THE PHOSPHORYLATION OF EUKARYOTIC RIBOSOMAL PROTEINS

by

Andrew D. Rankine

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To

my Parents

In the study of nature, as a philosopher has observed, the principles are certain general effects from primary causes from which spring countless secondary effects: the art of linking the first to the second is to walk sightless along a highway from which a thousand by-ways lead astray.

X. Bichat, 1805

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ABBREVIATIONS.

The standard abbreviations found in Biochem. J. (1975) 145, 1-20 are used throughout this thesis. The following additional abbreviations are also found in the text:

Ascites cells - Krebs II Ascites cells.

BHK cells - Baby Hamster Kidney, Litter 21, Clone 13 cells.

dibutyryl cyclic AMP - $N^6,O^{2'}$-dibutyryl adenosine 3':5'-monophosphate.

TEMED - N,N,N',N',-Tetramethylethylenediamine.

Bisacrylamide - N,N',-methylene bisacrylamide.

SDS - Sodium dodecyl sulphate.

PPO - 2,5-Diphenyloxazole.

POPOP - 1,4-Di[2-(5-phenyloxazolyl)]-benzene.

BBOT - 2,5-Di-(5-tert-butyl-2-benzoxazolyl)-thiophene.

1 E_{260} is that amount of ribosomes which, when dissolved in 1 ml. of H_2O, gives an absorbance of 1 at 260nm. in a 1 cm. light-path (1 E_{260} is approximately equivalent to 100µg of ribosomes).

Throughout this thesis, the centrifugal force on a sample during centrifugation, "g", is calculated based on the average radius of rotation of the centrifuge tube.

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ILLUSTRATIONS.

SUMMARY

The work described in this thesis extends studies on the phosphorylation of ribosomal proteins to Krebs Ascites and Baby Hamster Kidney cells. When these cells were incubated with [^{32}P]-orthophosphate, radioactivity became associated with ribosomal proteins, and was shown to be in the form of phosphoproteins by both chemical and enzymic criteria.

About 18 phosphoproteins were detected when protein extracted from 80S ribosomes was separated by polyacrylamide gel electrophoresis. However, many of these appeared to be non-ribosomal contaminants, for far fewer phosphoproteins were seen with purified ribosomes or ribosomal subunits. Five phosphoproteins were found on ribosomal subunits analysed by electrophoresis in gels containing sodium dodecyl sulphate, and three on gels containing urea at pH 4.5. This difference may have been due to some of the phosphoproteins being relatively acidic (and thus of low mobility at pH 4.5), or alternatively because of the poorer resolving power of the latter system of gel electrophoresis. The number of ribosomal phosphoproteins detected in this work is more than was originally reported for other tissues, but is still much less than has been found by workers who have phosphorylated ribosomes *in vitro* using protein kinases.

The ribosomal subunit on which a pair of phosphoproteins of low molecular weight was located,

differed for Ascites and Baby Hamster Kidney cells: the proteins were on the large subunit of Ascites cells, and on the small subunit of Baby Hamster Kidney cells. The most probable explanation of this result is that these proteins are at the interface between the ribosomal subunits. Such a location is consistent with the previously suggested role for these proteins in holding the subunits together as inactive monosomes.

Two-dimensional gel electrophoresis was used to try to identify unequivocally the ribosomal phosphoproteins. Only one protein was definitely identified by this method: a phosphoprotein of the small subunit of both Ascites and Baby Hamster Kidney cells, which was found to be the protein designated S6 in the standard nomenclature. The radioactivity associated with S6 comigrated with an anodic 'tail' of the protein, which apparently represents a number of increasingly phosphorylated derivatives of S6. This observation meant that it was possible to estimate the extent of phosphorylation of S6 merely by staining the protein of the phosphorylated derivatives, even when these contained no radioactivity.

Studies were performed to see whether the functional activity of ribosomes correlated in any way with the phosphorylation of ribosomal proteins (particularly S6). When protein synthesis was inhibited in Ascites cells, there was no change in either the specific radioactivity of the phosphate in the ribosomal protein, the number of phosphorylated proteins resolved

by one-dimensional gel electrophoresis, or the visible extent of phosphorylation of S6 in protein analysed by two-dimensional gel electrophoresis. This result contrasts with studies on the phosphorylation of S6 in other tissues, and may be due to high levels of phospho-protein phosphatase in Ascites cells. The phosphorylation of S6 was, however, very extensive in growing Baby Hamster Kidney cells, demonstrating that a high level of phosphorylation of S6 under physiological conditions does not require protein synthesis to be inhibited.

In studies to investigate the relationship between cyclic AMP and the phosphorylation of S6, it was found that dibutyryl cyclic AMP had no effect on the phosphorylation of S6 in Ascites cells, a result which again contrasts with those in other tissues. More significantly, the extent of phosphorylation of S6 in Baby Hamster Kidney cells under different growth conditions *in vivo* did not correlate with the cellular concentration of cyclic AMP, indicating that elevated levels of this nucleotide are not essential for the phosphorylation of S6 *in vivo*.

There was greater phosphorylation of S6 in growing, rather than resting, Baby Hamster Kidney cells, a situation which was not altered when the resting cells were stimulated to grow for short periods by the addition of fresh medium. S6 was also found to be extensively phosphorylated in the livers of young mice, but not in those of adult mice. These results are discussed in relation to a model in which ribosomes are

phosphorylated during synthesis and thereafter are normally slowly dephosphorylated. It is suggested that the phosphorylation of S6 may have a role in the assembly or extra-nuclear transport of ribosomes, or in the control of their lifespan in the cytoplasm.

SECTION 1.

INTRODUCTION.

1.1 THE EUKARYOTIC RIBOSOME.

1.1.1 The Structure of the Ribosome - General Aspects.

The translation of mRNA into protein requires the participation of about two hundred macromolecules - both RNA and proteins. This is many more than are required for the replication and transcription of DNA and presumably reflects the greater difficulty encountered in converting genetic information from nucleic acid to protein, than from nucleic acid to nucleic acid. Of the macromolecules involved in translation, about one-third are combined in the subcellular organelle which is the site of protein synthesis - the ribosome. The work presented in this thesis concerns one aspect of the structure and function of the ribosome.

