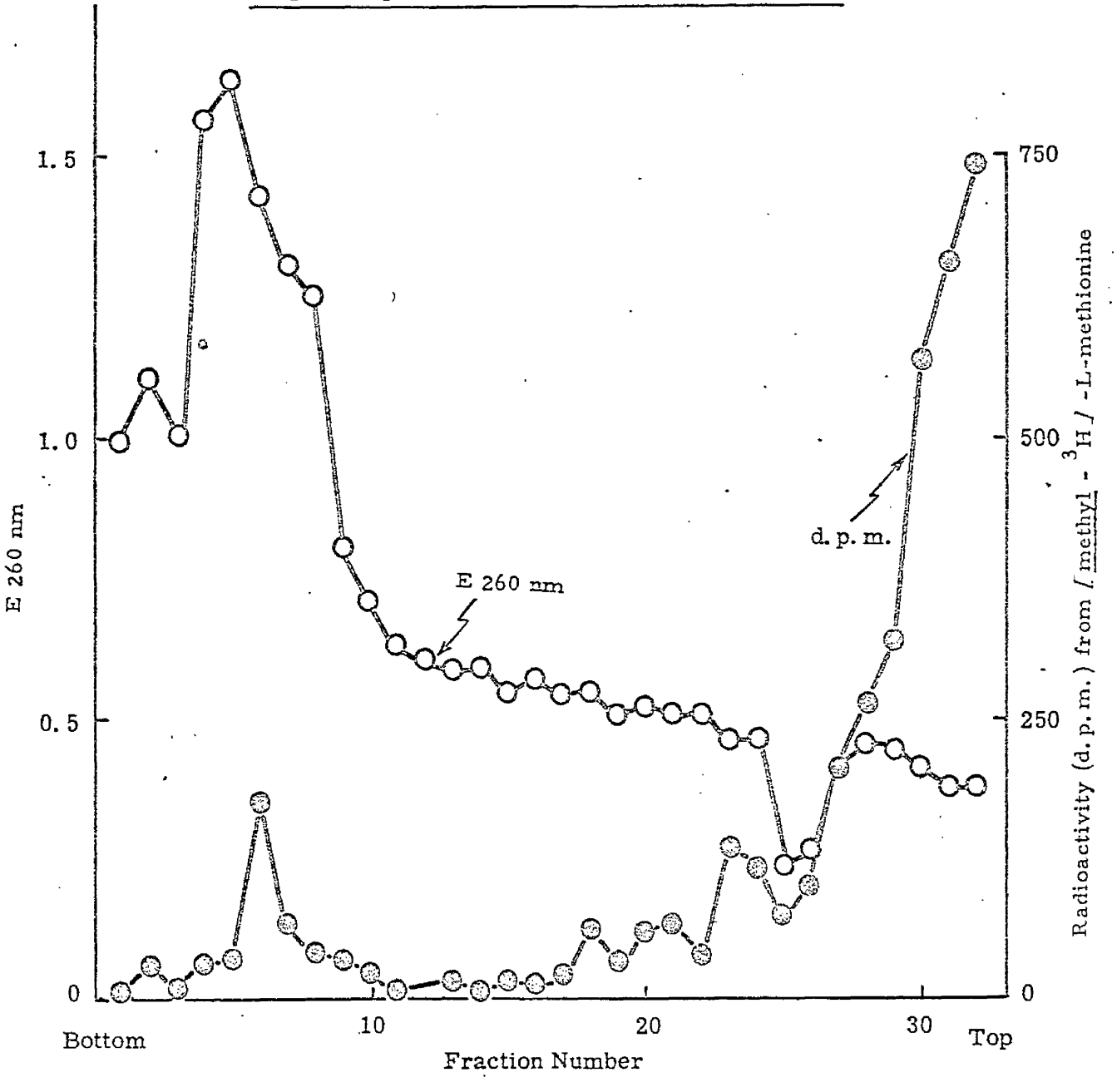
It was possible that methionine concentration had been a limiting factor for the methylation of nucleic acids in these experiments, although HSV grows normally in medium containing 7% normal methionine concentration (Section 1.4). A level of methionine which allowed normal cellular metabolism to proceed was therefore used for the growth of HSV to investigate a possible effect of interference with host methionine metabolism on HSV DNA methylation.

6.4. Virus Grown in 20% Normal Methionine Concentration

HSV was grown in EC2F [20% met] in the presence of 200 μC [methyl - ^{3}H] -L-methionine at a final specific activity of 193 μC/μmol. Supernatant virus (i.e. virus released from the cells into the medium) was harvested and washed twice with 0.005M-tris-HCl buffer, pH 7.4 and 0.14M - NaCl. DNA was extracted in 0.01M-tris-HCl buffer, pH 8.2 by the Marmur method (Marmur 1961), and purified and fractionated by CsCl density gradient centrifugation. Fractions containing viral DNA, separated from BHK21/C13 DNA (c.f. Figure 25), were combined, and, in order to remove traces of RNA or protein present in the DNA preparation, were incubated at 37° for 120 min in the presence of 0.5M-KOH. The pH of the solution was adjusted to 12.5 prior to recentrifugation in CsCl gradients (Vinograd, Morris, Davidson & Dove, 1963). At this pH, DNA is denatured, and has a higher density than the native form, but still fractionates according to base content. Thus, the u.v.-absorbing material equilibrated in the lower

Figure 30

Herpes Simplex Virus DNA fractionated at pH12.5



part of the gradient, but radioactivity did not follow exactly the same pattern (see Figure 30), and possibly indicates some host DNA contamination. Calculation of the incorporation of methyl groups from L-methionine into the u.v.-absorbing material (fractions 4 - 10), shown to be DNA by the Burton method (Burton, 1956), indicated that there is a minimum of 5×10^5 nucleotides per methylated nucleotide in HSV DNA. This takes into account the high background absorbance at 260 nm, and a methionine dilution factor (Section 1.6.), but makes no allowance for possible incorporation of label into thymine.

Thus negligible methylation of HSV DNA grown in both EC2F [7% met] and EC2F [20% met] was recorded. This is indicative of methionine not being a precursor for the methylation of HSV DNA, independent of its concentration in the growth medium.

HSV DNA, then, apparently is not methylated in infected BHK21/C13 cells and methylation of host DNA is inhibited after infection. Therefore it would appear that the virus contains or produces some means of prevention of the methylation of its DNA. Several possibilities of preventative means exist, such as the production of an inhibitor of DNA methylase, the production of an S-adenosylmethionine cleavage enzyme, or by inhibition of S-adenosylmethionine synthesis. These possibilities were tested in turn.

7. SEARCH FOR A DNA METHYLASE INHIBITOR IN BHK21/C13 CELLS INFECTED WITH HSV.

7.1. Assay of BHK21/C13 Cell DNA Methylase

Initially it was hoped to study BHK21/C13 DNA methylase, and possible inhibition of this enzyme, directly. There have been few reports of assays of mammalian DNA methylase, none of these being from BHK21/C13 cells. These methods (Burdon, Martin & Lal, 1967; Sheid, Srinivasan & Borek, 1968; Kalousek & Morris, 1968) were adapted for the preparation and assay of an enzyme extract from BHK21/C13 cells. The assay products were routinely extracted by the Marmur method (Marmur, 1961), and incubated with 0.5M-KOH to remove all contaminating RNA and protein which might have been methylated or might have trapped S-adenosylmethionine. The incorporation obtained was consistently in the region of 5 - 20 d.p.m., suggesting that either BHK21/C13 DNA methylase requires different assay conditions from other mammalian enzymes or that some of the incorporation obtained in the published data was not from methylated deoxynucleotides.

It was clear that information concerning inhibition of DNA methylase could not be obtained using the homologous system. It was therefore decided to try other enzyme sources.

7.2. The Effect of Extracts of Control and HSV-Infected BHK21/C13 Cells on Esch. coli DNA Methylase Activity

7.2.1. As assayed by Fujimoto, Srinivasan & Borek (1965)

Freshly grown Esch. coli B was disrupted by sonication in 0.02M-tris-HCl buffer, pH 8.0, 0.001 M-$MgCl_2$, 3×10^{-4}M-EDTA and 0.001M-2-mercaptoethanol and the 105,000 g supernatant used as enzyme source. The assay conditions described by the authors were followed exactly, and crude extracts of HSV-infected and uninfected BHK21/C13 cells were added. These extracts had been prepared by homogenization (Potter) of exponentially-growing cells 5 hours after infection with HSV (10 P.F.U./cell) or after mock-infection, in the above buffer. The assay was carried out as described by the above authors, but purification of the assay product by extraction by the method of Marmur (Marmur, 1961) and alkaline digestion was included before it was acid-precipitated on millipore filters for counting in toluene scintillant.

