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THE PHOSPHORYLATION OF RIBOSOMAL PROTEINS  
IN BABY HAMSTER KIDNEY FIBROBLASTS

by

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A Thesis Submitted for the Degree of  
Doctor of Philosophy

Department of Biochemistry,  
University of Glasgow.

May, 1982

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To Joanne, who I hope will be feeling better  
by the time this thesis finally comes to print.

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### Abbreviations

The abbreviations recommended by the Biochemical Journal (1981) in its Instructions to Authors (Biochem. J. 193, 4-27) have been used throughout this thesis with the following additions:-

BHK21/C13 cells	:	Baby Hamster Kidney Fibroblasts, litter 21, clone 13.
SDS	:	Sodium Dodecyl Sulphate
BSS	:	Balanced Salt Solution (2.1.3.1)
PBS	:	Phosphate Buffered Saline (2.1.3.2)
RSB	:	Reticulocyte Standard Buffer (2.1.3.3)
Protein Kinase	:	NTP - Protein Phosphotransferase
1 A <sub>260</sub> Unit	:	The quantity of material contained in 1ml of a solution which has an absorbance of 1 at 260nm, when measured in a cell with a 1cm light path. This is taken to be equivalent to :- 100µg of ribosomes, or 50µg of ribosomal RNA

The system of nomenclature for mammalian ribosomal proteins proposed by M<sup>c</sup>Conkey et al. (1979) was used in this thesis.

SUMMARY

This thesis describes studies directed towards understanding the function of the phosphorylation of a particular ribosomal protein - S6 - in eukaryotic cells.

Two experimental approaches to determine the extent of phosphorylation of ribosomal protein S6 were adopted. One involved labelling the cells with radioactive orthophosphate and quantitatively estimating the extent of phosphorylation of the ribosomes by measuring the radioactivity incorporated into the protein and the specific radioactivity of the presumed precursor, ATP. The other involved qualitative estimation of the phosphorylation by analysing the ribosomal proteins in a system of gel electrophoresis in which the phosphorylated derivatives of the protein are resolved from the unphosphorylated protein. This latter method had the advantage that it could be applied when it was not possible to label certain subcellular fractions of ribosomes with ( $^{32}\text{P}$ )-orthophosphate.

It had previously been found that the phosphorylation of ribosomal protein S6 was greater in pre-confluent hamster fibroblasts than in confluent fibroblasts. Part of this thesis describes attempts to determine whether this was due to there being a greater proportion of highly phosphorylated newly-synthesised ribosomes in the pre-confluent cells, as might have been expected if the function of the phosphorylation was in the nucleus.

The extent of phosphorylation of ribosomal protein S6 in nucleoli was first examined electrophoretically after labelling the cells with ( $^{35}\text{S}$ )-methionine, isolating the nucleoli and extracting their protein. However this approach was unsuccessful because of insufficient incorporation of radioactivity into the nucleolar ribosomal proteins. When

nucleoli were incubated in vitro with ( $^{32}\text{P}$ )-( $\gamma$ )-ATP, histones became labelled with ( $^{32}\text{P}$ ), showing that protein kinase activity was present. However no radioactivity could be detected in ribosomal protein S6, so that the extent (if any) of its phosphorylation could not be determined in this way.

The extent of phosphorylation of ribosomal protein S6 was also investigated in 'native' ribosomal subunits, as they include ribosomal subunits which have newly entered the cytoplasm from the nucleus. Proteins from 'native' 40S ribosomal subunits were again analysed by two-dimensional gel electrophoresis. The results here were complicated by the presence of non-ribosomal proteins, but seemed to indicate that ribosomal protein S6 was in a low state of phosphorylation in 'native' 40S subunits.

This conclusion was finally confirmed when the extent of phosphorylation of ribosomal protein S6 was compared in newly-synthesised and older cytoplasmic ribosomes. These two populations of ribosomes were identified by differentially labelling the cells with ( $^{35}\text{S}$ )-methionine, either for 20 minutes before harvesting (newly-synthesised ribosomes), or with a 3 hour pulse of ( $^{35}\text{S}$ )-methionine followed by a 'chase' with non-radioactive methionine for 2 days before harvesting (2-day old ribosomes). Electrophoretic analysis clearly showed that both newly-synthesised and 2-day old ribosomes had ribosomal protein S6 in a low state of phosphorylation. These results demonstrated that newly-synthesised ribosomes were no more phosphorylated than older ribosomes, in contrast to the original hypothesis. This therefore suggested that the proposal that the function of the phosphorylation was in the nucleus was incorrect.

The remainder of the work described in this thesis was concerned with the phosphorylation of ribosomal proteins in cells infected with, or transformed by viruses. It had been reported that in cells infected with several different viruses the ( $^{32}\text{P}$ )-labelling of ribosomal protein S6 increased, although no change in the position of electrophoretic migration of the protein was detected. The work described here clearly demonstrates that in cells infected with pseudorabies virus there is an increase of between 350% and 750% in the extent of phosphorylation of ribosomal protein S6, as estimated by both of the methods mentioned above.

In addition to the considerable increase in the phosphorylation of ribosomal protein S6, the phosphorylation of another ribosomal protein was also detected in the cells infected with pseudorabies virus. This was either S16 or S18, ribosomal proteins that are poorly resolved from one another electrophoretically. The phosphorylation of this protein has never been found in uninfected or mock-infected cells, suggesting that it may possibly be catalysed by a virus-induced protein kinase.

The phosphorylation of ribosomal proteins S6 and S16/18 was shown to be independent of the total cellular concentration of ATP and the intracellular concentration of cyclic-AMP. Although an increase of between 50% and 100% in the concentration of cyclic-AMP was detected in infected cells, a similar increase was also observed in mock-infected cells, suggesting that it was not responsible for the greater phosphorylation of ribosomal protein S6 in cells infected with pseudorabies virus.

The increase in the phosphorylation of ribosomal protein S6 was first detected at between 2 and 4 hours after infection. This preceded both the release of progeny virus from infected cells and the switch from

predominantly host to predominantly virus protein synthesis which were observed to occur at between 6 and 7 hours after infection.

When the cells were infected with herpes simplex virus type I there was a similar increase in the extent of phosphorylation of ribosomal protein S6 to that in cells infected with the related pseudorabies virus. In this case, however, ribosomal protein S16/18 did not become phosphorylated.

The phosphorylation of ribosomal proteins was also examined in cells transformed by Papova viruses. It was found that in cells transformed by polyoma virus and simian virus 40 there was an increase of approximately 230% in the extent of phosphorylation of ribosomal protein S6, but phosphorylation of ribosomal protein S16/18 was not detected.

The results do not allow a firm conclusion on the function of the phosphorylation of ribosomal protein S6 but provide a background against which proposals can be examined. Possible functions of the phosphorylation of ribosomal protein S6 discussed include both general and specific stimulation of host and/or viral protein synthesis.

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1. INTRODUCTION

## 1.1 THE RIBOSOME.

The ribosome is central in the translation of genetic information into proteins which are required for the structure as well as the functioning of the cell.

The first evidence for the existence of the discrete structures we now call ribosomes came from Claude (1941), who observed microscopic particles within chick and mammalian embryo cells. Studies by electron microscopy (Palade & Siekevitz, 1956) and electrophoresis (Petermann, Hamilton & Mizen, 1954) confirmed the existence of these structures, and established their nucleoprotein composition. Subsequently the term 'ribosome' was first used by Roberts (1958) to describe these ribonucleoprotein particles.

Direct evidence for the role of ribosomes in protein synthesis was shown by pulse-labelling experiments using radioactively labelled amino acids, in rat liver (Littlefield et al., 1955) and later in E. coli (M<sup>C</sup>Quillen, Roberts & Britten, 1959). In these studies it was found that the ribosome fraction was radioactively labelled much more rapidly than the bulk of the cytoplasmic proteins, indicating that ribosomes were the site of protein synthesis.

Ribosomes have been observed in all prokaryotes (reviewed by Jaskamus, Nomura & Davis, 1974; Brimacombe, Stoffler & Wittmann, 1978; Osawa & Hori, 1980) and in the cytoplasm of all eukaryotes (reviewed by Wool & Stoffler, 1974; Cox, 1977; Bielka & Stahl, 1978; Wool, 1979). In addition it has been shown that ribosomes, distinct from those in the eukaryotic cytoplasm, exist within the mitochondria (Borst & Grivell, 1971; Kroon, Agsteribbe & De Vries, 1972) and chloroplasts (Littleton,

1962). Moreover the ribosomes found in the eukaryotic cytoplasm can be of two classes, either 'free' or 'membrane bound' (Palade, 1956; Palade, 1975), the latter being responsible for the synthesis of proteins destined for secretion or insertion into biological membranes (reviewed by Sabatini & Kreibich, 1976).

#### 1.1.1 Constituents of the Ribosome.

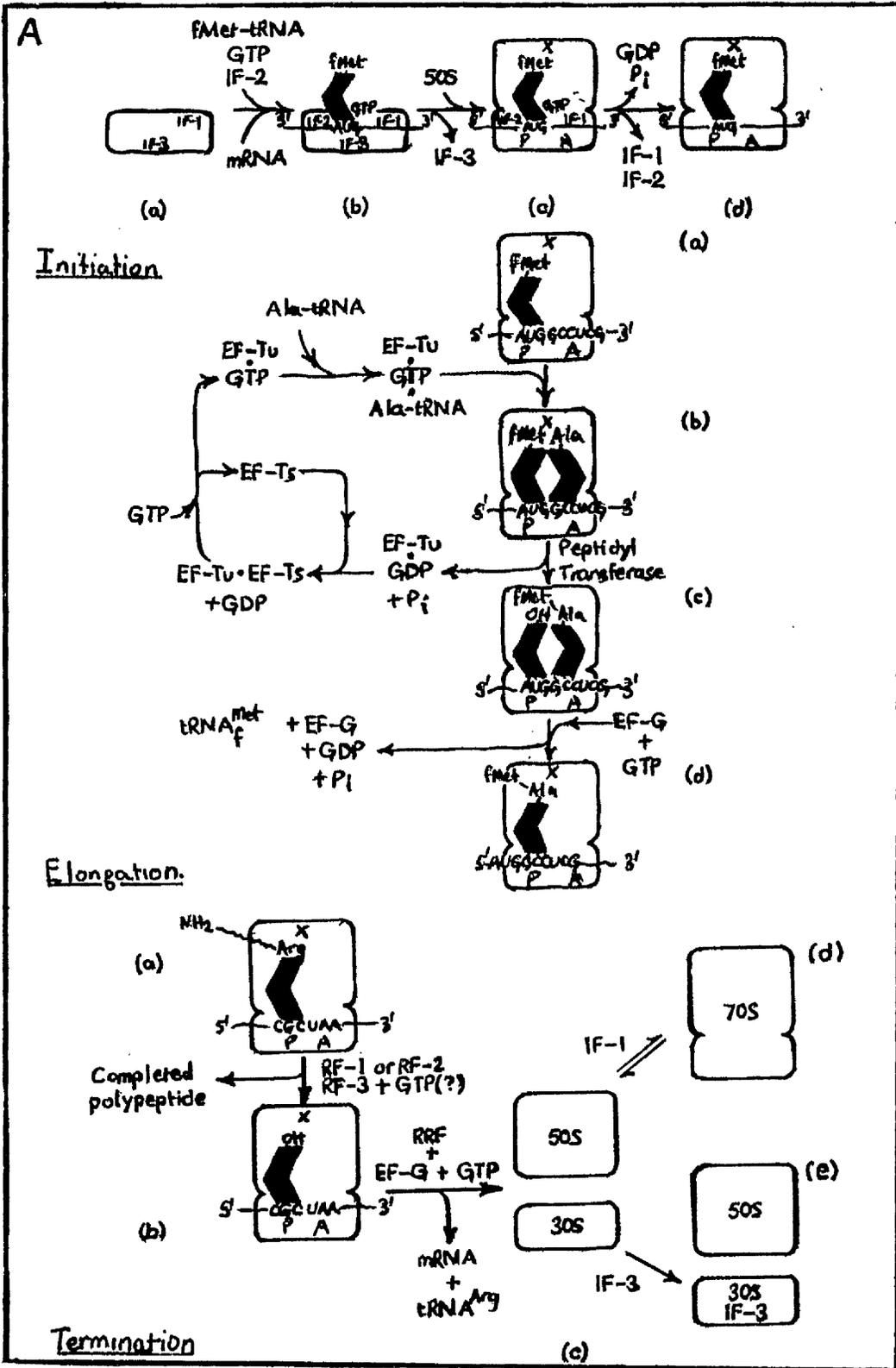
More is known about the structure of bacterial ribosomes and their components, than that of eukaryotes, with which the work described in this thesis is concerned. It is therefore intended to indicate only in outline what is known of bacterial ribosomes, this topic having been extensively reviewed (Cox, 1977; Wittmann, 1979; Nierhaus, 1982). The function of the ribosome in protein synthesis has also been extensively reviewed (Weissbach & Pestha, 1977; Weissbach, 1980). However, to orient the reader, summary schemes of prokaryotic and eukaryotic protein synthesis are presented in Figure 1.1.1.

The ribosome of E. coli has a sedimentation coefficient of 70S and is composed of two dissimilar subunits of sedimentation coefficients 30S and 50S (Tissieres et al., 1959). The ribosomes found in the cytoplasm of eukaryotes are larger than those of prokaryotes, having a sedimentation coefficient of 80S and being composed of subunits of sedimentation coefficients 40S and 60S (Petermann, 1964). The organelle ribosomes have a size similar to that of prokaryotes, with chloroplast ribosomes having a sedimentation coefficient of approximately 70S (Chua & Luck, 1974; Boynton, Gillham & Lambowitz, 1980). Mitochondrial ribosomes from fungi and higher plants have a sedimentation coefficient of 70-75S, whereas mitochondrial ribosomes from animal cells have a sedimentation coefficient of 55-60S (Chua & Luck, 1974; Boynton, Gillham & Lambowitz, 1980). Mammalian ribosomal subunits have a molecular weight estimated

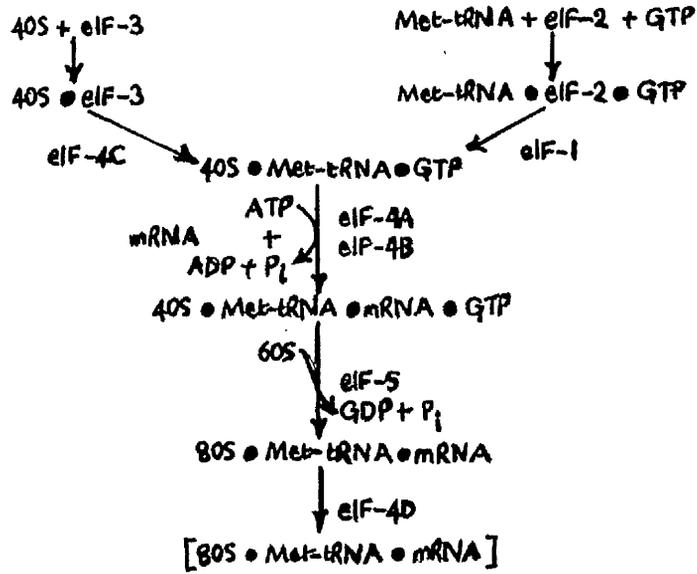
Fig. 1.1.1 Summary Schemes of Prokaryotic and Eukaryotic Protein  
Synthesis

- A. Prokaryotic protein synthesis
- B. Eukaryotic protein synthesis

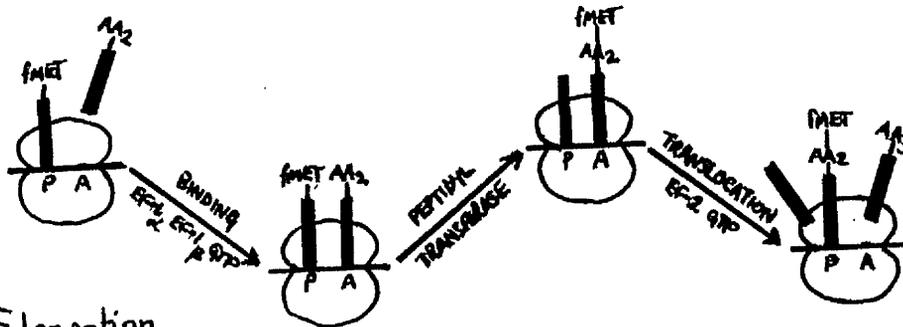
Reproduced from Weissbach (1980) and Adams et al. (1981).



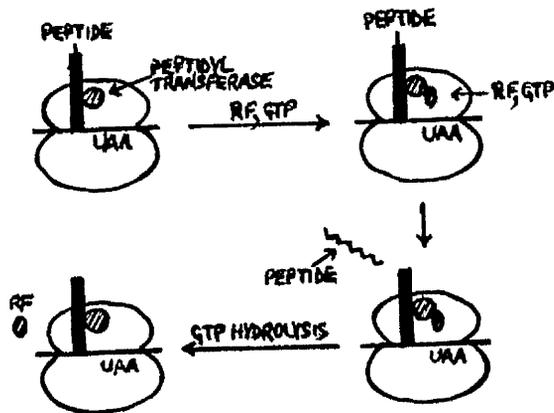
B



Initiation



Elongation



Termination

to be approximately  $1.5 \times 10^6$ , for the 40S subunit, and  $2.9 \times 10^6$ , for the 60S subunit (Hamilton, Pavlovec & Petermann, 1971). Although the term '80S ribosome' is commonly used to refer to eukaryotic ribosomes, it is not strictly accurate since the mass of the '80S ribosome' can vary from  $3.9 \times 10^6$  daltons in plants to  $4.55 \times 10^6$  daltons in mammals (Cammarano et al., 1972). This increased mass reflects an increase in the size of the 60S subunit, with the size of the 40S subunit having changed less during evolution.

Prokaryotic ribosomes have been found to contain three RNA molecules: 16S in the 30S subunit (Kurland, 1960), and 23S (Stanley & Block, 1965) and 5S (Rossett & Monier, 1963) in the 50S subunit. In contrast the ribosomes found in the cytoplasm of eukaryotes contain four species of RNA: 18S in the 40S subunit, and 28S, 5S and 5.8S in the 60S subunit. (reviewed by Maden, 1971). Chloroplast ribosomes contain similar RNA species to bacteria (ie. 16S, 23S and 5S) but like eukaryotic cytoplasmic ribosomes contain a second low molecular weight RNA, but of 4.5S instead of 5.8S (Dyer, Bowman & Payne, 1976). Mitochondrial ribosomes appear to be structurally more diverse, with those from fungi and higher plants containing two large RNAs and a 5S RNA whereas those from animal cells contain only the two large species of RNA (Chua & Luck, 1974; Boynton, Gillham & Lambowitz, 1980). As relatively little is known of these organelle ribosomes, they will not be considered further here.

The nucleotide sequence has been determined for each rRNA from both prokaryotes (Brownlee, Sanger & Burrell, 1967; Brosius et al., 1978; Brosius, Dull & Noller, 1980) and eukaryotes (Forget & Weissmann, 1967; Boseley et al., 1978; Veldman et al., 1981; Salim & Maden, 1981). Comparison of the sequence of 26S rRNA from yeast (Veldman et al., 1981) with that of

23S rRNA from E. coli (Brosius, Dull & Noller, 1980) does not reveal extended tracts of homology, the longest consisting of fourteen consecutive nucleotides. However, the overall homology in some stretches can be as high as 85%. There also appears to be little extensive homology between the sequences of 18S rRNA from Xenopus laevis (Salim & Maden, 1981) and the 16S rRNA from E. coli (Brosius et al., 1978) except near the 3' end. It has recently been suggested that the 5' end of 23S rRNA from E. coli is homologous to the 5.8S rRNA in eukaryotes (Nazar, 1980). Comparison of the sequence at the 5' end of 23S rRNA with the sequence of 5.8S rRNA from four different eukaryotes reveals approximately 50% homology (Jacq, 1981) and a similar level of homology was also observed between the sequences of E. coli 5S rRNA and the 5S rRNA from the same four eukaryotes (Jacq, 1981). In addition base complementarity has been suggested between the 5' end of 5.8S rRNA and the 3' end of 28S rRNA (Sollner-Webb & Reeder, 1979) and it has been observed that these two rRNAs are associated by hydrogen bonding in the 60S subunit (Pace, Walker & Schroeder, 1977). Models of the secondary structure for the rRNAs have been proposed (eg. Brimacombe, 1980; Noller et al., 1981; Stiegler et al., 1981; Veldman et al., 1981) some of which suggest considerably more homology between the prokaryote and eukaryote rRNAs.

Evidence from starch gel electrophoresis and carboxymethylcellulose chromatography suggest that ribosomes contain a large number of different proteins (Waller, 1964). This was later confirmed through analysis by polyacrylamide gel electrophoresis, and further chromatographic resolution of the proteins (Traut et al., 1967; Kaltschmidt et al., 1967; Hardy et al., 1969). However, since the many different components of the ribosome are not linked to one another by covalent bonds it has been necessary to define exactly what one means when a protein is referred to as a ribosomal protein. This is particularly

important since many proteins can bind to ribosomes both specifically (eg. protein synthetic factors in both prokaryotes and eukaryotes: Weissbach & Ochoa, 1976) and non-specifically (eg. E. coli ribonuclease: Waller, 1964).

A protein can be thought of as a ribosomal protein if it is assembled with rRNA and remains with the assembled ribosomal particle through successive rounds of protein synthesis. Zinker and Warner (1976) designated such proteins as class I ribosomal proteins. Such a theoretical definition is difficult to apply, and for practical purposes proteins are regarded as ribosomal if they remain with ribosomes after repeated washings with high concentrations of salt (Wool & Stoffler, 1974). Unfortunately the high concentrations of salt required to remove contaminating proteins can sometimes remove certain ribosomal proteins (Hardy, 1975). These (eg. protein S1 in E. coli: Van Dieijen et al., 1973) would fall into class III proteins of Zinker and Warner (1976), defined as proteins which can be removed from ribosomes by washing with high concentrations of salt. However this also includes proteins that are adventitiously adsorbed on to the ribosome. Zinker and Warner (1976) also proposed a class II of ribosomal proteins that can undergo exchange between ribosomes and a pool of soluble cytoplasmic protein in vivo. However the existence of such proteins is still somewhat controversial.

The ribosome of E. coli has been shown to contain 21 unique proteins in the 30S subunit, and 32 in the 50S subunit (reviewed by Wittmann, Littlechild & Wittmann-Liebold, 1980). Most ribosomal proteins are present in single copies in these ribosomes (Voynow & Kurland, 1971; Weber, 1972; Deusser, 1972; Hardy, 1975), one notable exception are the acidic proteins L7/L12 from the 60S subunit, (which differ only in

that the N-terminal serine residue of the former is acetylated: Terhorst et al., 1973) and are present in four copies per ribosome (Subramian, 1975). Ribosomes from different eukaryotic species have been reported to contain between 70 and 85 different proteins (reviewed by Wool, 1979). This variation in number might reflect genuine differences in the number of proteins in ribosomes from different species of eukaryotes (Wool & Stoffler, 1974) or may be due to difficulties in deciding whether or not a protein is a structural ribosomal protein (Warner et al., 1973).

The relative positions of migration of the ribosomal proteins on two dimensional gel electrophoresis has been the basis for nomenclature of ribosomal proteins (Kaltschmidt & Wittmann, 1970). A recent agreement on a standard nomenclature for mammalian basic ribosomal proteins has been made (McConkey et al., 1979) and this is the system used in the present study (cf. Fig. 2.2.7).

Eukaryotic ribosomal proteins have also been purified by chromatographic procedures (Wool, 1979). Rat liver ribosomal proteins have been separated by ion-exchange chromatography on carboxymethyl-cellulose, phosphocellulose and DEAE cellulose as well as by gel filtration (Tsurugi et al., 1977; Collatz et al., 1977; Lin, Tanaka & Wool, 1979). In this manner a total of 84 proteins were identified (reviewed by Wool, 1980), considerably more than had been previously estimated by two dimensional polyacrylamide gel electrophoresis (Wool & Stoffler, 1974). The identities of these proteins were determined by two dimensional gelelectrophoresis and their molecular weights estimated by one dimensional SDS gel electrophoresis. The number average molecular weight for the 34 40S subunit proteins was about 22,000 with a range of 11,200 to 39,000. The number average molecular

weight for the 50 proteins isolated from the 60S subunit was 21,000 with a range of 11,500 to 41,800. This gives the average molecular weight for a eukaryotic (rat liver) ribosomal protein of about 21,300 compared with the average for an E. coli ribosomal protein of approximately 18,000 (Wittmann et al., 1980).

However it must be considered that not all of these proteins are unique. Proteins S3a and S3b have almost identical tryptic peptide fingerprints, as do proteins S5 and S5', S16 and S19, S15 and S25, S18 and L23' and hence may represent single proteins. There is also evidence that some of the rat liver ribosomal proteins purified by Tsurugi et al. (1977), Collatz et al. (1977) and Lin, Tanaka and Wool (1979) could have been derived from others by proteolysis or other chemical modification (Leader & Mosson, 1980; Madjar & Traut, 1980).

Ribosomal proteins from prokaryotes and eukaryotes have been tested for immunological and functional cross-reactivity and it has been concluded that in general there is little homology between them (reviewed by Wool, 1979). However there is considerable evidence to suggest that certain acidic proteins of eukaryotic 60S subunits are structurally and functionally equivalent to proteins L7/L12 from E. coli ribosomes. Antiserum raised against proteins L7/L12 was shown to cross-react with 60S subunits from yeast (Wool & Stoffler, 1974), rat liver (Wool & Stoffler, 1974; Stoffler et al., 1974) and chicken liver (Howard, Smith & Gordon, 1976) and can also inhibit eukaryotic polyphenylalanine synthesis (Grasnuk, Nolan & Drews, 1977). Replacement of the acidic proteins in Artemia salina (Moller et al., 1975) or yeast (Richter & Moller, 1974) by E. coli proteins L7/L12 restored the activity of these ribosomes as well as did eukaryotic acidic proteins, indicating the functional equivalence of these proteins from different species. Antiserum raised

against the acidic protein eL12 from Artemia salina inhibits the EF-2 dependent GTPase reaction of Artemia salina ribosomes (Van Agthoven, Maasen & Moller, 1977), also indicates that its function is similar to that of L7/L12 of E. coli (Moller, 1974). Both E. coli proteins L7/L12 and the eukaryotic acidic proteins appear to be located at the subunit interface (Tischendorf, Zeickardt & Stoffler, 1975; Rankine & Leader, 1976; Horak & Schiffmann, 1977) and both appear to be present in multiple copies in ribosomes (Subramian, 1975; Van Agthoven et al., 1978; Kruiswijk, Planta & Magr, 1978), in contrast to most other ribosomal proteins which are found in only single copies in ribosomes. There is one other example of homology between prokaryotic and eukaryotic ribosomal proteins. Antisera raised against total eukaryotic 40S protein and eukaryotic ribosomal protein S6 inhibited polyphenylalanine synthesis in E. coli (Fischer, Stoffler & Wool, 1978). Although this suggests that eukaryotic ribosomal protein S6 (at least) is homologous to a protein of E. coli ribosomes, this latter has not yet been identified.

The complete amino acid sequences of all prokaryotic ribosomal proteins are known (reviewed in part by Wittmann, Littlechild & Wittmann-Liebold, 1980). In contrast, the sequence data for eukaryotic ribosomal proteins is mainly limited to the amino termini of several ribosomal proteins from rat liver (Wittmann-Liebold et al., 1979). One eukaryotic ribosomal protein the complete sequence of which has been determined is eL12 from Artemia salina (Amons, Pluijms & Moller, 1979). However, despite the apparent functional equivalence of this protein with proteins L7/L12 of E. coli, the sequence homology between them is very limited.

When eukaryotic ribosomal proteins from different tissues of the same organism were analysed by two dimensional electrophoresis essentially similar patterns were obtained (Martini & Gould, 1975; Fujisawa &

Elicein, 1975). There are minor differences between the patterns of ribosomal proteins from different species of higher eukaryotes (Ramjoun & Gordon, 1977; Issinger & Beier, 1978; Madjar & Traut, 1980) but more extensive differences between those of higher and lower eukaryotes (cf. eg. McConkey et al., 1979; Zinker & Warner, 1976).

#### 1.1.2 Synthesis and Assembly of Ribosomes.

The ribosomes of E. coli contain three RNA species and over fifty proteins and it appears that regulatory mechanisms ensure that the amounts of each ribosomal component synthesised are in the same stoichiometric ratio as found in the ribosome.

Production of equimolar amounts of all three ribosomal RNAs is ensured by the co-transcription of the genes for 16S, 23S and 5S rRNAs (Dunn & Studier, 1973; Ginsburg & Steitz, 1975). Ribosomal protein genes also appear to be found in clusters thus facilitating coordinate expression of these genes (reviewed by Nomura & Post, 1980). In addition, certain ribosomal proteins appear to have the ability to prevent the further translation of the polycistronic mRNA coding for their own and other ribosomal proteins, by direct interaction with the mRNA (Dean et al., 1981). In E. coli the synthesis of ribosomal RNA and ribosomal protein are tightly coupled. When protein synthesis is inhibited by amino acid starvation the rate of synthesis of both rRNA and the mRNA for ribosomal proteins is rapidly inhibited by the production of guanine tetraphosphate (ppGpp) (reviewed by Gausing, 1980). Thus under different conditions of growth the rate of synthesis of the ribosomes can be controlled by adjusting the levels of guanine tetraphosphate.

In eukaryotes the situation is somewhat more complex. The synthesis of ribosomes involves the coordinate expression of 28S, 18S, 5S and 5.8S

rRNAs as well as the genes coding for between 70-85 different proteins (Perry, 1972). The synthesis of 28S, 18S and 5.8S rRNA is coordinate since the genes for these RNA molecules belong to a single transcription unit (Maden et al., 1977; Long & Dawid, 1980). However the different genomic locations of the genes for 5S rRNA (Long & Dawid, 1980) and the widely dispersed positions of the ribosomal protein genes (D'Eustachio et al., 1981) indicates that more complex mechanisms, possibly involving feedback regulation (Pelham & Brown, 1980) are also involved. Moreover the complexity is increased since the 45S pre-rRNA transcription unit is transcribed by RNA polymerase I, whereas the 5S rRNA genes are transcribed by RNA polymerase III and ribosomal protein genes are transcribed by RNA polymerase II (Planta & Meyerink, 1980).

Eukaryotic ribosomal proteins are synthesised in the cytoplasm (Maisel & M<sup>C</sup>Conkey, 1971; Craig & Perry, 1971) and subsequently migrate to the nucleolus (Warner & Siero, 1967; Wu & Warner, 1971). Little is known about the way in which ribosomal proteins enter the nucleus (Warner et al., 1980). Although the nuclear envelope is studded with poly-ribosomes, it is clear that they are not enriched in mRNA for ribosomal proteins (Craig & Perry, 1971). Furthermore the complex structure of the nuclear envelope, with a double layer of membranes, a proteinaceous lamina, and tightly packed chromatin (Kirschner, Rusli & Martin, 1977) makes direct extrusion of newly formed proteins into the nucleus appear unlikely. There is also no direct evidence bearing on the question of whether the passage of proteins into the nucleus occurs by passive diffusion or by facilitated transport (Warner et al., 1980).

Nevertheless, Warner (1979) has shown that most newly synthesised ribosomal proteins were concentrated 10-15 fold in the nucleolus and 2-5 fold in the nucleoplasm. Pretreatment of cells with actinomycin D,

to deplete the nucleolar pool of pre-rRNA, had no effect on the concentration of newly formed ribosomal protein in the nucleus, but did lead to an increased amount in the nucleoplasm, at the expense of the nucleolus. This suggests that ribosomal proteins accumulate in the nucleus independently of the presence of pre-rRNA, but that binding to rRNA may account for the very high concentration found in the nucleolus. This conclusion is supported by the finding that most ribosomal proteins are found in equimolar amounts in the nucleolus (Phillips & M<sup>C</sup>Conkey, 1976).

Continued inhibition of rRNA synthesis by low doses of actinomycin D (this treatment specifically inhibits RNA polymerase I) shows that ribosomal proteins continue to be synthesised at a normal rate for 30 hours after the transcription of pre-rRNA had ceased (Warner, 1977). However, these proteins do not accumulate, but are degraded with half-lives of between 20-120 minutes, depending on the individual protein. Under normal conditions the ribosomal proteins are very stable, presumably because their assembly into ribosomes provides a structure in which the components are less susceptible to degradation (Warner, 1977). These findings suggest that the synthesis of mRNA for ribosomal proteins is independent of the synthesis of rRNA. In contrast to the continued synthesis of ribosomal proteins in the absence of rRNA, it appears that rRNA synthesis decreases in the absence of ribosomal proteins (Warner et al., 1980).

Under normal circumstances, the amount of each ribosomal protein synthesised appears to be within about 10% of the amount utilised (Warner, 1977; Gorenstein & Warner, 1977). This is remarkable if one considers the steps involved in determining how much of a protein is synthesised. Warner et al. (1980) have suggested that each ribosomal

protein could limit its own synthesis to some degree, possibly in a similar manner to that which occurs in E. coli (Dean et al., 1981).

In E. coli all three rRNAs as well as several tRNAs, found within the transcription unit, are synthesised as a single precursor of sedimentation coefficient 30S (reviewed by Schlessinger, 1980). This 30S pre-rRNA is cleaved to yield pre-23S, pre-16S and pre-5S rRNA (Ginsburg & Steitz, 1975), and these species are subsequently cleaved to yield the mature rRNAs in a process which requires the presence of ribosomal proteins (Sogin, Pace & Pace, 1977; Meyhack, Pace & Pace, 1977; Hayes & Vasseur, 1976). Two precursor particles have been described for the 30S subunit and three for the 50S subunit (Schlessinger, 1974). These precursor particles contain the pre-rRNA molecules, several ribosomal proteins as well as precursor proteins not present on the mature particles (Nierhaus, 1980).

Assembly of ribosomes in eukaryotes occurs in the nucleolus (Maden, 1968) and an 80S ribonucleoprotein particle can be isolated from nuclei of cells which are synthesising ribosomes (Warner & Siero, 1967). This 80S particle is thought to be the precursor for 40S and 60S subunits and contains the 45S pre-rRNA transcript as well as many 40S and 60S ribosomal proteins and some non-ribosomal proteins (reviewed by Warner et al., 1980). Maturation of the 80S particle gives rise to the 40S ribosomal subunit together with a 55-60S precursor particle (Warner & Siero, 1967; Kumar & Subramanian, 1974). During this maturation the 45S pre-rRNA transcript is cleaved to give 18S rRNA and a 32S pre-rRNA, a process which requires the presence of ribosomal proteins (Warner & Udem, 1972). The 55-60S precursor particle contains the 32S pre-rRNA, the 5S rRNA and approximately 60 proteins, about half of which appear to be ribosomal proteins since they were also found in mature 60S

ribosomal subunits (Kumar & Subramanian, 1975). Many of the non-ribosomal proteins found in the 55-60S particle were similar to those found in the 80S precursor particle and may perhaps be used repeatedly as scaffolding for ribosome assembly in the nucleolus (Warner et al., 1980). The 55-60S particle matures to give the 60S ribosomal subunit by further cleavage of the 32S pre-rRNA to 28S and 5.8S rRNAs and an alteration in the complement of associated proteins. Little is known regarding the migration of mature ribosomal subunits from the nucleus to the cytoplasm, except that it is a complex process (Warner et al., 1980).

The question as to whether cytoplasmic ribosomes undergo exchange with a cytoplasmic pool of free ribosomal proteins has been controversial. It was reported that up to 17% of the total ribosomal proteins in rat liver were present in free cytoplasmic pools, and that these free proteins could undergo a rapid exchange with those in ribosomes (Dice & Schminke, 1972). However repeated attempts (Wool & Stoffler, 1976; Warner, 1977) failed to demonstrate significant pools of most ribosomal proteins in the cytoplasm. Nevertheless it is clear that exchange of some ribosomal proteins does occur (Zinker & Warner, 1976; Warner & Gornstein, 1978) but this is likely to be restricted to a relatively small number of proteins.

Ribosomes are relatively stable structures with a half-life of approximately five days (Hirsch & Hiatt, 1966) and appear to turnover as whole units with the rRNAs and each of the ribosomal proteins being destroyed together (Tsurugi, Marita & Ogata, 1974).

### 1.1.3 Structure of the Ribosome.

The molecular structure of the ribosome has been investigated by a wide variety of physical and chemical techniques.

E. coli ribosomes have been reconstituted from purified ribosomal proteins and rRNA. This has been useful both in the study of protein topography and of the intermediates in the assembly of the ribosome (reviewed by Nierhaus, 1980). Total reconstitution of eukaryotic ribosomes has not yet been achieved, although there has been successful reconstitution of partially disassembled 60S subunits (Cox & Greenwell, 1976) and 40S subunits (Cox, 1981).

RNA-protein interactions have been studied in more detail by non-covalent binding of isolated E. coli ribosomal proteins to rRNA molecules (reviewed by Zimmerman, 1980). The specificity of binding of particular proteins to specific regions of the RNA was examined by the degree of protection afforded against ribonuclease (Douthwaite et al., 1979) or ketoxal (Garrett & Noller, 1979). In addition cross-linking of RNA to proteins has provided information on the proteins in the neighbourhood of RNA species within subunits (Wower et al., 1981). RNA-protein interactions have been examined in eukaryotic ribosomes in studies in which isolated ribosomal proteins were bound to immobilised RNA molecules. 5S rRNA (Metspalu et al., 1978; Ulbrich et al., 1980), 5.8S rRNA (Metspalu et al., 1978; Toots et al., 1979; Todokoro et al., 1981) and tRNA (Metspalu et al., 1978; Ulbrich et al., 1980) have all been studied in this manner. In these investigations it was consistently found that proteins L6 and L19 bound to 5S and 5.8S rRNA, suggesting the close proximity of these two RNA molecules. In addition a small number of 40S subunit proteins could also bind to 5.8S rRNA (Toots et al., 1979). As this RNA is a component of the 60S subunit this suggests it is located

at the subunit interface, although it is difficult to exclude the possibility of non-specific association of basic ribosomal proteins with RNA. Proteins within ribosomal subunits have also been cross-linked to rRNA and polyuridylic acid, either chemically (Svoboda & McConkey, 1978; Terao & Ogata, 1979a) or by irradiation with ultraviolet light (Reboud et al., 1978; Buisson et al., 1979; Terao & Ogata, 1979b; Reboud et al., 1980).