The ribosome is an electron-dense ribonucleoprotein particle found in the cytoplasm of cells (Jeener and Brachet, 1941; Palade, 1955). It comprises three different RNA molecules and a large number of proteins - about 70 in eukaryotes and 55 in prokaryotes. The prokaryotic ribosome has a sedimentation coefficient of 70S and is composed of two dissimilar subunits of sedimentation coefficients 30S and 50S (Tissières, et al., 1959) while the eukaryotic ribosome has a sedimentation coefficient of 80S, and is composed of subunits of sedimentation coefficients 40S and 60S (Peterman, 1964).

Although the process of translation is similar in both prokaryotes and eukaryotes (See Lengyel, 1974), the eukaryotic ribosome is appreciably larger, having, in mammals, an aggregate mass of 4.5×10^6 (Cammarano, et al.,

1972, a,b,c.) compared with 2.6×10^6 for the E.Coli ribosome (Tissières et al., 1959). Cammarano et al. (1972b) have determined the molecular weights of ribosomes from many diverse eukaryotic species, and found a range of 3.9×10^6 to 4.5×10^6. These differences are due to changes in the molecular weight of the larger subunit. This suggests that there has been a strong conservation of the size of the small subunit, but not the large subunit, during evolution.

Electron microscopy shows the small ribosomal subunits of eukaryotes to be elongated and slightly bent prolate ellipsoids about 230Å x 140Å x 115Å (Nonomura et al., 1971). The profile of the subunit is divided into "one-third" and "two-third" regions by a transverse partition, some 80Å from one end. The large ribosomal subunit has an approximately triangular skiff-shaped profile about 230Å on each side. The area of contact, both in eukaryotic ribosomes and in prokaryotic ribosomes, is sufficiently large to accommodate both tRNA and mRNA. The resistance of these RNA's to ribonuclease digestion while in complexes with ribosomes, is consistent with such a model (see Van Holde and Hill, 1974).

The site of synthesis of ribosomes is the nucleolus (Maden, 1968). Here, rRNA is transcribed and becomes associated with ribosomal proteins (which are synthesised in the cytoplasm - Heady and M^{c}Conkey, 1970) and first appear in the cytoplasm as ribosomal subunits (Girard et al., 1965). Ribosomes are quite long-lived in the cell, for Hirsch and Hiatt (1966) have shown that in rat liver, rRNA and ribosomal protein have a similar half-life of about five days.

In both prokaryotes and eukaryotes, ribosomes which are actively synthesising protein are found on polysomes. Generally, polysomes synthesising protein to be secreted from the cell are bound tightly to the membranes of the endoplasmic reticulum (Birbeck and Mercer, 1961), and vectorially transfer nascent polypeptides into the intercisternal space of the endoplasmic reticulum (Redman and Sabatini, 1966). However, membrane-bound polysomes have also been described in non-secreting cells (Andrews and Tata, 1971: Rosbash and Penman, 1971). In these cells, the polysomes are thought to be more loosely bound to the membrane, perhaps by the PolyA segment of their mRNA (Lande et al., 1975). These polysomes do not secrete proteins into the intercisternal space of the endoplasmic reticulum, and thus must perform a different function from tightly bound membrane polysomes.

There is only a small pool of free ribosomal subunits in the cytoplasm, and these exchange freely with polysomes (Henshaw et al., 1973). There is a much larger pool of free monosomes, but these do not readily enter polysomes or exchange with ribosomal subunits. This appears to contrast with the situation found in E.Coli, and suggests that the monosomes in eukaryotic cells may be a storage form of inactive ribosomes (Howard et al., 1970).

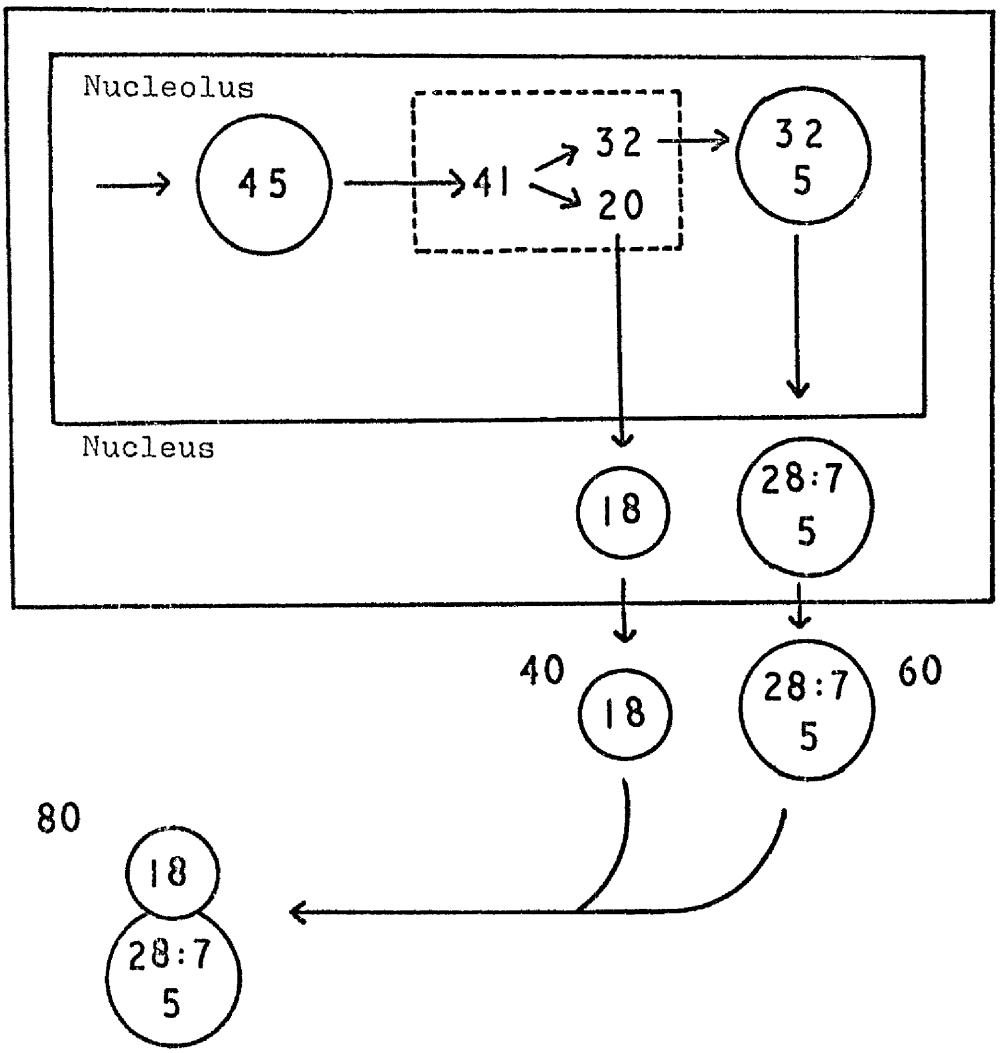


Fig. 1.1 Schematic representation of ribosome formation in eukaryotic cells. The numbers outside the particles represent the sedimentation coefficients of the particles themselves. The numbers inside the particles represent the sedimentation coefficients of the RNA species they contain. (Redrawn from Maden, 1971).