The results, summarized in Table XIV, suggested no inhibition of Esch. coli DNA methylase by either extract. To verify this finding a different assay system was used to measure Esch. coli DNA methylase.

(b) As assayed by Hausmann & Gold (1966)

The above enzyme (prepared as in Section (a)) and stored at 0° for 3 days was assayed by the method of Hausmann & Gold (1966) in

Table XIV

Effect of Extracts from Control and HSV Infected BHK21/C13 Cells on Esch. coli DNA Methylases

Addition to Assay	Average nmol methyl groups incorporated	
	Complete assay	Assay-DNA
None	1.1	0.29
None (0°)	0.47	
5 μl Infected Extract	0.88	
10 μl Infected Extract	1.06	
20 μl Infected Extract	1.22	
5 μl Control Extract	1.24	
10 μl Control Extract	1.35	
20 μl Control Extract	1.39	

DNA methylase activity was assayed by the method of Fujimoto, Srinivasan & Borek (1965), and the assay product purified as described in the text. Activity was expressed as nmol DNA methylated/mg protein. Cell extracts were prepared by homogenization of BHK21/C13 cells (uninfected and HSV-infected (10 P. F. U.)) 5 hours after infection.

Table XV

Effects of Extracts from Control and HSV-infected BHK21/C13 Cells on Esch. coli DNA Methylases

Addition to Assay	µg DNA/Assay	Average nmol incorporated per assay
None	0	0.11
None	50	0.17
None	100	0.27
None	200	0.30
None at 0°	200	0.09
5 µl Infected Extract	200	0.31
10 µl Infected Extract	200	0.32
20 µl Infected Extract	200	0.36
5 µl Control Extract	200	0.33
10 µl Control Extract	200	0.31

DNA methylase activity was assayed as described by Hausmann & Gold (1966), and the assay product purified as described in the text. Activity was expressed as nmol DNA methylated/mg. protein. Cell extracts were prepared by homogenization of BHK21/C13 cells (uninfected and HSV-infected (10 P.F.U./cell)) 5 hours after infection.

the presence of the above cell extracts which had been stored at -70° for 3 days. The assay product was extracted by the Marmur method (Marmur, 1961) and alkali-treated before acid-precipitation on millipore filters for counting in toluene scintillant. The results obtained are summarized in Table XV.

This experiment confirmed that crude extracts of BHK21/C13 cells, both uninfected and infected with HSV for 5 hours contained no inhibitor of Esch. coli DNA methylases. In fact, a possible slight stimulation of incorporation of ^{3}H from [methyl - ^{3}H] -S-adenosyl-methionine was obtained, this being slightly greater when uninfected rather than infected cell extracts were used. The stimulation is probably due to the presence of heterologous DNA in the extracts, and the small differences between control and infected cell extracts may be related to their relative DNA content, as host DNA appears to be at least partially degraded in cells infected with HSV.

Although no inhibitor of DNA methylase was detected under these assay conditions, the enzyme used was heterologous to the mammalian extracts, and so this system may not respond to an inhibitor designed for the BHK21/C13 enzyme.

After this work was carried out, further studies on mammalian DNA methylases were published (Kalousek & Morris, 1969) which tended to confirm the above findings that apparent incorporation of methyl groups

into mammalian DNA in vitro may have been present in contaminating material. With their system, these authors were able to measure an appreciable level of DNA methylase activity in rat spleen extracts. This mammalian DNA methylase assay system thus appeared to be suitable as a substrate for a putative DNA methylase inhibitor in HSV-infected BHK21/C13 cells.

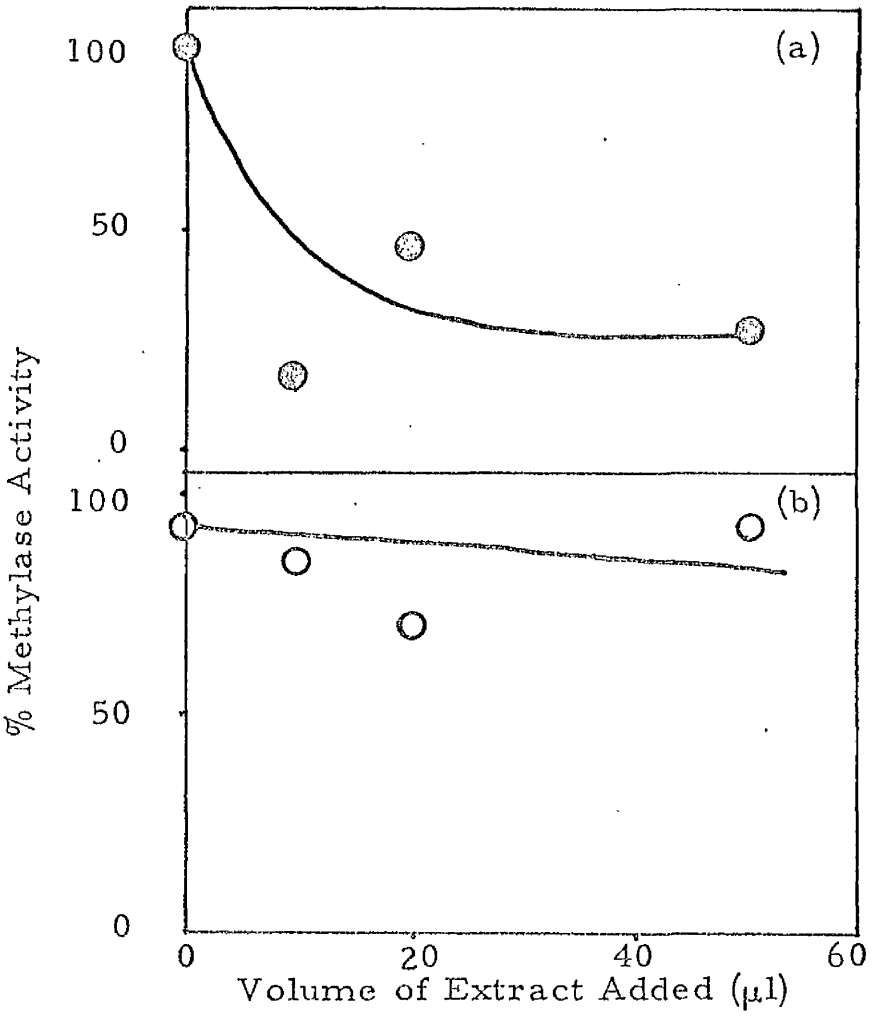
7.3. The Effect of Extracts of Control and HSV-Infected BHK21/C13 Cells on Rat Spleen DNA Methylase

The methods of preparation and of assay of the rat spleen DNA methylase were those of Kalousek & Morris (1969). For the assay, a volume of 0.2 ml was used, containing 10 μmol tris-HCl buffer, pH 7.8, 0.38 μg RNase, 40 μg Esch. coli B DNA, 50 μl enzyme preparation, and 0.5 μC[methyl - ^{3}H] - S-adenosylmethionine and cell extracts prepared as follows:

Confluent monolayers of exponentially growing BHK21/C13 cells were infected with HSV (50 P.F.U./cell), or were mock-infected. At 4 hours P.I. the cells were scraped off, washed with 0.1M-tris-HCl buffer, pH 7.8, 0.25M-sucrose and 4 x 10^{-4}M-MgCl2 and homogenized in the same buffer. This suspension was centrifuged for 10 min. at 5,000 g to give a nuclear pellet, and the supernatant centrifuged at 105,000 g for 60 min to give a soluble cytoplasmic fraction. The nuclear pellets were suspended in 0.01M-tris-HCl buffer, pH 7.8.

Figure 31

Effect of Cytoplasmic Extracts of Control and HSV-Infected BHK21/C13 Cells on Rat Spleen DNA Methylase



●—●, HSV-infected cell extract;

○—○, Control cell extract.

The results obtained from inclusion of the cytoplasmic fractions are summarized in Figure 31. These show that extracts of BHK21/C13 cells after 6 hours' HSV-infection inhibit rat spleen DNA methylase activity by about 50%. Control cell extracts showed no such inhibition. No inhibition of methylase activity was observed by either nuclear fraction, incorporation being in excess of that obtained without BHK21/C13 cell fraction addition.

Although it appeared from the above result that a cytoplasmic factor may prevent the methylation of HSV DNA in infected cells, it seemed worthwhile to investigate the possible involvement of additional factors. For example, in bacteriophage T3 infection of Esch. coli, the phage prevents methylation of its DNA by the production of an S-adenosyl-methionine cleavage enzyme (Gefter, Hausmann, Gold & Hurwitz, 1966). The presence of such an enzyme in BHK21/C13 cells after HSV infection was tested.