In the absence of X-ray crystallography a variety of techniques have been used to study the relative distribution of proteins on the ribosome. The most important of these will be briefly considered.

Cross-linking of E. coli ribosomal proteins with bi-functional agents (reviewed by Traut et al., 1980) has been useful in building maps of ribosomal protein topography. Cross-linking of eukaryotic ribosomal proteins has been performed in both the 40S subunit (Terao et al., 1980; Westermann, Gross & Bielka, 1980; Tolan & Traut, 1981) and 60S subunit (Uchiumi, Terao & Ogata, 1980).

Electron microscopy has been used to directly visualise the supra-molecular structure of ribosomal subunits from E. coli (Stoffler et al., 1980; Lake, 1980). Using 'immune electron microscopy' (electron microscopic visualisation of ribosomal subunits bridged by divalent antibodies to individual E. coli ribosomal proteins), it has been possible to locate individual ribosomal proteins on the surface of subunits. Three dimensional models of E. coli ribosomal subunits have been proposed based largely on these studies (Stoffler et al., 1980; Lake, 1980). Electron microscopy of eukaryotic ribosomal subunits indicate that they have essentially the same morphology as E. coli subunits despite their larger size (Doublik & Hellmann, 1978) although some

differences were also observed. A start has also been made to determine the position of ribosomal proteins on eukaryotic subunits by immune electron microscopy (Noll et al., 1978; Lutsch et al., 1979; Bommer et al., 1980).

Neutron scattering has been used to obtain information on the relative position of ribosomal proteins within the 30S subunit of E. coli (reviewed by Moore, 1980). This technique requires that ribosomal subunits be reconstituted using rRNA and purified ribosomal proteins, two of which have had the majority of their hydrogen atoms replaced by deuterium. The scattering of neutrons by these 'deuterated' proteins can generate information which can be used to determine the distance between them. Thus the relative positions of various ribosomal proteins has been determined and a corresponding model of ribosomal subunits built up (Ramakishman et al., 1981; Sillers & Moore, 1981). Such data has also been used to clarify discrepancies between the models of Stoffler et al. (1980) and Lake (1980), obtained from immune electron microscopy studies, and appears to favour the model of Lake (1980).

Affinity labelling and substrate-ribosome cross-linking have been used to identify the proteins involved at functional sites in the E. coli ribosome (reviewed by Cooperman, 1980). In eukaryotes, ribosomal proteins from rat liver have been cross-linked to initiation factor eIF-2 (Noll et al., 1978; Westermann et al., 1979). In particular proteins S3a and S6 have been cross-linked to the ternary initiation complex (Westermann, Nygard & Bielka, 1981) and antibodies raised against these proteins can block the binding of the ternary initiation complex to 40S subunits (Bommer et al., 1980). It has been suggested that these proteins are involved in the organisation of the P-site, a suggestion which is consistent with observations that proteins S3/S3a are located

near the mRNA entrance site (Stahl & Kobets, 1981) and that proteins S3a and S6 can be cross-linked to natural mRNA (Takahashi & Ogata, 1981), and protein S6 to polyuridylic acid (Terao & Ogata, 1979a,b). Proteins in rat liver and yeast 60S subunits have been labelled with analogues of puromycin (Bohm, Stahl & Bielka, 1979; Reboud et al., 1981) and tRNA (Perez-Gosalboz, Vasquez & Ballesta, 1978; Stahl et al., 1979). A consistent observation in all of these studies has been the labelling of protein L10, suggesting that this protein is located at the peptidyl-transferase centre of the ribosome.

#### 1.1.4. Control of Translation.

The function of the ribosome in protein synthesis has been presented diagrammatically in Figure 1.1.1. The control of this function is of more particular relevance to the work in this thesis.

In prokaryotes transcription and translation are tightly coupled and the control of the rate of protein synthesis generally occurs at the stage of transcription, although the control of the translation of coliphage RNA (Zinder, 1975) and that of ribosomal protein mRNAs mentioned above, provide exceptions. In eukaryotes, however, the sites of transcription and translation are separated by the nuclear membrane, and in view of this and the long half-lives of eukaryotic mRNAs it is not very surprising that there is more evidence for translational control of several types in eukaryotic cells.

In eukaryotes most mRNAs contain a polyA tail at the 3' terminus. It has been suggested that poly-A is required for the transport of mRNA from the nucleus to the cytoplasm (Weinberg, 1973), however the existence of mRNAs which lack poly-A (eg. histone mRNA) would argue against this idea. There is instead, reason to believe that the poly-A

is involved in translational regulation. It is clear that the poly-A region is not in itself necessary for translation of a mRNA, and histone mRNA which is translated in vivo is also actively translated in Krebs ascites cell free system (Gross et al., 1973) as is de-adenylated globin mRNA in several cell free systems (Sippel et al., 1974). However de-adenylated globin mRNA appears to be much less stable than adenylated globin mRNA for translation in Xenopus oocytes (Haez et al., 1974) and re-initiation of translation of de-adenylated ovalbumin mRNA was found to be much less efficient than that of polyadenylated ovalbumin mRNA (Doel & Carey, 1976). Thus poly-A may act by stabilising the mRNA, perhaps by protecting the 3' terminus from ribonucleolytic attack. In HeLa cells the poly-A sequence, found on the mRNA in the cytoplasm, becomes progressively shorter as the mRNA ages (Sheiness & Darnell, 1973) and it has been suggested that there is a minimum length of poly-A that is critical for mRNA stability and the eventual loss of this would leave the mRNA vulnerable to exonucleases (Brawermann, 1973).

In addition to the poly-A sequence at the 3' end of eukaryotic mRNAs, a 'cap-structure' (7 methyl guanosine:  $m^7pG$ ) is found at the 5' end of most eukaryotic mRNAs. There is some evidence to suggest that this 'cap-structure' is involved in the formation of mRNA-ribosome complexes and subsequently in the initiation of protein synthesis (Both et al., 1975). Supporting evidence comes from the findings that improperly capped mRNAs initiate at a much lower frequency (Both et al., 1975) and that cap analogues (eg.  $m^7pG$ : Roman et al., 1976; Suzuki, 1976) strongly inhibit the translation of capped mRNAs in cell free systems in vitro. A protein of molecular weight 24,000, which is not one of the established initiation factors although found as contamination in preparations of eIF-4B (Bergmann et al., 1979), can be cross-linked to mRNA bound in initiation complexes (Sonenberg et al., 1979). It has been suggested

that this protein may be involved in the recognition of the 'cap-structure'. However the 'cap-structure' does not appear to be an absolute requirement for initiation in eukaryotes since there are un-capped mRNAs (eg. polio and other viral mRNAs) which can be translated at high efficiency in vitro (Nomoto, Lee & Wimmer, 1976; Hewlett, Rose & Baltimore, 1976). The inactivation of 'cap-binding protein' by poliovirus would appear to be a strategy to favour the translation of its own mRNA.

Evidence from a number of sources demonstrate that the amount of protein synthesised in eukaryotic systems is not necessarily directly related to the molar concentration of mRNA present.

The various mRNAs of reovirus are synthesised in equimolar quantities in infected cells, whereas proteins made from these mRNA species may differ in quantity by a factor of 10 (Both et al., 1975).

Another example of differential mRNA translation is in the synthesis of  $\alpha$ - and  $\beta$ -globin. In intact cells these two peptides are translated from different mRNAs in a ratio of almost one to one. However the polyosomes carrying  $\beta$ -globin mRNA are significantly larger than those containing  $\alpha$ -globin mRNA (Hunt et al., 1968) and it has been shown that this is because there is more  $\alpha$ -globin mRNA than  $\beta$ -globin mRNA (Lodish, 1971). The rates of peptide elongation are the same for the two mRNAs (Lodish & Jacobsen, 1972) and it has been concluded that a rate-limiting step in peptide initiation must be more efficient for  $\beta$ -globin mRNA than for  $\alpha$ -globin mRNA.

Another example of translational control involves an aspect of the anti-viral state induced by interferon treatment. Exposure of virus

infected cells to interferon prevents the translation of virus mRNA while the translation of host mRNA is not affected (Yakobson et al., 1979). The mechanism by which interferon accomplishes this is not clear. A number of different effects of the protein synthetic machinery have been observed but none of them can easily explain the specificity of interferon against viral protein synthesis. These effects include the deacylation of leu-tRNA (Sen et al., 1976), the inhibition of mRNA methylation and capping (Sen et al., 1977), the activation of a specific eIF-2 protein kinase, enhanced by the preincubation of the cells with double stranded RNA (Revel et al., 1977) and the production of an oligonucleotide, pppA(2')p(5')A(2')p(5')A, inhibitor of translation (Kerr & Brown, 1978) which is synthesised from ATP by an enzyme requiring double stranded RNA. This oligonucleotide activates a ribonuclease (Ratner et al., 1977; Schmidt et al., 1978), but this appears to be active against host mRNA as well as viral mRNA. Since attempts, so far, have failed to reveal an interferon-induced inhibitor that is specific for viral protein synthesis in vitro, it has been suggested that there may be no such inhibitor but that the very features (whatever these may be) that give viral protein synthesis a selective advantage in vivo also make this more vulnerable than host protein synthesis to the inhibitors induced by interferon (Metz, 1975). Alternatively, it may be that the inhibitor is produced at its site of action, the viral RNA, and is degraded before reaching much of the cellular mRNA.

Clearly the inhibitory effects of different viruses on host cell protein synthesis need not, and probably do not, operate by a common mechanism. A mechanism specific for poliovirus RNA involves inactivation of the 'cap-binding protein', as mentioned above. Since poliovirus mRNA does not contain a 'cap structure' then its translation would not be

dependent upon 'cap-binding protein' and would be translated efficiently.

An alternative mechanism has been proposed by Carrasco and Smith (1976). These authors observed that viral mRNAs are translated best in vitro at relatively high monovalent cation concentrations, apparently through an effect on peptide initiation. In contrast to viral mRNA, host cell mRNA is translated best at lower salt concentrations. Thus viruses may cause changes in the outer membrane of the host cell that result in increased intracellular salt concentrations. Some indirect evidence for this mechanism has come from studies in which newly-infected cells can be made to synthesise proteins that are normally found late after infection with virus merely by transferring the cells to hypertonic medium (Kramer, 1980). However Egberts et al. (1977) have found that the change in ionic permeability in cells infected with mengovirus did not occur until after host protein synthesis had ceased. Moreover the differential translation of viral and host mRNAs at high ionic strength has not been observed with other viruses (Stevely & M<sup>C</sup>Grath, 1978). Thus the generality of the mechanism proposed by Carrasco and Smith (1976) is in doubt.

There is considerable evidence to suggest that there is a specific mechanism of translational control by haem in reticulocytes. This aspect of translational control involves the phosphorylation of eIF-2 and is therefore discussed in Section 1.2.2.

There are a number of other examples of the control of eukaryotic protein synthesis that appear to involve translation, although in the absence of mechanistic information these will not be considered here (reviewed by Austin & Clemens, 1980; Jagus et al., 1981). It is possible however that a proportion of cytoplasmic mRNAs are stored

in an inactive form in mRNP particles and can be recruited into poly-somes under certain circumstances.

## 1.2 PROTEIN PHOSPHORYLATION.

It has been known for some time that proteins can contain phosphate covalently bound at serine (Lipmann & Levine, 1932; Lipmann, 1933) or threonine residues (de Verdier, 1953). However these observations appeared of little interest until 1956 when Krebs and Fischer discovered that glycogen phosphorylase, the rate limiting enzyme in glycogenolysis, could be converted from a dephosphorylated 'b' form, the activity of which was dependent on 5'-AMP, to a phosphorylated 'a' form which was active in the absence of 5'-AMP. It was later shown that phosphorylase kinase (Krebs, Graves & Fischer, 1959), the enzyme which converts glycogen phosphorylase 'b' to 'a', and glycogen synthase (Friedman & Lerner, 1963), the rate limiting enzyme in glycogen synthesis, could also be interconverted between phosphorylated and dephosphorylated forms with subsequent alteration in activity.

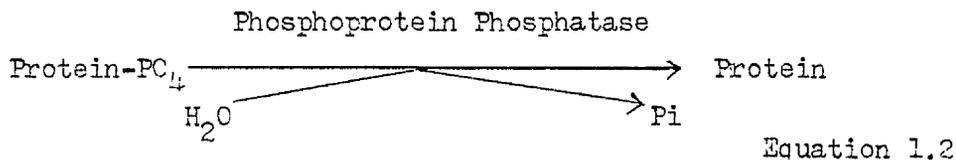
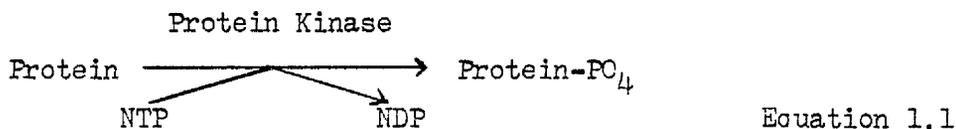
Despite the knowledge of these regulatory mechanisms in glycogen metabolism, it was not until the discovery of cyclic-AMP dependent protein kinase (Walsh, Perkins & Krebs, 1968) that it was realised that this kind of regulation occurred in other systems. More than one hundred phosphoproteins have now been identified, although for only some twenty-five of these has it been demonstrated that enzymic activity can be regulated by phosphorylation-dephosphorylation (reviewed by Krebs & Beavo, 1979).

Phosphorylation can either increase or decrease the enzymic activity of proteins. It has been suggested (Cohen, 1980) that in general enzymes involved in biodegradative pathways tend to be activated by phosphorylation, whereas enzymes involved in biosynthetic pathways are inactivated by phosphorylation. This could allow different metabolic

pathways to be regulated by the same protein kinases and phosphoprotein phosphatases.

### 1.2.1 Phosphoprotein Kinases and Phosphoprotein Phosphatases.

The phosphorylation-dephosphorylation process involves two types of enzymes, phosphoprotein kinases (protein kinases) and phosphoprotein phosphatases (Equations 1.1 & 1.2).



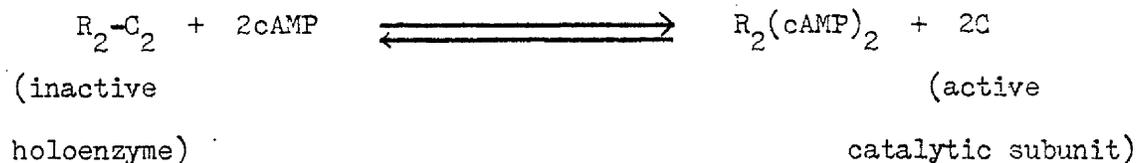
Protein kinases catalyse the transfer of the terminal phosphate from a triphosphate nucleotide donor, NTP, to a substrate protein (Equation 1.1).

In general NTP is ATP, although at least one protein kinase is known which also uses GTP.

Several protein kinases are regulated by direct interaction with regulatory agents which act as messengers in relaying signals from outside the cell. The first protein kinase shown to be regulated in this manner was a cyclic-AMP dependent protein kinase from rabbit skeletal muscle (Walsh, Perkins & Krebs, 1968). Cyclic-GMP dependent protein kinases were detected in lobster muscle (Kuo & Greengard, 1970) and later in several mammalian tissues (Lincoln, Dills & Corbin, 1977; Gill et al., 1976; Corbin & Lincoln, 1978). Enzymes have also been classified as calcium dependent protein kinases, of which two examples are known; phosphorylase kinase (Ozawa,

Hosoi & Ebashi, 1967) and myosin light chain kinase (Pires, Perry & Thomas, 1974). More recently it has been found that in normal cells and, more especially, in cells treated with interferon there are double stranded RNA dependent protein kinases (Lebleu et al., 1976; Ernst et al., 1976). A separate class of enzymes are the messenger independent protein kinases which appear to have no directly acting effectors. It cannot be excluded however that specific regulators of these enzymes may be found in the future. These latter protein kinases are usually named according to the substrates which they phosphorylate (eg. casein kinase, histone kinase etc.)

There are two types of cyclic-AMP dependent protein kinase (peak I and peak II), differing in their regulatory (R) subunits but having identical catalytic (C) subunits (Corbin, Keely & Park, 1975; Hofmann et al., 1975). It is generally accepted that both types of cyclic-AMP dependent protein kinase have the subunit structure  $R_2-C_2$ , and upon activation by cyclic-AMP dissociate to form dimers of the regulatory subunits and active monomers of the catalytic subunit (Equation 1.3).

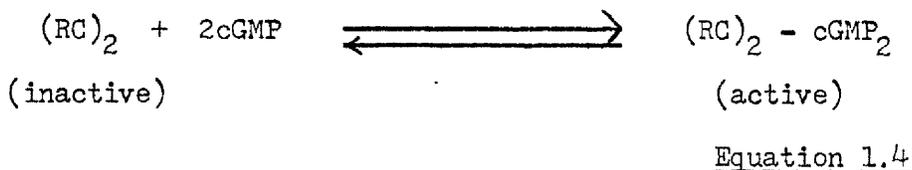


Equation 1.3

The reason for there being two cyclic-AMP dependent protein kinases is unclear since their substrate specificities are the same.

The cyclic-GMP dependent protein kinase is made up of two apparently identical subunits, but, unlike the cyclic-AMP dependent protein kinase does not contain separate catalytic and regulatory subunits

nor is it dissociated by its cyclic nucleotide activator (Gill et al., 1976; Inoue et al., 1976). See equation 1.4.



Two calcium dependent protein kinases are known, phosphorylase kinase (Ozawa, Hosoi & Ebashi, 1967) and myosin light chain kinase (Pires, Perry & Thomas, 1974). Phosphorylase kinase is a multi-subunit enzyme of the structure  $(\alpha\beta\gamma\delta)_4$ , where the  $\delta$  subunit is the calcium binding protein calmodulin (Cohen et al., 1978). This molecule of calmodulin is tightly bound to phosphorylase kinase and in the presence of calcium will stimulate its activity. Active myosin light chain kinase also contains a bound molecule of calmodulin allowing stimulation by calcium (Dabrowska et al., 1978; Yagi et al., 1978). In the absence of calmodulin however, myosin light chain kinase — in contrast to phosphorylase kinase — appears to have no basal activity (Adelstein & Klee, 1980). Apart from the stimulation of calcium dependent protein kinases, calmodulin stimulates several other enzyme activities ( reviewed by Cheung, 1980; Means & Dedman, 1980) and it is likely that calmodulin is the major intracellular receptor for calcium.

Almost all protein kinases phosphorylate serine or threonine residues, although the protein kinases from Retroviruses (Collett & Erickson, 1978; Sefton, Hunter & Raschke, 1981), Papovaviruses (Griffin, Sprangler & Livingston, 1979; Smith et al., 1979), and from cells treated with epidermal growth factor (Ushiro & Cohen, 1980; Cohen, Carpenter & King, 1980) phosphorylate tyrosine residues. The

susceptibility of particular serine or threonine residues to be phosphorylated, where there are a large number of potential sites available, appears to be largely determined by the amino acid sequence surrounding the phosphorylation site. Thus the phosphorylation site for cyclic-AMP dependent protein kinase contains two adjacent basic amino acids, at least one of which is arginine, immediately N-terminal to the residue that is phosphorylated. The sequence appears to be in one of the two forms:-

- Arg - Arg - X - Ser(P) -

or

- Lys - Arg - X - X - Ser(P) -

where X can be most amino acids (Cohen, 1980; Krebs & Beavo, 1979).

The dephosphorylation of phosphorylated proteins is catalysed by phosphoprotein phosphatases. A multifunctional phosphoprotein phosphatase capable of dephosphorylating a variety of protein substrates (including phosphorylase a, histones, glycogen synthase and phosphorylase kinase) has been obtained from various animal tissues (England, Stull & Krebs, 1972; Kato & Bishop, 1972; Zieve & Glinsmann, 1973). This enzyme (in contrast to the protein kinases) does not appear to be regulated by specific messenger molecules (Krebs & Beavo, 1979). Instead it appears to be regulated by competition between various phosphoprotein substrates, interaction with various protein inhibitors and by substrate-directed effects (eg. the specific interaction of ligands with the phosphoprotein substrates). This multifunctional phosphoprotein phosphatase can occur in two forms; the holoenzyme form consisting of two catalytic subunits complexed with one inhibitory subunit, and an active form consisting of catalytic subunits released by dissociation of the holoenzyme (Krebs & Beavo, 1979). There are two heat stable inhibitors

of this phosphatase (Huang & Glinsmann, 1975). Inhibitor I is of particular interest since it requires phosphorylation by cyclic-AMP dependent protein kinase to exert inhibitory activity. This provides a mechanism for amplifying the effects of cyclic-AMP since phosphorylation of inhibitor I results in the inactivation of the multifunctional phosphoprotein phosphatase.

Other specific phosphoprotein phosphatases have been described (Kikuchi, Tamura & Hiraga, 1977; Linn et al., 1969). It has been suggested that different substrate specific phosphatases may all contain the same non-specific catalytic subunit but different regulatory subunits, the latter being responsible for the substrate specificity (Imaoka et al., 1978).

### 1.2.2 The Role of Phosphorylation in Regulating the Activity of Proteins

The synthesis and degradation of glycogen provides the most well understood system in which the activity of proteins are regulated by phosphorylation - dephosphorylation (reviewed by Nimmo & Cohen, 1977; Cohen, 1980). One feature of this system is the highly coordinated manner in which the enzymes concerned are controlled by phosphorylation (Fig. 1.2.1).

The enzymes glycogen synthase and phosphorylase kinase can be phosphorylated at more than one site. Additional phosphorylations can alter the kinetics in ways that either differ from or extend the effects produced by the primary phosphorylation. This multi-site phosphorylation may involve different protein kinases acting at different sites. Six serine residues of glycogen synthase are, in fact, phosphorylated by three different protein kinases, thereby

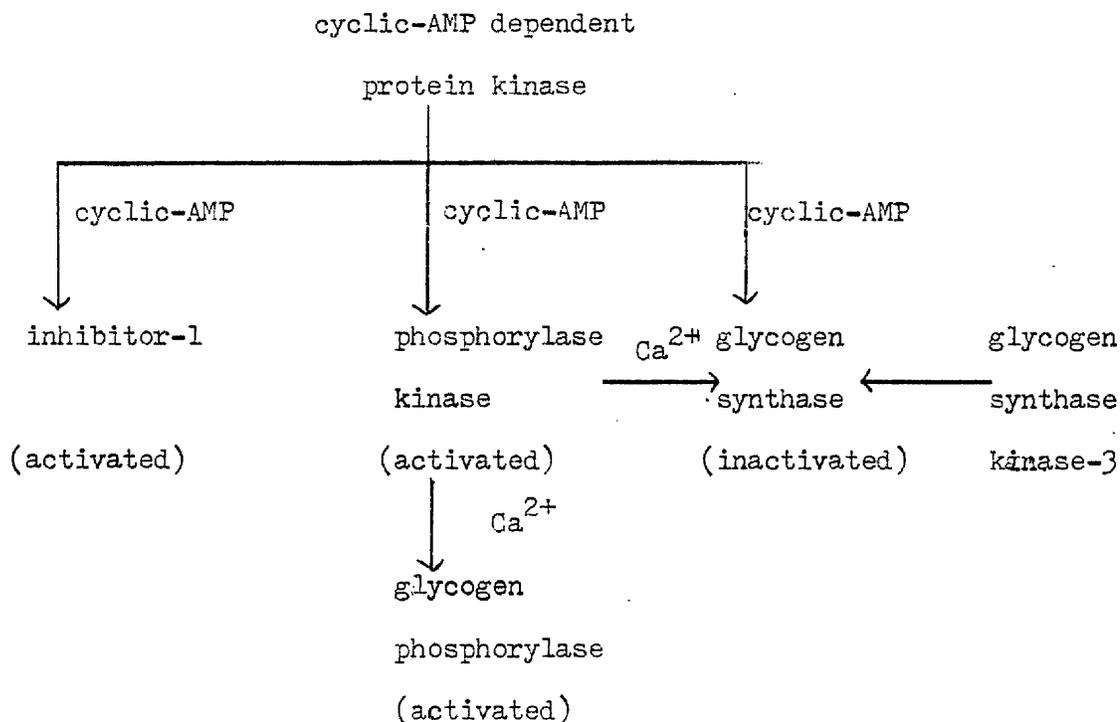


Figure 1.2.1

conferring sensitivity to more than one set of stimuli.

Examples of other enzymes regulated by phosphorylation have been reviewed by Krebs and Beavo (1979) and include: phosphofructokinase (Brand & Soling, 1975; Brand et al., 1976), pyruvate kinase (reviewed by Engstrom, 1978), fructose-1,6-diphosphatase (Riou et al., 1977), pyruvate dehydrogenase (Linn et al., 1969), hormone-sensitive lipase (Rizack, 1964), acetyl CoA carboxylase (Inoue & Lewenstein, 1972; Carlson & Kim, 1973), glycerophosphate acetyltransferase (Nimmo & Houston, 1978), hydroxymethylglutaryl CoA reductase (Berndt et al., 1976; Beg et al., 1978), tyrosine hydroxylase (Morgenroth et al., 1975; Yamauchi et al., 1978), phenylalanine hydroxylase (Abita et al., 1976), tryptophan hydroxylase (Hamon et al., 1978; Lysz & Sze, 1978), NAD-dependent glutamate dehydrogenase (Hemmings, 1978) and DNA-dependent RNA polymerase (Lincoln et al., 1977; Bell et al., 1977).

In certain systems phosphorylation appears to be essential for the functioning of proteins other than enzymes. When smooth muscle is stimulated electrically there is an increase in the cytoplasmic concentration of calcium ions leading to a calcium dependent phosphorylation of myosin P-light chain. This results in the interaction of myosin and actin with subsequent contraction. When the concentration of calcium ions falls the calcium dependent protein kinase becomes inactive allowing the phosphoprotein phosphatase to dephosphorylate the myosin P-light chain resulting in relaxation (Adelstein, 1978; England, 1980).

In other systems the precise role of protein phosphorylation has not been elucidated, although circumstantial evidence does suggest that the phosphorylation has important functions. Histone H1 shows a six fold increase in phosphate content between S-phase and prophase of the cell cycle (Bradbury et al., 1973). Experiments in vitro demonstrated that the highly phosphorylated prophase histone H1 was much more effective at aggregating DNA than the less phosphorylated S-phase histone H1 (Matthews, 1980). This suggests that phosphorylation of histone H1 may contribute to the process of chromosome condensation.

An example of protein phosphorylation, of more direct interest to the present study, is that of eIF-2, an initiation factor for eukaryotic protein biosynthesis. The discovery of this phenomenon came from studies of reticulocyte lysates in which it was observed that in the absence of sufficient quantities of haemin the rate of globin synthesis rapidly decreased (Bruns & London, 1965). This was associated with a disaggregation of polysomes and the disappearance of the initiation complex, Met-tRNA<sub>f</sub>-GTP-eIF-2, although normal concentrations of eIF-2, Met-tRNA<sub>f</sub> and GTP were present (Legon,

Jackson & Hunt, 1973). The inhibition of protein synthesis was accompanied by the formation of an inhibitor of translation, known as the haem controlled repressor (Maxwell & Rabinovitz, 1969), which was subsequently shown to have protein kinase activity, specifically phosphorylating the  $\alpha$  subunit of eIF-2 (Farrell, Hunt & Jackson, 1978). Although there is a strong correlation between the phosphorylation of eIF-2 and the inhibition of protein synthesis in crude reticulocyte lysates, in purer systems there is no decrease in the ternary initiation complex formation following phosphorylation of eIF-2. There is also no inhibition of the subsequent binding of this complex to the 40S ribosomal subunit (Trachsel & Staehlin, 1978; Safer & Anderson, 1978).

It has thus not yet been unequivocally established that the phosphorylation of eIF-2 inactivates this protein, although the circumstantial evidence is very persuasive. One possibility is that other factors (such as those described by de Haro and Cochoa, 1978; Das et al., 1979) are required before eIF-2 can form a stable initiation complex in vivo, and that in purified systems these factors are absent. Moreover, the formation of complexes between eIF-2, Met-tRNA<sub>f</sub> and GTP at physiological concentrations of the purified components in vitro is stoichiometric rather than catalytic, presumably due to the absence of these additional factors (Hunt, 1980). There has recently been support for the possibility that phosphorylation prevents the reformation of active eIF-2-GTP from the inactive eIF-2-GDP released from the ribosome (Clemens et al., 1982).

The haem controlled repressor is inactivated by stimuli other than haemin. These include high temperature, high hydrostatic pressure and oxidised glutathione.

A second protein kinase that can phosphorylate eIF-2 is activated by double stranded RNA. This protein kinase is present in reticulocyte lysates (Farrell et al., 1977) and in cell free systems which have been treated with physiological concentrations of interferon. This double stranded RNA activated inhibitor phosphorylates the same site(s) on the subunit of eIF-2 as the haem controlled repressor (Farrell et al., 1977).

### 1.3 THE PHOSPHORYLATION OF RIBOSOMAL PROTEINS.

The phosphorylation of ribosomal proteins in intact cells was first demonstrated independently by Kabat (1970) and Loeb and Blat (1970). Kabat showed that several phosphoproteins were present in ribosomes from rabbit reticulocytes whereas Loeb and Blat described a single phosphoprotein from rat liver. These reports were followed, shortly afterwards, by demonstration that ribosomal proteins could be phosphorylated in vitro by endogenous protein kinases (Kabat, 1971; Li & Amos, 1971) as well as by exogenous protein kinases (Walton et al., 1971; Eil & Wool, 1973).

Ribosomal phosphoproteins have now been detected, in vivo, in many mammalian species including rat (Loeb & Blat, 1970; Gressner & Wool, 1974a), rabbit (Kabat, 1970; Sanecka-Obacz & Borowski, 1974), mouse (Bitte & Kabat, 1972; Rankine & Leader, 1975), hamster (Leader, Rankine & Coia, 1976; Rupp, Humphrey & Shaeffer, 1976), bovine (Barden & Labrie, 1973) and human (Kaerlein & Horak, 1976; Lastick, Neilsen & McConkey, 1977). They are present in all tissues, so far examined, including liver (Loeb & Blat, 1970), kidney (Hill & Trachewsky, 1974), reticulocytes (Kabat, 1970), brain (Ashby & Roberts, 1975), adrenals (Roos, 1973), mammary gland (Majunder & Turkington, 1972), pituitary (Barden & Labrie, 1973) and in various tumour cells (Bitte & Kabat, 1972; Rankine & Leader, 1975; Stahl, Bohm & Bielka, 1974; Jolicoeur et al., 1974). Ribosomal phosphoproteins have also been detected in plants (Trewavas, 1973) as well as in primitive eukaryotes such as Saccharomyces cerevisiae (Grankowski & Gasior, 1975; Becker-Ursic & Davies, 1976; Zinker & Warner, 1976), Artemia salina (Van Agthoven, Maasen & Moller, 1977; Van Agthoven et al., 1978), Mucor racemosus

(Larsen & Sypherd, 1979; Larsen & Sypherd, 1980), Physarum polycephalum (Belanger, Bellemarie & Lemieux, 1979) and Tetrahymena pyriformis (Kristiansen, Plesner & Kruger, 1978; Kristiansen & Kruger, 1978).

Although there has been one report of ribosomal phosphoproteins in intact E. coli (Kurek, Grankowski & Gasior, 1972a) and one of ribosomal protein kinase (Kurek, Grankowski & Gasior, 1972b) others have found negligible phosphorylation of prokaryotic ribosomal proteins (Gordon, 1971; Rahmsdorf et al., 1973; Rahmsdorf et al., 1974).

Early analysis of ribosomal proteins showed considerable discrepancies in the number of phosphoproteins detected, this latter varying between eight (Majunder & Turkington, 1972) and one (Gressner & Wool, 1974a; Stahl, Bohm & Bielka, 1974). Overestimates in the number of ribosomal phosphoproteins arose from the ribosomes being contaminated with non-ribosomal phosphoproteins, which comigrate with ribosomal proteins on one dimensional gel electrophoresis. The introduction of two dimensional gel electrophoresis for the analysis of ribosomal proteins from E. coli (Kaltshmidt & Wittmann, 1970) and its subsequent application to the analysis of eukaryotic ribosomal proteins (Sherton & Wool, 1972) enabled the ribosomal proteins to be completely resolved from one another and to be differentiated from non-ribosomal contaminants by intensity of staining. Analysis by this method has established that, under normal circumstances, eukaryotic cells contain only two major ribosomal phosphoproteins.

One of the phosphoproteins has a molecular weight of 31,000, is a basic protein located in the 40S subunit and has been designated ribosomal protein S6 in the proposed uniform nomenclature for eukaryotic ribosomal proteins (McConkey et al., 1979). The other

phosphoprotein has a molecular weight of approximately 14,000, is an acidic protein (or proteins) located in the 60S subunit. Since it was not included in the above uniform nomenclature (McConkey et al., 1979) different laboratories have given it different designations; that of Rankine, Leader and Coia (1977) - L $\gamma$ - being used in this work.

Some other ribosomal proteins can become phosphorylated, but only under certain conditions. Thus proteins S2, S3 and L14 are phosphorylated in ascites cells incubated in medium containing glucose and amino acids (Leader & Coia, 1978a) or in medium lacking glucose but containing amino acids (Leader & Coia, 1978a). In HeLa cells infected with vaccinia virus there is more extensive phosphorylation of protein S2 and also phosphorylation of protein S16 (Kaerlein & Horak, 1976; Kaerlein & Horak, 1978), effects that were not found in cells infected with adenovirus (Blair & Horak, 1977), vesicular stomatitis virus (Marvaldi & Lucas-Lenard, 1977) or mengo virus (Rosnitschek, Traub & Traub, 1978). Trace phosphorylation of a number of other ribosomal proteins has also been reported (reviewed by Leader, 1980a).

Protein kinases capable of phosphorylating ribosomal proteins have been found in the cytosol of many cells including rat liver (Eil & Wool, 1971), reticulocytes (Traugh & Porter, 1976), yeast (Becker-Ursic & Davies, 1976), adrenal cortex (Walton & Gill, 1973) and corpus luteum (Azhar & Meron, 1975). Protein kinases have also been found associated with ribosomes, eg. reticulocytes (Kabat, 1971; Martini & Gould, 1973), trout testes (Jergil, 1972) and thyroid (Pavlovic-Hournac et al., 1973).

However in many of these studies the phosphorylated proteins were not characterised and the proteins phosphorylated could have been non-ribosomal. Others have shown that the pattern of phosphorylation in

cell free systems can be different from that observed in the intact cells (Traugh, Mumby & Traut, 1973; Walton & Gill, 1973; Ventamiglia & Wool, 1974). This indicates that it is necessary to fully characterise the proteins which are phosphorylated in cell free systems, and to demonstrate that these same proteins are phosphorylated in intact cells before the results can be regarded as significant.

### 1.3.1 The Acidic Phosphoprotein of the 60S Subunit.

Cawthon et al. (1974) detected a phosphoprotein of low molecular weight by one dimensional gel electrophoresis of protein from the 60S ribosomal subunit of reticulocytes or sarcoma cells. This phosphoprotein was later shown by two dimensional gel electrophoresis to be an acidic protein (or proteins), and has been observed in many mammalian tissues including mouse ascites cells (Rankine, Leader & Coia, 1977), BHK cells (Leader & Coia, 1978b), HeLa cells (Horak & Schiffmann, 1977), rat cerebral cortex (Roberts & Ashby, 1978) and L-cells (Houston, 1978). A low molecular weight acidic phosphoprotein of the 60S subunit has also been found in primitive eukaryotes such as yeast (Zinker & Warner, 1976; Otaka & Kobata, 1978) and Artemia salina (Van Agthoven, Maasen & Moller, 1977; Van Agthoven et al., 1978), indicating the evolutionary conservation of this phosphoprotein.

Two-dimensional gel electrophoretic analysis has shown a number of closely migrating phosphorylated and non-phosphorylated acidic species and it is now clear that there are at least two (but probably no more) acidic ribosomal proteins with discrete (but related) amino acid sequences (Zinker & Warner, 1976; Van Agthoven, Maasen & Moller, 1977; Leader & Coia, 1977; Horak & Schiffmann, 1977; Arpin, Madjar & Reboud, 1978; Otaka & Kobata, 1978; Houston, 1978; Van Agthoven et al., 1978). The acidic phosphoproteins eL12 and eL12' from Artemia salina have been

purified, and it has been found that the phosphoprotein eL12 contains a single phosphoserine residue, with two such residues in the slightly larger and more acidic distinct phosphoprotein eL12' (Van Agthoven et al., 1978). Up to three phosphorylation sites have been detected in the analogous phosphoprotein P2 from rat liver (Tsurugi et al., 1978).

There is considerable evidence to suggest that the acidic proteins of eukaryotic 60S subunits are structurally and functionally equivalent to proteins L7/L12 from E. coli ribosomes, as discussed above.

The observation by Zinker and Warner (1976) that the acidic phosphoprotein in yeast is added to ribosomes in the cytoplasm and that there exists a cytoplasmic pool of free acidic protein (Sanchez-Madrid & Ballesta, 1979; Zinker, 1980) suggest that this protein can undergo an exchange reaction between the ribosome and cytoplasm in vivo. Other evidence for an exchange reaction has been obtained by Kalthoff and Richter (1979) by injecting (<sup>3</sup>H)-labelled protein eL12 from Artemia salina into Xenopus laevis oocytes and observing the association of the labelled protein with the ribosome. Another interesting observation, in relation to an exchange reaction, is that the free acidic protein is unphosphorylated whereas this protein is phosphorylated when associated with the ribosome (Zinker, 1980).

This exchange reaction could act as a key step in the final stages of ribosome assembly and it is possible that phosphorylation of the acidic protein is essential for the ribosome to be functional. However this latter point requires testing. Alternatively, phosphorylation might increase the affinity of the acidic protein for the 60S subunit either after it has become bound or just before it does so. An increase in affinity for the 60S subunit following phosphorylation could account

for the lack of phosphorylated acidic protein in the free cytoplasmic pool in yeast (Zinker, 1980), even though protein kinase capable of phosphorylating this protein is present in the cytoplasm (Kudlicki, Grankowski & Gasior, 1976).