1.1.2 Ribosomal RNA.

The RNA component of the ribosome varies from 50% (in mammals) to 65% (in E.Coli) of its total mass. In eukaryotes, both 28S and 18S rRNA's are derived from a single transcriptional unit, 45S RNA (Perry et al., 1970; Scherrer et al., 1970) by the process of maturation shown in Fig. 1.1. A third RNA species, 5S RNA, is not derived from the precursor molecule in eukaryotes, although it does become associated with the ribosome at an early stage in its development in the nucleolus (Warner and Soeiro, 1967; Weinberg, 1973).

Ribosomal RNA's are extensively modified by methylation at specific sites. The 16S and 23S RNA's of ribosomes of E.Coli contain many methylated bases (Starr and Fefferman, 1964). In eukaryotes, 45S RNA is methylated predominantly at ribose residues, and this occurs during or after synthesis (Udem and Warner, 1972). In eukaryotes, the methylated residues are conserved during processing, and may play a role in the maturation of ribosomes (Maden and Salim, 1974).

Direct chemical evidence suggests that much of the rRNA is located on the outside of the ribosome (Cotter et al., 1967). This implies that the RNA is available to interact with the other components of the protein synthetic machinery outside the ribosome. Several lines of evidence suggest that rRNA does play an important role in protein synthesis. Disruption of the primary structure of the rRNA of E.Coli with ribonucleases (Lee and Quintanilla, 1972), or Colicin E3 (Bowman et al., 1971;

Senior and Holland, 1971) results in decreased binding of tRNA and mRNA to the ribosome. Similarly, studies in E.Coli with the antibiotic kasugamycin have shown that resistance to this antibiotic is conferred by lack of dimethylation of two adjacent adenine residues near the 3' - end of the 16S RNA (Helser et al, 1971, 1972).

Several studies have indicated that rRNA might be directly involved in the binding of tRNA and mRNA to ribosomes. It has been proposed that 5S RNA forms part of the site to which the TΨC loop of tRNA binds, both in prokaryotes (Erdmann et al, 1973) and in eukaryotes (Grummt, et al, 1974). It has also been observed that a reconstituted complex of 5S RNA with several ribosomal proteins has GTPase and ATPase activity (Gaunt - Klopfer and Erdmann, 1975). 16S RNA has been implicated in the binding of the preinitiation region of mRNA to ribosomes by base complementarity, through its 3' - terminus (Shine and Dalgarno, 1974, 1975), its 5' - terminus (van Knippenberg, 1975), and also through its ability to bind ribosomal protein S1, which is required for maximum binding of mRNA to the ribosome (Dahlberg and Dahlberg, 1975). 16S RNA has also been implicated in the binding to the ribosome of IF - 3, which is itself involved in the binding and recognition of mRNA (Gualerzi and Pon, 1973).

Finally, evidence for the direct involvement of rRNA in the function of the ribosome has been provided by Harris et al. (1973) and Greenwell et al. (1974) whose results suggest that 23S RNA may bind the 3' - CCA terminal triplet of tRNA to the peptidyl transferase centre

of the ribosome.

1.1.3 Ribosomal Proteins.

Initial hypotheses concerning the structure of the ribosome tended to regard it as being similar to the small spherical plant viruses, with a core of RNA, and a simple shell of repeating protein units. However, the electrophoretic studies by Waller and Harris (1961) on protein extracted from ribosomes of E.Coli showed that the protein could be resolved into more than twenty bands. Using the technique of two-dimensional polyacrylamide gel electrophoresis which involves separation by charge in the first dimension and by size in the second, it is now known that the 30S subunit of the ribosome of E.Coli contains 21 distinct protein species (referred to as S1 to S21) while the 50S subunit contains 34 (referred to as L1 to L34)(Kaltschmidt and Wittmann, 1970a; Hindennach et al, 1971a,b.). The ribosomal proteins of E.Coli are very basic, and Kaltschmidt (1971) has shown that about 70% of them have isoelectric points of pH10 or higher.

One difficulty is to define exactly what is meant by the term 'ribosomal protein'. A definition that has found wide acceptance is that ribosomal proteins are those which are present on the ribosome throughout its lifespan. This would exclude the initiation and other protein factors which are only concerned with one phase of protein synthesis. In practice, proteins present on the ribosome after washing

with 0.5M ammonium or potassium chloride are usually regarded as ribosomal.

The proteins of both subunits of the ribosomes of E.Coli have been isolated (Hindennach et al., 1971b) and antibodies raised against them. Lack of cross-reactivity showed all the proteins to be unique, with the exceptions of L7 and L12 which did cross-react (Stoffler and Wittmann, 1971). The amino acid sequences of proteins L7 and L12 have been determined, and it has been found that the only difference is the N-terminus: L7 having N-acetyl serine, while L12 has serine (Terhorst et al., 1973). Apart from L7/L12, the complete amino acid sequences of six other ribosomal proteins of E.Coli have been determined (Chen et al., 1975; Yaguchi, 1975) as well as substantial portions of all the others (Wittmann and Wittmann-Liebold, 1974). This work shows that there are no similar sequences of any significant length common between any of the proteins other than L7 and L12.

Estimates have been made of the number of copies of ribosomal proteins present per ribosome, and only for proteins L7 and L12 are there more than one copy (Voynow and Kurland, 1971). Some proteins of the 30S subunit appear to be present in a stoichiometry of less than one copy per ribosome, and it has been suggested that ribosomes are heterogeneous with respect to their protein complement (Kurland, 1970). However, some of these "fractional" proteins, e.g. S1, are necessary for protein synthesis, and it is possible that the stoichiometry observed was due to selective losses of surface ribosomal proteins during

preparation (Szer et al., 1975).