8. ASSAY OF S-ADENOSYLMETHIONINE CLEAVAGE ACTIVITY

BHK21/C13 cell cultures were infected with HSV at an input multiplicity of 10 P.F.U./cell for 5 and 9 hours, and harvested using versene. Cells were washed twice in 0.1M-tris-HCl buffer, pH 8.0, 0.14M-NaCl and 0.04M-2-mercaptoethanol and homogenized in 0.1M-tris-HCl buffer, pH 8.0. The assay volume of 0.5 ml contained

Table XVI

S-Adenosylmethionine Cleavage

Treatment of ^{14}C-labelled-S-Adenosylmethionine	Average c. p. m.	nmol cleaved
None	9, 074	
Absorbed to Amberlite	535	8
30 seconds, 0. 1N KOH	1, 138	17
30 minutes, 37° with BHK21/C13 cell extract	414	6
30 minutes, 37° with extract from BHK21/C13, infected 0 - 5 hours with HSV	384	6
30 minutes, 37° with extract from BHK21/C13, infected 0 - 9 hours with HSV	411	6
30 minutes, 0° with extract from BHK21/C13, infected 0 - 5 hours with HSV	231	3

S-adenosylmethionine cleavage enzyme was assayed by the method of Gefter, Hausmann, Gold & Hurwitz (1966).

100 μmol-tris-HCl buffer, pH 8.0, 8 μmol-2-mercaptoethanol and [methyl - ^{14}C] -S-adenosylmethionine (Gefter, Hausmann, Gold & Hurwitz, 1966). S-adenosylmethionine was removed after incubation by mixing twice with Amberlite CG - 50, leaving hydrolyzed material in the supernatant fraction. The results, shown in Table XVI suggested that cleavage of S-adenosylmethionine does not take place either during infection of BHK21/C13 cells by HSV or in the uninfected cells.

An additional means by which HSV could prevent methylation of its DNA is by the prevention of the synthesis of S-adenosylmethionine, which, in all systems so far studied, is the immediate donor of methyl groups for the methylation of nucleic acids. This possibility was therefore tested.

9. ASSAY OF S-ADENOSYLMETHIONINE SYNTHETASE ACTIVITY

S-adenosylmethionine synthetase was prepared by the method of Sheid & Bilik (1968) at 6 hours P.I. from BHK21/C13 cells either infected with HSV at an input multiplicity of 10 P.F.U./cell, or mock-infected, and assayed by the method of these authors (Sheid & Bilik, 1968). After 60 min incubation at 37° (when S-adenosylmethionine is still being formed linearly) averages of 0.204 and 0.210 μmol S-adenosylmethionine/mg protein had been synthesized by enzyme preparations from HSV-infected and control BHK21/C13 cells, suggesting no change in the level or rate of synthesis of S-adenosylmethionine at 6 hours P.I.

DISCUSSION

1. CONDITIONS FOR OPTIMAL INCORPORATION OF METHYL GROUPS FROM METHIONINE

1.1. The Effect of Sodium Formate on BHK21/C13 Cells

Sodium formate was found to be toxic at concentrations greater than 20mM which gives, in incubation medium, complete dilution of the methionine-derived tetrahydrofolate in the one-carbon pool. This may have been because of impurities in the sodium formate preparation or from an osmotic effect of the total resultant salt concentration in the medium.

1.2. The Effect of Methionine Concentration on Cell Growth and Metabolism

BHK21/C13 cells were found to be dependent on exogenously-supplied methionine for growth, for RNA synthesis and for DNA synthesis, each to a slightly different extent. This may perhaps be explicable in terms of the relative amounts of methionine required for different cellular processes. An endogenous source of methionine, even in cells which are not dividing, arises from a turnover of endogenous protein, as shown for HeLa cells (Eagle, Piez, Fleischman & Oyama, 1959; Vaughan, Soeiro, Warner & Darnell 1967), but a minimal level of exogenously-supplied methionine is clearly required to supplement this for each methionine-dependent cell process.

1.2.1. The effect of methionine concentration on DNA metabolism

DNA is required to be synthesized in relatively small amounts. However, it has been shown that this synthesis is methionine-dependent (Results, Section 1.3). This requirement may be (a) for methylation of the DNA or (b) for synthesis of some methionine-containing protein needed for replication. Lark (1968) has shown that, in Esch. coli, synthesis of DNA can only take place if template DNA is methylated, but that some synthesis can continue in the absence of methylation of this newly-synthesized DNA. In BHK21/C13 cells no significant deviation from normal in the extent of methylation of DNA was observed when cells were incubated in medium containing 7% normal methionine (Results, Section 1.6.3), a concentration which did not fully support DNA synthesis (Results, Section 1.3). This suggested that the limiting factor for cell growth was something other than the methylation of newly-synthesized DNA, perhaps a protein vital for the replicative process.

1.2.2. The effect of methionine concentration on RNA metabolism

RNA synthesis apparently became more methionine-dependent with time than did DNA synthesis (Figure 9). Even in cells which are not dividing there is RNA turnover, and thus RNA synthesis is able to continue by utilizing bases formed from breakdown of RNA. However, methylated bases are formed at the polynucleotide level and, in the absence of sufficient methionine they cannot be synthesized. For

example, in HeLa cells it has been shown that in effective methionine starvation ribosomal precursor RNA continues to be synthesized, but is deficient in methyl groups (Vaughan, Soeiro, Warner & Darnell, 1967) and complete ribosomes are not formed. Also, hypomethylated tRNA is formed in HeLa cells in methionine deprivation (Bernhardt & Darnell, 1969). In any case, the cell is likely to be able to function normally with partially depleted ribosomes and tRNA populations, since, at least in Esch. coli, mRNA is apparently not methylated (Moore, 1966).

1.2.3. The effect of methionine concentration on cell growth

It does not seem unreasonable to suggest that cell growth would be inhibited by some means other than by decreased DNA or RNA synthesis, and it is likely that lack of methionine for protein synthesis would affect cell growth. This would be consistent with results shown in Figure 8 for cell growth, as compared to Figure 9 for DNA and RNA synthesis in the presence of different methionine concentrations. These studies were carried out using different time-scales, however, and levels of methionine which were insufficient to support cell growth and division might also be proved to be insufficient to support RNA and DNA synthesis after longer periods of incubation of the BHK21/C13 cells in methionine-depleted medium.

1.2.4. The effect of methionine concentration on HSV growth

HSV grow normally in concentrations of methionine lower

than were required to support host cell metabolism (Results, Section 1.4), but still requires exogenously-supplied methionine for its replication (cf. pseudorabies virus (Kaplan, Shimono & Ben-Porat, 1970)). This suggests that the virus either incorporates methionine into a coat protein or into some other protein vital to its growth, e.g. an enzyme.

This result further suggested that either HSV proteins contain a smaller amount of methionine than host cell protein, or that there is a lower level of protein synthesis in infected cells, or that, after infection, there is a lower dependence on methylation of nucleic acids.

That the second possibility, at least, was true was shown in a study of methionine metabolism in HSV-infected cells (Results, Section 1.7), the findings of which were in agreement with those of Roizman, Borman & Rousta (1965) and Sydiskis & Roizman (1966) for the general pattern of protein metabolism in HSV-infected cells.

Subsequent work on the methylation of nucleic acids in HSV-infected cells (Results, Section 3 and 5) indicate that for both DNA and RNA there is a lower level of methylation relative to uninfected cells. Thus the possibility that there is a lower methionine-dependence in infection on this account might also be true.

2. TECHNIQUES FOR NUCLEIC ACID STUDY

2.1. MAK Fractionation

The technique used to fractionate nucleic acids derived from

cells infected with HSV in preliminary experiments was MAK column chromatography. Although theoretically this is a good method in that tRNA, rRNA and DNA are separated, in practice it did not yield sufficiently accurate results for the following reasons:

(a) the elution pattern was not sufficiently reproducible for accurate comparative studies,

(b) often DNA and RNA were insufficiently well separated, and

(c) the capacity of the column was low. This coupled with the low level of radioactivity incorporated into RNA and, especially, into DNA meant that often total counts were too low to be meaningful.

This problem of obtaining reproducible nucleic acid fractionation was solved by using a cell fractionation procedure (Results, Section 2).

In the study involving MAK fractionation (Results, Section 2.1) no step was included to remove radioactively-labelled methionine which may have been acylated to tRNA. This source of radioactivity may have masked the true level of methylation of this species. However, this source of error was removed in later studies either by treatment of the tRNA with pronase, or by incubation with 1.0M-tris-HCl buffer, pH 9.0 at 37°.