There has been little indication that the state of phosphorylation of the acidic phosphoprotein can be altered in intact cells, the protein being found generally in a state of almost complete phosphorylation (Leader & Coia, 1978b; Leader, Coia & Fahmy, 1978). This suggests that the protein may not have a dynamic regulatory function. One notable exception is the dephosphorylation of the acidic protein in Krebs ascites cells incubated in medium containing glucose and amino acids (Leader & Coia, 1978a).

### 1.3.2 Phosphorylation of Ribosomal Protein S6.

Ribosomal protein S6 from rat liver was the first ribosomal phosphoprotein to be identified by two-dimensional gel electrophoresis (Gressner & Wool, 1974a). The phosphorylation of protein S6 has subsequently been described in many other mammalian cells including hepatoma (Stahl, Bohm & Bielka, 1974), Krebs ascites cells (Rankine & Leader, 1975), HeLa cells (Kaerlein & Horak, 1976; Lastick, Neilsen & McConkey, 1977), L-cells (Marvaldi & Lucas-Lenard, 1977), reticulocytes (Traugh & Porter, 1976), cerebral cortex (Roberts & Ashby, 1978), Ehrlich ascites cells (Rosnitschek, Traub & Traub, 1978) and BHK cells (Leader, Rankine & Coia, 1976). The phosphorylation of protein S6 has also been observed in primitive eukaryotes such as Saccharomyces cerevisiae (Zinker & Warner, 1976; Hebert, Pierre & Loeb, 1977), Mucor racemosus (Larsen & Sypherd, 1979; Larsen & Sypherd, 1980), Physarum polycephalum (Belanger, Bellemarie & Lemieux, 1979) and Tetrahymena pyriformis (Kristiansen, Plesner & Kruger, 1978; Kristiansen & Kruger, 1978).

Protein S6 has been purified (Collatz et al., 1976), is basic in nature and has a molecular weight of 31,000. The phosphate has been shown to be present only at serine residues (Gressner & Wool, 1974a; Rankine, Leader & Coia, 1977; Hebert, Pierre & Loeb, 1977).

The extent of phosphorylation of protein S6 can vary (reviewed by Wool, 1979; Leader, 1980a). It was first shown by Gressner and Wool (1974a) that in regenerating rat liver, where protein S6 is highly phosphorylated, an anodal 'tail' of phosphorylated derivatives can be seen in the stained pattern of the gel when analysed by the two-dimensional gel electrophoresis method of Kaltschmidt and Wittmann (1970). This demonstration was possible since the first dimension of this system of electrophoresis separates the proteins on the basis of charge, and increased phosphorylation would alter the mobility of protein S6 during this separation by reducing its net positive charge. Gressner and Wool (1974a, 1974b) were able to resolve this 'tail' into 5-6 discrete derivatives, each presumably varying by the number of phosphorylated seryl residues. This was supported by the demonstration of a gradient in the specific ( $^{32}\text{P}$ )-radioactivity of the derivatives, this being highest in the most anodal derivative, and zero in the most cathodal derivative. Furthermore treatment of the most highly phosphorylated derivatives with alkaline phosphatase resulted in their position of electrophoretic migration changing to that of the unphosphorylated (and most cathodal) form of the protein.

Although protein S6 can be present in cells in a multiply phosphorylated form, under normal circumstances the protein is phosphorylated to a much lower extent (Gressner & Wool, 1974a; Leader, Rankine & Coia, 1976).

One type of stimulus which has been found to promote extensive phosphorylation of protein S6 is cyclic-AMP or agents which increase the intracellular concentrations of cyclic-AMP. Thus cells such as rat liver (Gressner & Wool, 1976a), rabbit reticulocytes (Cawthon et al., 1974), rat cerebral cortex (Roberts & Ashby, 1978) and hamster pancreatic islet tumour cells (Schubart et al., 1977) all respond in this manner to cyclic-AMP or derivatives of it such as dibutyryl cyclic-AMP. Phosphorylation of protein S6 can also be increased by treating cells with hormones that increase the intracellular concentration of cyclic-AMP. Examples include the effects of glucagon on rat liver (Blat & Loeb, 1971; Gressner & Wool, 1976a) or hamster pancreatic islet tumour cells (Schubart et al., 1977), or the effects of ACTH on tumour adrenal cells (Roos, 1973), or the effects of experimental diabetes induced by streptozotocin on rat liver (Gressner & Wool, 1976b). However in contrast, other cells do not show such a marked effect of cyclic-AMP on the phosphorylation of protein S6. Thus ascites cells (Rankine, 1976), glioma cells (Horak & Koschel, 1977) and yeast (Zinker & Warner, 1976) do not appear to respond to cyclic-AMP in this way, and in BHK cells (Leader & Coia, 1978c) and HeLa cells (Lastick & M<sup>C</sup>Conkey, 1978) the response is not always reproducible.

Another stimulus for the phosphorylation of protein S6 is the addition to cells of certain antimetabolites which are known to inhibit protein synthesis. Thus sodium fluoride (Kabat, 1970; Floyd & Traugh, 1981), puromycin (Gressner & Wool, 1974b) and cycloheximide (Gressner & Wool, 1974b; Kaerlein & Horak, 1976) all give such a response. These results however, can hardly be consistent with a hypothesis that the phosphorylation of protein S6 is involved in inhibition of protein synthesis because the phosphorylation of protein S6 is also increased in rapidly-growing cells, where the rate of protein synthesis is high. Thus pre-

confluent BHK cells contain protein S6 in a more highly phosphorylated state than do post-confluent cells (Leader, Rankine & Coia, 1976). An increase in phosphorylation of protein S6 is also found in HeLa cells after resuspension in fresh medium (Lastick, Neilsen & M<sup>C</sup>Conkey, 1977) or in chick embryo fibroblasts or quiescent Swiss mouse 3T3 cells after stimulation by serum (Haselbacher, Humbel & Thomas, 1979; Thomas, Siegmann & Gordon, 1979; Thomas et al., 1980). A similar enhancement in the phosphorylation of protein S6 has been observed in regenerating rat liver (Gressner & Wool, 1974a; Anderson, Grundholm & Sells, 1975; Scheinbuck, Sypherd & Moldave, 1974; Tas & Sells, 1978), although the situation here is more complex since partial hepatectomy also causes alterations in the intracellular concentration of cyclic-AMP (MacManus et al., 1972). However this association between extensive phosphorylation of protein S6 and a high rate of protein synthesis is not stringent since rat liver, which has a high rate of protein synthesis, has protein S6 in a low state of phosphorylation.

Another situation in which there is increased incorporation of (<sup>32</sup>P)-orthophosphate into protein S6 is HeLa cells infected with vaccinia virus (Kaerlein & Horak, 1976; Kaerlein & Horak, 1978). A similar phenomenon was observed in HeLa cells infected with adenovirus (Blair & Horak, 1977) and Ehrlich ascites cells infected with mengovirus (Rosnitschek, Traub & Traub, 1978), although not in L-cells infected with vesicular stomatitis virus (Marvaldi & Lucas-Lenard, 1977). However examination of the two-dimensional gels in these reports does not reveal any difference in the number or relative proportion of the phosphorylated derivatives of protein S6 and it is possible that the viruses merely stimulate the uptake of (<sup>32</sup>P)-orthophosphate by the cells.

It was difficult to conceive a function of the phosphorylation of

protein S6 in protein synthesis that could reconcile these apparently contradictory stimuli of cyclic-AMP, inhibition of protein synthesis and increased growth. The observation that increased phosphorylation of protein S6 occurred during rapid cellular growth suggested an alternative function in the synthesis of ribosomes (Leader, Rankine & Coia, 1976) which is very active under these conditions. Thus it was proposed that the phosphorylation may occur during synthesis of ribosomes in the nucleolus and be necessary for the assembly of the ribosomes or for their transport from the nucleus to the cytoplasm. In the cytoplasm slow dephosphorylation would occur unless, what were regarded as non-specific, cytoplasmic protein kinases were stimulated with cyclic-AMP. Since many of the antimetabolites mentioned above are known to influence the metabolism of cyclic-AMP (Appleman & Kemp, 1966; Wititsuwannakul & Kim, 1977; Ziv, Wagner & Stratman, 1978; Rall & Sutherland, 1962) their effects may be quite unrelated to their effects on protein synthesis, and be due to the alteration in the concentration of cyclic-AMP. In addition it is well known that sodium fluoride is a powerful inhibitor of many phosphoprotein phosphatases. The increased phosphorylation caused by these alterations may be fortuitous and have no real function.

The initial objective of the work described in this thesis was to test this hypothesis by determining whether the increased phosphorylation of the cytoplasmic ribosomes of rapidly-growing cells was due to a greater proportion of newly-synthesised ribosomes in these cells. If this proved correct it was planned to investigate the precise function and control of this phosphorylation.

## 2. MATERIALS AND METHODS

## 2.1 MATERIALS

### 2.1.1 Biological

#### (1) Cells

BHK21/C13 cells are an established line of Hamster kidney fibroblasts (MacPherson & Stoker, 1962). BHK21/C13/PyY (Stoker, 1962) and BHK 21/C13/SV28 (Wiblin & MacPherson, 1972) are established cell lines derived from BHK21/C13 cells by transformation with polyoma virus and simian virus 40 respectively.

#### (2) Viruses

Pseudorabies virus and herpes simplex virus are classified as members of the Herpesvirus group. Pseudorabies virus was originally obtained (Kaplan & Vatter, 1959), and subsequently passaged in culture. Herpes simplex virus type 1 (F strain) was derived from an original stock from Dr. Y. Becker (Hebrew University, Jerusalem).

### 2.1.2 Chemical

#### (1) General

All laboratory chemicals were analytical reagent (ANALAR) grade from British Drug House Ltd., Poole, Dorset except those listed below.

#### (2) Radiochemicals

These were purchased from Amersham International Ltd., Amersham, Bucks.

The radioisotopes were:

(<sup>32</sup>P)-orthophosphate supplied at 10mCi/ml.

(<sup>35</sup>S)-methionine containing 700-1,300 Ci/mmol.

(2(n)-<sup>3</sup>H)-methionine containing 8-10 Ci/mmol.

(<sup>32</sup>P)-(γ)-ATP containing 3,000-4,000 Ci/mmol.

<sup>14</sup>C-methylated protein mixture containing 0.833 $\mu$ Ci/ $\mu$ l of each of the following proteins:

Myosin 27 $\mu$ Ci/mg	(mol. wt. 200,000)
Phosphorylase-b 12 $\mu$ Ci/mg	(mol. wt. 92,500)
Bovine serum albumin 32 $\mu$ Ci/mg	(mol. wt. 69,000)
Ovalbumin 19 $\mu$ Ci/mg	(mol. wt. 46,000)
Carbonic anhydrase 8 $\mu$ Ci/mg	(mol. wt. 30,000)
Lysozyme 36 $\mu$ Ci/mg	(mol. wt. 14,300)

(8-<sup>3</sup>H)-cyclic-AMP containing 2-3Ci/mmol was part of a cyclic-AMP assay kit.

(3) Enzymes, Proteins and Amino Acids

Trypsin - Difco laboratories, Detroit, Michigan, USA.

Hexokinase and glucose-6-phosphate dehydrogenase - Boehringer-Mannheim,  
West Germany.

Bovine serum albumin - Armour Pharmaceutical Co. Ltd., Eastbourne.

Chymotrypsinogen - British Drug Houses Ltd., Poole, Dorset.

Cytochrome C - Miles Research Products, Spoke Poges, Slough.

Heparin - Evans Medical Ltd., Speke, Liverpool.

L-Glutamine - Sigma Chemical Co., St. Louis, USA.

(4) Nucleotides

Adenosine-monophosphate	} P-L Biochemicals Inc., Milwaukee, Wisconsin, USA.
Adenosine-diphosphate	
Adenosine-triphosphate	
Guanosine-monophosphate	
Guanosine-diphosphate	
Guanosine-triphosphate	
Adenosine-3'5'-cyclic-monophosphate	

Nicotinamide-adenine-dinucleotide-phosphate - Boehringer-Mannheim,  
West Germany.



Penicillin	}	Glaxo Pharmaceuticals Ltd., London.
Streptomycin		
Phenol Red	}	BDH Chemicals, Poole, Dorset.
Giemsa Stain		
L-Glutamine	-	Sigma Chemical Co. St. Louis, USA.

(8) Photographic Materials and Reagents.

DX 10 photographic developer	}	Eastman Kodak Co., Rochester, New York, USA.
FX 40 photographic fixer		
X ray (no screen) film (NS-2T)		
X-ray (screen) film (X-omat H)		

Cronex intensifying screens and cassettes - du Pont de Nemours & Co. Inc.  
Wilmington, Delaware, USA.

EN<sup>3</sup>HANCE (autoradiographic enhancer) - New England Nuclear, Boston, USA.

(9) Scintillation Reagents.

2,5-Diphenyloxazole (PPO)  
p-bis-[2-(5-phenyloxazole)]-benzene (POPOP)  
Triton X-100

Protosol (gel solubiliser) - New England Nuclear, Boston, USA.

(10) Miscellaneous Materials.

Spectropore 3 dialysis tubing - Spectrum Medical Industries Inc.,  
Los Angeles, USA.

Polyethyleneimine (PEI) impregnated cellulose - Macherey-Nagel & Co.,  
Düren, West Germany.

### 2.1.3 Composition of Standard Buffer Solutions.

(1) Balanced Salt Solution (BSS) was prepared according to Earle (1943) and contained 0.116M NaCl, 5.4mM KCl, 1mM MgSO<sub>4</sub>, 1mM NaH<sub>2</sub>PO<sub>4</sub>, 1.8mM CaCl<sub>2</sub> and 0.002% Phenol Red with the pH adjusted to 7.0 with 8.4% (w/v) NaOH. In normal use 50ml of a solution of ten times this concentration was diluted to 450ml with H<sub>2</sub>O, however on occasions the dilution was to 400ml and in this case was designated BSS/B. The solutions BSS (one-tenth Pi) and BSS (minus Pi) were BSS solutions in which the NaH<sub>2</sub>PO<sub>4</sub> concentration was 0.09mM and zero respectively.

(2) Phosphate Buffered Saline (PBS) was prepared according to Dulbecco and Vogt (1954) and contained 0.17M NaCl, 3.4mM KCl, 10mM NaHPO<sub>4</sub>, 2.4mM NaH<sub>2</sub>PO<sub>4</sub>, 0.49mM MgCl<sub>2</sub> and 0.68mM CaCl<sub>2</sub> at a pH of 7.4.

(3) Reticulocyte Standard Buffer (RSB) was prepared according to Penman (1966) and contained 10mM Tris-HCl (pH 7.5), 10mM KCl and 1.5mM Magnesium Acetate.

(4) Formal Saline consisted of a 4% (v/v) formaldehyde solution in 0.085M NaCl, 0.106M Na<sub>2</sub>SO<sub>4</sub>.

2.1.4 Formulation of Eagle's Minimum Essential Medium (MEM)

(Glasgow Modification)

This medium was described by Eagle (1959) and incorporates the Glasgow modification of MacPherson & Stoker (1962).

<u>MEM Amino Acids.</u>	<u>mg/l</u>	<u>mM</u>
L-Arginine	126.4	0.60
L-Cystine	24.0	0.10
L-Glutamine	292.0	2.00
L-Histidine	38.3	0.18
L-Isoleucine	52.5	0.40
L-Leucine	52.5	0.40
L-Lysine	73.1	0.40
L-Methionine	14.9	0.10
L-Phenylalanine	33.0	0.20
L-Threonine	47.6	0.40
L-Tryptophan	10.2	0.05
L-Tyrosine	36.2	0.02
L-Valine	46.9	0.40
---		
<u>Inorganic Salts &amp;</u>		
<u>other components</u>	<u>mg/l</u>	<u>mM</u>
CaCl <sub>2</sub> .6H <sub>2</sub> O	393.0	1.80
KCl	400.0	5.37
MgSO <sub>4</sub> .7H <sub>2</sub> O	200.0	0.81
NaCl	6,800.0	116.36
NaH <sub>2</sub> PO <sub>4</sub> .2H <sub>2</sub> O	140.0	0.90
NaHCO <sub>3</sub>	2,240.0	26.66
Glucose	4,500.0	25.00
Phenol Red	17.0	0.0017% (w/v)
---		

<u>MEM Vitamins</u>	<u>mg/l</u>
D-Calcium Pantothenate	2.0
Choline Chloride	2.0
Folic Acid	2.0
i-Inisitol	4.0
Nicotinamide	2.0
Pyradoxal HCl	2.0
Riboflavin	0.2
Thiamine	2.0

## 2.2 METHODS

### 2.2.1 Growth of BHK Cells

BHK21/C13 cells were grown as monolayers in rotating 80oz roller bottles in 180ml of Eagle's medium per bottle. This medium contained BSS/B, Eagle's minimum essential medium (Glasgow modification) (Section 2.1.4), 2.8g/l tryptose phosphate broth,  $10^5$  units/l penicillin, 100mg/l streptomycin, 100ml/l bovine calf serum and 40ml/l 5.6% (w/v)  $\text{NaHCO}_3$ . Cultures were seeded at  $2-3 \times 10^7$  cells per bottle and 'gassed' with 100ml 5% (v/v)  $\text{CO}_2$  in oxygen to maintain buffering capacity. The bottles were rotated at  $37^\circ\text{C}$  for 3 days, by which time the cells had just reached confluence (Fig. 2.2.1).

### 2.2.2 Passaging of BHK Cells

After cells had been grown for 3 days the medium was removed and the cells washed with 50ml 0.05% (w/v) trypsin, 0.02% EDTA in PBS (lacking  $\text{MgCl}_2$  and  $\text{CaCl}_2$ ). The solution was then removed leaving a small volume (ca. 5ml) in the bottle. The bottle was rotated at  $37^\circ\text{C}$  for approximately 5min until the cells began to detach from the glass. This process was aided by vigorous agitation of the bottle in a backwards and forwards motion. The cells were washed off the bottle and counted in a haemocytometer. Fresh cultures were reseeded from this cell suspension at  $2-3 \times 10^7$  cells per bottle.

Although the medium contained antibiotics, cells were checked for contamination by micro-organisms as follows. The cultures were examined for fungi and yeasts using Sabouraud's medium. Bacterial contamination was checked with blood agar plates. Contamination by Pleuropneumonia-like organisms (PPLO) was monitored by using PPLO plates. Any cultures showing positive reaction to these checks were discarded.

Fig. 2.2.1 Phase-Contrast Micrograph of Confluent BHK Cells.

BHK 21/C13 cells were routinely grown as monolayers in 80oz. roller bottles in 180ml of Eagle's medium (Section 2.2.1). For photographic purposes however, cultures were grown on 50mm petri dishes, and after the cells had reached confluence were fixed with 4% formal saline for 10-20min followed by staining with Giemsa stain for a further 10-20min. The cells were then viewed using a Zeiss phase-contrast microscope and photographed at a magnification of 110.



### 2.2.3 Infection of BHK cells with Pseudorabies Virus.

BHK cells grown to confluence for 3 days at 37°C were infected with 20pfu/cell of pseudorabies virus in 25ml of original medium and the virus allowed to adsorb to the cells for 1 hour at 37°C. After this adsorption period the medium was removed and replaced with 50ml of original medium and the infection allowed to continue at 37°C until harvesting.

### 2.2.4 Growth of Pseudorabies Virus.

Pseudorabies virus stocks were produced by infecting confluent BHK cells at a multiplicity of 1pfu/300 cells, and, after the initial adsorption period, allowing the infection to continue for a further 3 days. After this time all the cells had been infected and death of the cells and release of virus had occurred. This was evident from the rounding up and detachment of cells from the bottle.

The bottles were shaken gently to detach remaining cells from the glass surface, and the cell debris removed by centrifugation at 500g for 10min. The virus was sedimented by centrifugation at 20,000g for 90min in an MSE-18 centrifuge. The sedimented virus was resuspended in a small volume of Eagle's medium and a plaque assay carried out to determine the concentration of this virus stock.

The virus stock was stored as aliquots at -70°C.

### 2.2.5 Plaque Assay for Pseudorabies Virus.

Serial logarithmic dilutions of the pseudorabies virus stock solution were prepared in Eagle's medium (lacking serum). Dilutions of  $10^{-5}$ — $10^{-9}$  were used for most virus preparations. 50mm petri dishes were seeded with  $3 \times 10^6$  BHK cells, 4ml Eagle's medium added and the cells allowed to grow in a CO<sub>2</sub> incubator at 37°C. Four plates were used per dilution.

After the cells had grown for 24 hours the medium was removed and the cell monolayer infected with 0.2ml of the various virus dilutions, or 0.2ml of Eagle's medium as control. After adsorption of the virus to the cells for 1 hour, 5ml of Eagle's medium was added and the incubation continued for a further 2 hours. After this time 230ug heparin was added to each dish to prevent the virus spreading, and the cultures again returned to the incubator for a further 28 hours.

After this time the medium was removed from the cells and the infected monolayer fixed with 4% formal saline for 10-20min. After fixing, the monolayer was stained with Giemsa stain for 10-20min and then washed with distilled water. The plaques (Fig. 2.2.2) were viewed with a plate microscope and counted. Plates containing between 100-200 plaques were chosen and the average number taken over the 4 dishes was used in calculation of the virus concentration in the stock solution.

#### 2.2.6 Infection of BHK cells with Herpes Simplex Virus.

Infection with herpes simplex virus was carried out by the same procedure as that for pseudorabies virus except that the multiplicity of infection was 10pfu/cell.

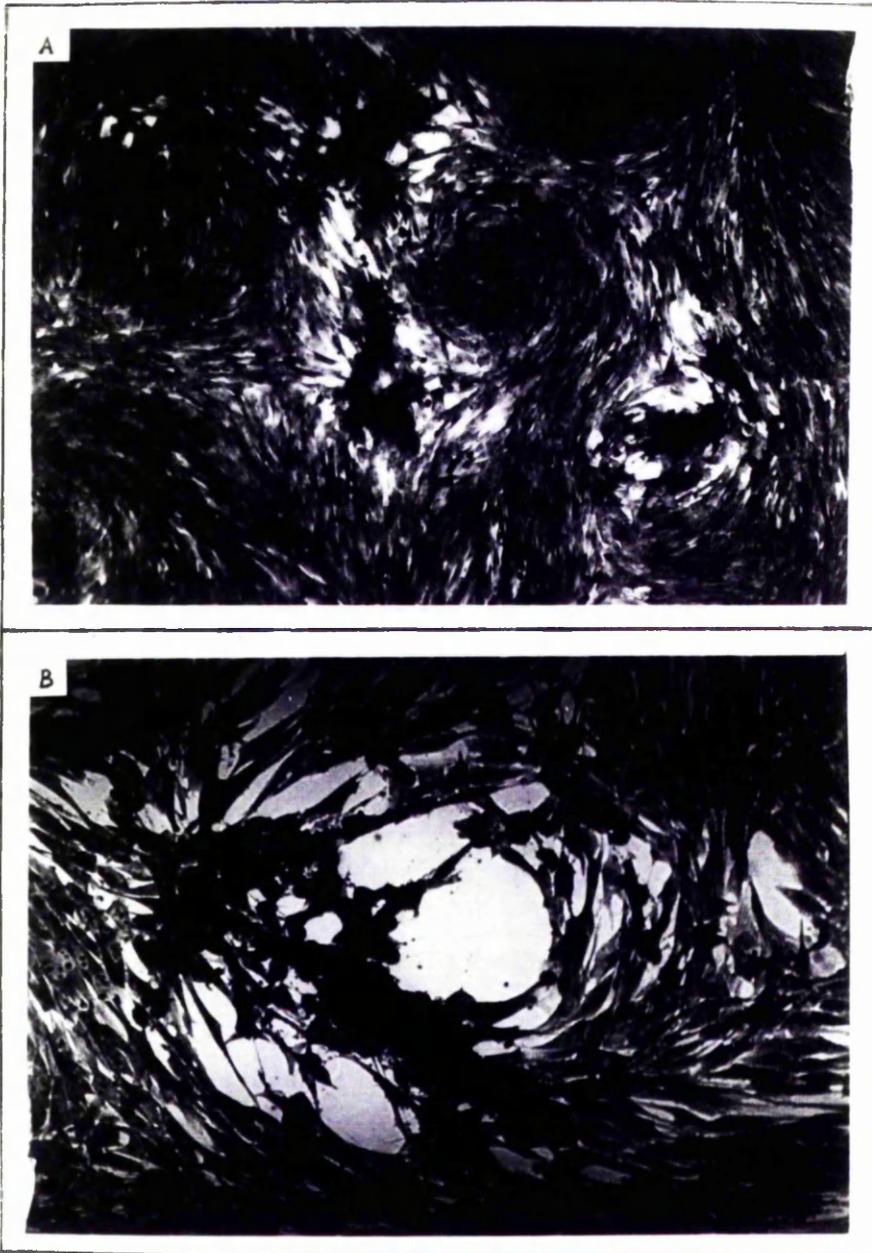
#### 2.2.7 Labelling of cells with $^{32}\text{P}$ -Orthophosphate.

The medium from the roller bottles was poured off and replaced with 50ml of Eagle's medium containing 0.09mM phosphate (one-tenth of the normal phosphate concentration) and lacking the tryptose phosphate broth supplement.  $^{32}\text{P}$ -orthophosphate (1-5mCi) was added to two bottles and the cells rotated at 37°C for 3 hours.

Fig. 2.2.2 Phase-Contrast Micrograph of Viral Plaques in BHK Cells  
Infected with Pseudorabies Virus.

BHK cells were grown in 50mm petri dishes until confluence and the cell monolayer infected with 0.2ml of the pseudorabies virus stock solution at various serial logarithmic dilutions. As described in Section 2.2.5 after incubation at 37°C for 28 hours the cells were fixed in 4% formal saline and stained with Giemsa stain. The viral plaques were normally viewed and counted using a plate microscope and photography carried out using a Zeiss phase-contrast microscope.

- A. Low magnification (x45) micrograph showing several viral plaques.
- B. Higher magnification (x110) micrograph showing close-up of a single plaque.



### 2.2.8 Labelling of cells with $^{35}\text{S}$ -methionine.

When cells were labelled with  $^{35}\text{S}$ -methionine the replacement medium contained  $2\mu\text{M}$  methionine (one-fiftieth of the normal methionine concentration) and the cells rotated at  $37^\circ\text{C}$  for between 15min and 3 hours.

### 2.2.9 Preparation of Cytoplasmic Ribosomes.

Ribosomes were prepared by a modification of the method of Ascione and Arlinghaus (1970). BHK cells were harvested into ice-cold PBS using a rubber scraper and pelleted at 500g for 5min. The cells were washed twice in PBS and then suspended in hypotonic medium containing 20mM Tris-HCl (pH 7.5), 50mM KCl, 5mM  $\text{MgCl}_2$  and 5mM  $\text{CaCl}_2$  and allowed to swell on ice for 10min. Then an equal volume of 1% Triton X-100 containing 0.55M KCl, 0.5M sucrose, 10mM  $\text{MgCl}_2$ , 5mM  $\text{CaCl}_2$  and 1mM EDTA was added. The ribonuclease inhibitor, dextran sulphate was added to a final concentration of 50 $\mu\text{g}/\text{ml}$  and, after allowing to stand on ice for 5min, the cells were lysed by 6-10 strokes of a teflon/glass homogeniser. The nuclear fraction was removed by centrifugation at 1500g for 10min, and 10% (w/v) sodium deoxycholate was added to the supernatant to a final concentration of 0.25% to disperse cytoplasmic membranes. This was then centrifuged at 165,000g for 2 hours in a Beckman Ti50 rotor. The sedimented ribosomes were further purified by suspension in a buffer containing 10mM Tris-HCl (pH 7.5), 10mM KCl, 1mM  $\text{MgCl}_2$  and 0.1mM EDTA and sedimenting through a cushion of 2M sucrose, dissolved in the same buffer, at 105,000g for 16 hours in a Beckman Ti50 rotor.

A yield of about 500  $A_{260}$  units of ribosomes was generally obtained from the  $3 \times 10^9$  cells (from twelve roller bottles) often used as starting material. These were stored at  $-70^\circ\text{C}$ .

### 2.2.10 Preparation of Ribosomal Subunits by Dissociation at High Ionic Strength.

This type of ribosomal subunit (hereafter used without qualification) was prepared by a modification of the method of Leader and Wool (1972). Ribosomes were suspended at 4°C in 10mM Tris-HCl (pH 7.5), 80mM KCl and 5mM MgCl<sub>2</sub>. The concentration of KCl was adjusted to 500mM by addition of one-half of a volume of 1.25M KCl and the suspension clarified by centrifugation at 1000g for 5min. To promote separation of the subunits and remove nascent peptide, the clarified suspension was incubated at 37°C for 15min with 0.1mM puromycin and 20mM 2-mercaptoethanol. Aliquots of the suspension containing approx. 80 A<sub>260</sub> units of ribosomes were then layered directly onto 37ml of a linear 10-30% (w/v) sucrose gradient containing 10mM Tris-HCl (pH 7.6), 300mM KCl, 5mM MgCl<sub>2</sub> and 20mM 2-mercaptoethanol.

After centrifugation at 96,000g for 4 hours at 28°C in a Beckman SW27 rotor, the gradients were forced, by means of a peristaltic pump, through a Gilford flow-cell on a Gilford model 240 recording spectrophotometer analysing at 260nm, and the subunit fractions indicated in Fig. 2.2.3 collected. The pooled 40S or 60S fractions from 6 gradients were sedimented at 177,000g for 16 hours in a Beckman Ti60 rotor.

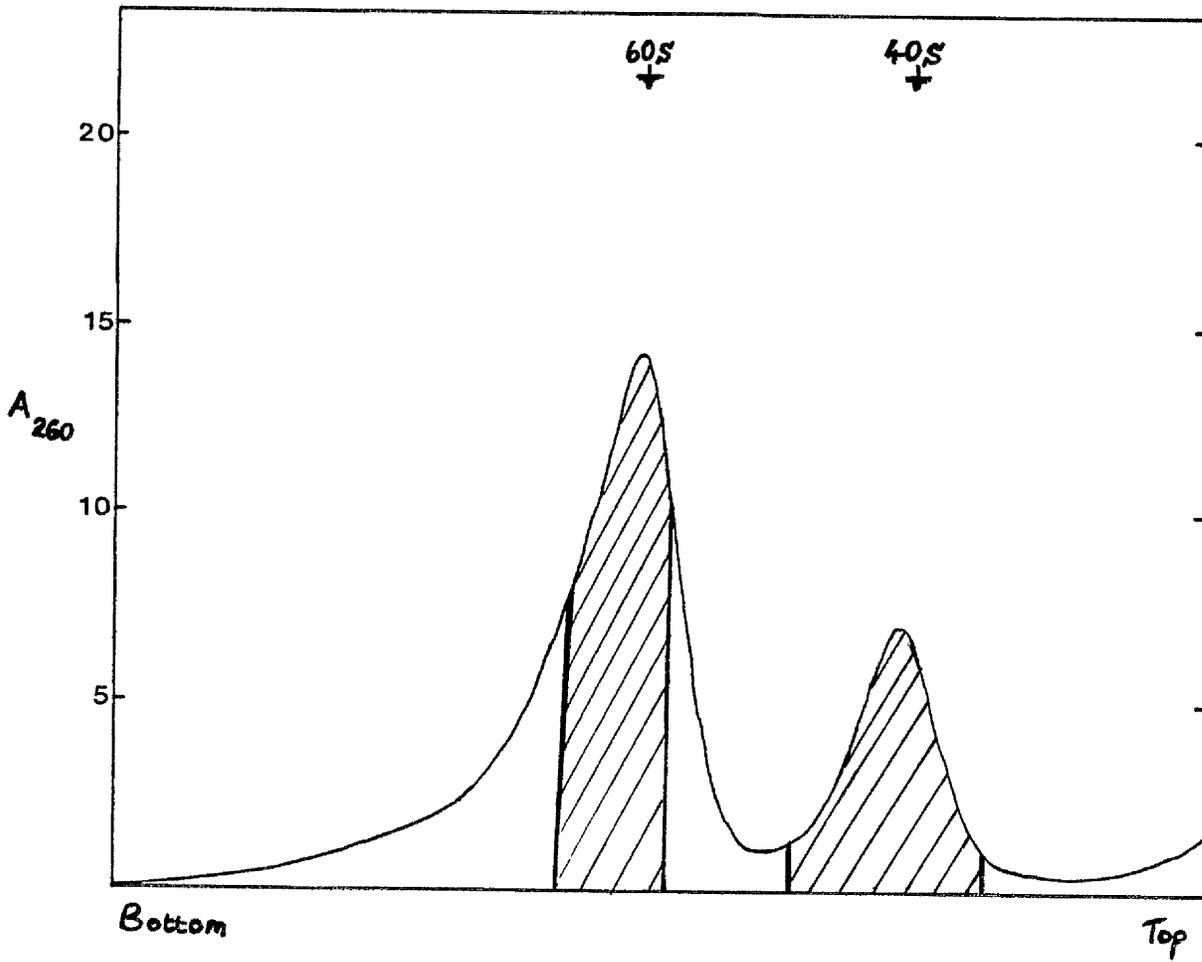
### 2.2.11 Preparation of 'Native' Ribosomal Subunits.

A post-nuclear supernatant was prepared as described in section 2.2.9, except that the volumes of hypotonic buffer and lysing solution were reduced to about one-quarter to give a final volume of approx. 10ml. Aliquots (1.7ml) were layered over 37ml of a 20-40% linear sucrose gradient containing 20mM Tris-HCl (pH 7.6), 25mM KCl, 2mM MgCl<sub>2</sub>, 0.1mM EDTA and 20mM 2-mercaptoethanol. After centrifugation at 65,000g

Fig. 2.2.3 Separation of BHK Ribosomal Subunits by Dissociation at High Ionic Strength.

Ribosomal subunits were separated on 10-30% (w/v) sucrose density gradients containing 10mM Tris-HCl (pH 7.6), 300mM KCl, 5mM MgCl<sub>2</sub> and 20mM 2-mercaptoethanol (2.2.10).

The shaded regions below the subunit peaks indicate the portions of each gradient collected as 60S and 40S subunits.



for 16 hours at 0°C in a Beckman SW27 rotor, the gradients were analysed as described in section 2.2.10 and the fractions indicated in Fig. 2.2.4 were sedimented at 177,000g for 16 hours in a Beckman Ti60 rotor.

#### 2.2.12 Extraction of Protein from Ribosomes and Ribosomal Subunits.

Protein was extracted from ribosomes and ribosomal subunits by a modification of the method described by Sherton and Wool (1974a). Ribosomes or ribosomal subunits were suspended at a concentration of approx. 75  $A_{260}$  units/ml in a buffer containing 10mM Tris-HCl (pH 7.7) and 600mM magnesium acetate. To this was added 2 vols. glacial acetic acid and the mixture stirred at 0°C for 1 hour. The precipitated ribosomal RNA was removed by centrifugation at 10,000g for 10min in a Eppendorf microcentrifuge. The supernatant was decanted and the yield of protein increased by re-extraction of the pellet with the acetic acid/Tris/magnesium acetate mixture as before. The supernatants were pooled and dialysed for 24 hours against 1 litre of 7% acetic acid with 5-6 changes, and then lyophilised. To ensure maximum retention of protein the dialysis tubing was acetylated according to the method of Craig (1967) before use or alternatively Spectropore 3 tubing (Spectrum Medical Industries), which does not require pretreatment, was used.

Overall yields of protein were quite variable but generally between 0.5-1.0mg of both 40S and 60S ribosomal protein was obtained from  $2-3 \times 10^9$  BHK cells.

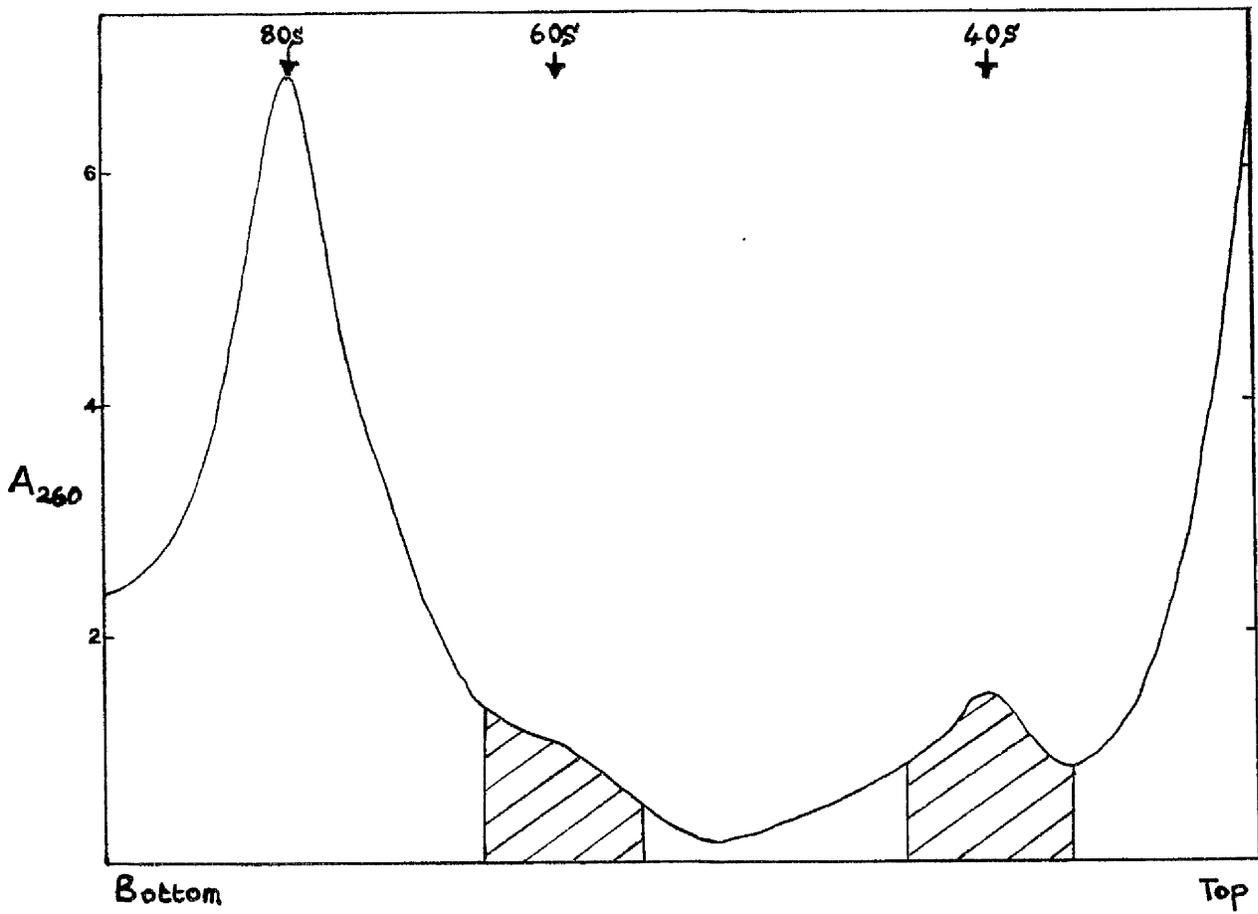
#### 2.2.13 Preparation of Nucleoli from BHK cells.