The topography of the ribosomes of E.Coli has been studied by several different techniques in the last few years. Bifunctional reagents have been used to link and define neighbouring proteins (see Traut et al., 1974). Several investigators have digested ribosomes by mild ribonuclease treatment and analysed the proteins present in the subunit fragments (Morgan and Brimacombe, 1973; Roth and Nierhaus, 1973). The results of these studies have been found to correlate well with the map which describes the order in which ribosomal proteins attach to pre-ribosomal particles during ribosome assembly, and the interdependency of different proteins in this process (see Nomura and Held, 1974). Zimmerman (1974) has described the interactions between ribosomal proteins and RNA's, and defined the sites on the RNA at which the proteins bind.

An approach to the assignment of functions to individual ribosomal proteins has been made using affinity labels. These include analogues of mRNA, GTP, peptidyl tRNA and antibiotics (see Cantor et al., 1974). Photo-affinity labelling of the ribosomal proteins of E. Coli participating in EF-G dependent hydrolysis of GTP indicated the involvement of proteins L7/L12 (Maasson and Moller, 1974), which had previously been cross-linked to EF-G (Acharya et al., 1973). Although proteins L7/L12 seem to be intimately related to the function of elongation factors, they are not part of the GTPase itself, as a ribonucleoprotein particle which has uncoupled GTPase activity does not contain L7/L12 (Gaunt-Klopfer and Erdmann, 1975).

In contrast to the great deal which is known about the proteins of the bacterial ribosome, little is known about eukaryotic ribosomal proteins. The eukaryotic ribosome contains about 70 proteins, as determined by two-dimensional polyacrylamide gel electrophoresis (Sherton and Wool, 1972, 1974b; Wolfle et al., 1972; Martini and Gould, 1971; Howard et al., 1975). There are about 40 proteins in the 60S subunit and about 30 in the 40S subunit, and their patterns on two-dimensional gels are completely different from the patterns produced by the ribosomal proteins of E.Coli. Moreover, immunological studies show no cross-reactivity between ribosomal proteins of rat liver and E.Coli, the only exception being that Wool and Stoffler (1974) have found that rat liver proteins L40 and L41 are structurally homologous to proteins L7 and L12 of E.Coli. This suggests that since L7/L12 have been conserved throughout evolution, they play an important role in protein synthesis, and is consistent with the assignment of a function for them in the activity of elongation factors.

The molecular weights of ribosomal proteins from rat liver (Lin and Wool, 1974; Terao and Ogata, 1975) and rabbit reticulocytes (Howard et al., 1975) have been estimated. These three determinations are in quite good agreement, with molecular weights ranging from 10,000 to 40,000, with a number average of 22,000 for the proteins of the 40S subunit; and 10,000 to 55,000 with a number average of 28,000 for the proteins of the 60S subunit. It is evident from these figures, that eukaryotic ribosomes contain not only more proteins, but larger ones than those

of E.Coli, where the number average molecular weights are 14,000 for the 30S subunit, and 16,000 for the 50S subunit (Bickle and Traut, 1971).

Ribosomal proteins vary from species to species (Martin and Wool, 1969) and there are marked differences between such distantly related species as rat and tetrahymena. Having analysed ribosomal proteins by two-dimensional gel electrophoresis, Delaunay et al. (1974) correlated the comigrating spots of two species and the age of the evolutionary branch point leading to those species. For example, proteins of different mammalian species may comigrate almost exactly, while plant ribosomes may only have 20 - 25 spots comigrating with mammals.

Minor differences in the two-dimensional gel patterns of ribosomal protein from different tissues of the same species may be accounted for by contamination by non-ribosomal proteins, and the variable subunit location of some proteins, which can result from different methods of preparation (Sherton and Wool, 1974b.).

The reconstitution of active eukaryotic ribosomal subunits from RNA and proteins has yet to be achieved. This may be a reflection of the fact that the initiation of eukaryotic ribosome assembly is on the 45S precursor rRNA (Weinberg and Penman, 1970) and it may be that only 45S RNA contains the information for assembly which is lacking in 18S and 28S RNA. However, there are strong interactions between the proteins and RNA in the 40S subunit of rat liver ribosomes, since 7 proteins will bind individually to 18S RNA (Westermann and Bielka, 1974).

Recent studies have provided initial information regarding the surface topography of the eukaryotic ribosome. Leader (1974, 1975) and Wolfle (1974) have used enzymic iodination by lactoperoxidase to show that most rat liver ribosomal proteins are at least partially on the surface of the ribosomal subunits. Westermann et al. (1974) have studied the protection of ribosomal proteins from chemical reaction by the various ribosomal substrates (e.g. tRNA, initiation factors) in order to define the proteins at the various sites on the ribosome. Further progress in this area will be facilitated by the purification of the individual ribosomal proteins.

1.1.4 Control of the Function of the Ribosome.

In the prokaryotic cell there is little requirement for control of the rate of protein synthesis after transcription, and indeed transcription and translation are tightly coupled. However, in eukaryotes, mRNA is much longer lived (Singer and Penman, 1973) and the site of transcription and translation are separated. It would therefore be advantageous for the eukaryotic cell to be able to control directly the rate of protein synthesis, and indeed there is evidence for several types of translational control, some of which involve the structure of the ribosome itself.

There are a number of metabolic circumstances which cause a decrease in the rate of protein synthesis, disaggregation of polysomes and concomitant appearance of inactive monosomes in the cytoplasm. When the cells

are returned to normal conditions, the cytoplasmic monosomes re-enter polysomes and protein synthesis resumes. This occurs rapidly, and in the presence of Actinomycin D, suggesting that pre-existing mRNA is being used, and that control is being exerted at the translational level. Examples of such conditions include the following: amino acid starvation (Vaughan et al., 1971; van Venrooij et al., 1970) or serum deprivation (Hassell and Engelhardt, 1973) of cultured cells, the arrest of protein synthesis at mitosis (Fan and Penman, 1970; Stanners and Becker, 1971), the control of globin synthesis by haem (Adamson et al., 1972; Gross and Rabinowitz, 1972), and the control of muscle protein synthesis by insulin (Wettenhall et al., 1974).

There are some other examples of translational control which fall outside this general scheme. These include the inhibition of host cell protein synthesis by viruses (Martin and Kerr, 1968) and the inhibition of viral mRNA translation by interferon (Falcoff et al., 1973). Phytohaemagglutinin is thought to stimulate protein synthesis in lymphocytes through an effect on translation (Kay et al., 1975) and hormones such as ACTH (Garren et al., 1965), and growth hormone (Barden and Korner, 1972) may also exert translational control of protein synthesis.