2.2. Method of Measurement of the Methylation of Newly-synthesized DNA and RNA

These studies were undertaken to measure the extent of methylation of newly-synthesized nucleic acid after HSV-infection of BHK21/C13 cells. To this end, cultures were incubated in the presence of [methyl - ^{14}C]-L-methionine and a tritiated nucleoside (uridine or thymidine) labelled so as to prevent entry of isotope into DNA or RNA respectively.

2.2.1. DNA

It proved possible in the case of DNA to correlate on a molar basis incorporation of methyl groups and thymidine with the extent of methylation of newly-synthesized DNA. In the calculation of the levels of synthesis and methylation several factors were taken into consideration. These were:

1). possible cellular dilution of exogenously-supplied radioactively-labelled thymidine,

2). the molar relationship between thymidine and DNA nucleotides,

3). a time lag between synthesis and methylation of DNA,

4). cellular dilution of exogenously-supplied radioactively-labelled methionine,

5). incorporation of methyl groups from methionine into thymidine, and

6). incorporation of methyl groups into other nucleotides.

1). In a study of synchronized L929 mouse fibroblast cells, Adams (1969) has shown that these cells have nuclear thymidine pools and that, on average throughout the cell cycle, there are 150 pmol thymidine/10^6 cells. Assuming comparable pool sizes in BHK21/C13 cells, this would mean a dilution factor for exogenously-supplied thymidine of about 1.02 when these cells are incubated in medium containing 5×10^{-6}M thymidine. A similar value can be obtained from the results of a study of calf thymus nucleic acid precursors (Potter, Schlesinger, Buettner-Janusch & Thompson, 1957). Adams (1969) also showed that equilibration of exogenous thymidine with the acid-soluble pool was achieved within 10 minutes of its addition to the culture medium. These facts therefore suggest negligible cell dilution of added thymidine under the conditions used in the present experiments. The contribution of factors 2) -6) have been discussed elsewhere (Results, Sections 1.6.3 and 1.1). It was further assumed that added thymidine was not diluted out by de novo synthesis.

The results of this study showed that the DNA base content of BHK21/C13 cells includes 1 mole% 5-methylcytosine.

In the study of BHK21/C13 DNA methylation (Results, Section 5.1) methyl groups were incorporated into thymidine. The origin of these groups could be (a) from the tetra-hydrofolate one-carbon pool, although this seems unlikely since the basic pyrimidine ring structure did not

become labelled; (b) from deamination of 5-methylcytidine in the later stages of DNA formation - this has been found to be unlikely, at least in HeLa cells (Burdon & Adams, 1969) - ; (c) from deamination of 5-methylcytidine and from thymidine released during RNA turnover; (d) from deamination of cytidine followed by methylation of the uridine formed, or methylation of incorporated uridine at the polynucleotide level of DNA; or (e) if methyl groups of thymidine could exchange with those of methionine. At the present time, no evidence is available to resolve this problem.

2.2.2. RNA

The relationship between the incorporation of added radioactively-labelled uridine and RNA synthesis was found to be more difficult to assess than that for DNA. It was routinely found that levels of incorporation of uridine were much lower than could be expected. The most likely explanation for this would be that added label was being diluted out intracellularly. This dilution could be either (a) endogenous or (b) exogenous.

Dilution by factor (a) could arise from added cytidine. This was included in incubation medium to prevent the incorporation of label from labelled cytidine via labelled uridine into the rapidly-turned-over CCA-terminus of tRNA. However, it has been shown that when exogenous uridine is provided to cell preparations from mouse and human tissues

the reaction catalyzed by uridine kinase is rate-limiting in the utilization of the uridine, and is inhibited by UTP and, especially, by CTP (Cohen, 1968). In anticipation of possible dilution of uridine by cytidine an experiment was carried out to measure uridine incorporation in the presence of different levels of cytidine. No apparent dilution was detected. No other component of the medium was likely to dilute out radioactively-labelled uridine.

Dilution by factor (b) could arise from two sources. A study in B. subtilis has shown that uracil derivatives are in rapid equilibration with one another (Nierlich & Vielmetter, 1968). However, these workers have shown that turnover of RNA contributes to the pools, and that UDP and UTP form part of a cycle of mRNA turnover to which UMP is not a component. If this were the case in BHK21/C13 cells also, UTP from turnover of RNA might effectively dilute out UTP from uridine added in the medium. The second possible endogenous source of uridine is by de novo synthesis. It is possible that enzymes for the processing of uracil to UTP are closely linked to those for its synthesis, so that UTP from this source might also dilute out UTP from added uridine.

Whatever the cause, any dilution of radioactively-labelled uridine would give a misleading, low value for the synthesis of RNA during the labelling period, and therefore a spuriously high percentage of methylated

nucleotide would appear to be present in this RNA, as little dilution of added radioactive methionine occurs (Results, Section, 1.6). It is likely, however, that difficulty in assessment of RNA synthesis is caused by endogenous dilution of uridine, either by de novo synthesis, or by breakdown of pre-existing RNA.

3. TRANSFER RNA

3.1. Transfer RNA Synthesis and Methylation in HSV-Infection

A soluble RNA fraction from HSV-infected BHK21/C13 cells has been shown to be virus-specified (Subak-Sharpe & Hay, 1965) and there is evidence to suggest that at least part of this is tRNA (Subak-Sharpe, Shepherd & Hay, 1966). For these species to differ in the above tests from their host-cell counterparts requires them to have a different base sequence with probably associated secondary or tertiary structural alterations. The synthesis of enzymic and structural proteins in HSV-infected cells may require methylase enzyme activity to methylate newly-synthesized tRNA or alternatively, some enzyme activity may be required to alter the secondary or tertiary structure of pre-existing tRNA molecules to that of tRNA molecules suited to the needs of the virus. In the present study both tRNA methylation and tRNA methylase activity were found to vary in the course of infection of BHK21/C13 cells with HSV.

3.1.1. Early in infection

It was found (Results, Section 3) that an apparent initial increase followed by a rapid decrease in the methylation of newly synthesized 4S RNA was observed in BHK21/C13 cells after infection with HSV. The timing of these alterations was dependent on the multiplicity of infection used.

The initial increase in methylation over synthesis of this species of RNA could result from any of the following factors:-

1. If methylation lagged behind the synthesis of tRNA as it does in the case of DNA in HeLa cells (Burdon & Adams, 1969), and there was an inhibition of host tRNA synthesis soon after infection with HSV, an apparent increase in methylation over synthesis would be detected because of the methylation of previously synthesized tRNA. However, the lag for the conversion of precursor tRNA to tRNA in BHK21/C13 cells is 10 minutes (Burdon, Martin & Lal, 1967), and, at the multiplicity of infection used in the present study, synthesis of 4S RNA remains at the same level as in uninfected cells up to 3 hours P.I. in BHK21/C13 cells (Subak-Sharpe, Shepherd & Hay, 1966; Results, Section 3) and in HEp-2 cells (Wagner & Roizman, 1969).

2. RNA synthesis was in fact higher than was evident from uridine incorporation. This could arise from either an increased de novo

synthesis of uridine soon after infection or from degradation of host RNA. To determine whether or not the former applies would require a study of the enzymes involved in uridine biosynthesis after HSV infection. It is interesting to note that although the incorporation of isotopes into rRNA species was uniformly low, there did appear to be a slight increase in the level of methylation over synthesis of rRNA at the same time as it occurred in 4S RNA. It is possible, therefore, that this was a result of a general effect on uridine incorporation and that at that time isotope-incorporation of RNA precursor did not reflect true RNA synthesis. Perhaps comparison of the above results with measurement of RNA synthesis using radioactively-labelled phosphate would help to elucidate this problem, as would autoradiographic analysis of the bases of the tRNA methylated in the early stages of HSV-infection (cf. Results, Section 2.3). On the other hand, breakdown of host RNA, which can also be correlated with multiplicity of infection, does take place, but the time-course of this is not yet known.

3. It does seem probable that if tRNA is required to be synthesized, it should take place at this time, i.e. prior to the synthesis of the bulk of virus-induced or virus-coded proteins, and we cannot rule out the possibility that it is this tRNA which is being synthesized with a methyl group content which, relative to synthesis, is higher than that of control cell tRNA.

3.1.2. Later in infection

In exponentially-growing BHK21/C13 cells a reduction in both synthesis and methylation of 4S RNA occurred later in HSV infection, and, especially at later times, the fall in methylation was greater than the fall in synthesis (Results, Section 3.1). This drop in synthesis could be caused by an increased de novo synthesis of uridine, an increased reutilization of the products of RNA degradation, or a combination of these, but it is most likely to be related to an inhibition of host 4S RNA synthesis.