Nucleoli were prepared from BHK cells by a modification of the methods of Murumatsu et al. (1974) and Knowler (1976).

Fig. 2.2.4 Separation of BHK 'Native' Ribosomal Subunits

'Native' ribosomal subunits were separated on 20-40% (w/v) sucrose density gradients containing 20mM Tris-HCl (pH 7.6), 2mM MgCl<sub>2</sub>, 0.1mM EDTA and 20mM 2-mercaptoethanol (2.2.11).

The shaded regions below the subunit peaks indicate the portions of each gradient collected as 'native' 60S and 'native' 40S subunits.



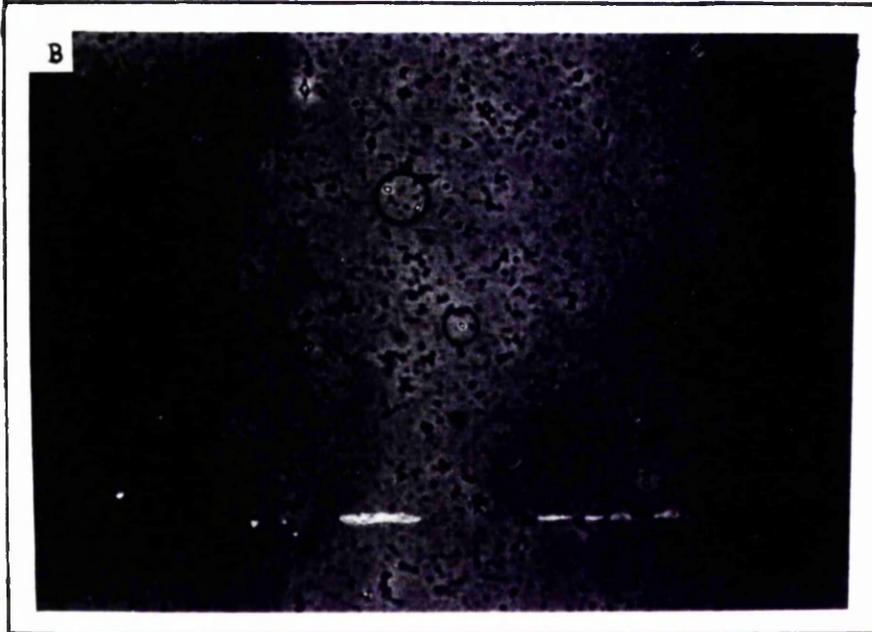
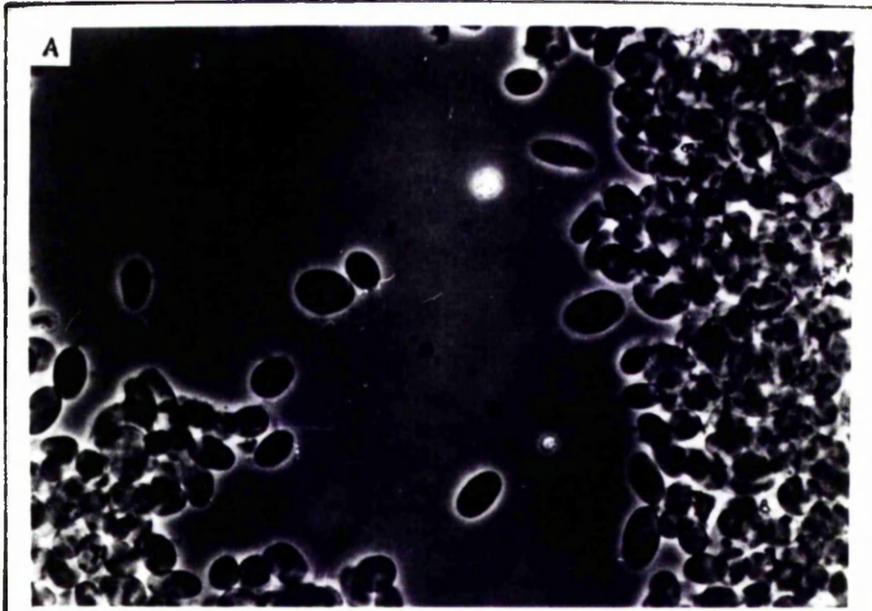
Cells were harvested into ice-cold PBS using a rubber scraper and centrifuged at 500g for 5min. The cells were washed twice in PBS and then suspended in 10 vols. of one-sixth RSB and allowed to swell on ice for 15min. The swollen cells were sedimented at 500g for 5min and resuspended in one-sixth RSB and the detergent NONIDET P40 (NP40) added to a final concentration of 0.3%. The cells were kept on ice for a further 15min and then disrupted with three strokes of a teflon/glass homogeniser. The nuclear fraction was sedimented by centrifugation at 1,000g for 5min and then resuspended in RSB by gentle shaking. An equal volume of 0.5M sucrose in RSB was added and the suspension gently mixed. The suspension was underlayered with one-half of a vol. of 0.32M sucrose in RSB and centrifuged at 1,000g for 5min. The purified nuclei were examined under a Zeiss phase-contrast microscope to check for possible damage. The purified nuclei (Fig. 2.2.5A) were suspended in a small volume (ca. 2ml) of 0.34M sucrose, 0.05mM MgCl<sub>2</sub> and sonicated at 0°C using an MSE ultrasonicator at 200W for 1.5--2min with 15 second bursts at 1 minute intervals. The sonicate was examined under a Zeiss phase-contrast microscope to check that the nuclei had been broken (Fig. 2.2.5B), and underlayered with 1 vol. of 0.88M sucrose, 0.05mM MgCl<sub>2</sub> and centrifuged at 2000g for 20min. The nucleolar pellet was again checked for contamination by nuclei before being stored at -70°C until required.

Protein was extracted from nucleoli by the method used for ribosomal subunits (section 2.2.12).

Fig. 2.2.5 Phase-Contrast Micrographs of Nuclei and Nucleoli Isolated  
from BHK Cells

Nuclei and nucleoli were isolated from confluent BHK cells as described in Section 2.2.13. The preparations were viewed and photographed using a Zeiss phase-contrast microscope.

- A. Micrograph showing nuclei at a magnification of 180.
- B. Micrograph showing sonicated nuclear preparation at a magnification of 180. Nucleoli are identified inside circles.



## 2.2.14 Gel Electrophoresis.

### (1) One Dimensional SDS Tube Gels.

SDS tube gels were prepared by the method of Laemmli (1970). The lower separation gels were cast the day before use and contained 12.5% (w/v) acrylamide, 0.4% (w/w) methylene-bis-acrylamide, 0.1% SDS, 0.03% TEMED, 37mM Tris-HCl (pH 8.5) and 0.05% ammonium persulphate.

Upper stacking gels were cast on the day of use and contained 3% (w/v) acrylamide, 0.06% (w/w) methylene-bis-acrylamide, 12mM Tris-HCl (pH 8.5), 1% SDS, 0.03% TEMED and 0.04% ammonium persulphate.

The lengths of the separation gel and stacking gel were 8cm and 1.5cm respectively and their diameter was 6mm. Protein samples were in a mixture containing 50mM Tris-HCl (pH 8.5), 2% SDS, 5% (v/v) 2-mercapto-ethanol and 0.001% Bromophenol Blue and were completely reduced and denatured by heating at 100°C for 2min. Immediately prior to electrophoresis 0.5 vol. glycerol was added.

The electrophoresis buffer contained 25mM Tris-HCl (pH 8.5), 0.192M glycine and 0.1% SDS. The gels were subjected to electrophoresis at 4mA/gel for about 1.5—2.0 hours until the Bromophenol Blue dye marker was about 1cm from the bottom of the gel. The gels were then removed from their tubes and processed as described in section 2.2.15. Typical results for the analysis of 40S and 60S subunits are shown in Fig. 2.2.6.

### (2) One Dimensional SDS Slab Gels.

All solutions were as for tube gels (above) except that in some cases the separation gel contained 10% (w/v) acrylamide.

Fig. 2.2.6 One-Dimensional SDS Gels of Ribosomal Proteins

Ribosomal proteins (approx. 30 $\mu$ g) from the 40S and 60S ribosomal subunit were subjected to one-dimensional SDS gel electrophoresis.

(2.2.14.1).

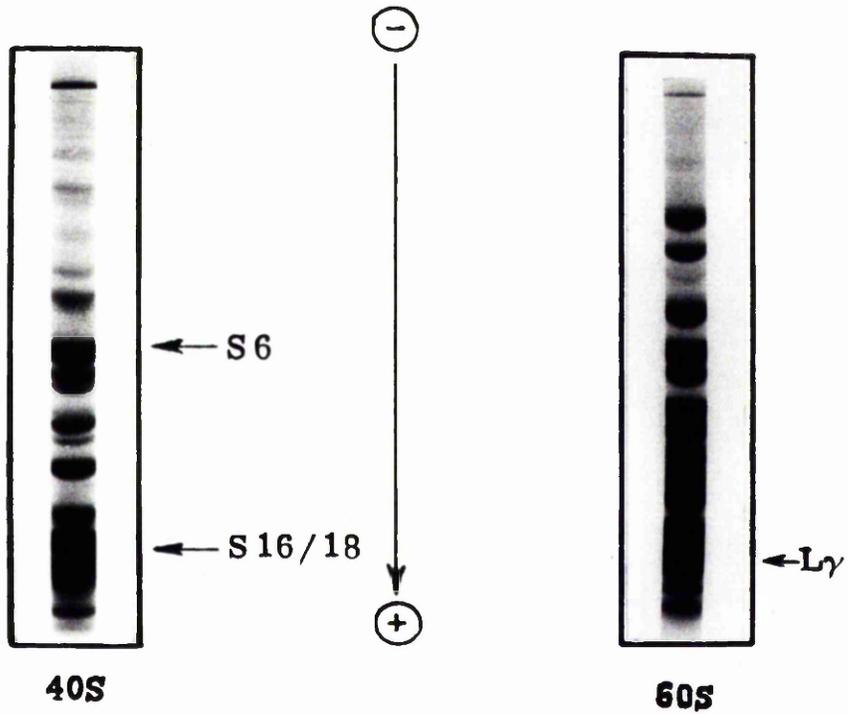
A. 40S subunit proteins

B. 60S subunit proteins

Also indicated are the positions of the major ribosomal phosphoproteins described in this work.

<u>Phosphoprotein</u>	<u>Mol. Wt.</u>
S6	31,000
S16/18	18,000
L8	14,000-14,500

The molecular weights of the phosphoproteins were calculated from Fig. 2.2.8A.



Sample wells were made by inserting a polythene comb into the stacker gel prior to polymerisation. The dimensions of the separation gel and stacker gel were 18cm x 18cm x 1mm and 1.5cm x 18cm x 1mm respectively.

Electrophoresis was carried out at 14mA for 16 hours until the tracker was about 2cm from the bottom of the gel. The gels were processed as described in section 2.2.15.

### (3) Two-Dimensional Kaltschmidt/Wittmann Gels.

The method of Kaltschmidt and Wittmann (1970) was modified to incorporate the alterations described by Lastick and M<sup>c</sup>Conkey (1976). The first dimensional gel contained 6M urea, 4% (w/v) acrylamide, 0.13% (w/v) methylene-bis-acrylamide, 0.2M Tris-HCl (pH 8.7), 0.26M boric acid, 10mM EDTA and 0.2% TEMED. Polymerisation was catalysed by the addition of 50 $\mu$ l of 10% (w/v) ammonium persulphate per 10ml of gel solution. Lyophilised ribosomal protein was prepared for electrophoresis by dissolving at a concentration of 10mg/ml in a solution containing 8M urea, 20mM Tris-HCl (pH 8.2), 26mM boric acid, 1mM EDTA and 5% (v/v) 2-mercaptoethanol. The protein was completely reduced by heating the solution at 60°C for 10min. Generally 100 $\mu$ g of protein was analysed in each gel. Electrophoresis was towards the cathode at 3mA/gel for 3 hours using cytochrome C (on a parallel gel) as a marker (Leader 1980), with an electrophoresis buffer containing 60mM Tris, 78mM boric acid, 3mM EDTA pH 8.6. Gels were carefully removed from the tubes and frozen in hexane at -70°C until further processing. Just before annealing to the second-dimensional gel they were equilibrated with 6M urea, 0.35M acetic acid and 5% (v/v) 2-mercaptoethanol at room temperature for less than 5min.

The second dimensional gel contained 6M urea, 15% (w/v) acrylamide, 0.47% (w/v) methylene-bis-acrylamide, 0.44M acetic acid, 25mM KOH and 0.5% (v/v) TEMED pH 4.05. Polymerisation was catalysed by the addition of 3ml of 10% (w/v) ammonium persulphate per 100ml of gel solution.

Once the first dimensional gel had been equilibrated, it was annealed in contact with the slab gel by means of a solution containing 6M urea, 0.35M acetic acid, 5% 2-mercaptoethanol and 1% agarose. Electrophoresis in the second dimension was towards the cathode at 8mA/gel for 16 hours in a buffer containing 93mM glycine, 13mM acetic acid (pH 4.05). Gels were removed from the glass plates and processed as described in Section 2.2.15.

The standard electrophoretic pattern for ribosomal proteins, showing the nomenclature used (McConkey et al., 1979) is shown in Fig. 2.2.7.

#### 2.2.15 Processing of Gels

##### (1) Staining of Gels

SDS gels and two-dimensional gels were stained for 2-3 hours at 37°C in 0.1% Coomassie Brilliant Blue R-250 in 50% (v/v) methanol and 10% (v/v) acetic acid.

##### (2) Destaining of Gels

Gels were destained for 24-48 hours by diffusion in 7.5% acetic acid at 37°C.

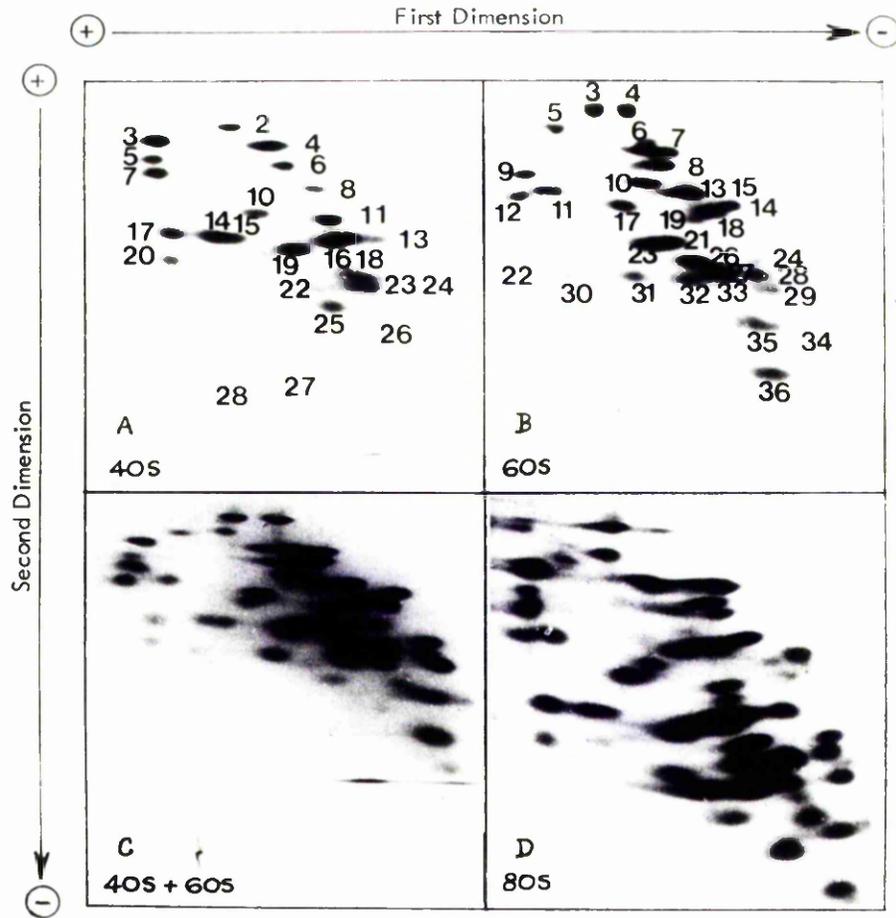
##### (3) Autoradiography of Gels

Tube gels and two-dimensional gels (but not SDS slab gels) were sliced longitudinally to give 1mm thickness and then dried on to

Fig. 2.2.7 Two-Dimensional Kaltschmidt/Wittmann Gel Electrophoresis  
of Ribosomal Proteins.

Ribosomal protein (100 $\mu$ g) was subjected to two-dimensional gel electrophoresis as described in Section 2.2.14.3. The nomenclature of the ribosomal proteins was according to M<sup>c</sup>Conkey et al. (1979).

- A. Protein from 40S subunit
- B. Protein from 60S subunit
- C. Protein from 40S and 60S subunit mixture
- D. Protein from 80S monoribosomes



Whatman 3MM filter paper under suction from an electrical vacuum pump (Speedivac-Edwards). Autoradiography was on Kodirex X-ray film for 3-4 weeks for ( $^{32}\text{P}$ )-labelled gels, or 8-12 weeks for ( $^{35}\text{S}$ )-labelled gels.

#### (4) Fluorography of Gels

Gels were sliced to give 1mm thickness and soaked in 'Enhance' (NEN) for 1 hour at 20°C with stirring. The light sensitive material was precipitated by agitation in water for 1 hour and the gels dried on to Whatman 3MM filter paper under vacuum as before. Fluorography was on Kodirex film for 4-6 weeks at -70°C for ( $^{35}\text{S}$ ) or ( $^{14}\text{C}$ )-labelled gels.

#### (5) Densitometry of Gels

Densitometric records of SDS tube gels and of sections from two-dimensional gels were obtained using the linear transport accessory to the Gilford 240 spectrophotometer at 586nm.

#### (6) Quantitative Measurement of Radioactivity in Gels

SDS tube gels were sliced transversely into 2mm slices using a multiple razor blade assembly and solubilised in 0.5ml of 90% (v/v) 'Protosol' (NEN) at 37°C for 16 hours. The radioactivity was determined in 4.5 ml of scintillant containing 4g 2,5 diphenyl-oxazole (PPO) and 0.2g p-bis-[2-(5-phenyloxazole)]-benzene (POPOP) per litre of toluene in a Packard scintillation spectrometer.

Radioactivity in two-dimensional gels was determined by cutting out the protein spots using an extra fine scalpel and treating as before.

#### 2.2.16 Determination of Molecular Weights of Proteins in SDS Gels.

The molecular weights of the proteins were estimated by electrophoresing molecular weight standards in gels parallel to those with ribosomal proteins. For SDS tube gels the standards used were bovine serum albumin (molecular weight 69,000) chymotrypsinogen (molecular weight 25,750) and cytochrome C (molecular weight 12,400). For SDS slab gels the standards used were myosin (molecular weight 200,000), phosphorylase-b (molecular weight 92,500), bovine serum albumin (molecular weight 69,000), ovalbumin (molecular weight 46,000), carbonic anhydrase (molecular weight 30,000) and lysozyme (molecular weight 14,300). The molecular weight of the ribosomal proteins could then be estimated by the method of Weber and Osborn (1969). Typical calibration curves are shown in Fig. 2.2.8.

#### 2.2.17 Determination of the Concentration of Protein.

The concentration of protein was measured by the dye binding assay of Bradford (1976) modified by Spector (1978). This method is simpler and less prone to interference by laboratory reagents eg. urea, than the method of Lowry et al. (1951). The assay was based on the quantitative binding of protein to Coomassie brilliant blue G-250. The Coomassie brilliant blue G-250 solution was made up at a concentration of 0.01% (w/v) in 0.95% (v/v) ethanol and 8.5% (w/v) phosphoric acid. An aliquot of the protein solution (containing up to 10 $\mu$ g of protein) was mixed with 1ml of this reagent and the absorbance at 595nm measured in a spectrophotometer. A standard curve was constructed using known amounts of bovine serum albumin. A typical example is shown in Fig. 2.2.9.

Fig. 2.2.8 Determination of Molecular Weights of Proteins in SDS Gels

The molecular weights of sample proteins were obtained by comparison with proteins of known molecular weight which were electrophoresed in parallel gels.

A. Proteins used were:-

	<u>Mol. Wt.</u>
Bovine serum albumin	69,000
Chymotrypsinogen	25,700
Cytochrome-C	11,700

B. Proteins used were:-

	<u>Mol. Wt.</u>
Myosin	200,000
Phosphorylase-b	92,000
Bovine serum albumin	69,000
Ovalbumin	46,000
Carbonic anhydrase	30,000
Lysosyme	14,300

The relative mobility ( $R_f$ ) was calculated by comparing the distance of migration of the proteins with that of the tracker dye Bromophenol Blue.

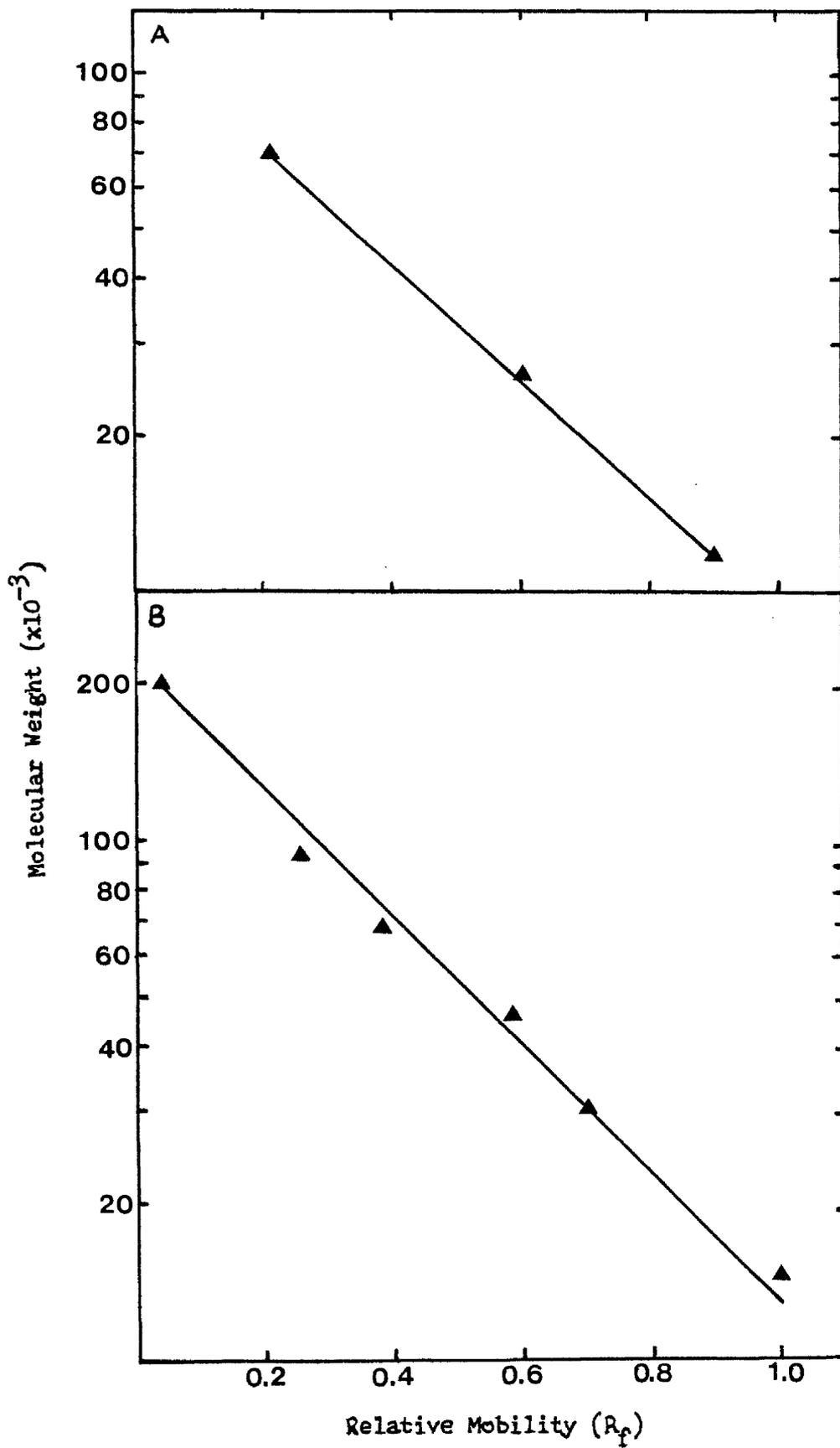
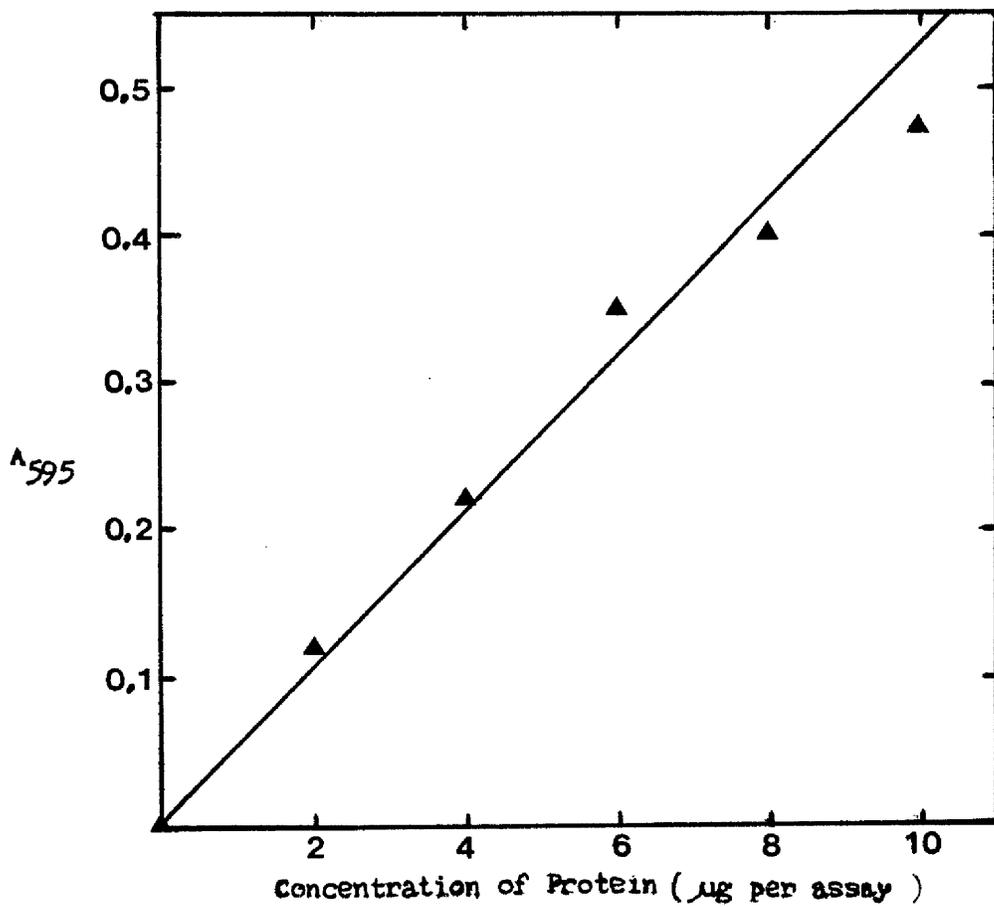


Fig. 2.2.9 Measurement of the Concentration of Protein.

The concentration of protein was measured by the dye-binding assay of Bradford (1976) modified by Spector (1978) (Section 2.2.19). A standard curve was constructed using known amounts of bovine serum albumin and plotting these against the corresponding absorbance measurements at 595nm.



### 2.2.18 Extraction of Nucleotides from BHK Cells

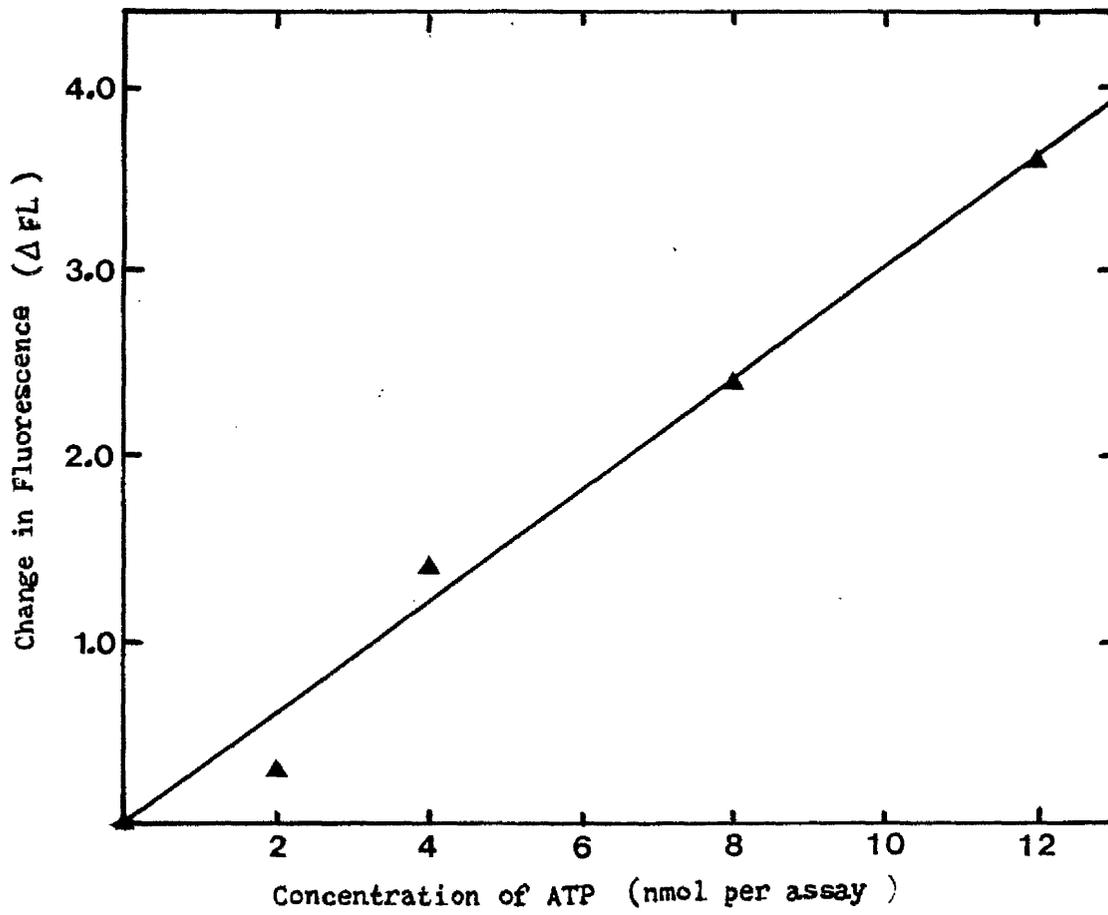
Cells grown for 3 days at 37°C were harvested by scraping into ice-cold 0.4N perchloric acid. The cell slurry was homogenised with ten strokes of a glass tissue grinder. The homogenate was left on ice for 30min, and the material precipitable by perchloric acid removed by centrifugation at 2,000g for 5min. The supernatant was decanted and neutralised with 0.72M KOH, 0.16M KHCO<sub>3</sub> and the KClO<sub>4</sub> precipitate was allowed to develop for 15min at 0°C. The KClO<sub>4</sub> precipitate was removed by centrifugation at 2,000g for 5min and the decanted supernatant stored at -20°C.

### 2.2.19 Measurement of the Concentration of ATP in Nucleotide Extracts

The concentration of ATP in the perchloric acid extract was determined by a method utilising coupled enzymatic reactions ultimately involved in a reduction of the ~~nicotinamide~~ nucleotide NADP. The method involves the quantitative phosphorylation of glucose by ATP with hexokinase and the simultaneous measurement of NADP reduction in the presence of glucose-6-phosphate dehydrogenase (Estabrook et al., 1967). An aliquot of the neutralised perchloric acid extract (usually 0.1 or 0.2 ml) was diluted to 1ml with a reaction mixture containing 70mM Tris-HCl (pH 7.5), 70mM MgCl<sub>2</sub>, 2mM glucose and 2mM NADP. The sample was placed in a cuvette in a fluorimeter and an aliquot of glucose-6-phosphate dehydrogenase added to ensure that any glucose-6-phosphate present in the sample was removed. After establishment of a suitable baseline of fluorescence at 460nm, an aliquot of hexokinase was added and the increase in fluorescence associated with NADP reduction determined. Other samples containing known amounts of ATP were also tested and the fluorescence change in the perchloric acid extract compared with these standard values. A typical standard curve of fluorescence change versus ATP concentration is shown in Fig. 2.2.10.

Fig. 2.2.10 Measurement of the Concentration of ATP.

The concentration of ATP in perchloric acid extracts of cells was determined by a method utilising coupled enzymatic reactions ultimately involved in the reduction of the *nicotinamide* nucleotide NADP (Estabrook et al., 1967), (Section 2.2.19). A standard curve was obtained by measuring the fluorescence change obtained using known amounts of ATP.



#### 2.2.20 Measurement of Radioactivity of (<sup>32</sup>P)-ATP.

This was determined by separation of the nucleotides (5 $\mu$ l) by chromatography on polyethyleneimine (PEI) cellulose using 0.85M KH<sub>2</sub>PO<sub>4</sub> (pH 3.4) as solvent (Cashel, Lazzasini & Kalbacher, 1969). The nucleotides ATP, ADP, GTP, and GDP (5 $\mu$ l of 5mM) were also included as standards and their positions on the PEI cellulose plate after chromatography circled when viewed under a UV lamp. The tracks containing the <sup>32</sup>P-labelled nucleotide mixtures were cut out and subjected to autoradiography using Kodirex X-ray film for 24-48 hours (Fig. 2.2.11) to locate the ATP which was then cut out and its radioactivity determined by scintillation spectrometry using a scintillant containing 660ml toluene, 330ml 2-methoxyethanol, 4g 2,5 diphenyloxazole (PPO) and 0.1g p-bis-[2-(5-phenyloxazole)]-benzene (POPOP) per litre.

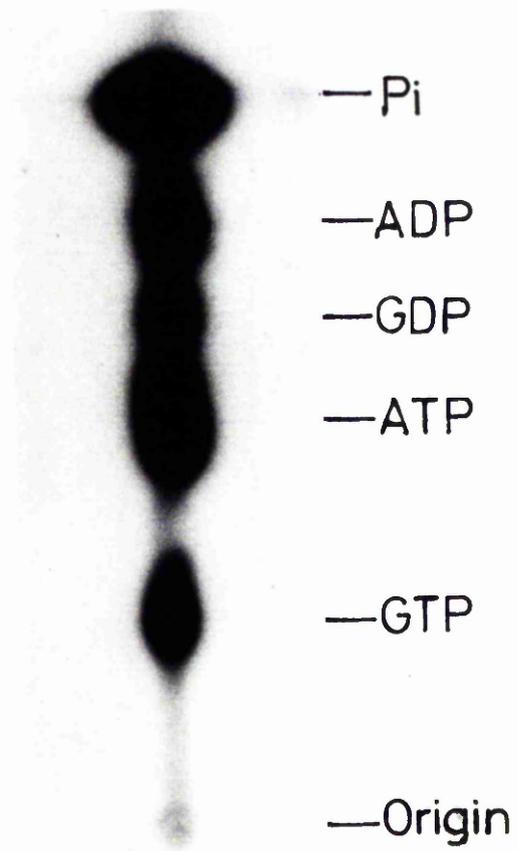
#### 2.2.21 Measurement of the concentration of Cyclic AMP.

The concentration of cyclic AMP in the neutralised perchloric acid extracts of cells was determined by means of a competitive protein binding assay (Gilman, 1970) involving the use of <sup>3</sup>H-cyclic AMP and a cyclic AMP binding protein (Radiochemical Centre, Amersham.)

The incubation mixture contained an aliquot of the perchloric acid extract, <sup>3</sup>H-cyclic AMP, cyclic AMP binding protein in a buffer containing 50mM Tris-HCl (pH 7.5) and 4mM EDTA, giving a total volume of 200 $\mu$ l. The binding was carried out at 4°C for 2 hours, after which time, 100 $\mu$ l of activated charcoal, in the same buffer, was added to remove unbound cyclic AMP. After centrifugation at 10,000g for 10min the radioactivity associated with the supernatant was determined by scintillation spectrometry with a scintillant containing 667ml toluene, 333ml TritonX-100, 5.5g 2,5 diphenyl-

Fig. 2.2.11 PEI Cellulose Chromatography of (<sup>32</sup>P)-Labelled Nucleotides.