In some of these cases cited, the evidence suggests that the ribosome is not directly involved. For example, in the control of globin synthesis by haem, a specific initiation factor IF-MP is involved (Clemens et al., 1974), and in interferon action an inhibitor of tRNA is

implicated (Content et al., 1974). In the case of at least one viral inhibition of protein synthesis (the effect of encephalomyocarditis on mouse plasmacytoma cells) it appears that the structure of the viral mRNA allows it to bind to host cell ribosomes with much greater facility than host cell mRNA (Lawrence and Thach, 1974). In many other cases, the mechanism of translational control is unknown, but in a few instances there is evidence for the direct involvement of the ribosome itself. For example, Nakaya et al. (1974) found that changes in the 60S subunit of ribosomes from the skeletal muscle of diabetic rats cause the ribosomes to be more susceptible to dissociation by EIF-3. Similarly following hypophysectomy in the rat there is a decrease in the ability of the ribosome of the liver to bind aminoacyl-tRNA (Barden and Korner, 1972). Although these results imply changes in the structure of the ribosome, such changes have not been demonstrated directly. There have been claims, however, for differences in the protein complement of free and membrane-bound ribosomes, and that these differences mediate the partition of mRNA's between these two types of ribosomes (Fehlmann et al., 1975). However, it is possible that more subtle changes in protein structure could be involved in the control of ribosome function. One such alteration is phosphorylation - the subject of this thesis. The importance of the phosphorylation of proteins is considered in the following section, while Section 1.3 considers the phosphorylation of ribosomal proteins.

1.2 PHOSPHORYLATION OF PROTEINS.

1.2.1 Phosphoproteins - Occurence and Significance.

Phosphoproteins are ubiquitous in the eukaryotic cell and are found in many regulatory roles in metabolism. The first phosphoproteins which were recognised as such were the animal nutritive proteins casein and phosvitin. Enzymes were found which catalysed the transfer of the terminal phosphate of nucleoside triphosphates to the hydroxyl groups of serine or threonine residues in proteins, and these were called protein phosphotransferases, or, trivially, protein kinases (Burnett and Kennedy, 1954).

It was initially thought that the phosphorylation of proteins such as casein or phosvitin was a non-specific procedure to allow phosphate to be transported into the animal embryo (Williams and Sanger, 1959). However, it was later discovered that phosphorylation of enzymes could modify their activity, thus forming an important part of the cellular response to a number of hormones. The central role which phosphoproteins play in the glycogenolysis induced by adrenalin or glucagon in liver is now well established (Rall *et al.*, 1957; Sutherland and Rall, 1960). Krebs *et al.* (1966) have described a cascade mechanism for the regulation of glycogenolysis in muscle in which adrenalin, stimulating adenyl cyclase, causes production of cyclic AMP which then accelerates the phosphorylation and activation of phosphorylase kinase. The latter enzyme catalyses the conversion of phosphorylase b to phosphorylase a, when Ca^{2+} is available, and glycogenolysis ensues (see Fischer *et al.*, 1971).

Phosphoproteins are also intimately involved in glycogen synthesis. Glycogen synthetase, which catalyses the transfer of glycosyl moieties from UDPG to glycogen (Larner and Villar-Palasi, 1971) appears to be inactivated by phosphorylation (Friedman and Larner, 1963). The regulation of many soluble enzymes such as pyruvate dehydrogenase and Acetyl CoA carboxylase is mediated through the interconversion of active and inactive forms by phosphorylation (see Segal, 1973). Phosphoproteins are thus directly involved in the regulation of some of the central pathways of intermediary metabolism.

Nuclear proteins have also been found to be subject to phosphorylation, and this raises the possibility of a role for phosphorylation in the control of gene activity. Histones as well as non-histone proteins are phosphorylated (Langan, 1968a), and different kinases are involved depending on the substrate. The general physiological significance of the phosphorylation of histones remains unclear, but it has been suggested that during spermatogenesis these modifications may be involved in dissociating the histone from DNA to make way for protamine (Sung and Dixon, 1970).

There would seem to be a positive correlation between the phosphorylation of the lysine-rich histone, H1, slightly lysine-rich histone, H2A, and cell replication (Balhorn et al., 1972a,b,c). There is an increased rate of phosphorylation of histone H1 during S phase (Marks et al., 1973) followed by rapid dephosphorylation after mitosis (Lake, 1973). Balhorn et al. (1975) have found

that in rapidly growing hepatoma cells, histones H1 and H5 are phosphorylated rapidly after synthesis, but more slowly once bound to the chromosome. The phosphorylation of histones H1, H2A and H5, which contain large amounts of lysine, therefore, seem to be intimately involved in DNA replication and gene activation (see Elgin and Weintraub, 1975).

The non-histone proteins of the nucleus have also been implicated in the control of gene activation (see Kleinsmith, 1975). This hypothesis is based on the observations that the non-histone proteins exhibit tremendous heterogeneity (Garrard et al., 1974), and are tissue specific (Teng et al., 1971). The fact that these are also subject to phosphorylation by a heterogeneous group of protein kinases found in the nucleus (Kish and Kleinsmith, 1974) suggests that here too, phosphorylation may be important.

Another class of proteins which have been found to contain phosphoproteins are those of membranes. This phosphorylation of membrane proteins is thought to mediate ion transport, for example, in retinal membrane (Kuhn et al., 1973; Frank et al., 1973) and in erythrocyte membranes (Roses and Appel, 1973). The permeability of membranes to metabolites and other substances such as synaptic transmitters is also thought to be mediated by phosphorylation of proteins in the membrane (Chang and Quatrecasas, 1974; Greengard and Kebabian, 1974).

Phosphorylation of proteins may also be involved in the process of viral infection of cells. Thus protein

kinase activity has been observed in several viruses, including Semliki Forest virus (Tan and Sokol, 1974) and Sindbis virus (Waite et al., 1974). These viruses and several others such as SV40 (Tan and Sokol, 1972) and adenovirus (Russell et al., 1972) also contain phosphoproteins. The role of viral phosphoproteins remains unclear, but it seems likely that viral protein kinases are involved in the modification of host cell metabolism in order to facilitate viral replication.