The fall in methylation relative to synthesis of "4S" RNA could be 1. a false impression of decreased methylation in tRNA or 2. a true fall in methylation of tRNA.

1. It is possible that the fall in methylation relative to synthesis of "4S" RNA is not as great as was apparent from the results obtained in all systems, and especially in the "serum-depleted" system. Any apparent decrease in methylation could arise from two sources. Firstly, a decrease would be magnified by incorporation of methyl groups from non-radioactive methionine derived, for example, from the breakdown of protein, although this possibility seems unlikely from protein metabolism studies (Results, Section 1.7). Secondly, a decrease in the extent of methylation of newly-synthesized RNA would also be magnified

by the presence of RNA other than tRNA in the 4S fraction after infection. Transfer RNA is the species of RNA with the highest methylated nucleotide content, and so the presence of any other species such as *r*RNA or mRNA (host or virus) which had been synthesized and rapidly broken down during the pulse-time would have diluted out the methylation. It is also possible that some new species of non-methylated, or poorly-methylated "4S" RNA is synthesized in infected cells. It has been shown, for example, that a large amount of 5S RNA appears late in adenovirus-2 infection of KB cells (Reich, Forget, Weissman & Rose, 1966) and there is an accumulation of precursor tRNA and "5S" RNA in pseudorabies virus-infected BHK21/C13 cells (Shepherd, 1969). These, perhaps along with other small RNA molecules which are also low in methylated nucleotide content, might be synthesized in HSV-infected cells. Fractionation of the RNA synthesized in "serum-depleted" cells around 7 - 9 hours P.I., using a system which resolves low molecular weight RNA species would be required to determine the nature of this RNA. To determine whether virus-coded RNA is present in this material which did appear to be partly slightly larger than 4S (Results, Section 3) would require studies of its ability to anneal to HSV DNA. Use of this technique should, in fact, be made at different times after infection to determine whether any virus-coded RNA is methylated.

2. That there is a true fall in the extent of methylation of tRNA in the later stages of HSV-infection is supported by the autoradiographic analysis of soluble RNA 6 hours P.I. (Results, Section 2.3). The fact that base-methylation was inhibited in a non-uniform manner suggests decreased methylase activity at this stage of the growth of the virus. As will be discussed later, altered levels and patterns of tRNA methylation could allow HSV to control metabolism in the cell.

These alterations in the levels of "tRNA" methylation can be correlated with variation in tRNA methylase activity in HSV-infected cells.

3.2. Transfer RNA Methylase in HSV-infection

3.2.1. Early in infection

It was clear from autoradiographic analysis of both tRNA methylated *in vivo* in BHK21/C13 cells and *Esch. coli* tRNA methylated *in vitro* by an extract from BHK21/C13 cells that several tRNA methylases are normally present in BHK21/C13 cells. The presence or absence of spots on the autoradiograms from the tRNA assay products as compared to those obtained from BHK21/C13 tRNA methylated *in vivo* could arise from nucleotide sequences being absent or present in *Esch. coli* tRNA relative to BHK21/C13 t RNA. Thus different dinucleotides (NmpN) could result from hydrolysis of the methylated tRNA.

The tRNA methylase activity in exponentially-growing cells fell about 5-fold when BHK21/C13 cells were incubated in "serum-depleted"

medium (Results, Section 4.3). This decreased activity allows detection of any small elevation of enzyme activity -caused, for example, by virus infection - which would perhaps not be detected in exponentially-growing cells. Thus, at an early stage in HSV-infection of "serum-depleted" BHK21/C13 cells (Results, Section 4.3) there was a slight increase in tRNA methylase activity, as measured in vitro in the 105,000 g supernatant fraction in infected cells relative to uninfected cells (Figure 23). This 2-fold elevation was evident only after 3-hours' infection of serum-depleted cells. It is not known at present whether or not higher levels are reached slightly earlier in infection.

The "mixing" experiments carried out in both systems (Results, Section 4.3) showed incorporation in excess of that expected. It is possible that this incorporation could have arisen from methylation of heterologous tRNA, at sites not methylated by enzymes normally present in BHK21/C13 cells, suggesting again that, perhaps, some tRNA methylase activity was associated with HSV-infection at an early stage, and remained at least until 6 hours P.I. It is not known whether this new activity was required to methylate new tRNA or to supermethylate pre-existing tRNA.

However this may be, it was clear that there was no major increase or decrease in tRNA methylase activity at early times after HSV infection. There is no evidence as to whether the small increase

in enzyme activity was virus-induced or virus-coded.

3.2.2. Later in infection

The increase in total tRNA methylase activity at each stage of infection was transient, and levels had returned to those of uninfected cells in "serum-depleted" cultures by 6 hours P.I. By this time total tRNA methylase activity had fallen below that of uninfected exponentially-growing cells (Results, Section 4.2.). It is not known whether, (a) this is a general decrease in enzyme activity or whether, (b) it is restricted to the methylation of only some bases or nucleotides. Indications are that the latter situation is more likely to be true because, (1), no suggestion of an inhibitor was obtained from "mixing" experiments (Results, Section 4.3), (2), slight differences in the nucleotides methylated in vivo were indicated from autoradiographic studies (Results, Section 2), and (3), inclusion of cycloheximide in the incubation medium of exponentially-growing cells for 9 hours indicated that the tRNA methylases were relatively stable.

From these findings, it would appear that tRNA methylase activity (whether or not it was virus-induced or virus-coded) continued to be present in HSV-cells, at least up to 6 hours P.I.

3.3. Conclusions on Transfer RNA Methylation in HSV-infection

The general findings on RNA synthesis and methylation indicated that there was an initial slight increase followed by a significant

decrease in the level of methylation of newly synthesized 4S RNA, with concomitant slight alterations in tRNA methylase activity in BHK21/C13 cells after infection with HSV. At least part of the decreased methylation of the RNA was caused by synthesis of RNA (perhaps of new species) which was deficient in methyl groups and which may or may not be tRNA.

What significance these findings have to HSV infection is difficult to assess, but it is likely to be linked to the suggested role of methylation of tRNA in control of protein metabolism.

3.4. Role of Methylation of Transfer RNA

Much of the interest in tRNA is linked to its possible role in cellular metabolic regulation. Because of its "linking" role in protein metabolism (Figure 4), control of the form and abundance of this RNA species will be important to the economy of the cell. Theories have been proposed which suggest that control of protein synthesis could be mediated by tRNA, including the "modulation" hypothesis (Ames & Hartman, 1963), the "abundance" hypothesis (Yamane, 1965; Anderson, 1969), the "control by synthesis" hypothesis (Subak-Sharpe & Hay, 1965) and the "adaptor modification" hypothesis (Sueoka & Kana-Sueoka, 1964). It is to this last theory that methylation of tRNA is relevant, and discussion will therefore be confined to it.

That different aspects of tRNA function are structure-dependent has been shown by a variety of methods (Gartland & Sueoka, 1966; Lindahl, Adams, Geroch & Fresco, 1967; Yegian & Stent, 1969; Muench, 1969; Mehler, 1970), and tRNA function can be lost by the alteration of a single base, e.g. pseudouridine (Siddiqui, Krauskopf & Ofengand, 1970).

It is likely that methylated nucleotides play important roles in the maintenance of the structural integrity of tRNA molecules, e.g. by alteration of the affinity of one base for another (Pillinger, Hay & Borek, 1969).

There are conflicting reports concerning reactivity in acylation of methyl-deficient tRNA molecules relative to fully methylated tRNA. Reduced ability to accept amino acids has been reported (Peterkofsky, 1964; Shugart, Chastain, Novelli & Stulberg, 1968), but unaltered efficiency has also been reported (Peterkofsky, Jesensky, Bank & Mehler, 1964; Borek & Srinivasan, 1966), and unaltered or slightly increased ability of phenylanine tRNA has recently been reported (Biezunski, Giveon & Littauer, 1970). Clearly, further work using purified tRNAs is required to determine the role of methyl groups of tRNA in aminoacylation.

A double role for the methyl groups of tRNA is made possible by their presence in the anticodon itself (Matthews, 1963) and elsewhere

in the anticodon loop. Altered binding efficiency has been described for phenylalanine tRNA and leucine tRNA from Esch. coli (Capra & Peterkofsky, 1966, 1968; Stern, Gonano, Fleissner & Littauer, 1970). The altered properties are probably caused by the absence of the large hydrophobic methyl group, in or near the anticodon.