BHK cells were incubated with (<sup>32</sup>P)-orthophosphate and nucleotides extracted with perchloric acid as described in Section 2.2.18. The nucleotides were separated by chromatography on polyethyleneimine (PEI) cellulose using 0.85M KH<sub>2</sub>PO<sub>4</sub> as solvent. The tracks containing the (<sup>32</sup>P)-labelled nucleotides were cut out and subjected to autoradiography using Kodirex X-ray film for 24-48 hours as described in Section 2.2.20.



oxazole and 0.1g p-bis-[2-(5-phenyloxazole)]-benzene per litre.

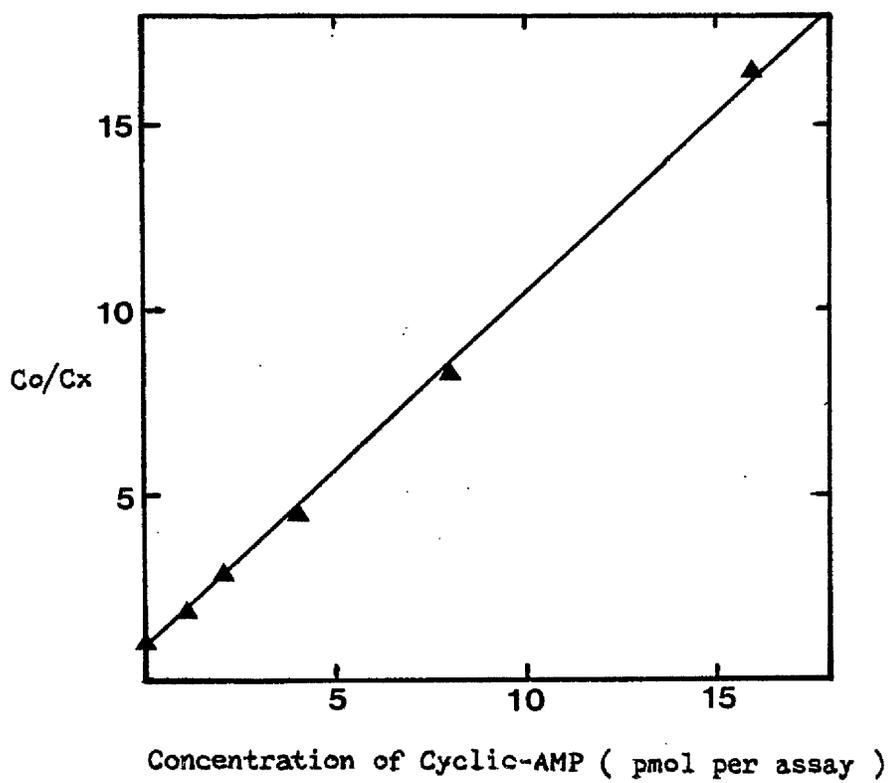
The radioactivity in each sample ( $C_x$ ) was compared with that from a control sample containing no added non-radioactive cyclic AMP ( $C_0$ ). The ratio of  $C_0/C_x$  was then calculated for each sample. A calibration curve with the  $C_0/C_x$  ratio obtained with known amounts of non-radioactive cyclic AMP was constructed and used to estimate the amount of cyclic AMP in the cell extracts. A typical calibration curve is shown in Fig. 2.2.12.

#### 2.2.22 Measurement of Protein Content of Cells.

The precipitate obtained after centrifugation of the perchloric acid treated cells (section 2.2.18) was dissolved in 10ml of a buffer containing 8M urea, 20mM Tris-HCl (pH 8.2), 1mM EDTA and 5% (v/v) 2-mercaptoethanol. The concentration of protein was then measured by the dye-binding assay described in section 2.2.17.

Fig. 2.2.12 Measurement of the Concentration of Cyclic-AMP.

The concentration of cyclic-AMP in perchloric acid extracts of cells was determined by means of a competitive protein binding assay (Gilman, 1970) involving the use of  $^3\text{H}$ -cyclic-AMP and a cyclic-AMP binding protein (Section 2.2.21). The binding of  $^3\text{H}$ -cyclic-AMP to the cyclic-AMP binding protein was measured in the presence of an aliquot of the perchloric acid extract which contained an unknown amount of non-radioactive cyclic-AMP. The associated radioactivity in each sample ( $C_x$ ) was then compared with that from a control sample containing no added non-radioactive cyclic-AMP ( $C_0$ ). The ratio  $C_0/C_x$  was then calculated for each sample. A calibration curve was constructed using the  $C_0/C_x$  ratios obtained after the addition to the incubation mixture of known amounts of non-radioactive cyclic-AMP.



### 3. RESULTS

3.1 THE PHOSPHORYLATION OF RIBOSOMAL PROTEIN S6 IN BHK CELLS  
TRANSFORMED BY PAPOVA VIRUSES.

It had been previously demonstrated (Leader, Rankine & Coia, 1976) that ribosomal protein S6 was more highly phosphorylated in pre-confluent BHK 21/C13 cells than in cells that had reached confluence. It was assumed that this difference was due to the difference in the rates of growth of the cells and it was therefore suggested that the phosphorylation might be related to the greater synthesis of ribosomes during more rapid growth. However the results of that study did not exclude the possibility that the difference in cell density might instead be responsible for the difference in phosphorylation. Before proceeding to investigate whether the phosphorylation was related to synthesis of ribosomes it was decided to see whether an effect of cell density could be excluded.

An opportunity for doing this was suggested by the availability of certain lines of BHK cells that had been transformed by papova viruses. These have different growth characteristics from the untransformed parent: at confluence they overgrow the monolayer faster than the untransformed cells, thus having a higher cell density as well as a higher rate of growth. The cells used in this investigation were the normal BHK 21/C13 cells (MacPherson & Stoker, 1962), BHK 21/C13/PyY cells (a line obtained by transformation with polyoma virus: Stoker, 1962) and BHK 21/C13/SV28 cells (a line obtained by transformation with simian virus 40: Wiblin & MacPherson, 1972).

The cells were grown for three days, by which time they had reached confluence. Both untransformed and transformed cells were still growing at this stage although the transformed cells were at a higher

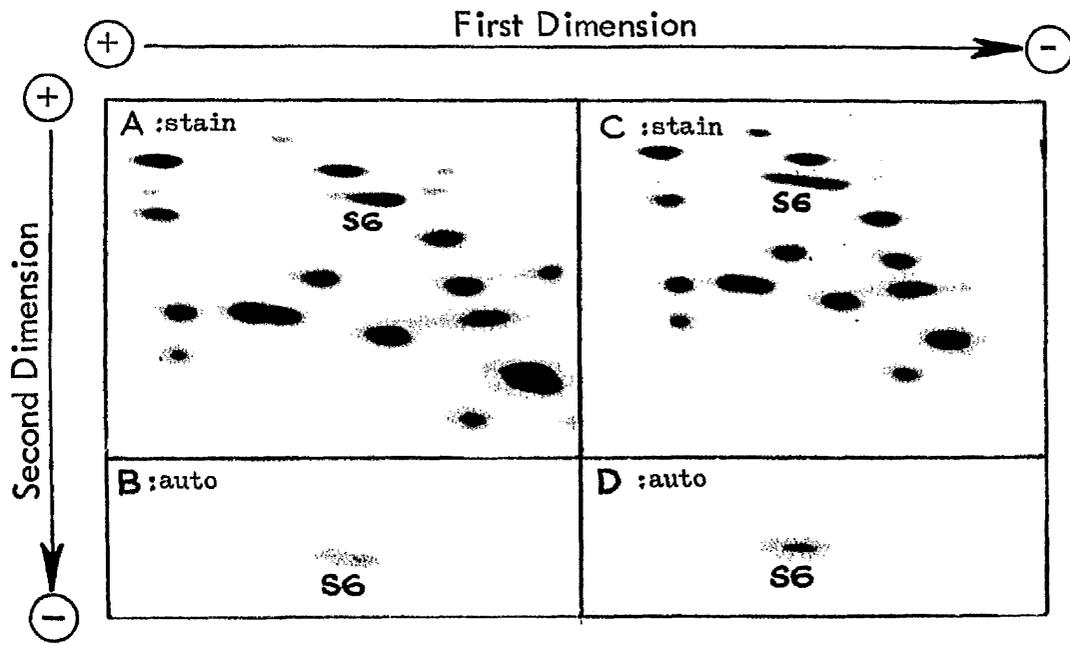
cell density because overgrowth had occurred. The cells were incubated for 3 hours with ( $^{32}\text{P}$ )-orthophosphate and then harvested. Ribosomes and their subunits were isolated from the cells (as described in Sections 2.2.9 and 2.2.10) and protein extracted from these (Section 2.2.12). Protein from the 40S ribosomal subunit was subjected to two-dimensional gel electrophoresis (Section 2.2.14.3). The results for normal cells and cells transformed by polyoma virus are shown in Fig. 3.1.1.

It can be seen from the pattern of staining that in the transformed cells there is a greater proportion of the ribosomal protein S6 migrating at a more anodal electrophoretic position than in normal cells. Examination of the corresponding autoradiographs (Fig. 3.1.1 (B) & (D)) indicates that the radioactivity associated with protein S6 is also situated to the anodal side of the position of electrophoretic migration of the bulk of the protein from normal cells. This is more clearly seen in Fig. 3.1.2 which shows a quantitative analysis of the distribution of radioactivity in protein S6. This was obtained by cutting out the section of two-dimensional gel containing protein S6 and its derivatives, scanning the section densitometrically, slicing into 3mm portions and determining the radioactivity in each portion. This showed that there was an increase of 140% in incorporation of ( $^{32}\text{P}$ )-orthophosphate accompanying this redistribution of protein to more anodal derivatives. When these values were converted to moles of phosphate per mole of ribosomal protein S6, taking into account differences in the specific radioactivity of ( $^{32}\text{P}$ )-( $\gamma$ )-ATP (which was presumed to be the phosphate donor), values of 0.16 and 0.51 moles of phosphate per mole of ribosomal protein were obtained for normal and transformed cells respectively. This was an increase in the extent of phosphorylation of protein S6 in transformed cells of 230%.

Fig. 3.1.1 The Phosphorylation of Ribosomal Protein S6 in Normal BHK Cells and Cells Transformed by Polyoma Virus.

Normal cells (BHK 21/C13) and cells transformed by polyoma virus (BHK 21/C13/PyY) were grown for 3 days and then incubated with 5mCi of ( $^{32}\text{P}$ )-orthophosphate, at a concentration of 100 $\mu\text{Ci/ml}$ , in Eagle's medium containing 0.09mM phosphate (one-tenth the normal phosphate concentration) and from which tryptose phosphate broth had been omitted. The cells were harvested and their ribosomes and ribosomal subunits prepared (Sections 2.2.9 & 2.2.10). Ribosomal proteins were extracted (Section 2.2.12) and 100ug subjected to two-dimensional gel electrophoresis (Section 2.2.14.3).

- A. 40S protein from BHK 21/C13 cells: stained gel
- B. 40S protein from BHK 21/C13 cells: autoradiograph
- C. 40S protein from BHK 21/C13/PyY cells: stained gel
- D. 40S protein from BHK 21/C13/PyY cells: autoradiograph



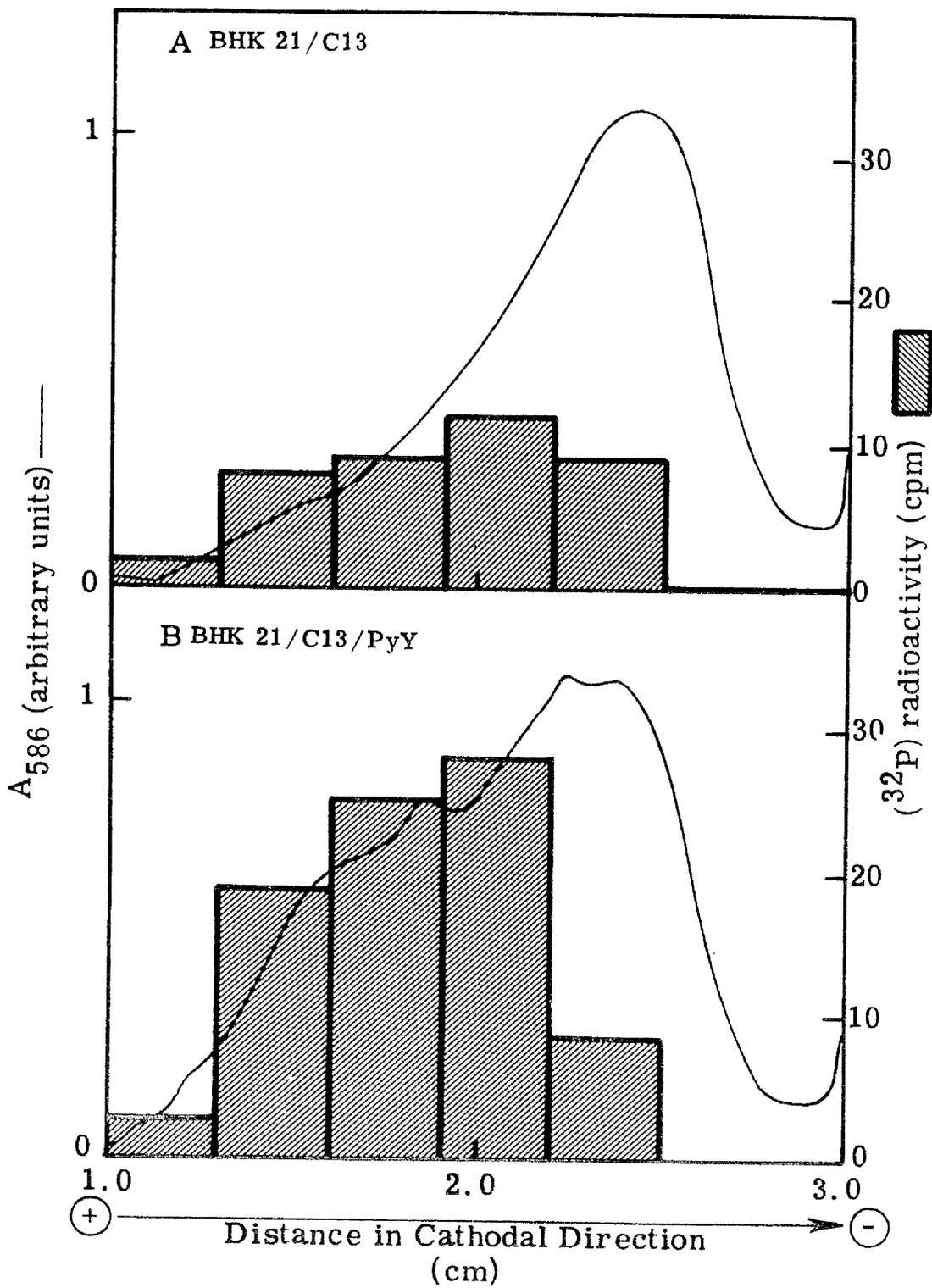
BHK 21/C13

BHK 21/C13/PyY

Fig. 3.1.2 Distribution of (<sup>32</sup>P)-Radioactivity and Dye-Stain in  
Electrophoretically Separated Ribosomal Protein S6 from  
Normal BHK Cells and Cells Transformed by Polyoma Virus

The labelling of cells, their fractionation and gel electrophoresis of their 40S ribosomal proteins were as described in the legend to Fig. 3.1.1. The section of two-dimensional gel containing ribosomal protein S6 was cut out and the distribution of dye-stain monitored by absorbance at 586nm (solid line). The gel fragment was sliced and each slice solubilised in 0.5ml of 90% Protosol (NEN.) at 37°C for 16 hours. After solubilisation 4.5ml of a scintillant mixture (Section 2.2.15.6) was added and the radioactivity in each slice measured by scintillation spectrometry at an efficiency of 70%.

- A. Protein S6 from BHK21/C13 cells.
- B. Protein S6 from BHK21/C13/PyY cells.



This confirms that the phosphorylated derivatives of protein S6 migrate more slowly in the first dimension than the unphosphorylated form of the protein, a result well documented by others (Gressner & Wool, (1974(a)); Leader, Rankine & Coia, (1976); Lastick, Neilsen & M<sup>C</sup>Conkey, (1977); (Haselbacher, Humbel & Thomas, (1979)). Thus Fig. 3.1.1 and Fig. 3.1.2 demonstrate that protein S6 is more phosphorylated in cells transformed by polyoma virus than in normal cells. It should also be noted that the extent of phosphorylation of protein S6 in confluent normal cells would appear to be relatively low, as only a small proportion of the stained material migrates to an electrophoretic position coincident with the position of the radioactivity.

Similar experiments were performed on cells transformed by simian virus 40, although in this case the cells were not labelled with (<sup>32</sup>P)-orthophosphate. This was a valid approach because Figs. 3.1.1 and 3.1.2 had shown that it is possible to distinguish preparations of ribosomes with protein S6 phosphorylated to different extents just by the relative position of electrophoretic migration of the stained protein S6.

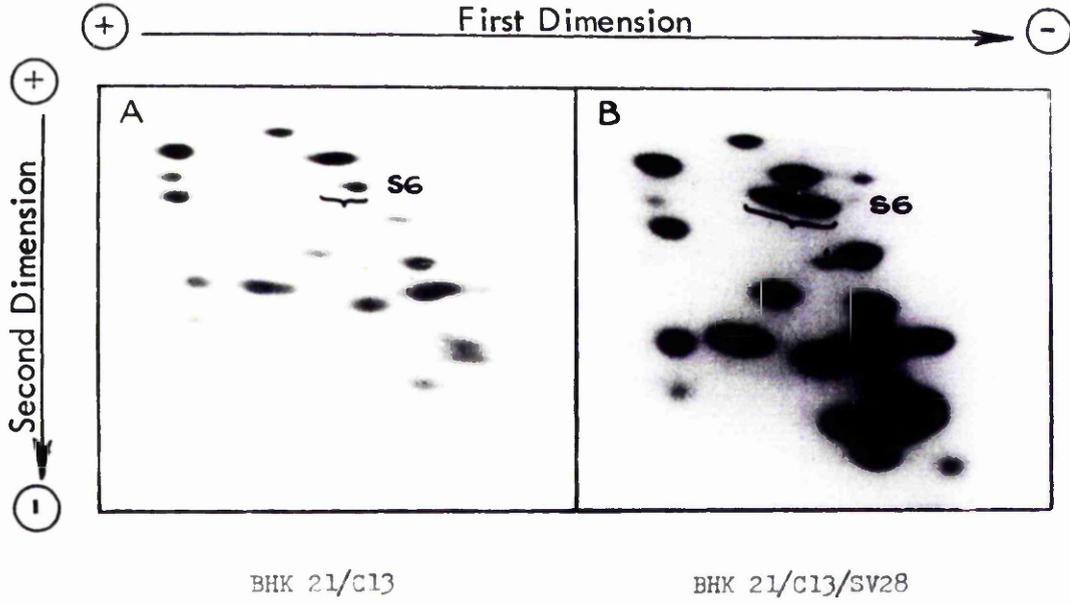
Ribosomal protein from the 40S subunit of normal BHK 21/C13 cells and cells transformed by simian virus 40 (BHK 21/C13/SV28) was subjected to two-dimensional gel electrophoresis (Fig. 3.1.3). Here also part of the stained protein S6 from the transformed cells has been displaced to the more anodal position at which the phosphorylated derivatives of the protein are known to migrate.

Thus it is clear that in this study, as in the one by Leader, Rankine and Coia,(1976) using pre-confluent and confluent BHK cells, the

Fig. 3.1.3 The Phosphorylation of Ribosomal Protein S6 in Normal  
BHK Cells and Cells Transformed by Simian Virus 40.

Normal (BHK 21/C13) and simian virus 40 transformed cells (BHK 21/C13/SV28) were grown for 3 days and then harvested. Their ribosomes and ribosomal subunits were prepared (Section 2.2.9 & Section 2.2.10) and ribosomal proteins extracted (Section 2.2.12), and 100ug of these subjected to two-dimensional gel electrophoresis (Section 2.2.14.3)

- A. 40S protein from BHK 21/C13 cells.
- B. 40S protein from BHK 21/C13/SV28 cells.



greater phosphorylation of ribosomal protein S6 was found in the more rapidly growing cells. In the previous study, however, these were the ones with the lower cell density (pre-confluent cells), but here they are the ones with the higher cell density (transformed cells). This rules out cell density as the correlate with phosphorylation in these two studies. The difference in phosphorylation of protein S6 observed between normal cells and those transformed by papova viruses cannot be due to increased concentrations of cyclic AMP since the cyclic AMP concentrations are generally lower in virus-transformed cells, including those transformed by polyoma and simian virus 40 (Ryan and Heidrick, 1974). It therefore seems likely that the difference in growth rate is the factor determining the difference in phosphorylation.

### 3.2 THE PHOSPHORYLATION OF RIBOSOMAL PROTEIN S6 IN RELATION TO THE ASSEMBLY OF RIBOSOMES AND THEIR TRANSPORT FROM THE NUCLEUS.

The correlation between growth rate and the extent of the phosphorylation of protein S6 (section 3.1) strengthened the possibility that the phosphorylation might be related to the greater proportion of newly synthesised ribosomes in more rapidly growing cells. Experiments were therefore performed to test the hypothesis (Leader, Rankine and Coia, 1976) that the phosphorylation of protein S6 occurs and functions in the nucleolus during assembly of the ribosomes or their transport from the nucleus.

#### 3.2.1 The Extent of Phosphorylation of Ribosomal Protein S6 in the Nucleolus.

The most direct approach to testing the hypothesis that the phosphorylation of protein S6 is of importance in the assembly of ribosomes in the nucleolus or in their transport from the nucleus to the cytoplasm, was to determine whether S6 was highly phosphorylated in the nucleolus. It was decided not to attempt to isolate nucleolar ribosomal precursor particles directly because these appear to lose some of the proteins of the small subunit during isolation (Shepherd and Maden, 1972). Instead the approach adopted was to examine total nucleolar protein. There were two problems to be overcome here. Firstly labelling in vivo with  $^{32}\text{P}$ -orthophosphate does not result in sufficient radioactivity in nucleolar ribosomal proteins for subsequent detection. This is because nucleolar ribosomal proteins form only a small proportion of total cellular ribosomal proteins, large amounts of which must be prepared to detect  $^{32}\text{P}$ -labelled phosphoproteins. Secondly, histones are the predominant basic proteins

of the nucleolus and nucleolar ribosomal proteins are not detectable in a stained electrophoretogram of total nucleolar proteins. In order therefore, to detect nucleolar ribosomal proteins in the nucleolar protein population, an attempt was made to label them preferentially. As histones contain a smaller percentage of methionine residues than ribosomal proteins (0.55% compared with 2.3%: Isenberg, (1979); Low, Wool and Martin, (1969)), it was thought that incubating cells with  $^{35}\text{S}$ -methionine would label them much less than ribosomal proteins. The ribosomal proteins might then be detectable and the extent of phosphorylation of protein S6 could then be estimated by comparison of the position of two-dimensional electrophoretic migration of its  $^{35}\text{S}$ -methionine radioactivity, with the position of electrophoretic migration of the phosphorylated and unphosphorylated forms of protein S6 (cf. Fig. 3.1.2).

Such analysis of nucleolar proteins is shown in Fig. 3.2.1. It was evident from this figure, which in fact contained the most radioactivity of three similar experiments, that insufficient incorporation of radioactive precursor into nucleolar ribosomal protein had taken place, even though incorporation into cytoplasmic ribosomes, from the same preparation, was sufficient to be detected by autoradiography (Fig. 3.2.2). To detect the nucleolar ribosomal proteins adequately, either application of more total protein or increasing the amount of  $^{35}\text{S}$ -methionine might seem indicated. However the amount of protein already being applied to the gels was near their capacity and the amount of  $^{35}\text{S}$ -methionine being used (2.5 mCi) was near the limits of economy. Moreover the predominant radioactive spots were in the position at which histones are known to migrate (see below). It was therefore decided to explore other approaches to the question.

Fig. 3.2.1 Two-Dimensional Gel Electrophoresis of Nucleolar  
Proteins Isolated from BHK Cells Incubated with  
(<sup>35</sup>S)-Methionine

BHK cells were grown for 3 days and then incubated with 2.5mCi of (<sup>35</sup>S)-methionine, at a concentration of 25μCi/ml, in Eagle's medium containing 2μM methionine (one-fiftieth the normal methionine concentration). The cells were harvested and their nuclei prepared (Section 2.2.13). Nucleoli were isolated from these (Section 2.2.13) and their total protein extracted (Section 2.2.12). An aliquot containing 100μg protein was mixed with 100μg unlabelled 80S ribosomal protein, as carrier, and the mixture subjected to two-dimensional gel electrophoresis (Section 2.2.14.3).

- A. Stained pattern of nucleolar protein + 80S ribosomal protein.
- B. Autoradiograph of nucleolar protein labelled with (<sup>35</sup>S)-methionine.

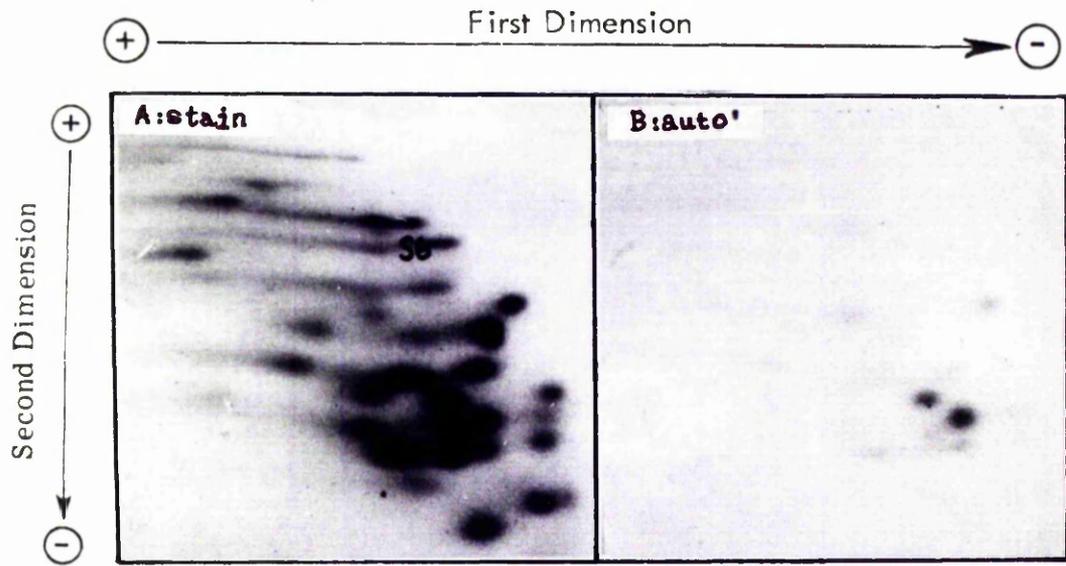
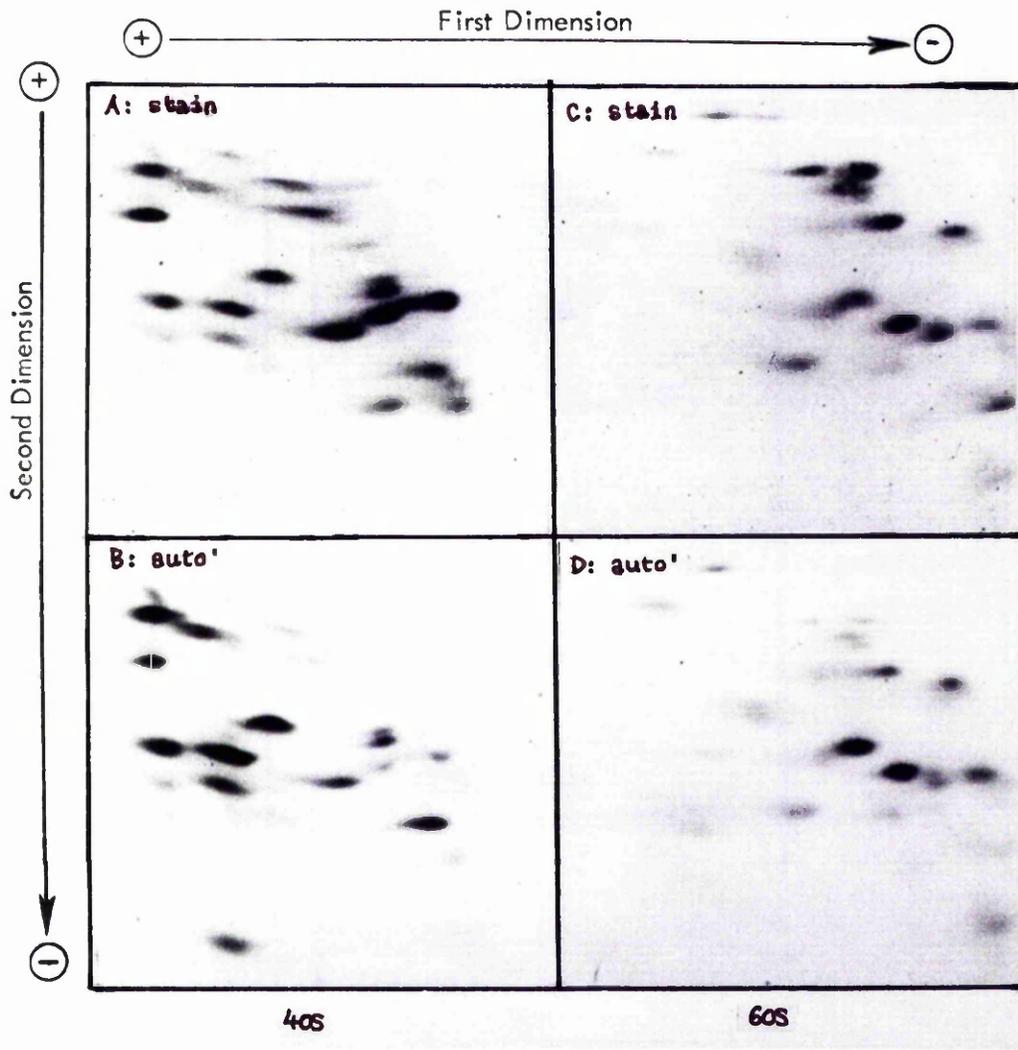


Fig. 3.2.2 Two-Dimensional Gel Electrophoresis of Ribosomal  
Proteins isolated from BHK Cells incubated with  
(<sup>35</sup>S)-Methionine

BHK cells were grown and labelled as described in Fig. 3.2.1. The cells were harvested and their nuclei prepared (Section 2.2.13). The post-nuclear supernatant was used to prepare ribosomes (Section 2.2.9). From these, ribosomal subunits were prepared (Section 2.2.10) and their proteins extracted (Section 2.2.12) and 100 $\mu$ g subjected to two-dimensional gel electrophoresis (Section 2.2.14.3).

- A. 40S ribosomal protein: stained gel
- B. 40S ribosomal protein: autoradiograph
- C. 60S ribosomal protein: stained gel
- D. 60S ribosomal protein: autoradiograph



One alternative method adopted to examine the phosphorylation of protein S6 in nucleoli was to incubate the latter with  $^{32}\text{P}$ -( $\gamma$ )-ATP to see whether phosphorylated derivatives of protein S6 became labelled. By incubating the nucleoli with radioactive precursor in vitro it was hoped to overcome the problem of obtaining sufficient incorporation of radioactivity into ribosomal protein mentioned above. Two-dimensional gel electrophoretic analysis of nucleoli labelled in such a manner is shown in Fig. 3.2.3. It can be seen that, although a protein had become labelled, this was not ribosomal protein S6 but a protein of lower molecular weight, in the region of S23/24. This  $^{32}\text{P}$ -labelled protein is most likely one of the histones since their charge and size characteristics are such that they would be expected to be found in the lower half of a Kaltschmidt/Wittmann gel. Supporting such an identification is the fact that on rare occasions, the 40S ribosomal proteins were contaminated by proteins migrating to this position of the two-dimensional gel and these were also labelled with  $^{32}\text{P}$ -orthophosphate (Fig. 3.2.4). This was ascribed to contamination of the ribosomes by nuclei and contamination of the extracted ribosomal protein by histone. It therefore seems most likely that the  $^{32}\text{P}$ -labelled protein observed in Fig. 3.2.3 was due to phosphorylation of one of the histones.

The experiment of Fig. 3.2.3 does confirm (Kang, Olson & Busch, (1974), Grummt, (1974), Grummt & Grummt, (1974), Ballal et al., (1975), Sakuma et al., (1978) and Kawashima & Izawa, (1977)) that protein kinase activity is present in the nucleolus. However it is difficult to assess, without purification and further study, whether this is a specific histone kinase, or whether it can also phosphorylate protein S6. This is possible despite the fact that protein S6 was not phosphorylated here, because histones, being present in a much

Fig. 3.2.3 Phosphorylation of Nucleolar Proteins in vitro

BHK cells were grown for 3 days and their nucleoli prepared (Section 2.2.13). These were labelled in vitro by incubation with 25 $\mu$ Ci ( $^{32}$ P)-( $\gamma$ )-ATP at 250 $\mu$ Ci/ml in a buffer containing 250mM sucrose, 100mM Tris-HCl (pH 7.5), 100mM NaCl, 20mM MgCl<sub>2</sub>, 6mM NaF and 10mM 2-mercaptoethanol. After incubation for 15min at 37°C the reaction was stopped by the addition of 1 vol. of glacial acetic acid and 0.2 vol. of 1M MgCl<sub>2</sub>, and the protein extracted as in section 2.2.12. The protein (100 $\mu$ g) was subjected to two-dimensional gel electrophoresis or mixed with 100 $\mu$ g non-radioactive 80S ribosomal protein and then subjected to gel electrophoresis (Section 2.2.14.3).

- A. Nucleolar protein: stained gel
- B. Nucleolar protein: autoradiograph
- C. Nucleolar protein + 80S carrier protein: stained gel
- D. Nucleolar protein + 80S carrier protein: autoradiograph

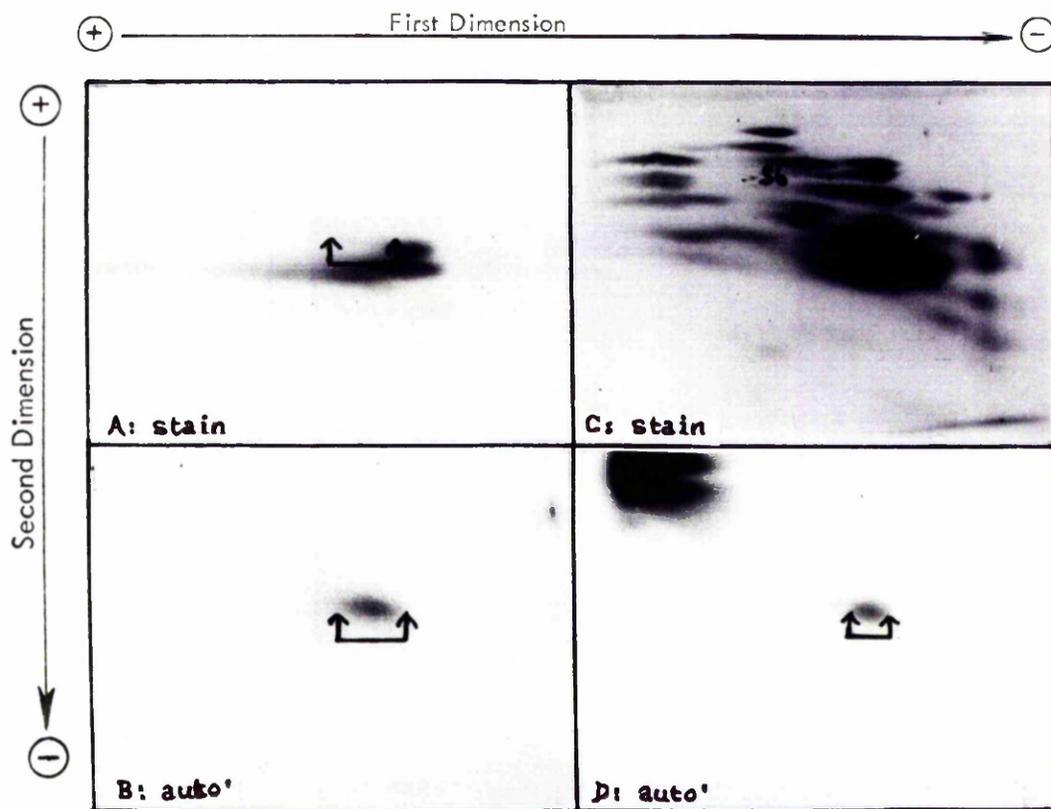
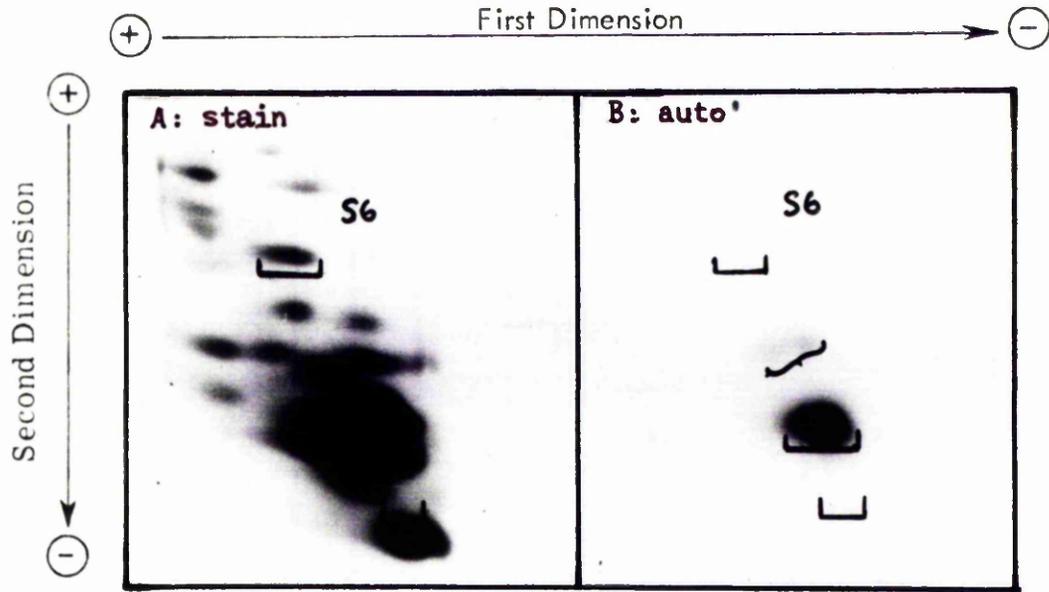


Fig. 3.2.4 Two-Dimensional Gel Electrophoresis of (<sup>32</sup>P)-Labelled Ribosomal Proteins Showing Contamination with other Basic Phosphoproteins.