It is thus evident that the reversible phosphorylation of proteins is an important method of regulating their biological function. The elucidation of the metabolism of phosphoproteins is clearly an important part of our understanding of metabolic regulation in the cell.

1.2.2 Protein Kinases.

Protein kinases are to be found in eukaryotic cells and some viruses, but their presence in prokaryotic cells is more doubtful (see Rubin and Rosen, 1975). They may be classified as "cyclic nucleotide-dependent" or "cyclic nucleotide-independent". These terms should be taken to mean "dependent" for maximal activity since most protein kinases exhibit low basal activity in the absence of added cyclic nucleotide. Moreover, most protein kinases are activated by more than one cyclic nucleotide, but investigations have centred on their relationships with cyclic AMP and cyclic GMP, since these are the predominant cyclic nucleotides found in the cell.

Brostrom et al. (1970) and Gill and Garren (1970)

were the first to propose that cyclic AMP-dependent protein kinases are composed of two dissimilar subunits, the regulatory and the catalytic subunits. When these subunits are in combination, the kinase is inactive. Cyclic AMP promotes dissociation of the subunits to yield a complex of the regulatory subunit with cyclic AMP, and a free enzymically active catalytic subunit. In addition there may be other factors which affect the activity of the catalytic subunit. Protein inhibitors found in mammalian tissues bind to the catalytic subunit, inhibiting its enzymic activity and its ability to recombine with regulatory subunits (Walsh et al., 1971; Ashby and Walsh, 1972). Also ATP has been found to inhibit the cyclic AMP-dependent dissociation of the regulatory and catalytic subunits (Haddox et al., 1972) and also promote dissociation of the regulatory subunit-cyclic AMP complex (Brostrom et al., 1971).

Protein kinases which are independent of cyclic nucleotides are also known. It may be argued that these could merely be free catalytic subunits of cyclic nucleotide-dependent enzymes. However, this is unlikely to be so, as many kinases show a pattern of activity that is completely unrelated to changes of cyclic nucleotide concentrations in the cell, for example during the cell cycle (Shepherd et al., 1971; Oey et al., 1974). Moreover, protein kinases are also active in higher plants, where cyclic nucleotides appear to be absent.

The specificity of protein kinases towards their substrates is not well understood. The main reason for

this is that reports have seldom dealt with pure enzyme preparations. Certainly, many tissues have been found to contain two or more separable kinase activities. According to Kish and Kleinsmith (1974) bovine liver nuclear protein kinases may comprise up to 50 unique species which have different substrate and cyclic nucleotide dependencies, thus showing an extraordinary degree of functional heterogeneity. The situation is complicated further by the finding that catalytic and regulatory subunits from a wide variety of organisms and tissues can interact to form hybrid enzymes (Miyamoto et al., 1973; Takai et al., 1974). Because of the heterogeneity of the preparation, the activity of protein kinases in vitro tends to be more dependent on assay conditions such as pH (Jergil and Dixon, 1970) or ionic strength (Reimann et al., 1971) than on the substrate. This is presumably because one set of assay conditions will favour one enzyme species while inhibiting the activity of others.

1.3.1 The Phosphorylation of Eukaryotic Ribosomal Proteins
1.3 THE PHOSPHORYLATION OF EUKARYOTIC RIBOSOMAL PROTEINS.

1.3.1 The Phosphorylation of Eukaryotic Ribosomal Proteins in vivo.

Kabat (1970) first observed that when rabbit reticulocytes were incubated for 60 mins. with $[^{32}P]$ - orthophosphate, there was extensive incorporation of ^{32}P into the microsomal fraction of the cell, especially into the o-phosphoserine and o-phosphothreonine residues of proteins. The ribosomes were washed in a buffer of high ionic strength to remove any non-ribosomal proteins adventitiously bound to the ribosomes, and the protein extracted. This protein was subjected to electrophoresis in polyacrylamide gels containing SDS, and it was found that radioactivity comigrated with structural ribosomal proteins.

Among the 70 or so eukaryotic ribosomal proteins, Kabat (1970) initially found two phosphoproteins. One occurred on the 60S subunit (in early papers referred to as Si - later as protein I) and a second on the 40S subunit (referred to as F - later protein II). Of two other labelled bands found on the gels, one was ascribed to a tightly bound non-ribosomal protein and the second to a small molecule, perhaps a nucleotide.

Ribosomal phosphoproteins are not restricted to reticulocytes. Kabat (1970) stated that ribosomal phosphoproteins could also be found in various tissues from chicken embryo. Blat and Loeb (1971) observed labelling of ribosomal protein in rat liver after injection of $[^{32}P]$ - orthophosphate. They analysed this on polyacrylamide gels

and found one labelled band. It is interesting to note that the phosphorylation of ribosomal proteins is confined to eukaryotes, as they have been shown to be absent from prokaryotic ribosomes (Gordon, 1971).

1.3.2 Phosphorylation of Eukaryotic Ribosomal Proteins *in vitro*.

Certain ribosomal proteins can be phosphorylated *in vitro* by incubating ribosomes or ribosomal subunits with cyclic AMP-dependent, or - independent protein kinases. The kinases used were either those found bound to the ribosomes (Kabat, 1971; Li and Amos, 1971) or those present in the cytosol (Loeb and Blat, 1970; Walton *et al.*, 1971; Eil and Wool, 1971) and catalysed the transfer of the terminal phosphate of ATP to serine and threonine residues in ribosomal proteins. There is, however, some uncertainty as to whether the ribosomal proteins which are phosphorylated *in vitro* are necessarily phosphorylated *in vivo*. For example, the number of proteins which can be phosphorylated *in vitro* is often greater than the number phosphorylated *in vivo*. Thus, although Kabat (1970,1971) observed similar phosphorylation *in vitro* and *in vivo*, Eil and Wool (1971) reported that up to 12 proteins were phosphorylated *in vitro*, when separated subunits were used as substrates. It is possible that the lack of tRNA, mRNA and factors for protein synthesis may make the ribosome more susceptible to phosphorylation *in vitro*. Another difficulty is the fact that in no case has any enzyme which will phosphorylate ribosomal protein *in vitro* been shown to be specific

for that substrate, and although protein kinase activity has been found to be directly associated with ribosomes, it can be removed by washing in buffers containing 0.5 - 1M KCl (Kabat, 1971; Eil and Wool, 1971; Li and Amos, 1971).