Methylation in the 2'-OH position of the ribose moiety enhances ordered structures (Bobst, Cerutti & Rottmann, 1969) and so may play a role in the function of tRNA.

Although at present the situation is far from clear, it is probable that depending on their nature and position in tRNA, methyl groups will be found to differ in their role from one species of tRNA to another, and that in each case they will cause subtle modifications of one or another of the functions of tRNA in protein synthesis (a) aminoacylation (b) efficiency of binding to ribosomes, or (c) alterations in coding response. From this, it can be seen how regulation of protein synthesis could be partly controlled by modifying specific tRNA molecules, thus altering the translation of genes. Support for this theory comes from (a) the reported distribution of cytokinins (which are growth-promoting in plants) in tRNA species (Armstrong, Burrows, Skoog, Roy & Söll, 1969, (b) by altered tRNA and tRNA methylase activity in tumours as compared to normal tissue (Weinstein, 1968; Baliga, Borek, Weinstein & Strinivasan, 1969), (c) by the differences in tRNA populations and tRNA methylases

in differentiating as compared to non-differentiating tissue (Zeikus, Taylor & Buck, 1969; Baliga, Srinivasan & Borek, 1965; Simon, Glasky & Rejal, 1967; Hancock, McFarland & Fox, 1967) and (d) by the carcinogenic effect of some alkylating agents which result in methylation of DNA and RNA (Lawley & Brookes, 1963).

3.5. Transfer RNA Methylation and Virus Infection

There are several reports of the appearance of new tRNA species, of altered tRNA methylation patterns, and of altered tRNA methylase activities in host cells after virus infection, as, for example, in Esch. coli infected by its bacteriophages (Introduction, Section 7.9). Phages T2 and T4 greatly stimulate tRNA methylase activity early in infection, whilst λ phage first inhibits, and then removes the inhibition of tRNA methylases later in infection. On the other hand bacteriophage T3 inhibits tRNA methylase activity by inducing an S-adenosylmethionine cleavage enzyme. It is clear from results obtained in the present study that HSV does not resemble any of these viruses since (a) any increased tRNA methylase activity in the early stages of infection was very slight in comparison to that after T2-and T4-infection of Esch. coli, (b) no inhibitor of tRNA methylase could be detected, yet a dialyzable inhibitor was found in Esch. coli after λ phage-infection, (c) no S-adenosyl-methionine cleavage activity could be detected after HSV-infection (Results, Section 8).

Alterations in the tRNA population and in tRNA methylating activity also occur after virus infection of higher organisms. For example, avian RNA-containing tumour viruses may contain 4S RNA similar to that of the host cell (Erikson, 1969), may give rise to quantitative and qualitative differences in the tRNA population in the infected cell (Trávniček & Ríman, 1970) or may induce new tRNA methylases (Hacker & Mandel, 1969). Thus it seems that avian tumour viruses behave similarly to the animal tumour viruses (Introduction, Section 7) in that they are able to induce new tRNA methylase enzymes within the cells of tumours which they induce. Thus they elicit a modified pattern of methylation in tRNA, either to cope with the synthesis of new tRNA species or to change the methylation pattern of pre-existing host tRNA. The ability to induce at least one tRNA methylase is likely to be shared by the non-oncogenic virus, polio-virus, to catalyze the formation of 6-methyladenine which is absent in host tRNA before infection but is present after infection (Grado, Friedlender, Ihl & Contreras, 1968).

Again it can be said that HSV does not resemble any of these viruses in the effect it has on the tRNA population and on tRNA methylases. Perhaps, though, it is to be expected that a DNA-containing non-oncogenic virus should behave differently from this RNA virus and from oncogenic viruses. An initial enhancement followed by a steady decline in 4S RNA methylation has been reported in the case of FMDV (Ascione & Vande

Woude, 1969) but these changes have not been correlated with alterations in tRNA methylases. Thus HSV and FMDV cannot yet be compared.

4. RIBOSOMAL RNA

4.1. Function of Methylation in Ribosomal RNA

As already described (Introduction, Section 5.2) methylation of ribosomal RNA appears to be involved in the maturation of this molecule, as methyl groups are conserved in the formation of 18S and 28S from 45S RNA and lack of methylation inhibits ribosome formation. Very little further methylation can take place after the formation of these rRNA molecules.

Methylated nucleotides may have two functions in ribosomes, the first being a structural role, and the second being involved in the binding of tRNA and mRNA to the ribosomes. This latter role was suggested from work on phenylalanyl-tRNA in Esch. coli (Kaji & Nakada, 1967), although fully methylated RNA is not the only requirement in binding (Traub, Hosokawa, Craven & Nomura, 1967).

4.2. Ribosomal RNA and Virus Infection

Inhibition of rRNA methylation is widespread after virus infection. For example, after T4 phage infection ribosomal RNA methylation was greatly reduced (Boezi, Armstrong & DeBacker, 1967), and rRNA methylation is inhibited more rapidly than synthesis after FMDV-infection (Ascione & Vande-Woude, 1969).

4.3. Ribosomal RNA and HSV-infection

In the present study rapid inhibition of synthesis of rRNA was noted, this being greater and more rapid when a multiplicity of infection of 50 P.F.U./cell was used than when 10 P.F.U./cell was used for infection, as it has previously been observed (Wagner & Roizman, 1969). No significant variation in the extent of methylation of newly-synthesized rRNA was detected in either exponentially-growing or "serum-depleted" BHK21/C13 cells infected with HSV. This is, perhaps, to be expected, as rRNA synthesizing and methylating enzymes are located in the nucleolus (Greenberg & Penman, 1966), and this organelle is degraded by HSV (Schwartz & Roizman, 1969).

However, some synthesis of "new" RNA species migrating in the 10 - 20S region of agarose gels was indicated, especially when HSV was grown in "serum-depleted" BHK21/C13 cells (Results, Section 3.2). The time of synthesis of this material (3 - 7 hours P.I.) supports the possibility that this may be virus-coded mRNA as detected previously (Hay, Kötteles, Keir & Subak-Sharpe, 1966). These species contain a negligible level of methylation, which is also a feature of mRNA. From the vast inhibition of synthesis of RNA in exponentially-growing cells (Results, Section 3.1) it seems unlikely that this material is host rRNA. The true nature of this RNA has yet to be determined.

5. DNA

5.1. Host Cell DNA

Mammalian DNA is methylated to an extent of about 1 mole% nucleotide. It has been shown (Results, Section 1.6.3 and Discussion, Section 2.2.1) that this level of methylation in the DNA of BHK21/C13 cells can be measured by relating the incorporation of methyl groups from methionine to the incorporation of a DNA precursor.

5.2. Inhibition of Host Cell DNA Methylation by HSV.

The extent of methylation as well as that of synthesis of DNA has been shown to be inhibited in BHK21/C13 cells after infection with HSV (Results, Section 5.2). This inhibition was progressive with infection, being much greater after 9 hours than after 5 hours, suggesting that not all of the cells were initially infected, with the result that some cells continued to synthesize and methylate DNA normally for some time. Much of the fall in methylation was caused by the synthesis of viral DNA. But, when host cell DNA was separated from viral DNA, calculation still showed that the level of methylation of host DNA itself was decreased after infection, implicating inhibition of host DNA methylation in the early stages of virus infection. The involvement of an inhibitor of DNA methylation was indicated, and this suggestion was supported by the inhibition of rat spleen DNA methylase by the cytoplasmic fraction from HSV-infected BHK21/C13 cells (Results, Section 7.3).

However, at present, the possibility cannot be ruled out that the apparent inhibition of the rat spleen enzyme was caused by degradation of substrate DNA by the action of DNase, whose activity is increased in cells infected with HSV (Keir & Gold, 1963; Russell et al., 1964). However, although this explanation might be true for in vitro measurement of methylation, it is unlikely to hold for the inhibition of host DNA methylation in vivo.

It has been shown that inhibition was not directed towards S-adenosylmethionine synthetase activity (Results, Section 9), nor was there cleavage of S-adenosylmethionine in infected cells (Results, Section 8). These findings were supported by the results obtained for tRNA methylase activity (Results, Section 4 and above). Nevertheless, it cannot be stated decisively at this stage whether the inhibitor acts preferentially on DNA methylase, or whether it acts on all nucleic acid methylases, inhibition occurring only at later stages of virus growth. Consideration of "mixing" experiments with tRNA methylase (Results, Section 4.3), which, even at later stages in virus growth showed no inhibition of tRNA methylase, and of the negligible extent of methylation of HSV DNA supports the former possibility. As already discussed, it is not possible at this time to assay directly for an inhibitor of BHK21/C13 DNA methylase.