BHK cells were grown for 3 days and incubated with 5mCi of (<sup>32</sup>P)-orthophosphate, at a concentration of 50μCi/ml, in Eagle's medium containing 0.09mM phosphate (one-tenth the normal phosphate concentration). The cells were harvested, their ribosomes isolated (Section 2.2.9) and ribosomal subunits prepared (Section 2.2.10). Ribosomal protein was extracted from these (Section 2.2.12) and 100μg subjected to two-dimensional gel electrophoresis (Section 2.2.14.3).

- A. 40S protein: stained gel
- B. 40S protein: autoradiograph



greater quantity than ribosomal proteins, would tend to be phosphorylated in preference.

### 3.2.2 The Extent of Phosphorylation of Ribosomal Protein S6 in 'Native' 40S Subunits.

Another approach was to examine the extent of phosphorylation of protein S6 in 'native' 40S subunits which include the ribosomal subunits which have newly entered the cytoplasm from the nucleus.

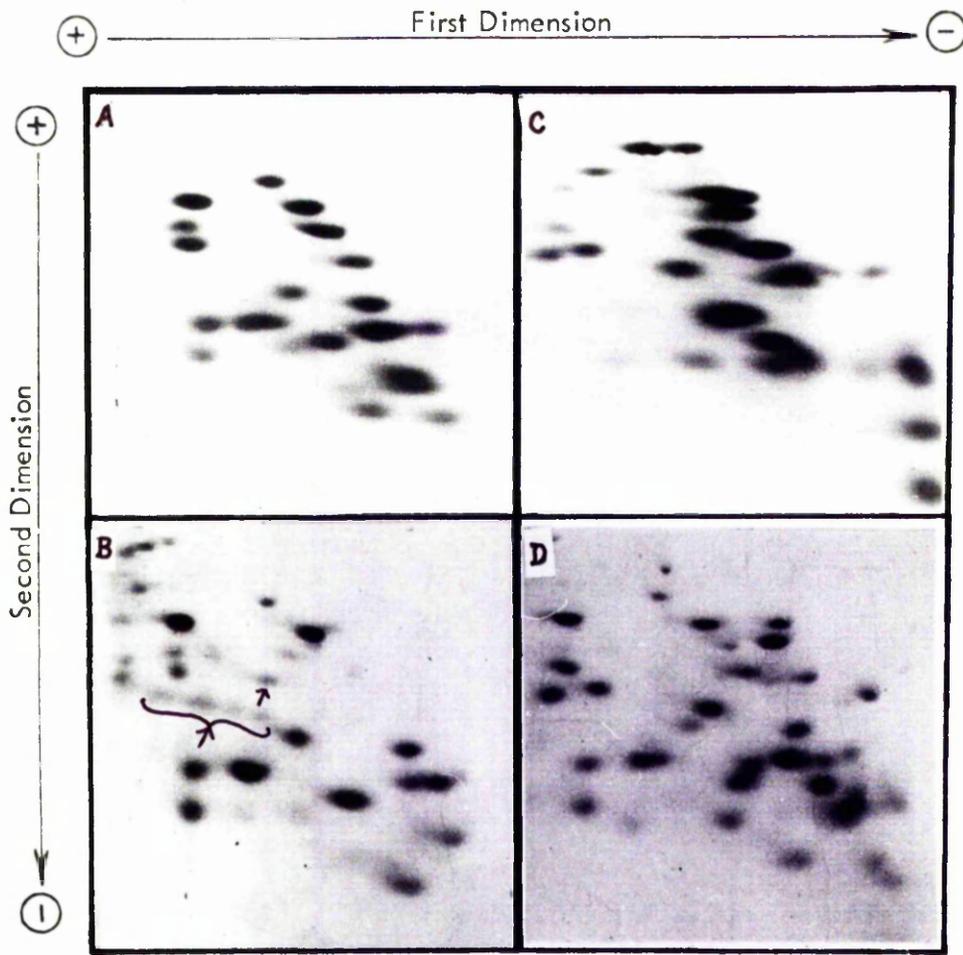
'Native' subunits can be separated from other cytoplasmic forms of ribosomes in the post-nuclear supernatant by sucrose density centrifugation. The concentrations of  $K^+$  and  $Mg^{2+}$  in the gradients were low (25mM and 2mM respectively) to maintain the integrity of 80S monoribosomes. After collection of the 'native' subunits their total protein was extracted (section 2.2.12) and subjected to two-dimensional gel electrophoresis (section 2.2.14.3) as were proteins from subunits prepared in the normal manner of dissociation at high ionic strength (Fig. 3.2.5).

It can be seen from frames A and B of Fig. 3.2.5 that the electrophoretic patterns of the two types of subunits are quite similar, although several additional proteins appear unique to the 'native' subunits (arrows). In the 'native' subunits very little protein was detected at the position of unphosphorylated protein S6, in contrast with subunits prepared at high ionic strength. There was also a weakly stained spot at a position where one might expect one of the more phosphorylated derivatives of protein S6. This may indicate either that protein S6 is highly phosphorylated in 'native' 40S subunits or that protein S6 had not entered the gel (as sometimes happened) and a non-ribosomal protein of the 'native' subunits migrated fortuitously in this region.

Fig. 3.2.5 Two-Dimensional Gel Electrophoresis of Proteins from  
'Native' Ribosomal Subunits and Ribosomal Subunits  
Prepared at High Ionic Strength.

BHK cells were grown for 3 days and harvested for the preparation of subunits by dissociation of ribosomes at high ionic strength, ribosomes were prepared from the post-nuclear supernatant (Section 2.2.9) and ribosomal subunits prepared by centrifugation through a 10-30% linear sucrose gradient containing 500mM  $K^+$ , 5mM  $Mg^{2+}$  and puromycin (Section 2.2.10). 'Native' subunits were prepared from the post-nuclear supernatant by centrifugation through a 10-40% linear sucrose gradient containing 25mM  $K^+$  and 5mM  $Mg^{2+}$  (Section 2.2.11). Protein was extracted from both these types of ribosomal subunits (Section 2.2.12) and subjected to two-dimensional electrophoresis (Section 2.2.14.3).

- A. protein from 40S subunits prepared at high ionic strength.
- B. protein from 'native' 40S subunits.
- C. protein from 60S subunits prepared at high ionic strength.
- D. protein from 'native' 60S subunits.



Evidence supporting this latter interpretation was obtained by examination of the electrophoretic migration of proteins from 'native' 60S subunits (Fig. 3.2.5., frame (D)), which shows considerable cross-contamination with 40S subunit proteins, as others have also found (Sunkvist & Howard, 1974). Here protein S6 was clearly present in the position of electrophoretic migration of the unphosphorylated protein.

### 3.2.3 Determination of the Relative Phosphorylation of Protein S6 at Different Times after the Synthesis of Ribosomes.

As 'native' subunits do not contain exclusively newly-synthesised subunits, but also subunits produced by dissociation of terminating polysomes, the suggestion of a low extent of phosphorylation of protein S6 in 'native' subunits did not prove that newly synthesised ribosomes were not extensively phosphorylated. An alternative approach to the question was to compare the newly-synthesised and older ribosomes directly to see if the phosphorylation of protein S6 was different in the two populations. If the greater phosphorylation of cytoplasmic ribosomes in more rapidly growing cells is due to the greater proportion of newly-synthesised ribosomes, then in less rapidly growing cells (for example, confluent untransformed BHK cells), then the newly-synthesised ribosomes should be much more highly phosphorylated than the bulk of ribosomes. The method of distinguishing the two types of ribosomes in the total cytoplasmic population was as follows.

Newly-synthesised ribosomes were labelled preferentially by taking cells that had grown for three days and incubating these with <sup>35</sup>S-methionine for 20min and then harvesting. Older ribosomes were labelled preferentially by taking cells that had grown for one day and incubating these with <sup>35</sup>S-methionine for 3 hours and then 'chasing' the

radioactivity out of the amino acid pool, with a medium containing unlabelled methionine, for 2 more days. The relative extents of phosphorylation of protein S6 in the two types of ribosomes were then determined by comparing the position of electrophoretic migration of the radioactively labelled protein S6 after two-dimensional gel electrophoresis .

Such electrophoretic analysis of protein from the 40S subunit of both types of labelled ribosomes is shown in Fig. 3.2.6. Inspection of the autoradiographs indicates that the majority of the radioactivity in protein S6, from both newly-synthesised and two-day old ribosomes, coincides with the bulk of the stained protein, which is in the position of the unphosphorylated parent protein.

A more quantitative analysis of the distribution of radioactivity along protein S6 was obtained by cutting out the portion of two-dimensional gel containing protein S6 and its derivatives, scanning the section densitometrically, slicing into 3mm portions and determining the radioactivity in each portion. Fig. 3.2.7 shows this analysis, which again indicates that the majority of the radioactivity resides with the unphosphorylated form of protein S6.

A double-labelling experiment of a single culture of cells, in which newly-synthesised and older ribosomes were preferentially labelled using ( $^{35}\text{S}$ ) and ( $^3\text{H}$ )-methionine respectively, gave essentially similar results (Fig. 3.2.8), although somewhat less clear-cut because of the high background radioactivity on the ( $^3\text{H}$ )-labelling.

As considered at greater length in the discussion, the conclusion drawn from these experiments is that protein S6 is no more extensively

Fig. 3.2.6 The Extent of Phosphorylation of Ribosomal Protein S6  
at Different Times After Synthesis of Ribosomes.

Newly synthesised ribosomes were preferentially labelled by growing BHK cells for 3 days and incubated for 20min with 2.5mCi of (<sup>35</sup>S)-methionine, at a concentration of 25µCi/ml, in Eagle's medium containing 2µM methionine (one-fiftieth the normal methionine concentration). The cells were then harvested and their ribosomes isolated (Section 2.2.9).

Older ribosomes were preferentially labelled by growing BHK cells for 1 day and incubating for 3 hours with 2.5mCi of (<sup>35</sup>S)-methionine, at a concentration of 25µCi/ml, in Eagle's medium containing 2µM methionine (one-fiftieth the normal methionine concentration).

This medium was replaced by Eagles medium containing 0.1mM unlabelled methionine (the normal concentration) and the cells grown for 2 more days. The cells were then harvested and their ribosomes isolated (Section 2.2.9).

Ribosomal subunits were prepared (Section 2.2.10 ) from both types of ribosomes, and their proteins extracted (Section 2.2.12) and 100µg subjected to two-dimensional gel electrophoresis (Section 2.2.14.3).

- A. protein from subunits labelled for 20min with (<sup>35</sup>S)-methionine: stained gel.
- B. protein from subunits labelled for 20min with (<sup>35</sup>S)-methionine: autoradiograph.
- C. protein from subunits labelled for 3 hours with (<sup>35</sup>S)-methionine and chased in unlabelled methionine for 48 hours: stained gel.
- D. protein from subunits labelled for 3 hours with (<sup>35</sup>S)-methionine and chased in unlabelled methionine for 48 hours: autoradiograph.

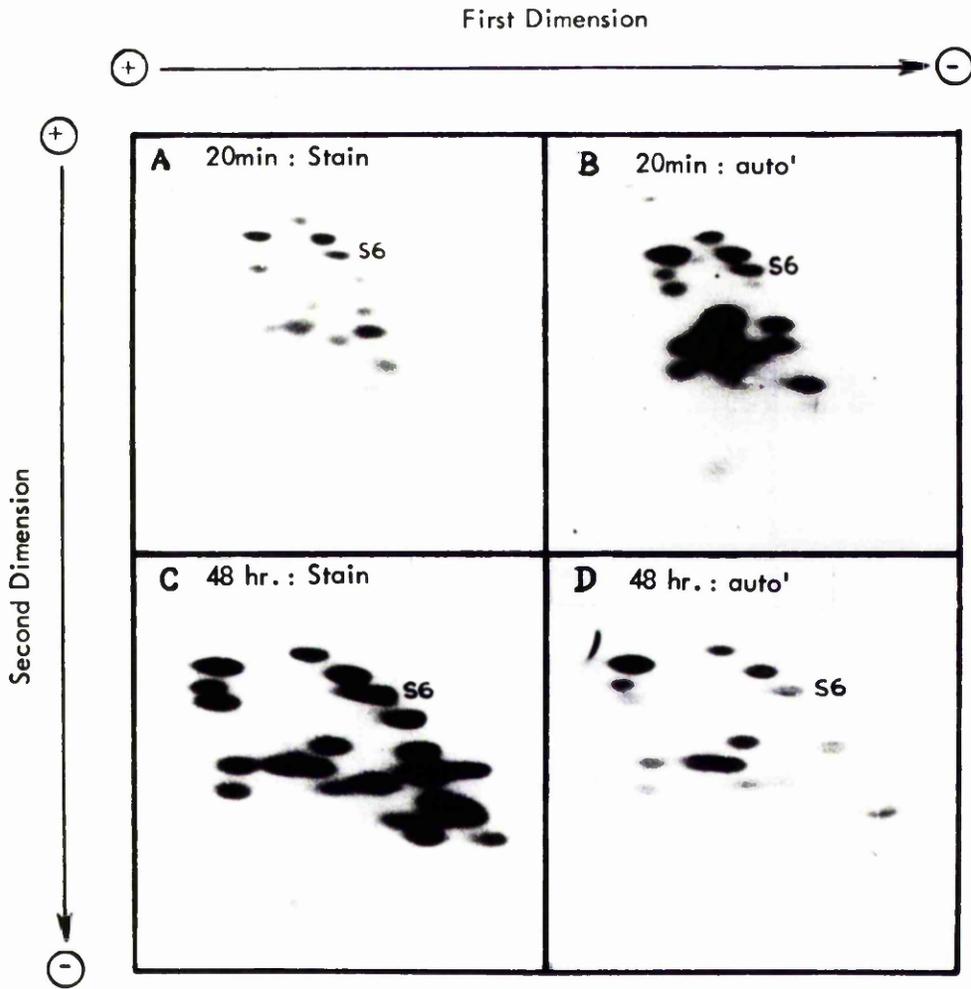


Fig. 3.2.7 Distribution of (<sup>35</sup>S)-Radioactivity and Dye-Stain in Electrophoretically Separated Protein S6 from Differentially Labelled BHK Ribosomes

The growth of cells, their labelling, fractionation and the gel electrophoresis of their 40S ribosomal proteins were as described in the legend to Fig. 3.2.6.

The section of two-dimensional gel containing ribosomal protein S6 was cut out and the distribution of dye-stain monitored by the absorbance at 586nm (solid line). The gel fragment was sliced and each slice solubilised in 0.5ml of 90% Protosol (NEN) at 37°C for 16 hours. After solubilisation 4.5ml of a scintillant mixture (Section 2.2.15.6) was added and the (<sup>35</sup>S)-radioactivity measured by scintillation spectrometry at an efficiency of 60%.

- A. Protein S6 from cells labelled for 20min with (<sup>35</sup>S)-methionine.
- B. Protein S6 from cells labelled for 3 hours with (<sup>35</sup>S)-methionine and chased in unlabelled methionine for 48 hours.

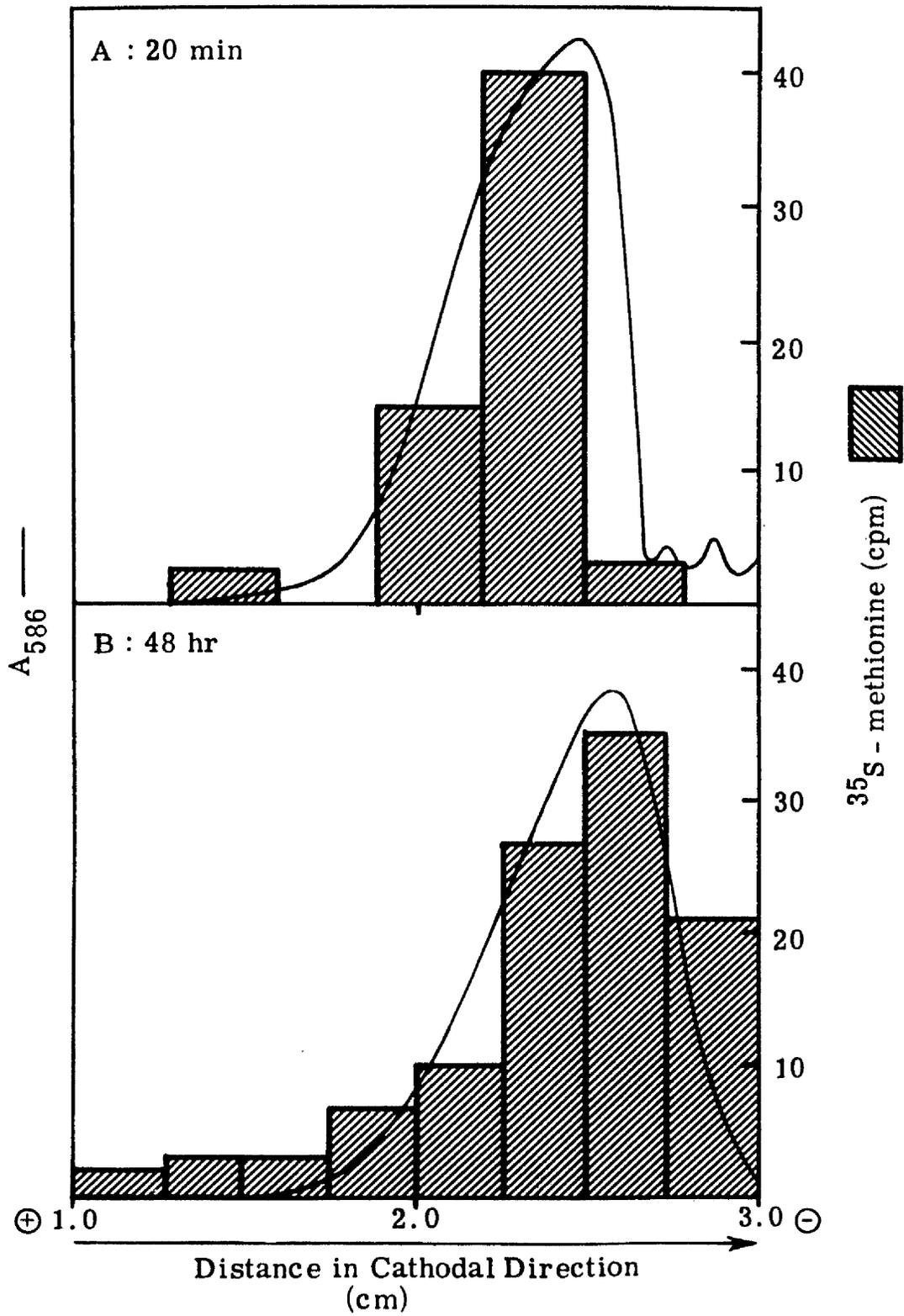
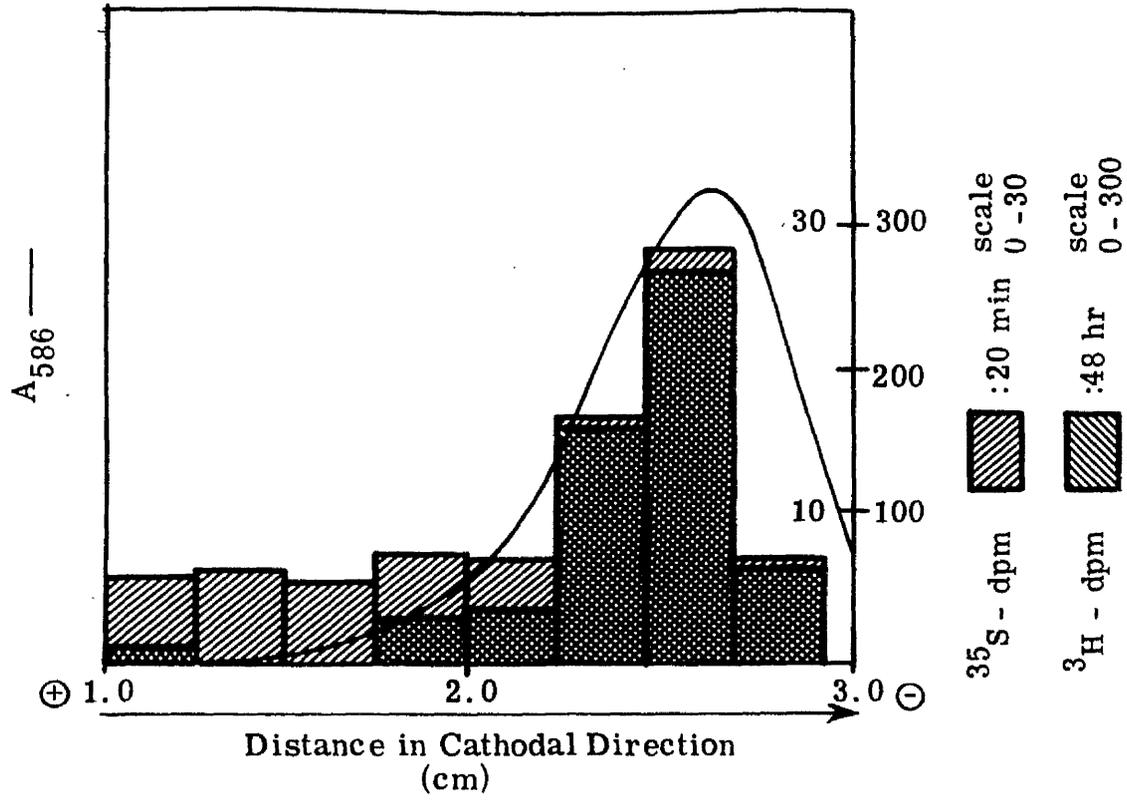


Fig. 3.2.8 Distribution of (<sup>3</sup>H) and (<sup>35</sup>S)-Radioactivity and Dye-Stain in Electrophoretically Separated Ribosomal Protein S6 from Differentially Labelled BHK Ribosomes.

BHK cells were grown for 1 day and 'older' ribosomes differentially labelled by incubation for 3 hours with 5mCi of (<sup>3</sup>H)-methionine, at a concentration of 50µCi/ml, in Eagle's medium containing 2µM methionine (one-fiftieth the normal methionine concentration). The cells were then grown for 2 more days, in Eagle's medium containing the normal concentration (0.1mM) of unlabelled methionine. Newly-synthesised ribosomes were differentially labelled by incubation for 20min with 2.5mCi of (<sup>35</sup>S)-methionine, at a concentration of 25µCi/ml, in Eagle's medium containing 2µM methionine (one-fiftieth the normal methionine concentration) and the cells immediately harvested. Ribosomes and their subunits were prepared (Sections 2.2.9. & 2.2.10), ribosomal protein extracted (Section 2.2.12) and 100µg subjected to two-dimensional gel electrophoresis (Section 2.2.14.3).

The section of two-dimensional gel containing ribosomal protein S6 was cut out and the distribution of dye-stain monitored by absorbance at 580nm (solid line). The gel fragment was sliced and each slice solubilised in 0.5ml of 90% Protosol (NEN) at 37°C for 16min. After solubilisation 4.5ml of a scintillation mixture (Section 2.2.15.6) was added and the (<sup>3</sup>H) and (<sup>35</sup>S)-radioactivity measured by scintillation spectrometry. The amount of 'carry-over' (<sup>35</sup>S)-radioactivity in the (<sup>3</sup>H) channel was 8% and the efficiency of counting for (<sup>3</sup>H) and (<sup>35</sup>S) was 25% and 59% respectively. The tritium (<sup>3</sup>H) disintegrations per min (dpm) were calculated according to the following equation.

$${}^3\text{H}_{\text{dpm}} = {}^3\text{H}_{\text{cpm}} - 8\% {}^{35}\text{S}_{\text{dpm}} \times {}^3\text{H}_{\text{eff}}$$



phosphorylated in newly-synthesised ribosomes than in two-day old ribosomes. This suggests that the postulated role for phosphorylation of protein S6 in nuclear assembly or extra-nuclear transport of ribosomes is incorrect and it was decided not to pursue this line of study further.

### 3.3 THE PHOSPHORYLATION OF RIBOSOMAL PROTEINS IN BHK CELLS INFECTED BY VIRUSES OF THE HERPES CLASS.

In view of the results suggesting that phosphorylation of protein S6 was not involved in the synthesis of ribosomes, attention was turned to other systems which might throw light on the function of this phosphorylation. As outlined in the Introduction, it was thought that further examination of cells infected by viruses was justified, and studies were undertaken on viruses of the Herpes class which infect BHK cells, the ribosomes of which had already been characterised in this work. Initially it was decided to concentrate on the swine virus, pseudorabies virus, which is more convenient for experimentation as it has a shorter replication cycle than, for example herpes simplex virus.

#### 3.3.1 The Phosphorylation of Ribosomal proteins in BHK cells Infected with Pseudorabies Virus.

BHK cells were grown for 3 days and infected with pseudorabies virus (as described in section 2.2.3). The virus was allowed to adsorb for one hour and the medium then replaced by 50ml of the original medium. The infection was allowed to continue for various times, with the time of infection quoted being taken from the initial addition of the virus. Mock-infected cells were treated in exactly the same manner except that the virus was replaced by a similar volume of Eagle's medium. The cells were incubated for 3 hours with ( $^{32}\text{P}$ )-orthophosphate and then harvested. Ribosomes and ribosomal subunits were prepared (sections 2.2.9 and 2.2.10) and their proteins extracted (section 2.2.12) and subjected to two-dimensional gel electrophoresis (section 2.2.14.3).

Such electrophoretic analysis of protein from the 40S ribosomal subunit of cells infected for 5 hours with pseudorabies virus and

uninfected cells is shown in Fig. 3.3.1. Similar comparison of labelled protein from cells infected with pseudorabies virus (in this case for 6 hours) and mock-infected cells is shown in Fig. 3.3.2.

It can clearly be seen that there was a considerable increase in the amount of ( $^{32}\text{P}$ )-radioactivity incorporated into ribosomal protein S6 from the virus infected cells compared with that in the uninfected cells (Fig. 3.3.1) or the mock-infected cells (Fig. 3.3.2). This increase in radioactive incorporation was accompanied by a marked anodal displacement of most of the stained S6 protein from the position to which it migrates in uninfected or mock-infected cells. This indicates that there is a considerable increase in the extent of phosphorylation of the protein. In addition a further ribosomal protein, either S16 or S18, has become labelled with ( $^{32}\text{P}$ )-ortho-phosphate in the cells infected with virus. This protein was never labelled in either uninfected or mock-infected cells.

The relationship between the phosphorylation of ribosomal protein S6 and its electrophoretic mobility is better illustrated in Fig. 3.3.3, which shows the 40S ribosomal proteins from cells infected for 9 hours with pseudorabies virus. At this time the overall extent of phosphorylation of protein S6 was rather less than that at 5 or 6 hours after infection, with some stained material being present at the original position of the protein as well as at anodal positions of intermediate displacement. However in another experiment the extent of phosphorylation of protein S6 at 9 hours after infection was comparable with that at 6 hours after infection (data not shown). Fig. 3.3.4 shows quite clearly that the majority of the ( $^{32}\text{P}$ )-radio-

Fig. 3.3.1 The Phosphorylation of 40S Ribosomal Proteins in Cells Infected for 5 hours with Pseudorabies Virus and in Uninfected Cells.

BHK cells were grown for 3 days and infected with pseudorabies virus (20pfu/cell). After 2 hours of infection the cells were incubated, for a further 3 hours, with 5mCi of ( $^{32}\text{P}$ )-orthophosphate, (at a concentration of 50 $\mu\text{Ci/ml}$ ) in Eagle's medium containing 0.09mM of phosphate (one-tenth the normal phosphate concentration) and from which tryptose phosphate broth had been omitted. Uninfected cells were labelled in a similar manner. The cells were then harvested and their ribosomes and ribosomal subunits isolated (Section 2.2.9 and Section 2.2.10). Ribosomal proteins were extracted (Section 2.2.12) and 100 $\mu\text{g}$  subjected to two-dimensional gel electrophoresis (Section 2.2.14.3)

- A. 40S protein from uninfected cells: stained gel.
- B. 40S protein from uninfected cells: autoradiograph.
- C. 40S protein from cells infected for 5 hours with pseudorabies virus: stained gel.
- D. 40S protein from cells infected for 5 hours with pseudorabies virus: autoradiograph.

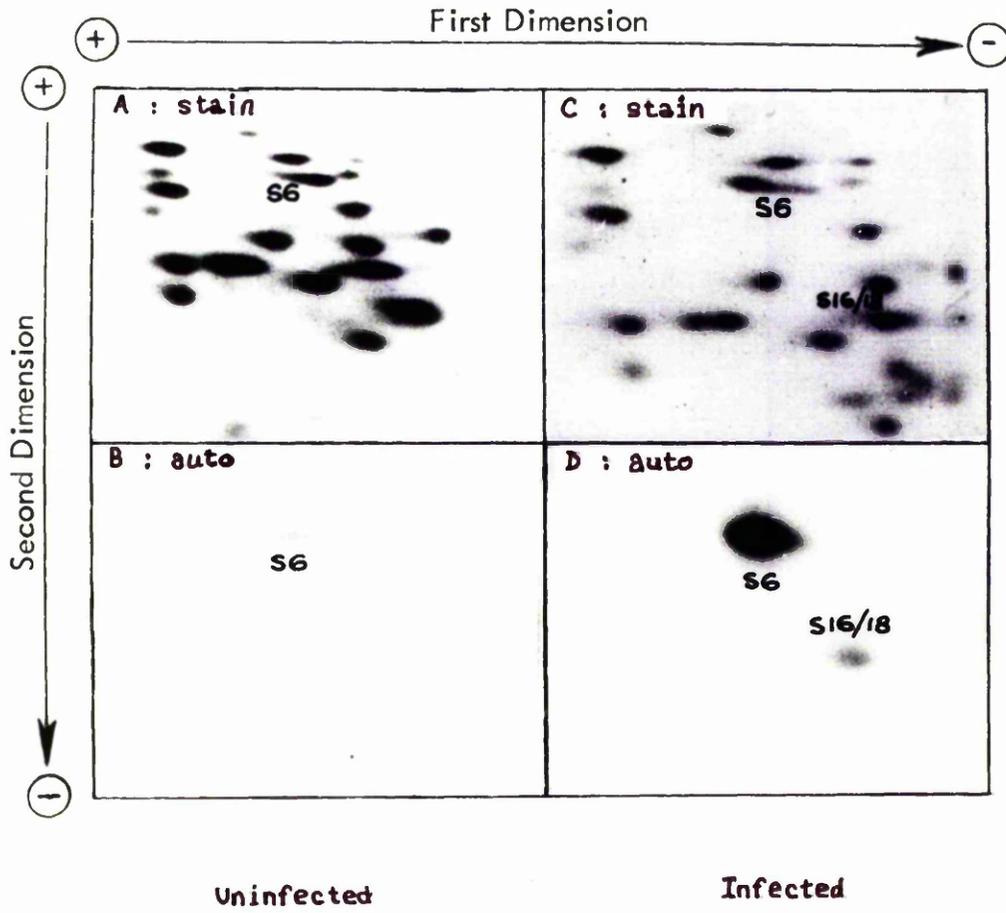


Fig. 3.3.2 The Phosphorylation of 40S Ribosomal Proteins in Cells  
Infected for 6 Hours with Pseudorabies Virus and in  
Mock-Infected Cells.

BHK cells were grown for 3 days and infected with pseudorabies virus (20pfu/cell), or mock-infected with a similar volume of Eagle's medium. After 3 hours of infection (or mock-infection) the cells were incubated for a further 3 hours with 5mCi of ( $^{32}\text{P}$ )-orthophosphate (at a concentration of 50 $\mu\text{Ci/ml}$ ) in Eagle's medium containing 0.09mM phosphate (one-tenth the normal phosphate concentration) and from which tryptose phosphate broth had been omitted. The cells were then harvested and their subunits prepared (Sections 2.2.9 & 2.2.10). Ribosomal protein was extracted (Section 2.2.12) and 100 $\mu\text{g}$  subjected to two-dimensional gel electrophoresis (Section 2.2.14.3).

- A. 40S protein from mock-infected cells: stained gel.
- B. 40S protein from mock-infected cells: autoradiograph
- C. 40S protein from cells infected for 6 hours with pseudorabies virus: stained gel.
- D. 40S protein from cells infected for 6 hours with pseudorabies virus: autoradiograph.

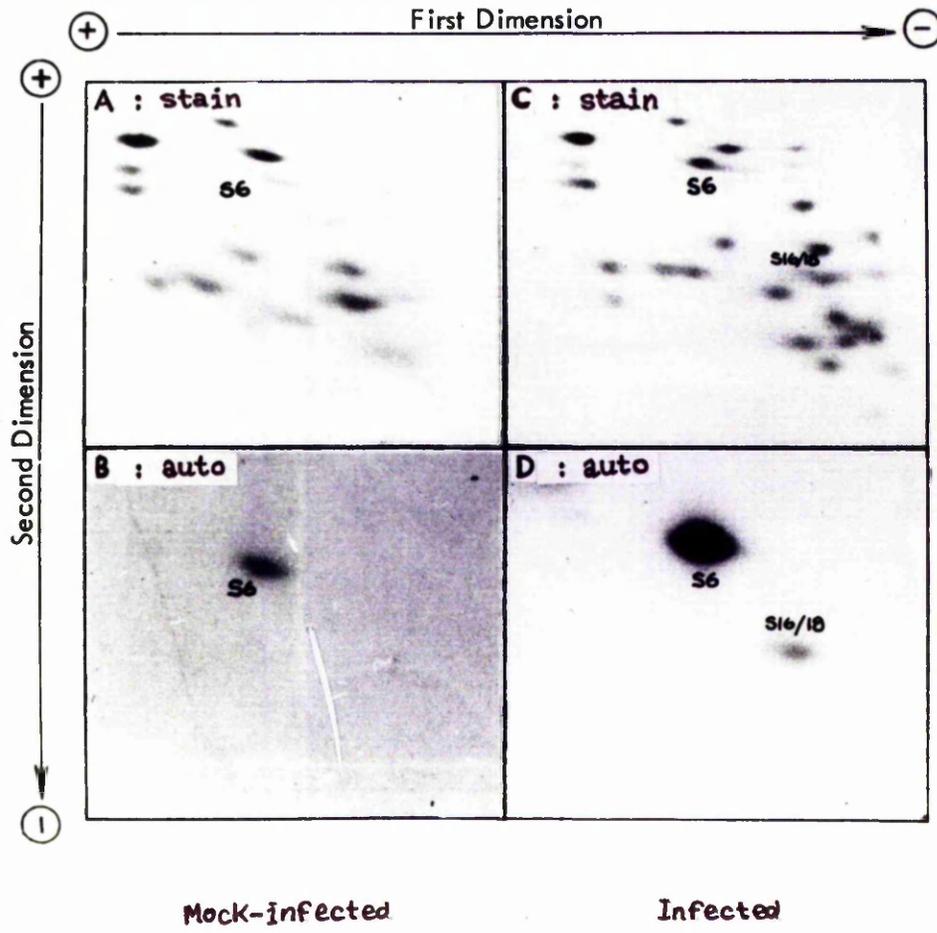


Fig. 3.3.3 The Phosphorylation of 40S Ribosomal Proteins in Cells  
Infected for 9 hours with Pseudorabies Virus.

BHK cells were grown for 3 days and infected with pseudorabies virus (20pfu/cell). After infection for 6 hours the cells were incubated (for a further 3 hours) with 5mCi of ( $^{32}$ P)-orthophosphate, at a concentration of 50 $\mu$ Ci/ml, in Eagle's medium containing 0.09mM phosphate (one-tenth the normal phosphate concentration) and from which tryptose phosphate broth had been omitted. The cells were then harvested and their ribosomes and ribosomal subunits isolated (Sections 2.2.9 & 2.2.10). Ribosomal protein was extracted (Section 2.2.12) and 100 $\mu$ g subjected to two-dimensional gel electrophoresis (Section 2.2.14.3).

- A. 40S subunit protein: stained gel.
- B. 40S subunit protein: autoradiograph.