1.3.3 Function and Control of Phosphorylated Ribosomal Proteins.

Kabat (1970) originally reported that the phosphoprotein of the 60S subunit of rabbit reticulocyte ribosomes which he called Si (later I) was only phosphorylated on single ribosomes. Since single ribosomes are thought to be a storage form for inactive ribosomes (see Section 1.1), this result suggested the intriguing possibility that the phosphorylation of protein Si may play a role in inactivating the ribosome and excluding them from the ribosome cycle. Consistent with this idea was the observation that phosphorylation of Si has a slow turn-over rate.

Labelling of protein F of the 40S subunit of rabbit reticulocyte ribosomes was stimulated by sodium fluoride. This occurred even in the presence of cycloheximide, a potent inhibitor of protein synthesis which prevents the "runoff" of ribosomes from the mRNA, thus dissociating phosphorylation of protein F from disaggregation of polysomes by sodium fluoride. Likewise, the phosphorylation of protein Si was not influenced by sodium fluoride. The interpretation of these results was difficult, but they seemed to link phosphorylation of protein F with the inhibition of protein synthesis.

At this time there were two other reports which

described physiological effects on the phosphorylation of ribosomal proteins *in vivo*. Blat and Loeb (1971) found that injection of glucagon into rats increased the incorporation of ^{32}P into liver ribosomes. However, in this work, the labelled proteins were less well characterised and the location of the protein on the ribosomal subunits was not examined. Moreover, since a different type of gel electrophoresis was used, correlations with the work of Kabat could not be made. The results of Blat and Loeb (1971) did, however, raise the possibility that cyclic AMP might be a mediator of phosphorylation of ribosomal proteins, a possibility which Kabat (1970) had earlier discounted. Similarly, Correze *et al.* (1972) found that thyroid hormones stimulated the labelling of rat liver ribosomal proteins by $[^{32}P]$ - orthophosphate, but did not analyse the labelled protein by gel electrophoresis.

The only other relevant report at this time was an apparent physiological effect on the activity of the protein kinase associated with ribosomes. Li and Amos (1971) found that labelling by $[^{32}P]$ - orthophosphate of the ribosomal protein of chick embryo fibroblasts *in vitro* was higher when the ribosomes (and associated kinase) were from cells which had been grown in the presence of calf serum than when these were from cells grown without serum.

1.3.4 The Aims of the Project.

The work described in this thesis is intended to extend our knowledge of the phosphorylation of ribosomal proteins. The first objective was to ascertain the number

of proteins phosphorylated *in vivo*, to try to reconcile this with the number of proteins phosphorylated *in vitro*. It was further hoped to determine whether the phosphorylation of ribosomal protein was stimulated by the inhibition of protein synthesis as previously suggested from experiments with sodium fluoride, by performing comparative studies with other, more specific, inhibitors of protein synthesis. Lastly, it was decided to examine physiological conditions which might be expected to affect phosphorylation of proteins, especially situations where the concentrations of cyclic AMP were changed, or the rate of protein synthesis altered.

In pursuing these objectives, two different types of cells were used. Krebs II Ascites cells were used because they are more typical of eukaryotic cells than the non-nucleated reticulocyte, in which Kabat (1970) had only found two phosphorylated ribosomal proteins. These cells also had the advantage that they were easier to label than rat hepatocytes, in which Blat and Loeb (1971) had found one ribosomal phosphoprotein. To study physiological effects on the phosphorylation of ribosomal protein, Ascites cells were unsuitable, as their viability outside the mouse peritoneum is limited. Instead, cultured BHK fibroblasts (a non-neoplastic cell) were used.

SECTION 2.

EXPERIMENTAL.

2.1 MATERIALS.

2.1.1 Biological.

Mice used for the growth of Krebs II Ascites cells were female albinos of the Porton strain. They were 7 - 8 weeks old on inoculation, and weighed 25 - 30g. Krebs II Ascites cells are neoplastic cells derived from the solid Krebs II carcinoma, and are grown in the peritonea of mice as a homogeneous suspension in the ascitic fluid (see Klein and Klein, 1951). BHK cells are an established line of hamster fibroblasts (Macpherson and Stoker, 1962).

2.1.2 Chemical.

(a) General. Triton X-100, 2-mercaptoethanol, PPO, POPOP, and BBOT were obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks; β-alanine, toluene and methoxyethanol from British Drug Houses Chemicals, Poole, Dorset; and alkaline phosphomonoesterase (E.C.3.1.3.1.) Calf Intestinal Mucosa, DL-o-phosphoserine, DL-o-phosphothreonine, and ammonia-free glycine from Sigma Chemical Co. Ltd., Kingston-Upon-Thames, Surrey. Dibutyryl cyclic AMP was purchased from Boehringer Corp. (London) Ltd., and 3 isobutyl-1-methylxanthine was a gift from Dr. J.P. Durham. All other chemicals were reagent grade chemicals from either British Drug Houses or Sigma. Prior to use, reagent grade sucrose was treated with Norit A activated charcoal to remove material that absorbed at 260nm. (Stirewalt et al., 1967).

(b) Radiochemicals. These were purchased from the Radiochemical Centre Ltd., Amersham, Bucks. $[^{32}P]$-orthophosphate

was supplied in 10mCi lots and contained 70-90 Ci/mg P. L-[4,5-^{3}H]- Leucine contained 55 Ci/mmol.

(c) Inhibitors of Protein Synthesis. Puromycin dihydrochloride and cycloheximide were obtained from Nutritional Biochemicals Corp., Indianapolis, Ind. U.S.A. Sodium fluoride was a reagent grade chemical from British Drug Houses, and pactamycin was a gift from Dr. G.S. Ponken, Upjohn Corp., Kalamazoo, Mich., U.S.A.

(d) Reagents for Electrophoresis. The following chemicals were obtained from the Eastman-Kodak Co., Rochester, N.Y., U.S.A: Riboflavin, methylene-bisacrylamide, TEMED, ammonium persulphate and Bromophenol Blue. Acrylamide was supplied by Koch-Light, SDS by British Drug Houses Chemicals, and Coomassie Brilliant Blue R-250 by Sigma. Pyronin Y and Amido Black (Naphthalene Black 10B) were supplied by G.T.Gurr Ltd., London. Urea was supplied by Carlo Erba S.A., Milan. Before use, an 8M solution of urea was passed through a column of Bio-rad AG501-X8 resin (Bio-rad Laboratories, Richmond, Calif., U.S.A.) to remove accumulated cyanates.