5.2.1. Source of inhibitor

There are several possible sources of an inhibitor of DNA methylase in HSV-infected cells. For example, (a) in the viral envelope, (b) in the viral coat protein, (c) a component of the nucleocapsid or (d) a molecule induced by the virus soon after infection of the cell. That inhibition of rat spleen DNA methylase was detected by the inclusion of a cytoplasmic fraction from infected cells does not disqualify any of these possibilities. Although such an inhibitor would be expected to act in the nucleus, it need not originate or be concentrated there. Also, in the preparation of the cell fractions, hypotonic buffer was used to disrupt the cells, and this may have allowed diffusion of a soluble substance from the nucleus into the cytoplasm. In any case, activity of traces of inhibitor in the nucleus would have been masked by methylation of the DNA added in this fraction (Results, Section 7.3). Lack of detection of inhibition of Esch. coli DNA methylase in which a total cell homogenate was used (Results, Section 7.2) could also be explained by this type of phenomenon or by the inhibitor's specificity for a mammalian DNA methylase. Measurement of the inhibitory action of HSV-infected cell fractions prepared by different methods, and of purified virions and/or viral constituents might help to locate the DNA methylase inhibitor.

Another possible explanation for the inhibition of methylation of host DNA in HSV-infected cells is that the virus itself or a virus-induced substance cancels out the action of an activator of DNA methylase. It has been suggested that two factors, an activator and an inhibitor, are involved in the regulation of levels of DNA methylation in Esch. coli (Falaschi & Kornberg, 1965), the inhibitor being a lipopolysacharide of the bacterial cell wall. The presence of an inhibitor of DNA methylase in the membranous fraction of rat hepatoma nuclei has been reported (Datta & Datta, 1969).

5.3. HSV DNA

It has been shown that there was negligible incorporation of label into HSV DNA when this virus was grown in BHK21/C13 cells in the presence of [methyl - ^{3}H]-L-methionine, and this indicated that there was less than one methyl group per virus particle (Results, Section 6). However, despite the low incorporation of methyl groups, it was clear that in each case a small number of counts did appear to be associated with viral DNA, and when the virus was grown in both 7% and 20% normal methionine concentration, this small amount of viral DNA-associated radioactivity occurred on the lighter side of the viral DNA band. This material could have two origins - (a) if HSV DNA were slightly heterogeneous, and allowed incorporation into material of higher A + T base content, or (b) a small piece of host DNA with high

G + C base content consistently banding in this region. With respect to the first possibility, there are no known reports of heterogeneity of HSV DNA, but there is the possibility of some incorporation into the DNA in the form of thymidine. Synthesis and methylation of BHK21/C13 cell DNA in the early stages of infection would have led to the formation of some labelled thymidine (Results, Section 5.1). Subsequent breakdown of this DNA and reutilization of its constituents by the virus could have led to the formation of labelled HSV DNA. With respect to the second possibility, BHK21/C13 DNA is of heterogeneous bouyant density, so after fragmentation there is possible overlap of the two DNAs in the gradients used.

If the small fraction of labelled DNA banding with viral DNA were to prove to be host-cell DNA its origin would be of interest. Polyoma virus DNA has been shown to be non-methylated, but a small piece of (methylated) host DNA can be encapsidated within polyoma virus coats (Kaye & Winocour, 1967). It is possible that this is a property shared by other mammalian viruses. However, if this does apply to HSV, the amount of host DNA must be extremely small, unless the majority of it represents a fraction of host DNA of high G + C content synthesized after viral inhibition of DNA methylation has commenced, or a piece of DNA synthesized and methylated normally before viral infection.

This DNA might also represent a small fraction of host DNA

outwith the virus particle but in very close association with it. This point could be determined by stringent purification of the DNA, for example by density gradient or chromatographic fractionation and repeated DNase treatment, before extraction and analysis of viral DNA.

5.4. Non-methylation of HSV DNA

HSV produces an inhibitor of DNA methylase so its DNA can escape methylation by host-cell DNA methylase, but perhaps other safeguards are included during infection. It is possible, for example, that there are no sites on HSV DNA which are acceptable to a BHK21/C13 enzyme for methylation. Given a purified mammalian DNA methylase assay and sufficient pure, intact HSV DNA this possibility could be tested, although it seems unlikely.

Another mechanism by which HSV DNA remains non-methylated might follow if host DNA methylase is in close association with DNA polymerase and/or nuclear histones, and if DNA is therefore required to have such an association before it can be methylated. Then it would be unlikely that HSV DNA would form such an association since it codes for a DNA polymerase, distinct from that of its host, which synthesizes viral DNA in the soluble rather than the chromatin fraction of nuclei of infected cells. That histones are intimately involved in mammalian DNA methylase reactions has recently been proved, at least for Krebs II

ascites tumour cells (Burdon, personal communication). This also may be the means whereby HSV prevents the methylation of host DNA by dissociating newly-synthesized host DNA from the histone, thereby effectively destroying the host DNA methylase activity. In addition, it is most unlikely that any HSV DNA would be synthesized by the host DNA polymerase, and therefore be available for methylation in the above suggested complex. It has been shown that, at least in the case of Esch. coli, methylation of template DNA is required for DNA replication to occur (Lark, 1968).

5.5. Function of DNA Methylation

DNA methylation and DNA methylases of different host cells and their viruses have been discussed previously (Introduction, Sections 3.4 - 3.9). The effects of methylation of bases on the structure of polynucleotide complexes have also been discussed (Introduction, Section 3.3). But, despite the large volume of work which has been carried out, little is known about the functions of methyl groups in DNA. However, several roles for the methyl groups of DNA have been suggested, such as:

1. Methyl groups might give an identity to the DNA of an organism so that "foreign" DNA would be recognized as such and thus be removed from the recipient cell (Srinivasan & Borek, 1964). Certainly in bacteria this kind of identification may well operate. Also, bacterial host-specificity may be conferred on phage by methylation at specific

sites in its DNA (Arber & Dussoix, 1962; Arber, 1965; Meselson & Yuan, 1968). This modification activity represents the first example of a DNA methylase whose biological function is known. It is linked to restriction by a DNA endonuclease. This relationship between modification and restriction was also suggested by Billin (1968) and has recently been discussed by Arber & Linn (1969). In this modifying role methylation may protect DNA from DNase activity, in other circumstances it may be responsible for making DNA susceptible to this enzyme. For example, alkylation of coliphage T7 DNA with methyl-methansulphate forms sites at which nuclease can act (Strauss & Robbins, 1968).

Nuclease activity is induced in HSV-infected BHK21/C13 cells (Morrison & Keir, 1967). Host cell DNA is broken down, whilst HSV DNA is apparently not affected by this enzyme(s) (Perera, personal communication). It is possible that, by analogy to the above situation, this enzyme specifically acts at sites at or near methylated bases, and that HSV DNA remains unmethylated to escape this degradation.

2. A possible role for methylation as a form of punctuation in DNA is attractive but has been questioned for the following reasons:- (a) such a role would be expected to be enhanced more in organisms high in the evolutionary scale, yet methylation is not related in extent to

this scale (Borek & Srinivasan (1966), and b) even at a level of 1 mole% nucleotide there are too many methyl groups present to make this a possible function, unless this role is involved only when the methylated nucleotide has specific nearest neighbour bases.

3. Methylation of DNA bases has been suggested as a possible guide for the copying or non-copying of a strand of DNA by DNA polymerase. In Esch. coli $15T^-$ methionine starvation does not permit the continuation of DNA replication beyond the point in the chromosome at which methylation ceases. This inhibition can be reversed by methylation but not by ethylation of the DNA (Lark, 1968). This author suggests that a recognition system dependent upon methylation may exist which normally acts as a regulatory mechanism during DNA synthesis. However, coliphage T2, which is normally methylated, when grown in cells co-infected with phage T3 (which induces cleavage of S-adenosyl-methionine) is quite normal in growth and recombination (Gefter, Hausmann, Gold & Hurwitz, 1966). In this case methylation is involved in the continuation of DNA synthesis, but involvement of methylation of DNA in terminating or not allowing DNA synthesis can equally well be envisaged. Thus HSV-coded DNA polymerase would synthesize its own DNA but not that of its host. Since, in addition, there might be little or no control over this synthesis, synthesis of HSV DNA would be rapid

and would continue (until stopped by depletion of precursors) to the advantage of the virus in self-replication.