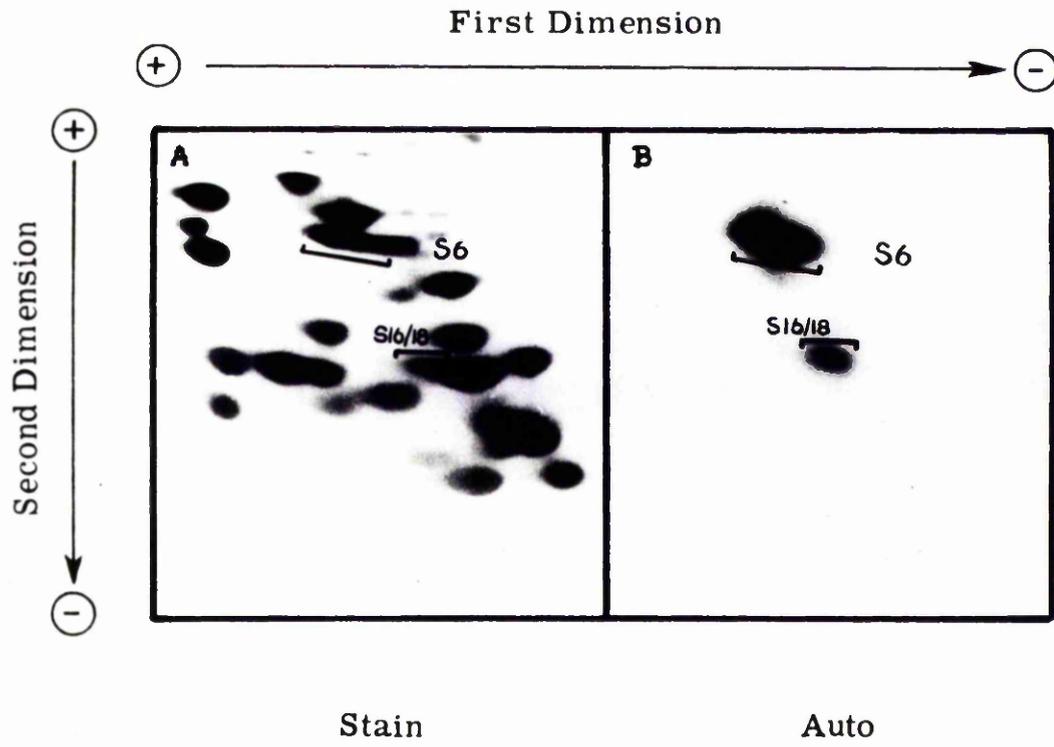
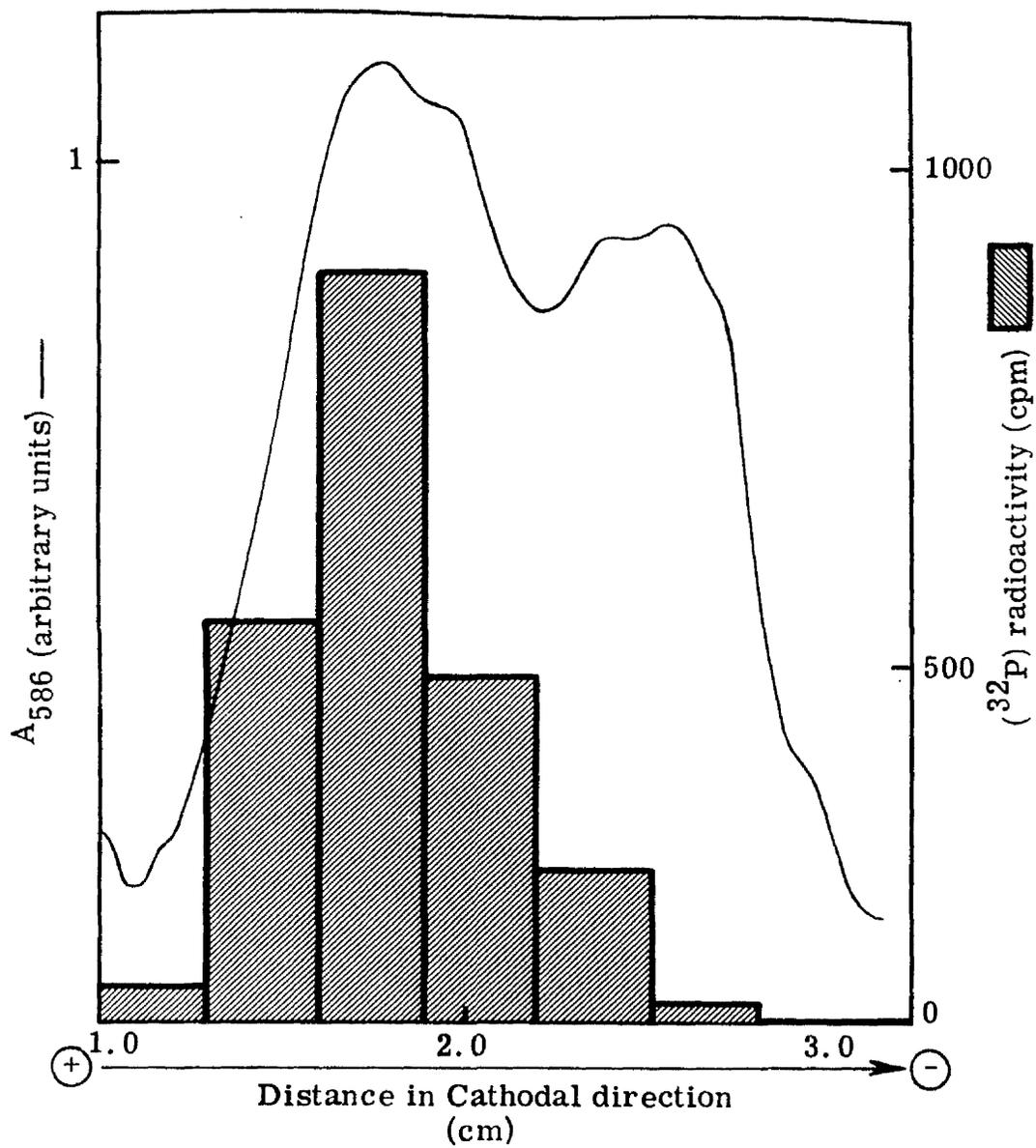


Fig. 3.3.4 Distribution of ( $^{32}\text{P}$ )-Radioactivity and Dye-stain along Ribosomal Protein S6 from Cells Infected for 9 hours with Pseudorabies Virus.

The section of two-dimensional gel containing protein S6 from Fig. 3.3.3 was cut out and the distribution of dye-stain monitored by the absorbance at 586nm (solid line). The gel fragment was sliced and each slice solubilised in 0.5ml of 90% Protosol (NEN) at 37°C for 16 hours. After solubilisation 4.5 ml of a scintillant mixture (Section 2.2.15.6) was added and the ( $^{32}\text{P}$ )-radioactivity in each slice measured by scintillation spectrometry at a efficiency of 70%.



activity resides in the peak of the most anodal derivatives of protein S6, very little being associated with the most cathodal peak.

Fig.3.3.3 also shows, more clearly than Fig. 3.3.1 and Fig. 3.3.2, a distinct appearance of stained protein to the anodal side of the position to which proteins S16 and S18 migrate in uninfected cells. This stained protein coincides with the blackened region on the autoradiograph and thus represents the phosphorylated form of protein S16/18. It is difficult to assess whether the displacement of this stained protein is due to protein S16/18 being more phosphorylated at nine hours after infection or whether it is merely a result of the application of a greater quantity of protein to this particular two-dimensional gel.

Two-dimensional gel electrophoretic analysis of 60S ribosomal proteins from cells infected for 6 hours with pseudorabies virus is shown in Fig. 3.3.5. No significant labelling of the 60S basic ribosomal proteins is evident. Although two radioactive spots are seen on the autoradiograph, these are clearly due to contamination with 40S proteins since their position coincides with that of protein S6 and protein S16/18 on the stained gel.

### 3.3.2 Attempts to Determine the Stoichiometry of Phosphorylation of Proteins S6 and S16/18.

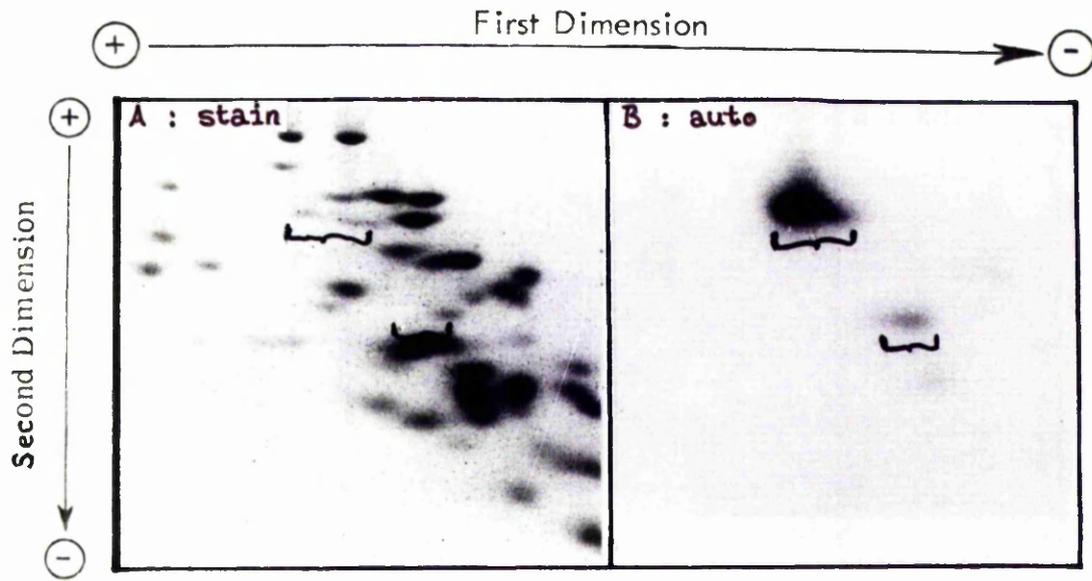
The changes in phosphorylation of protein S6 in cells infected with pseudorabies virus are clearly of considerable magnitude, and, at least at 5 and 6 hours after infection, involve most of the molecules of protein S6 in the ribosomal population. However, the predominance

Fig. 3.3.5 Two-dimensional Gel Electrophoresis of 60S Ribosomal Protein from Cells Infected for 6 hours with Pseudorabies Virus.

BHK cells were infected with pseudorabies virus for 6 hours and labelled ribosomal protein prepared as described in legend to Fig.

3.3.2. Ribosomal protein from the 60S subunit (100 $\mu$ g) was subjected to two-dimensional gel electrophoresis (Section 2.2.14.3).

- A. 60S subunit protein: stained gel.
- B. 60S subunit protein: autoradiograph.



of a single species in Fig. 3.3.1 and Fig. 3.3.2 and the incomplete resolution of the different species in Fig. 3.3.3 make it difficult to make a quantitative estimate of the phosphorylation. Comparison with published work in which all the various derivatives of protein S6 were present and resolved (Gressner & Wool, 1974(b); Treolar, Treolar & Kisilevsky, 1977; Thomas, Siegmann & Gordon, 1979) suggests that the major species of protein S6 found in the cells infected with virus (Fig. 3.3.1 and Fig. 3.3.2) contain 4 or 5 phosphoryl groups per molecule of protein S6.

To attempt a direct quantitative estimation, the amount of radioactivity incorporated into ribosomal protein S6 and protein S16/18 was determined by cutting out the proteins from the two-dimensional gel and measuring the radioactivity by scintillation spectrometry. Such analysis (Table 3.3.6) shows that there was a 7-12 fold increase in ( $^{32}\text{P}$ )-labelling of protein S6 in cells infected with virus compared with mock-infected cells. Although comparison between Experiment I and Experiment II indicates that there was a greater incorporation of radioactivity into protein S6 in mock-infected cells from Experiment II, a similar difference was seen for the 9 hour infection between the two experiments. This reflects the fact that the amount of radioactivity incorporated into ribosomes varied from one experiment to another. This can, to some extent, be ascribed to the variations in the amounts of ( $^{32}\text{P}$ )-orthophosphate and/or cells used in different experiments. It was therefore necessary to calculate the specific radioactivity of ( $^{32}\text{P}$ )-( $\gamma$ )-ATP (the presumed phosphate precursor), and use this value to calculate the number of moles of phosphate incorporated per mole of each ribosomal protein. These values had to be further adjusted to take account of the fact that all of the protein applied to the gel does not enter either the first

Table 3.3.6 The Radioactivity of Proteins S6 and S16/18 in Cells  
Infected with Pseudorabies Virus and in Mock-Infected  
Cells

The section of two-dimensional gel containing proteins S6 and S16/18 in Fig. 3.3.2 and Fig. 3.3.3 were cut out and solubilised in 0.5ml of Protosol (NEN) at 37°C for 16 hours. After solubilisation 4.5ml of a scintillant mixture (Section 2.2.15.6) was added and the ( $^{32}\text{P}$ )-radioactivity measured by scintillation spectrometry at an efficiency of 70%.

Table 3.3.6

	CONDITIONS	RIBOSOMAL PROTEINS	( <sup>32</sup> P)-cpm RECOVERED
Experiment I	Mock-Infection	S6	10 ± 3
		S16/18	0 ± 0
	9 hr. Pseudorabies Virus Infection	S6	74 ± 23
		S16/18	72 ± 6
Experiment II	Mock-Infection	S6	138 ± 16
		S16/18	4 ± 2
	6 hr. Pseudorabies Virus Infection	S6	1766 ± 43
		S16/18	216 ± 21
	9 hr. Pseudorabies Virus Infection	S6	1049 ± 31
		S16/18	208 ± 14

or the second dimension of the two-dimensional gel system. This was calculated from the relative incorporation of radioactivity into protein S6 when similar amounts were applied to two-dimensional gels and SDS gels (for which 100% recovery was assumed). The value of  $3.2 \pm 0.3$  was obtained as the mean from four separate determinations.

The results from Experiment I and Experiment II of Table 3.3.6 were treated in this manner and the adjusted values shown in Table 3.3.7. In one experiment (Experiment I), where infection was for 9 hours, the values obtained were 1.02 moles and 0.2 moles of phosphate per mole of ribosomes from infected and mock-infected cells respectively. These values are too low in absolute magnitude in comparison with the values estimated from the position of migration of the various phosphorylated derivatives of the protein. However, they may reflect the relative difference in phosphorylation of the protein between the two conditions. Higher values of 2.94 moles and 5.41 moles of phosphate per mole of protein S6 at 6 hours and 9 hours after infection respectively, were found in Experiment II. These values for protein S6 are nearer the 4-5 moles of phosphate estimated from the position of its electrophoretic migration. However the different values for 6 and 9 hours after infection are clearly inconsistent with the visual estimation since in this experiment the proportion of protein S6 in the most highly phosphorylated derivatives was approximately the same at these times after infection (data not shown).

One possible explanation for this difference in values could be a difference in the amount of protein S6 entering the two-dimensional gel. There is a tendency to obtain decreased amounts of (or even to lose) some of the larger 40S ribosomal proteins, especially proteins

Table 3.3.7 The Specific Radioactivity of Ribosomal Proteins  
(S6 and S16/18) and ( $\gamma$ )-ATP from Cells infected with  
Pseudorabies Virus and from Mock-Infected Cells.

Virus infection and cell labelling were as described in Fig. 3.3.2 and Fig. 3.3.3.

Specific Radioactivity of ( $\gamma$ )-ATP:- ATP concentration was measured in a neutralised perchloric acid extract of ( $^{32}\text{P}$ )-labelled cells by a fluorometric assay (Section 2.2.19). The ( $^{32}\text{P}$ ) radioactivity associated with ATP was measured by scintillation spectrometry after separation of nucleotides by chromatography using PEI cellulose (Section 2.2.20). The specific activity of ( $\gamma$ )-ATP was expressed as  $10^3$  cpm per nanomole ( $\gamma$ )-ATP. The calculation assumes that during a 3 hour incubation with ( $^{32}\text{P}$ )-orthophosphate the  $\alpha$ -phosphate of ATP was unlabelled and that the ( $\beta$ ) and ( $\gamma$ )-phosphates become labelled to the same extent (Kabat, 1972).

Specific Radioactivity of Ribosomal Proteins:- Preparation of ribosomes, ribosomal subunits and protein extraction was as previously described (Sections 2.2.9, 2.2.10 & 2.2.12). The ribosomal protein concentration was measured by the dye-binding assay of Bradford (1976) (Section 2.2.17) and 100ug was subjected to two-dimensional gel electrophoresis (Section 2.2.14.3). The ( $^{32}\text{P}$ )-radioactivity associated with proteins S6 and S16/18 was measured by scintillation spectrometry as described in Fig. 3.3.6. The value was corrected ( $\times 3.2$ ) to compensate for recovery of radioactivity from two-dimensional gels as compared with SDS gels, for which 100% recovery was assumed. The specific radioactivity of ribosomal protein was expressed as  $10^3$  cpm in ribosomal protein per mole of ribosomes. The calculation assumes that there is 1 mole of the particular ribosomal protein per mole of ribosomes and that the total molecular weight of the 40S subunit proteins was  $0.78 \times 10^6$  daltons.

Table 3.3.7

Experiment	CONDITIONS	(γ)-ATP specific radioactivity (x10 <sup>3</sup> cpm/nmol (γ)-ATP)	PROTEIN S6		PROTEIN S16/18	
			specific radioactivity (x10 <sup>3</sup> cpm/mol ribosomes)	moles PO <sub>4</sub> per mol ribosomes	specific radioactivity (x10 <sup>3</sup> cpm/mol ribosomes)	moles PO <sub>4</sub> per mol ribosomes
Experiment I	Mock-Infection	4.48	0.90	0.20	0.00	0.00
	9 hr. Pseudorabies Virus Infection	5.83	6.11	1.05	5.92	1.02
Experiment II	Mock-Infection	27.10	17.22	0.64	0.00	0.00
	6 hr. Pseudorabies Virus Infection	31.13	91.45	2.94	11.14	0.36
	9 hr. Pseudorabies Virus Infection	10.03	54.27	5.41	10.72	1.07

S2 and S6 (see Fig. 3.3.2 and Fig. 3.3.3). This may be a result of relatively poor solubility of these proteins. Another problem is that the values for the specific radioactivity of the ( $^{32}\text{P}$ )-( $\gamma$ )-ATP were measured at the time of harvesting the cells. However the relevant value is that at the time of phosphorylation. Although this would be expected to be within 2 hours of the measurement, a subsequent decline in the specific activity of the ( $^{32}\text{P}$ )-( $\gamma$ )-ATP, together with relatively slow turnover of phosphate might explain the difference between the values at 6 and 9 hours after infection.

The low values obtained in Experiment I (Table 3.3.7) may, in part, be due to these considerations but in addition incomplete reduction of the protein could have also contributed. It was subsequently shown (Leader & Mosson, 1980) that the anomalous migration of certain ribosomal proteins observed in some of the work of Leader and co-workers could be ascribed to oxidation of proteins, especially during the first dimensional gel electrophoresis, resulting in intra-molecular disulphide bridges. As oxidation is also known to produce inter-molecular disulphide bridges, the higher molecular weight species formed would have had greater difficulty in entering the second dimension. As a similar anomalous migration to that described by Leader & Mosson, (1980) was observed in this particular experiment (data not shown) such losses could clearly have been a factor in the low values obtained.

The variability in values obtained from measurements of the phosphate content of the proteins based on radioactive incorporation clearly limits their utility. Moreover there has been a report of compartmentalisation of the ATP precursor pool for ribosomal protein S6 from the main ATP pool (Thomas et al., 1979), further complicating

matters. It would therefore appear that the extent of phosphorylation, obtained from the relative mobility of the protein during two-dimensional gel electrophoresis, though less quantitative, is more reliable.

### 3.3.3 Relationship of the Phosphorylation of Ribosomal Proteins to other Biochemical Events occurring in BHK Cells after Infection with Pseudorabies Virus.

It was important to determine the time at which the increase in phosphorylation of the ribosomal protein occurred after infection so that comparison with other biochemical events resulting from the infection could be made.

#### 3.3.3.1 Time at which Ribosomal Proteins are Phosphorylated in Infected Cells.

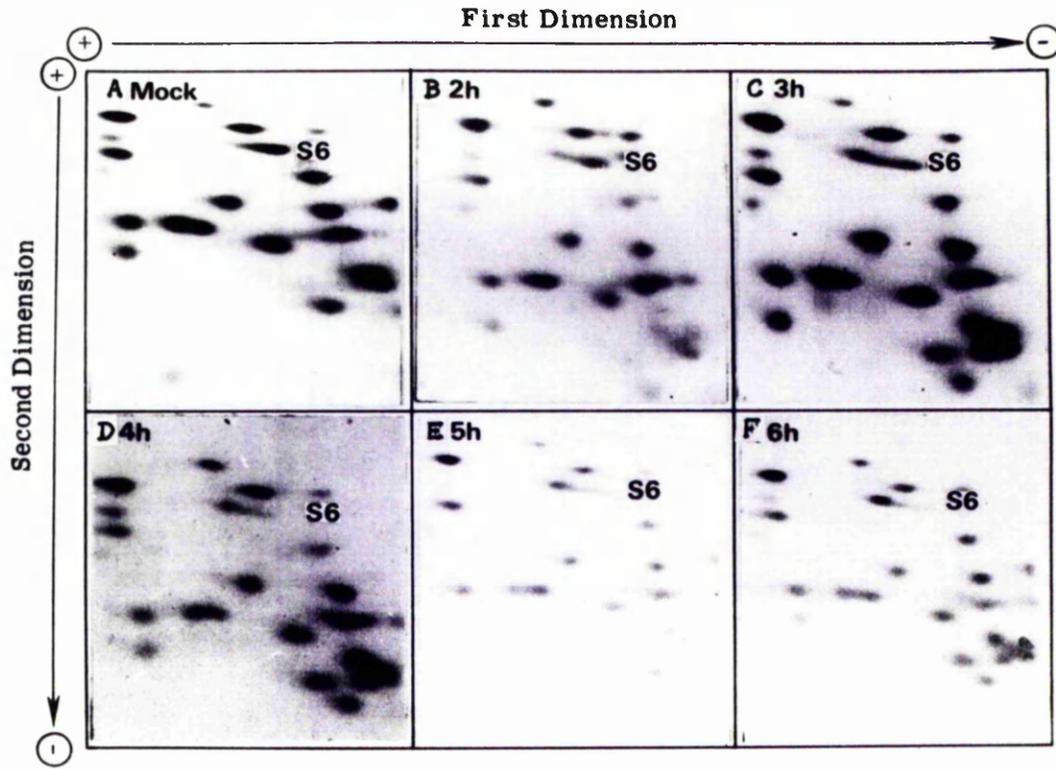
Two-dimensional gel electrophoretic analysis of proteins from the 40S ribosomal subunit of cells infected for various times with pseudorabies virus are shown in Fig. 3.3.8. The relative extent of phosphorylation of ribosomal protein S6 can be seen from its position of electrophoretic migration. At 2 hours after infection (Frame B), protein S6 is no more phosphorylated than in uninfected cells (Frame A). By 3 hours after infection (Frame C) a stage has been reached in which material is seen at both the most anodal and cathodal electrophoretic positions with a smaller amount of material in between. By 4 hours the majority of the stained protein S6 has been displaced to the position of most highly phosphorylated derivatives, and this situation is maintained until at least 6 hours after infection. In one experiment the overall extent of phosphorylation had declined by 9 hours to about the level at 3 hours (see Fig. 3.3.3) but in another experiment (not shown) no decline was seen at 9 hours after

Fig. 3.3.8 The Time of Phosphorylation of Ribosomal Protein S6  
in BHK Cells following Infection with Pseudorabies Virus.

BHK cells were grown for 3 days and infected with pseudorabies virus (20pfu/cell). After various periods of infection the cells were harvested, their ribosomes isolated (Section 2.2.9) and these separated into ribosomal subunits (Section 2.2.10). The proteins were extracted from these (Section 2.2.12) and 100µg subjected to two-dimensional gel electrophoresis (Section 2.2.14.3).

The figure shows the stained pattern of 40S subunit proteins from:-

- A. mock-infected cells
- B. cells infected for 2 hours
- C. cells infected for 3 hours
- D. cells infected for 4 hours
- E. cells infected for 5 hours
- F. cells infected for 6 hours



infection.

The time of phosphorylation of protein S16/18 is more difficult to assess. This is because the protein can be labelled with  $^{32}\text{P}$ -ortho-phosphate without there being any visible alteration in the electrophoretic position of stained protein on the two-dimensional gels (Fig. 3.3.1 and Fig. 3.3.2). Although table 3.3.7 suggests an increase in phosphorylation between 6 and 9 hours, as previously discussed for protein S6, these data would appear unreliable.

### 3.3.3.2 Release of Progeny Virus from Infected Cells

Infection was carried out as previously described (Section 2.2.3) but after the 1 hour adsorption period the cell sheet was washed carefully to remove excess virus. Periodically, throughout the infection, aliquots of medium were removed and the amount of viable virus determined by plaque assay (Section 2.2.5).

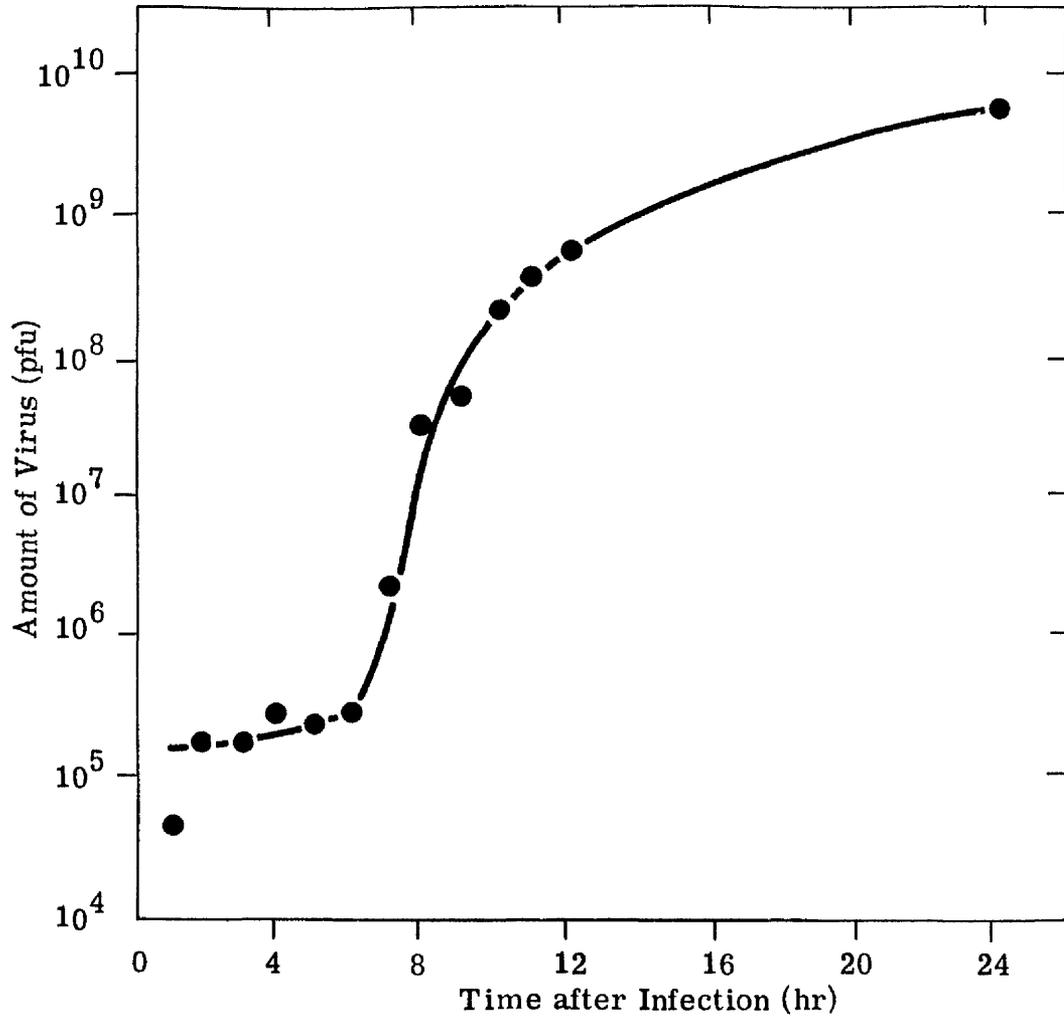
The amount of virus released from the cells at different times after infection is shown in Fig. 3.3.9. The progeny virus first appeared in the medium between 6 and 7 hours after infection. The most rapid release of virus occurred between 7 and 11 hours, although production of virus continued slowly thereafter. Clearly the phosphorylation of ribosomal proteins S6 and S16/18 precedes the first release of progeny virus.

### 3.3.3.3 Appearance of New Proteins

The early stages of expression of the viral genome are indicated by the appearance of proteins not detected in uninfected cells. To examine this, cells were infected with pseudorabies virus and the infection allowed to continue for various lengths of time. Fifteen mins. before harvesting the cells were incubated with  $^{35}\text{S}$ -methionine.

Fig. 3.3.9 Infection of BHK Cells with Pseudorabies Virus: Time-  
Course of Release of Virus into the Medium.

BHK cells were grown for 3 days and infected with pseudorabies virus (20pfu/cell), as described in section 2.2.3. After the 1 hour adsorption period the medium was removed and the cell sheet washed 3 times with 50ml of Eagle's medium to remove excess virus. Fresh Eagle's medium (50ml) was added and an aliquot (0.5ml) removed periodically throughout the infection. The amount of viable virus in each aliquot was determined by plaque assay (Section 2.2.5).



to label proteins being synthesised at that specific time during the infection. Cytoplasmic proteins were then isolated, reduced with 2-mercaptoethanol and analysed by one-dimensional SDS polyacrylamide gel electrophoresis.

Fluorographic analysis of the labelled proteins (Fig. 3.3.10) showed that new (presumably viral) proteins were first detected at about 6 hours after infection. They continued to be synthesised until at least 12 hours after infection.

The new proteins have been given the designation 'V' in Fig. 3.3.10 and include several strongly labelled bands (V-3,4,7,9,10) as well as others labelled to a lesser extent (V-1,2,5,6,8,11). The apparent molecular weights of these proteins are shown in Table 3.3.11 and compared with values for pseudorabies virion proteins obtained by Stevely (1975).

A decline in the synthesis of most proteins in uninfected cells (designated 'H' in Fig. 3.3.10) could first clearly be seen at approximately 7 hours after infection, and by 9 to 12 hours after infection the synthesis of most host proteins had virtually ceased. This is evident in several bands (designated H1-7 and H9-11). However it may be seen that at least one host protein (H8) continues to be synthesised at an unchanged rate during virus infection, as others have previously observed (Marsden, Crombie & Subak-Sharpe, 1976). From the size (approximately 46,000) and predominance of this protein it is likely to be actin (Bray & Thomas, 1975).

#### 3.3.3.4 The Concentration of Cyclic-AMP in Infected Cells.

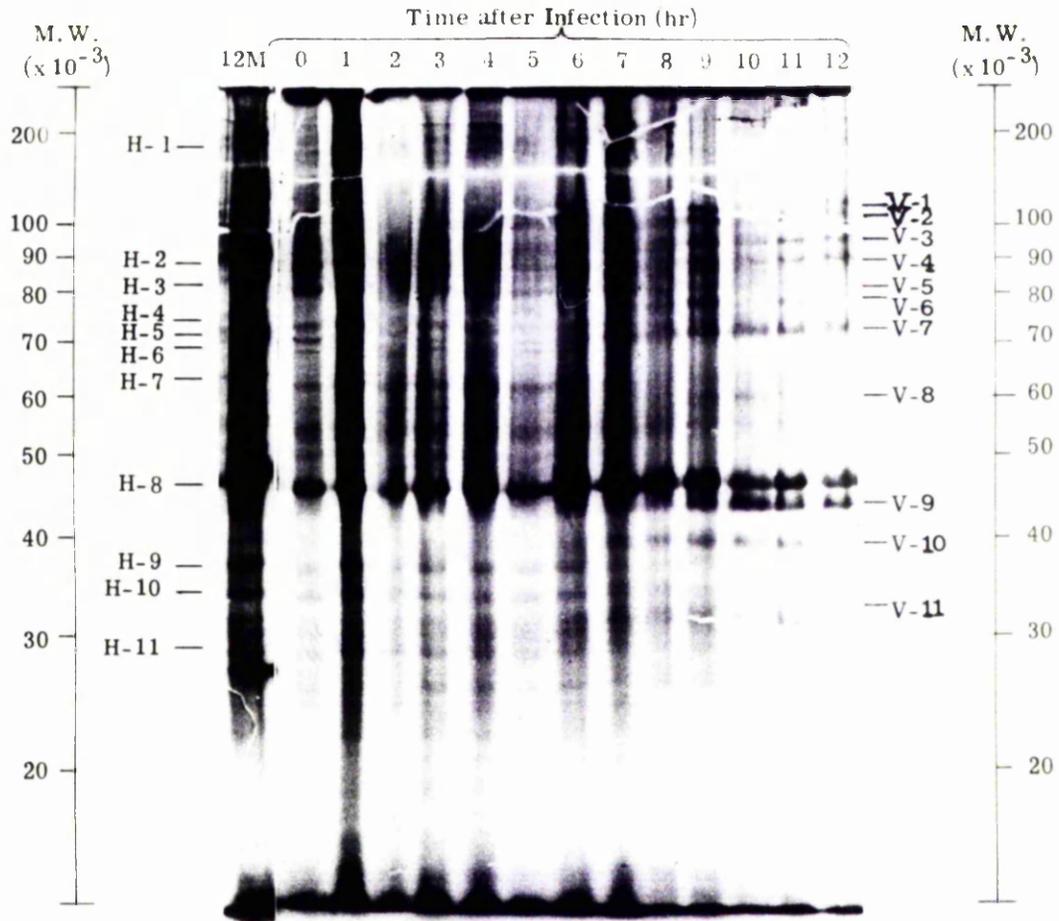
The ability of cyclic-AMP to increase the extent of phosphorylation

Fig. 3.3.10 Proteins Synthesised in BHK Cells after Infection with Pseudorabies Virus.

BHK cells were grown for 3 days until confluence and infected with pseudorabies virus (20pfu/cell) for various lengths of time. Fifteen minutes before harvesting, the cells were incubated with 20 $\mu$ Ci ( $^{35}$ S)-methionine, at a concentration of 4mCi/ml, in Eagle's medium containing no non-radioactive methionine. The post-nuclear supernatant was prepared as described in Section 2.2.9 except that the final volume was reduced to 0.5ml. An equal volume of a solution containing 1% (w/v) SDS and 5% (v/v) 2-mercaptoethanol was added and the protein completely reduced and denatured by heating at 100°C for 5 min. An aliquot of each sample containing 100,000cpm of ( $^{35}$ S)-radioactivity was then subjected to one-dimensional SDS gel electrophoresis (Section 2.2.14.2) using a slab gel containing 10% (w/v) acrylamide.

A ( $^{14}$ C)-methylated protein mixture (Amersham International Ltd.) was used as molecular weight markers: these were myosin (mol. wt. 200,000), phosphorylase-b (mol. wt. 92,500), bovine serum albumin (mol. wt. 69,000), ovalbumin (mol. wt. 46,000), carbonic anhydrase (mol. wt. 30,000) and lysozyme (mol. wt. 14,300). The molecular weight scale was calculated from a standard curve such as that shown in Fig. 2.2.2(B).

Proteins synthesised in uninfected and mock-infected cells were designated H(1-11), whereas proteins synthesised only after virus infection were designated V(1-11). The slot marked 12M is of a sample from cells which had undergone mock-infection for 12 hours.



Proteins Synthesised in BHK Cells after Infection  
with Pseudorabies Virus

Table 3.3.11 Comparison of the Molecular Weights of the 'Virus-Induced' Proteins with Pseudorabies Virus Virion Proteins Obtained by Stevely (1975)

The molecular weights of the 'virus-induced' proteins (designated V-1 to V-11 in Fig. 3.3.10) were calculated from Fig. 2.2.8(B) and are compared with the molecular weights of the pseudorabies virus virion proteins calculated by Stevely (1975).

Brackets cover proteins for which correspondence is not clear.

Table 3.3.11

Molecular Weight ( $\times 10^{-3}$ )	
'virus-induced' proteins (this work)	virion proteins (Stevely, 1975)
-	230
V-1 135 } V-2 130 } V-3 115 }	{ 150 { 140 { 120 { 115
-	110
V-4 92	92
V-5 84	85
V-6 80	82
V-7 69	72
V-8 62	63
-	56
V-9 44	45
V-10 40	41
-	36.5
V-11 32	32
-	29
-	27
-	22.5
-	20.5

of ribosomal protein is well documented (see Introduction), and there has been a report of elevated concentrations of cyclic-AMP in cells following infection with herpes simplex virus (Bittlingmaier, Schneider & Falke, 1977). It was therefore important to measure the concentration of cyclic-AMP in cells infected with pseudorabies virus to determine whether an increase in the concentration of cyclic-AMP could be responsible for the increased phosphorylation of protein S6.

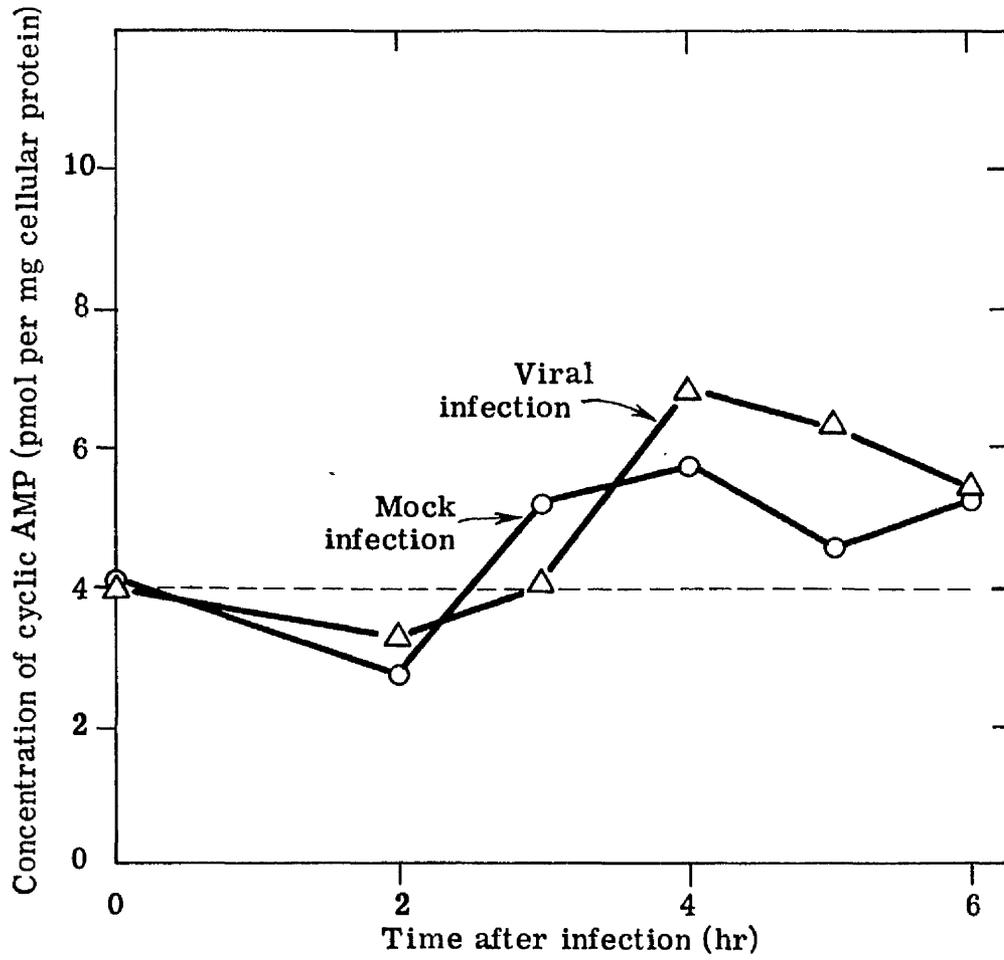
Neutralised perchloric acid extracts were prepared from cells (Section 2.2.18), which had been infected by virus or had been mock-infected for various lengths of time. The concentration of cyclic-AMP was then measured in these extracts by the competitive binding assay described by Gilman (Section 2.2.21). Results of this analysis (Fig. 3.3.12) show that there was no clear difference between the concentration of cyclic-AMP in cells infected with pseudorabies virus or mock-infected, for up to 6 hours after infection. However the concentration of cyclic-AMP in both infected and mock-infected cells did rise above the initial concentration after 3 hours of infection (or mock-infection), reaching a maximum at 4 hours and slowly declining to about the original concentration by 6 hours after infection. This increase was reproducible, being found in a second experiment, although in this case the maximum concentration of cyclic-AMP was obtained at 2 hours, and not 4 hours after infection.