4. A mechanism similar to that described above can be suggested for control of DNA-dependent RNA polymerase activity. Support for the involvement of DNA methylation in this activity has been given by the decreased in vitro functional template activity, in an RNA polymerase reaction, of calf thymus DNA methylated in vitro by 1-nitroso-3-nitroso-1-methylguanidine (Lingens, Sussmuth, Wacker & Chandra, 1967). On the other hand, the presence or absence of 6-methyladenine in T2 DNA and T4 DNA had no effect on the enzymatic synthesis of RNA, nor did it affect RNA chain initiation (Novogrodsky, Gefter, Maitra, Gold & Hurwitz, 1966).

5. Some forms of methylation of DNA, e.g. 7-methylguanosine formed from carcinogenic alkylating agents can form anomalous base pairing with adenosine, thus causing misreading of DNA in its synthesis and in the synthesis of RNA, and has thus been implicated in tumour formation and activity (Brookes & Lawley, 1960; Colburn & Boutwell, 1968). Mutation by base-pair deletions can also be stimulated by erroneous DNA methylation (Lawley & Brookes, 1968).

It is thus possible that the lack of methylation in HSV DNA is required for specific synthesis of viral DNA by viral DNA polymerase;

uncontrolled, rapid synthesis of viral DNA; or preferential breakdown of host DNA by induced DNase activity. Present studies with isolated enzyme extracts and HSV DNA may determine the validity of these suggested roles, as it should be possible to methylate purified HSV DNA by either enzymic (mammalian or bacterial) or chemical means.

In vitro methylation of bacteriophage T7 converts it into a substrate for a specific nuclease from M. lysodeikticus, the breaks in single strands of DNA occurring at or near the sites of methylation (Strauss & Robbins, 1968). A similar alteration of susceptibility to some nuclease attack might be found after in vitro methylation of HSV DNA, and perhaps its ability to act as template for HSV-induced DNA polymerase, as measured in vitro.

With the recent publication of an infectivity assay for HSV DNA (Lando & Ryhiner, 1969), modified HSV DNA could be tested for alteration in biological activity. It would be of interest to discover whether it were to resemble tobacco mosaic virus in the degree of mutagenicity which was invoked after methylation of its RNA (Singer & Fraenkel-Conrat, 1969), or polyoma virus, the plaque-forming ability of whose DNA was unaltered after in vitro methylation (Winocour, Kaye & Stollar, 1965).

6. APPLICATIONS

This study, in which the level of methylation of RNA and DNA was measured during infection of BHK21/C13 cells by HSV, represents

the first of its kind for animal viruses. Although Winocour, Kaye & Stollar (1965) reported no significant difference in the incorporation of methyl groups into RNA after polyoma virus-infection, they did not measure methylation of newly-synthesized species, nor did they measure enzyme activity. Further studies are planned in which animal viruses, both related and unrelated, can be compared with respect to their effect on the methylation of nucleic acids.

As already discussed, the T-even bacteriophages are apparently similar with respect to their base content and host-specificity, yet differ widely with respect to their effects on methylation.

HSV is a large DNA-containing virus with a high G + C content yet is not methylated. If methylation at C-5 of cytosine is employed to differentiate certain cytosine residues in mammalian DNA, then one might expect HSV DNA to be highly methylated. That it is not suggests that methylation of DNA has a more specific function, such as termination of DNA and/or RNA synthesis. One might, then, speculate that animal DNA-containing viruses will not generally be methylated. This is supported by the fact that pseudorabies has very little methylated base in its DNA (Hay, personal communication).

Speculation might go on from here to suggest that viruses which differ in methylated DNA content might have different origins from those

which do not, but this is not supported by the similarity in methylation, yet probable different origin (Subak-Sharpe, et al., 1966) of polyoma virus and HSV.

HSV DNA apparently escapes methylation by means of an inhibitor. The method of inhibition in the case of polyoma virus is not yet known.

Regardless of the means of inhibition in other systems it would be valuable to locate and isolate the inhibitor of HSV DNA methylation, as this substance could be used in several studies, for example as follows.

Little is known of the function of methylation of DNA in mammalian systems. Is it, for example, involved in the control of DNA replication? In the past it has been difficult to differentiate between effects of lack of methylation in the DNA and shortage of methionine for protein production. Use of an isolated DNA methylase inhibitor could be made to determine which possibility is true.

Different types of mammalian DNA are methylated to different extents. For example, the methylated base-content of satellite DNA is higher than the majority of nuclear DNA (Salomon, Kaye & Herzberg, 1969) whilst that of sperm-cell DNA is much lower (Vanyushin, Tkacheva & Belozersky, 1970). Although use of these different DNAs might be made in studies of the role of the methylated bases in in vitro

studies, use of DNA of which the methylated base content has been controlled by means of an inhibitor might provide additional information.

HSV also causes differences in the levels of methylation of tRNA after infection, with concomitant slight changes in total tRNA methylase activity. This supports the theory that tRNA methylation is important to the economy of the cell. If the tRNA methylase activity induced in the early stages of infection is virus-coded, it is probable that control of tRNA methylation is an important form of control in cells, as the virus is unlikely to waste information on a mechanism which is not beneficial to its own rapid production.

Further speculation on the role of tRNA methylation in uninfected and infected animal cells, and its possible role in the "take over" of infected cells by the virus, awaits further knowledge of the tRNA species methylated in HSV-infection and of tRNA methylation and tRNA methylase activity following infection by other animal viruses.

SUMMARY

This study was carried out to determine the effect of infection of mammalian cells by herpes simplex virus, a large deoxyribonucleic acid (DNA)-containing animal virus, on the methylation of newly-synthesized nucleic acids.

1. Conditions for optimal incorporation of radioactively-labelled methyl groups from methionine into nucleic acids which did not seriously affect cell and virus metabolism were worked out.

2. Ribonucleic acid (RNA) from baby hamster kidney (BHK21/C13) cells, mock-infected or infected with herpes simplex virus and pulse-labelled with radioactive uridine and methionine at intervals during viral growth, was extracted from the cell cytoplasm, and fractionated by electrophoresis on two percent agarose gels.

3. A greatly modified method from that published for agarose gels gave reproducible sharp resolution of the main RNA species using small amounts of RNA.

4. Synthesis of ribosomal RNA as measured by uridine incorporation was rapidly inhibited after infection of both exponentially-growing and "serum-depleted" BHK21/C13 cells. In the latter system, metabolism is reduced compared to "normal", so that virus-induced processes are more readily detected. The level of methylation of newly-synthesized ribosomal RNA did not vary significantly.

5. In the early stages of herpes simplex virus infection synthesis of the small RNA species, 4S, did not vary significantly. In the later stages of infection of exponentially-growing cells "4S" RNA synthesis declined, but in "serum-depleted" cells it increased, reaching a peak seven to nine hours after infection. The extent of methylation of this RNA which may consist of a mixture of "new" RNA, including transfer RNA species, was very much lower than that of uninfected cells.

6. A slight increase in methylation of newly-synthesized 4S RNA with a concomitant slight increase in transfer RNA methylase activity, occurred early in infection. It is not known if these increases can be correlated with the methylation of new species of transfer RNA or super-methylation of pre-existing transfer RNA. Autoradiographic studies suggested that methylation patterns were altered slightly after herpes simplex virus infection.

7. Enzyme "mixing" experiments suggested that the additional enzyme activity might be virus-induced or virus-coded, and that it continued into stages of infection when total transfer RNA methylase activity had decreased. No transfer RNA methylase inhibitor could be detected.

8. New species of RNA, tentatively identified as messenger RNA, which were methylated to a negligible extent, appeared three to seven hours after virus-infection.

9. BHK21/C13 DNA was shown to be methylated via methionine at a level of one mole percent nucleotide, this being present as 5-methylcytosine. Incorporation of methyl groups into thymine from methionine was detected, but the mechanism involved is not known.

10. Infection of BHK21/C13 cells with herpes simplex virus produced progressive inhibition of methylation of newly-synthesized DNA. The DNA of the virus, purified and separated from host DNA by equilibrium density centrifugation was shown to be non-methylated at the poly-nucleotide level.

11. The mechanism whereby herpes simplex virus DNA escapes methylation was shown to be neither by inhibition of S-adenosylmethionine synthetase activity nor by cleavage of S-adenosylmethionine.

12. No BHK21/C13 DNA methylase activity could be measured by any of the available assay systems, but inclusion of cell fractions from control and virus-infected BHK21/C13 cells in the assay of rat spleen DNA methylase suggested the presence of an inhibitor of mammalian DNA methylase in the cytoplasm of herpes simplex virus-infected cells. The origin and nature of this inhibitor are undetermined.

13. It is possible that alteration of methylation patterns of nucleic acids could be a means used by viruses to gain control of host cell metabolic processes.

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