#### 3.3.3.5 The Concentration of ATP in Infected Cells.

It has been shown recently (Larsen & Sypherd, 1980) that an increase in the phosphorylation of ribosomal protein S6 was accompanied by an increase in the total cellular concentration of ATP. Here studies were with dimorphic fungus Mucor racemosus, under culture conditions

Fig. 3.3.12 The Concentration of Cyclic-AMP in Cells Infected with Pseudorabies Virus.

BHK cells were grown for 3 days and infected with pseudorabies virus (20pfu/cell). Cells were taken at various times after infection and extracted with perchloric acid (Section 2.2.18). This extract was neutralised and aliquots (50 $\mu$ l) used to determine the concentration of cyclic-AMP by the competitive binding assay described by Gilman (1970) (Section 2.2.21). Two cultures were used for each time point and the concentration of cyclic-AMP in the perchloric acid extract from each culture was measured in duplicate. The protein content of the precipitate obtained after the perchloric acid extraction was determined as described in Section 2.2.22. The concentration of cyclic-AMP was then expressed as pmol/mg cellular protein.



which led to accelerated growth or after changes in the gaseous environment which led to cellular morphogenesis. This raised the possibility that the extent of phosphorylation was related to the concentration of ATP, the presumed phosphate donor for the phosphorylation of protein S6, and that this might be the case in other systems.

Neutralised perchloric acid extracts were prepared from cells (Section 2.2.18), which had been infected with pseudorabies virus (or mock-infected) for various lengths of time. The concentration of ATP was then measured by coupled enzymatic reactions, ultimately involving the quantitative reduction of NADP to NADPH (Section 2.2.19). The results of this analysis (Fig. 3.3.13) showed that the concentrations of ATP in both cells infected with pseudorabies virus or mock-infected cells were not significantly altered for up to 6 hours after infection.

#### 3.3.4 The Phosphorylation of Ribosomal Proteins in BHK Cells Infected with Herpes Simplex Virus Type I.

It was of interest to know whether the increase in phosphorylation of proteins S6 and S16/18 was restricted to cells infected with pseudorabies virus, or whether it extended to cells infected with other herpes viruses. Thus experiments were performed to measure the extent of phosphorylation of ribosomal proteins in cells infected with herpes simplex virus type I.

Electrophoretic analysis of <sup>32</sup>P-labelled ribosomal protein from the 40S subunit of cells infected for 13.5 hours with herpes simplex virus are shown in Fig. 3.3.14. It is evident from this figure that in cells infected with herpes simplex virus the majority of protein S6 has

Fig. 3.3.13 The Concentration of ATP in Cells Infected with Pseudorabies Virus.

BHK cells were grown for 3 days and infected with pseudorabies virus (20pfu/cell). Cells were taken at various times after infection and extracted with perchloric acid (Section 2.2.18). An aliquot (100-200 $\mu$ l) of the neutralised extract was taken and the ATP concentration measured by a coupled enzymatic reaction which ultimately results in the quantitative reduction of NADP to NADPH (Section 2.2.19). Two cultures were used for each time point and the concentration of ATP in the perchloric acid extract from each culture was measured in duplicate. The protein content of the precipitate obtained after the perchloric acid extraction was determined as described in Section 2.2.22. The concentration of ATP was then expressed as nmol/mg cellular protein.

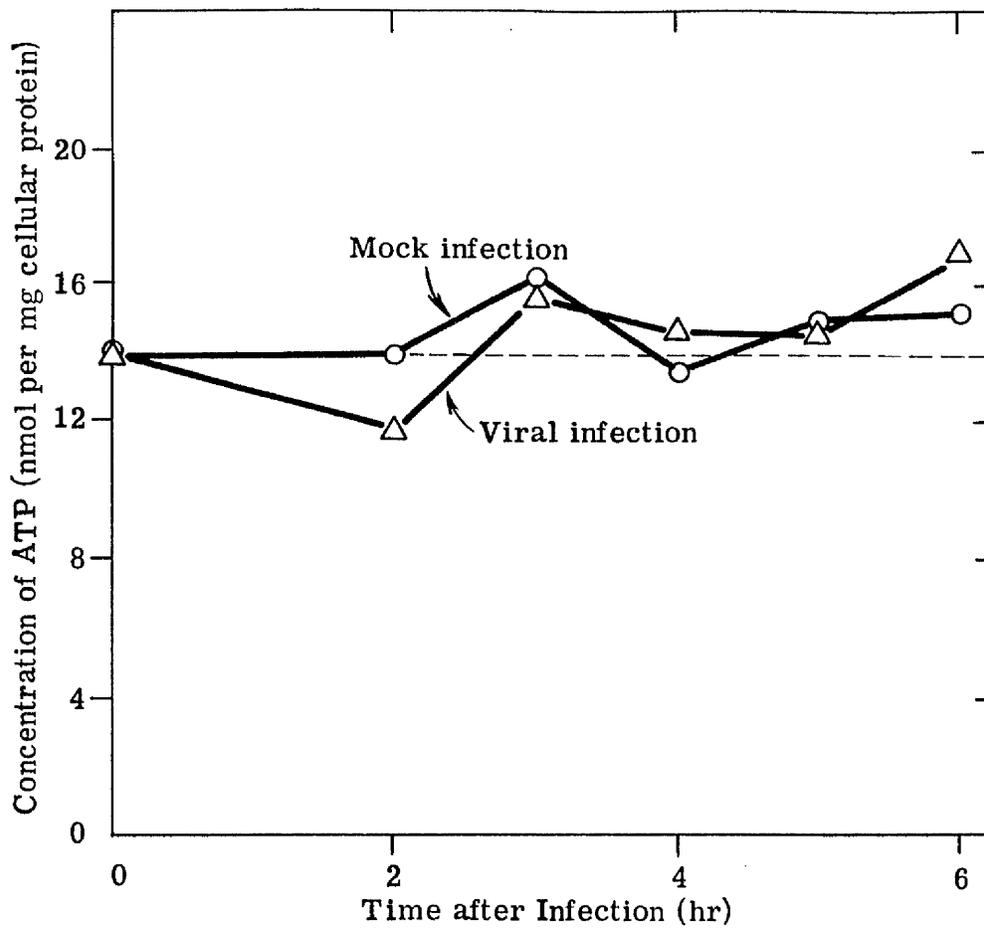
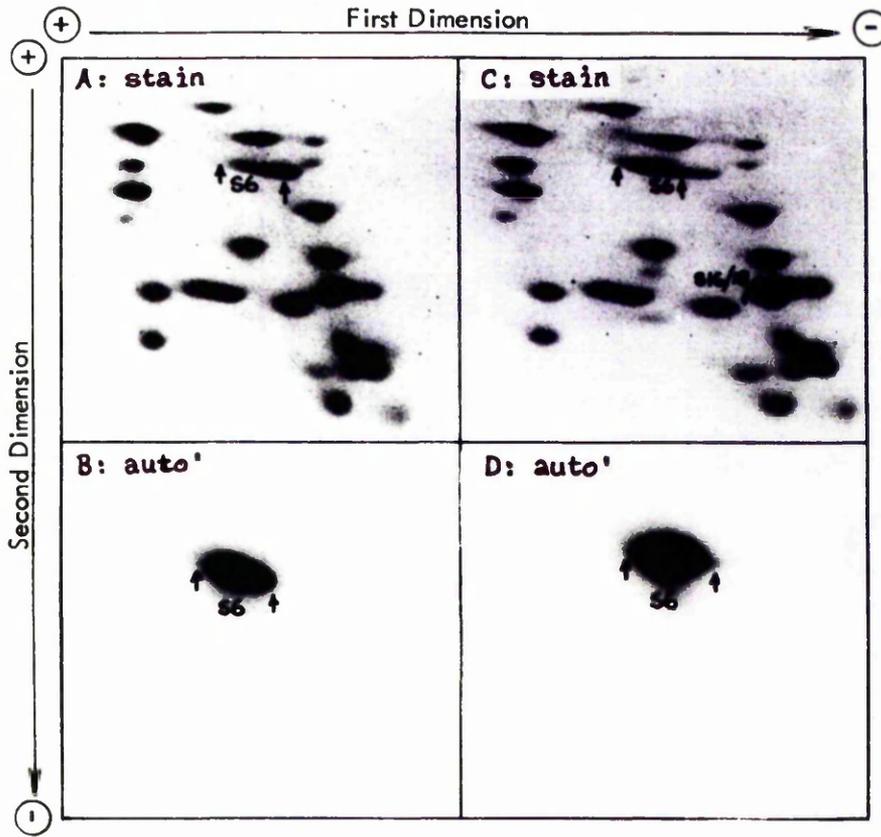


Fig. 3.3.14 The Phosphorylation of 40S Ribosomal Proteins in Cells  
Infected for 13.5 hours with Herpes Simplex Virus  
Type I and in Mock-Infected Cells.

BHK cells were grown for 3 days and infected with herpes simplex virus type I (10pfu/cell) or mock-infected with a similar volume of Eagle's medium. After 10.5 hours of infection the cells were incubated for a further 3 hours with 5 mCi of ( $^{32}\text{P}$ )-orthophosphate (50 $\mu\text{Ci/ml}$ ) in Eagle's medium containing 0.09mM phosphate (one-tenth the normal phosphate concentration) and from which tryptose phosphate broth had been omitted. The cells were then harvested and their ribosomes and ribosomal subunits isolated (Section 2.2.9 and Section 2.2.10). Protein was extracted from these (Section 2.2.12) and 100 $\mu\text{g}$  was subjected to two-dimensional electrophoresis (Section 2.2.14.3)

- A. 40S protein from mock-infected cells: stained gel.
- B. 40S protein from mock-infected cells: autoradiograph.
- C. 40S protein from cells infected for 13.5 hours with herpes simplex virus type I: stained gel.
- D. 40S protein from cells infected for 13.5 hours with herpes simplex virus type I: autoradiograph.



Mock-infected

HSV-1 Infected

again been displaced to the position of its most highly phosphorylated derivatives. However, it is clear that, in contrast to cells infected with pseudorabies virus, ribosomal protein S16/18 did not become labelled with  $^{32}\text{P}$ -orthophosphate after infection with herpes simplex virus, at least at this time.

Results of more quantitative analysis of the distribution of  $^{32}\text{P}$ -radioactivity and dye-stain in electrophoretically separated protein S6 substantiating this conclusion, are shown in Fig. 3.3.15.

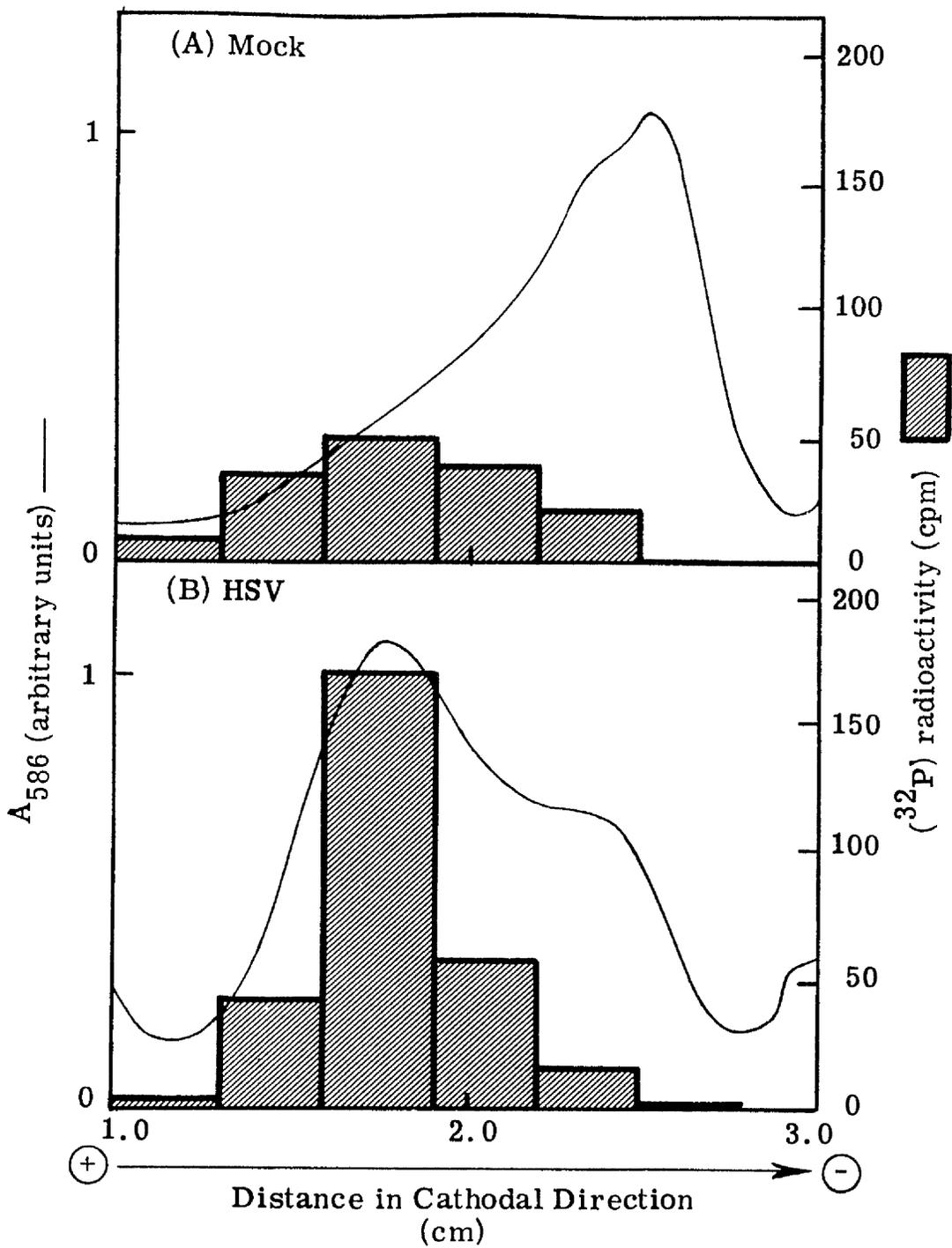
Quantitative measurement of the phosphate content of protein S6 (cf. Section 3.3.2) gave values of 0.64 and 4.0 moles of phosphate per mole of protein S6 from mock-infected cells and cells infected for 13.5 hours with herpes simplex virus respectively. These values are consistent with the position of the dye-stain in protein S6 in two-dimensional gels. They are also similar to the values calculated in Experiment II of Table 3.3.7 for cells infected with pseudorabies virus. However in view of the lack of reproducibility previously described for this type of measurement (Section 3.3.2) this may be purely fortuitous.

Fig. 3.3.15 Distribution of ( $^{32}\text{P}$ )-Radioactivity and Dye-stain in Electrophoretically Separated Ribosomal Protein S6 from Cells Infected for 13.5 hours with Herpes Simplex Virus Type I, and from Mock-Infected Cells.

Virus infection, cell labelling, fractionation and gel electrophoresis were as described in Fig. 3.3.14.

The section of two-dimensional gel containing protein S6 was cut out and the distribution of dye-stain monitored by absorbance at 586nm (solid line). The gel fragment was sliced and each slice solubilised in 0.5ml of 90% Protosol (NEN) at 37°C for 16 hours. After solubilisation 4.5ml of a scintillant mixture (Section 2.2.15.6) was added and the ( $^{32}\text{P}$ )-radioactivity in each slice measured by scintillation spectrometry at an efficiency of 70%.

- A. Protein S6 from mock-infected cells.
- B. Protein S6 from cells infected for 13.5 hours with herpes simplex virus type I.



#### 4. DISCUSSION

#### 4.1 THE PHOSPHORYLATION OF RIBOSOMAL PROTEIN S6 IN RELATION TO THE ASSEMBLY OF RIBOSOMES AND THEIR TRANSPORT FROM THE NUCLEUS.

One part of the work described in this thesis was performed to test a particular hypothesis. This was that the difference in phosphorylation of ribosomal protein S6 observed in rapidly-growing and slowly-growing cells was a result of different proportions of newly-synthesised ribosomes in the total cytoplasmic population under the different growth conditions. This proposed greater phosphorylation of ribosomal protein S6 in newly-synthesised ribosomes was envisaged in terms of a function for this phosphorylation in the assembly of the ribosomes in the nucleolus or their transport from the nucleus to the cytoplasm.

Of the various approaches adopted to investigate this question, only those relating to the newly-synthesised ribosomes found in the cytoplasm gave satisfactory results. Thus, by differential labelling with radioactive amino acids to identify the proteins of newly-synthesised and two-day old ribosomes, it was found that in both cases ribosomal protein S6 had electrophoretic properties characteristic of the unphosphorylated form of the protein (Fig. 3.2.6 - Fig. 3.3.8). This clearly shows that newly-synthesised ribosomes do not have ribosomal protein S6 in a highly phosphorylated state.

Work with native ribosomal subunits, which include newly-synthesised ribosomes that have recently entered the cytoplasm from the nucleus, also suggested that protein S6 was not highly phosphorylated in these ribosomal species (Fig. 3.2.5). This result is consistent with the work of Bitte and Kabat, (1972), in which protein was analysed only by one-dimensional gel electrophoresis. A ( $^{32}\text{P}$ )-labelled band II (probably corresponding to protein S6) was found to contain no more

radioactivity in native ribosomal subunits than in subunits derived from polysomes by dissociation at high ionic strength. Stronger corroborative evidence can be found by inspection of the work of Sundkvist and Howard (1974) in which the proteins from native 40S subunits (somewhat confusingly designated 'washed-free 40S subunits') were analysed by two-dimensional gel electrophoresis. There protein S6 was clearly found in the position of migration of the unphosphorylated form of the protein.

These results demonstrate that the difference in phosphorylation of protein S6 in ribosomes from rapidly-growing and slowly-growing cells is not due to there being more new ribosomes in rapidly-growing cells, as the original hypothesis proposed. Nevertheless these results do not exclude the possibility that protein S6 is highly phosphorylated in the nucleolus but is rapidly, rather than slowly, dephosphorylated upon entering the cytoplasm. However, there is no evidence to suggest this, and certain work that has appeared more recently (Auger-Buendia & Tavitian, 1979; Auger-Buendia, Longuet & Tavitian, 1979; Lastick, 1980) would seem to argue against it. Auger-Buendia and co-workers showed that proteins from nucleolar 80S pre-ribosomal particles, labelled with ( $^{35}\text{S}$ )-methionine, contained protein S6 which migrated to the position of the unphosphorylated form of the protein in two-dimensional gel electrophoresis. The work of Lastick (1980) also showed that ( $^{35}\text{S}$ )-labelled 80S pre-ribosomal particles contain protein S6 in a low state of phosphorylation as judged by its electrophoretic migration. Olson et al. (1974) attempted to study the phosphorylation of nucleolar pre-ribosomal proteins more directly and found that certain proteins were phosphorylated when nucleoli were incubated in vitro with ( $^{32}\text{P}$ )-( $\gamma$ )-ATP. However the system of electrophoretic analysis used in that work does not allow any

correlation of the resolved spots with ribosomal proteins by the other systems of electrophoresis on which the standard nomenclature is based. Indeed, the first dimension of that system is so acidic that it cannot even be ascertained whether the phosphorylated proteins were, in fact, ribosomal. However, the work of Olson et al., (1974) does bear, in one respect, upon the question under consideration as the phosphoproteins labelled in the nucleoli were not the same as those phosphorylated in cytoplasmic ribosomes labelled in vivo. This implies that the proteins phosphorylated in pre-ribosomal particles do not include protein S6, and suggests that protein S6 is not phosphorylated in the nucleolus.

The reason why no direct analysis of the phosphorylation of protein S6 in nucleolar pre-ribosomal particles was attempted in the study described in this thesis was the report of Shepherd and Maden (1972) that a number of ribosomal proteins, including S6 are not present on these particles. Presumably this was due to loss of the proteins under the conditions of their isolation, and this loss did not occur under the conditions used later by Auger-Buendia and Tavitian, 1979; Auger-Buendia, Longuet and Tavitian, 1979 and Lastick, 1980.

It is a little surprising that protein S6 appears not to be phosphorylated in the nucleolus given that histone kinase is clearly present (Fig. 3.2.3; Kang, Olson & Busch, 1974), and given that in assays in vitro histone kinases usually phosphorylate protein S6 (Traugh & Porter, 1976; Centiempo et al., 1981). It is also surprising in view of results from Grummt, (1974) which showed that the addition of ribosomal protein stimulated the incorporation of ( $^{32}\text{P}$ ) into nucleoli by 130% during incubation in vitro with ( $^{32}\text{P}$ )-( $\gamma$ )-ATP, although in

that study the phosphoproteins were not characterised. One possibility is that there is a high level of a specific protein S6 phosphatase in the nucleolar preparation, although there are no data to indicate whether this is in fact the case.

#### 4.2 PHOSPHORYLATION OF RIBOSOMAL PROTEINS IN CELLS INFECTED BY VIRUSES.

The latter part of the work in this thesis addressed itself to a different question, namely the phosphorylation of ribosomal protein S6 in cells infected by viruses. Results clearly demonstrate that in cells infected with pseudorabies virus there is a dramatic increase in the extent of phosphorylation of ribosomal protein S6, as well as the phosphorylation of ribosomal protein S16/18, a protein not found phosphorylated in uninfected or mock-infected cells (Fig. 3.3.1 — Fig. 3.3.3).

Other workers have found increases in the incorporation of ( $^{32}\text{P}$ )-orthophosphate into ribosomal protein S6 following infection with a variety of viruses, including vaccinia virus (Kaerlein & Horak, 1976; Kaerlein & Horak, 1978), adeno virus (Blair & Horak, 1977) and mengo virus (Rosnitschek, Traub & Traub, 1978). However, in none of these studies was there a clear change in the position of electrophoretic migration of the protein indicative of an increase in the absolute extent of its phosphorylation. In contrast, the work presented in this thesis clearly shows a marked alteration in the position of the majority of the stained ribosomal protein S6 to that of the highly phosphorylated anodal derivatives of the protein.

The fact that the increased ( $^{32}\text{P}$ )-labelling of ribosomal protein S6 found after infection of cells with other viruses (such as vaccinia) was not associated with a high extent of absolute phosphorylation of the ribosomal protein may be because of an alteration in the specific radioactivity of the ATP precursor pool, or in the rate of turnover of the phosphoryl groups on ribosomal protein S6. However, it must also be considered whether the low absolute phosphorylation of the isolated

ribosomes, in these circumstances, is an artifact due to phosphatase action during the isolation of the ribosomes, and it may be merely fortuitous that such a dephosphorylation did not occur during isolation of ribosomes from BHK cells in this work. Thus, for example, a five-fold difference in labelling of isolated ribosomes after infection of HeLa cells with vaccinia virus (Kaerlein & Horak, 1978) could represent a difference between say, 0.1 and 0.5 moles of phosphate per ribosome which would not have been detectable as a change in the electrophoretic migration of ribosomal protein S6. Such a difference in the isolated ribosomes might actually have reflected a difference between say, 1 and 5 moles of phosphate per ribosome in the intact cells. This alternative deserves consideration because in HeLa cells infected by vaccinia virus there are clearly absolute increases in the extent of phosphorylation of other ribosomal proteins (S2 and S16). Against such a general model of phosphorylation of protein S6 in cells infected by viruses, is the report of Marvaldi and Lucas-Lenard, (1977) that there was no increase in the incorporation of ( $^{32}\text{P}$ )-orthophosphate into ribosomal protein S6 following infection of L-cells with vesicular stomatitis virus. Whether this would be a genuine exception to the proposed generalisation or whether the design of the experiments resulted in failure to detect any changes, remains to be seen.

It has also been shown in this thesis that infection with herpes simplex virus type 1 resulted in a dramatic increase in the extent of phosphorylation of ribosomal protein S6 although ribosomal protein S16/18 did not become labelled (Fig. 3.3.14 and Fig. 3.3.15). During the course of this work Fenwick and Walker, (1979) reported related studies with herpes simplex virus types 1 and 2. They found that there was a slight increase in the incorporation of ( $^{32}\text{P}$ )-orthophosphate into a small

ribosomal subunit protein of molecular weight 36,000, when analysis was by one-dimensional gel electrophoresis. This protein is likely to be ribosomal protein S6 (molecular weight 31,000) since it was also labelled, to a lesser extent, in mock-infected cells. The work of Fenwick and Walker (1979) is also consistent with the results in this thesis, in that no protein of the molecular weight of protein S16/18 became labelled during infection with herpes simplex virus, although infection was for 5 hours, rather than the 13.5 hours in this work. These authors in fact concentrated their attention on a phosphoprotein of molecular weight 48,000 found only in the infected cells, and they concluded that this was a protein of the small ribosomal subunit. However, this is unlikely to be the case in view of the fact that the largest protein of the small ribosomal subunit has a molecular weight of 39,000 (Wool, 1980). This would explain why the phosphoprotein of molecular weight 48,000 was not seen on the two-dimensional gels used for the separation of ribosomal proteins in the present study (Fig. 3.3.14). Instead this protein is likely to be a non-ribosomal protein, that remains closely associated with the ribosomes and is possibly an initiation factor (cf. Floyd, Merrick & Traugh, 1979; Traugh, 1981).

The differences in phosphorylation pattern observed with different viruses raise the possibility that these phosphorylations are catalysed by protein kinases not present in uninfected cells. Such kinases might arise in a variety of different ways; they could be brought into the cell in the virion, translated from viral mRNA during infection, or even translated from new host mRNA, the synthesis of which is provoked by the virus.

The virions of a large number of viruses have been reported to contain protein kinases (Strand & August, 1971; Hatanaka, Twiddy & Gilden, 1972; Tan & Sokol, 1974). Most relevant to the present question is that they have also been reported in vaccinia virus (Poaletti & Moss, 1972; Kleiman & Moss, 1975) and herpes simplex virus (Rubenstein, Gravell & Darlington, 1972; Lemester & Roizman, 1980). However, the observation that several hours elapse after the initial infection before the increase in phosphorylation of ribosomal protein S6 (at least in cells infected with pseudorabies virus, Fig. 3.3.8) tends to argue against such a virion kinase being responsible for this phosphorylation. In general, it is unclear whether such virion kinases have any role in the infectious cycle and they may merely be host enzymes trapped during encapsulation. In the case of herpes simplex virus however, a protein kinase has been found tightly associated with the virion and is only accessible to exogenous substrates in vitro after the virion has been disrupted with detergent (Lemester & Roizman, 1980). Whether or not this protein kinase becomes accessible to host proteins during the infectious cycle is not yet clear.

There is definite evidence for protein kinases being coded by the viral genome in the case of Retroviruses (Collett & Erickson, 1978; Erickson et al., 1979; Sefton, Hunter & Raschke, 1981) and Papovaviruses (Griffin, Sprangler & Livingston, 1979; Smith et al., 1979; Tijan & Robbins, 1979). However, these kinases are known to be responsible for the transformed state that can be produced by these tumour viruses (Pawson et al., 1980; Lau, Krzyzek & Faras, 1981; Sefton et al., 1980) and, indeed the protein kinases are extraordinary in that they phosphorylate tyrosine residues (Eckhart, Hutchinson & Hunter, 1979; Levinson et al., 1980; Reynolds, Van de Ven & Stephenson, 1980; Sefton, Hunter & Raschke, 1981) and not serine residues.

Admittedly it has been shown here that cells transformed by Papova-viruses contain protein S6 in a more highly phosphorylated state than in untransformed cells (Section 3.1) and in another report (Decker, 1981), published at the same time as some of this work (Kennedy & Leader, 1981), similar results were obtained in cells transformed with Retroviruses. However, as had previously been found with phosphorylation of protein S6 in other circumstances (Gressner & Wool, 1974(a); Rankine, Leader & Coia, 1977; Hebert, Pierre & Loeb, 1977) this phosphorylation is of serine residues (Decker, 1981) and would not therefore seem to be catalysed by the phospho-tyrosine kinase. This conclusion must, however, be qualified in the light of a recent report which indicates that the kinase from murine sarcoma virus, although autophosphorylating at tyrosine residues, appears to phosphorylate cellular proteins at serine residues. Until this has been confirmed with the Rous Sarcoma (src) kinase the stimulus for the phosphorylation of protein S6 in these cells will remain unclear.

Although new protein kinase activities have been reported in cells productively infected with adeno virus (Lassam et al., 1979) and herpes simplex virus (Blue & Stobbs, 1981), it cannot yet be decided whether these are coded by the viral or host genomes, nor is it yet known whether these protein kinases can phosphorylate ribosomal proteins and, if so, with what specificity.

#### 4.3 THE FUNCTION OF THE PHOSPHORYLATION OF RIBOSOMAL PROTEIN S6.

The greater phosphorylation of ribosomal protein S6 found previously in pre-confluent cells (Leader, Rankine & Coia, 1976) has been shown in this work not to be due to the lower cell density compared to that of the less phosphorylated confluent cells (Section 3.1). It thus appears likely that the more rapid rate of growth is the key factor here. This would be consistent with observations in certain other experimental systems. Thus, Lastick, Nielsen and M<sup>C</sup>Conkey (1977) observed increased phosphorylation of protein S6 when suspension HeLa cells were stimulated to grow by the addition of fresh serum. A similar increase in the phosphorylation of protein S6 was observed when stationary chick embryo fibroblasts or mouse 3T3 cells were stimulated to grow by the addition of insulin, insulin-like growth factor or serum (Haselbacher, Humbel & Thomas, 1979; Thomas, Siegmann & Gordon, 1979; Thomas et al., 1980); when PC12 nerve-like cells were stimulated with growth factors (Halegoua & Patrick, 1980) or following fertilisation of sea urchin eggs (Ballinger & Hunt, 1981). A similar correlation between the phosphorylation of protein S6 and growth rate may exist in regenerating rat liver (Gressner & Wool, 1974(a)) although here other factors (eg. increases in cyclic AMP) may be involved. As the hypothesis that the phosphorylation functions during the assembly or transport of ribosomes from the nucleus has been found to be untenable (Section 4.1), it is necessary to consider what other explanation might be consistent with the high phosphorylation during cellular growth.

The most obvious possibility is that the increased phosphorylation of protein S6 in rapidly growing cells is related to the high rates of protein synthesis in such cells. The reason that this idea had seemed

unable to account for the results in BHK cells (Leader, Rankine & Coia, 1976) was the fact that no change in phosphorylation of protein S6 was observed on 'shift-up' and 'shift-down' of protein synthesis.

Although there is in fact no reason necessarily to expect decreased phosphorylation of protein S6 when protein synthesis is inhibited, one would have expected increased phosphorylation when confluent cells were stimulated to grow again. It is possible that there was in fact an increase in the phosphorylation of protein S6 in the stimulated confluent BHK cells, but that this was obscured by dephosphorylation of the ribosomes during their isolation. Such dephosphorylation might have arisen from an elevated phosphoprotein phosphatase activity associated with the high cell density, and it may be significant that much lower cell densities were employed in the experiments of others, described above.

However the suggestion of a role for the phosphorylation of protein S6 in protein synthesis is still confronted by the other problems that originally led to the consideration of alternative hypotheses. These are that some stimuli that increase the phosphorylation of protein S6 either have no effect on protein synthesis (eg. cyclic AMP, Cawthon *et al.*, 1974; Gressner & Wool, 1976; Lastick & M<sup>C</sup>Conkey, 1980; Floyd & Traugh, 1981) or in fact inhibit protein synthesis (eg. puromycin, cycloheximide and NaF, Gressner & Wool, 1974 (b) ; Cawthon *et al.*, 1974; Lastick & M<sup>C</sup>Conkey, 1980; Floyd & Traugh, 1981). Moreover in rat liver, where the rate of protein synthesis is high, there is little phosphorylation of protein S6 (Gressner & Wool, 1974(a)). Furthermore protein synthetic experiments in vitro do not show any differences between ribosomes with high or low levels of phosphorylation of protein S6 (Eil & Wool, 1973). Despite this, more and more circumstantial data has accumulated which show that, in tissue culture cells, a

high degree of phosphorylation of protein S6 is associated with a higher rate of protein synthesis. Especially relevant are the results of Thomas et al. (1980) which demonstrated that although cycloheximide, which blocked protein synthesis, had no effect on the phosphorylation of protein S6 stimulated by serum, both processes were inhibited by theophylline. These authors offered the explanation that the stimulation in phosphorylation of protein S6 was a necessary prerequisite for the increase in protein synthesis, and not a consequence of it. Further, the observation of protein S6 in a higher state of phosphorylation in polysomes than in monosomes has been made by several groups (Bitte & Kabat, 1972; Kruppa & Martini, 1978; Leader & Coia, 1978c). In addition, protein S6 has been located near the initiation site on the ribosome (Westermann, Nygard & Bielka, 1981) and has been cross-linked to polyuridylic acid (Terao & Ogata, 1979a & b) and to a natural mRNA (Takahashi and Ogata, 1981).

To try to reconcile a role for the phosphorylation of protein S6 in protein synthesis with the results mentioned above, an alternative hypothesis has been proposed by Leader (1980a). He suggests that phosphorylated protein S6 need only stimulate protein synthesis under certain conditions, when the rate of protein synthesis is limited by the step which phosphorylation of protein S6 affects. Thus phosphorylation of protein S6 may be neither necessary for protein synthesis (eg. in rat liver) nor necessarily increase protein synthesis (eg. after stimulation with cyclic-AMP), if the control step is not limiting. This might explain the failure of Eil and Wool (1973) to detect any differences in the protein synthetic activity of ribosomes with protein S6 in high and low states of phosphorylation, if the experiments were not carried out at the various limiting concentrations of all the different components. The above hypothesis could also explain the increased phosphorylation of protein S6 that occurs following

pathological liver injury (Gressner & Greiling, 1977; Gressner & Greiling, 1978(a); Gressner & Greiling, 1978(b); Gressner, 1980; Leader, 1980(c)) if general protein synthesis was stimulated in an attempt to maximise protein synthesis under conditions where the overall rate is reduced.

An alternative way of reconciling the conflicting data however, is to suggest that phosphorylation of protein S6 may stimulate the translation of specific mRNAs rather than increasing the synthesis of all proteins. Such specific mRNAs would be assumed to have a higher affinity for phosphorylated protein S6 than other mRNAs. Thus, this could occur even when the rate of protein synthesis is inhibited or when no overall increase in protein synthesis is observed. It is well known, for example, that cyclic AMP causes the induction of specific gluconeogenic enzymes in liver without affecting total protein synthesis (Shrago et al., 1963; Yeung & Oliver, 1968).

How then can these two models accomodate the results presented in this thesis, showing that infection of cells by pseudorabies virus and herpes simplex virus increases the phosphorylation of protein S6? Two kinds of response may be envisaged.

Firstly the phosphorylation of protein S6 may have been promoted by the virus to its own advantage. Here, an effect on the interaction of specific mRNAs with the ribosome might favour the virus if viral mRNAs had a greater affinity than the majority of host mRNAs for binding to ribosomes with phosphorylated protein S6. This might also explain why certain host messages, (presumed to be of the high affinity class), continue to be translated long after other host messages have stopped being translated (see eg. band H8 in Fig. 3.3.10). However, if viral

mRNAs were to preferentially bind to ribosomes with phosphorylated protein S6 then one would need to explain why the phosphorylation of protein S6 precedes the switch from host to predominantly viral protein synthesis by three to four hours.

One possibility is that the time lag in viral protein synthesis is not real, but that phosphorylation of protein S6 occurs at this time to aid the synthesis of early viral proteins, most of which are involved in controlling the viral infection (Hones & Roizman, 1974; Jones & Roizman, 1979). As such proteins are usually required in only small amounts, one would expect a low concentration of these early viral mRNAs in the infected cell. The postulated greater affinity of these viral mRNAs for ribosomes with phosphorylated protein S6 would therefore allow them to compete better with the much higher concentration of lower affinity host mRNA. Thus, this small amount of viral protein synthesis would not alter appreciably the overall rate of host protein synthesis, nor would it be detectable in Fig. 3.3.10. The shut-off of host protein synthesis would only become detectable later in infection when the much greater amount of high affinity viral mRNAs, coding for the major structural proteins, appear in the infected cells.

Paradoxically, it is also possible to envisage the phosphorylation of protein S6 as a defence mechanism by the host, even though this ultimately fails. Two kinds of response may be considered. Firstly the phosphorylation of protein S6 could stimulate general protein synthesis in an attempt to maximise protein synthesis under conditions where the overall rate is reduced, in a similar manner to the defence mechanism that could be imagined to occur with the phosphorylation of protein S6 during pathological liver injury (as previously discussed).

Alternatively, the host response could be to stimulate the synthesis of specific key host proteins which are essential for the survival of the cell or for defence against the infection. However, if this is the case it cannot be excluded that the virus may be using this response to direct the specific synthesis of its own proteins.

Although the difference between the phosphorylation of protein S6 in normal cells and those transformed by viruses has been discussed in terms of the different growth rates of these cells, the above considerations suggest the possibility of a more direct role in viral transformation. Thus one could imagine that the virus has directed the phosphorylation of protein S6 to promote the specific synthesis of proteins necessary for the establishment and maintenance of the transformed state.

Clearly, direct experiments testing the relative affinities of different mRNAs for ribosomes with phosphorylated and non-phosphorylated protein S6 are needed to settle this question.

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