(e) Protein Standards. Proteins used as molecular weight markers for SDS gels were: Bovine serum albumin, Fraction V from Armour Pharmaceutical Co. Ltd., Eastbourne; horse heart cytochrome c from Miles Research Products, Stoke Poges, Slough; and chymotrypsinogen A from Cambrian Chemicals, Croydon, Surrey.

(f) Autoradiography. Kodirex KD 54T X-Ray film, Kodak DX-80 developer and FX-40 Fixer were used throughout.

2.2 Composition of Standard Buffer Solutions.

(a) Balanced Salt Solution (BSS) was prepared according to Earle (1943) and contained 0.116M NaCl, 5.4mM KCl, 1mM $MgSO_4$, 1mM NaH_2PO_4, 1.8mM $CaCl_2$ and 0.002% phenol red. The pH was adjusted to 7.0 with 8.4% (w/v) $NaHCO_3$. It was stored as a 10 fold concentrate, 50ml being diluted to 450ml with distilled water immediately before use. 50ml of 10 fold BSS diluted to 400ml is designated as BSS/B, and BSS (minus phosphate) is BSS from which NaH_2PO_4 has been omitted.

(b) Phosphate Buffered Saline (PBS) was prepared according to Dulbecco and Vogt (1954) and contained 0.17M NaCl, 3.4mM KCl, 10mM NaH_2PO_4, 2.4mM KH_2PO_4, 0.49mM $MgCl_2$ and 0.68mM $CaCl_2$, the pH being adjusted to 7.4.

(c) Tris Buffered Saline (TBS) contained 35mM Tris-HCl (pH 7.5) and 146mM NaCl.

(d) 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.

(e) Medium K contained 30mM Tris-HCl (pH 7.5), 125mM KCl, 5mM magnesium acetate and 5mM 2-mercaptoethanol.

TBS, RSB and Medium K were stored as 10 fold concentrated solutions at - 10°C to prevent bacterial contamination.

Fig. 2.3.2.1 Formulation of Eagle's MEM.

Amino Acid.	Concentration (mg/l)
L-arginine.HCl	126.40
L-cystine	24.02
L-glutamine	292.30
L-histidine.HCl.H_2O	38.30
L-isoleucine	52.50
L-leucine	52.50
L-lysine.HCl	73.06
L-methionine	14.90
L-phenylalanine	33.02
L-threonine	47.64
L-tryptophan	10.20
L-tyrosine	36.22
L-valine	46.90

Vitamins	Concentration (mg/l)	Salts and other Components	Concentration (mg/l)
D-Ca pantothenate	2.00		
Choline chloride	2.00	$CaCl_2$	200
Folic acid	2.00	D-glucose	4,500
i.inositol	4.00	$MgSO_4$	98.0
Nicotinamide	2.00	KCl	400
Pyridoxal.HCl	2.00	NaCl	6,800
Riboflavin	0.20	NaH_2PO_4	140
Thiamine.HCl	2.00	Phenol red (Na)	10

METHODS.

2.3 Growth of Cells.

2.3.1 Growth of Krebs II Ascites Cells.

Ascites cells were maintained by the intraperitoneal injection of 0.2ml of ascitic fluid (approx. 25 x 10^6 cells) into mice at 7 day intervals.

2.3.2 Growth of BHK Cells.

BHK-21/C13 cells were cultured as monolayers in rotating 80oz. roller bottles according to the method of House and Wildy (1965). 180ml of Eagle's medium (Busby et al., 1964) were used per bottle. This medium contains BSS/B, Eagle's Minimal Essential Medium (MEM) (Fig. 2.3.2.1), 2.8g/l Tryptose phosphate broth (Difco bacto), 10^5 units/l Penicillin, 100mg/l Streptomycin, 100ml/l calf serum (Gibco Bio-cult, Glasgow) and 40ml/l 5.6% (w/v) $NaHCO_3$. The bottles were "gassed" with 50ml 5% (v/v) CO_2 in oxygen to maintain the buffering capacity of the medium. The bottles were seeded with 5 x 10^7 cells and rotated at 37°C for 2 days. The cells were removed from the glass with a rubber scraper, suspended in Eagle's medium, and dispersed into further bottles. Although the medium contains antibiotics, cells were tested 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 agar plates. Any cultures showing positive reactions to these checks were discarded.

The cells grew exponentially for three days, and cells which are designated 'growing' were harvested after two or three days' growth. They reached confluence after about four days' growth, and cells designated 'resting' were harvested after four to eight days' growth.

2.4 Labelling of Cells.

2.4.1 Labelling of Ascites Cells.

Seven days after inoculation, mice were killed by cervical dislocation. The abdominal skin was swabbed with 70% alcohol and drawn back. The ascitic fluid was removed into precooled tubes and diluted with ice-cold TBS. Very bloody tumours were rejected. All subsequent operations were carried out at 0-4°C. The cells were washed in TBS by being repeatedly pelleted by centrifugation at 1,000g for 10mins. and resuspended in TBS until virtually no red blood cells remained (usually 2-3 centrifugations). The cells were then resuspended at a concentration of 10^7 cells/ml in 400ml of a medium containing BSS (minus phosphate), 10% (v/v) calf serum (minus phosphate), 10^5 units/l Penicillin, 100mg/l Streptomycin, and buffered with Tris-magnesium citrate (Hogan and Korner, 1968). Carrier-free [^{32}P]-orthophosphate was then added to a final concentration of 0.05mCi/ml, and the cells incubated at 37°C for 3h, with constant stirring in a 2 litre conical flask containing 200ml of culture.

2.4.2 Labelling of BHK Cells.

The medium from two roller bottles was poured off, and replaced with 50ml of Eagle's medium, supplemented with

10% (v/v) calf serum, but lacking inorganic phosphate. 5mCi [^{32}P]-orthophosphate was added to each bottle and the cells rotated at 37°C for 3h.

2.5 Preparation of Ribosomes.

2.5.1 Preparation of Ribosomes from Ascites Cells.

Ribosomes were prepared from Krebs II Ascites cells as described by Mathews and Korner (1970). After incubation, Ascites cells were pelleted by centrifugation at 1,000g for 10mins. and resuspended in a minimal volume of TBS. To this was added 1.5 vols. of RSB and the cells transferred to a Dounce glass/glass homogeniser, and suspended with two up-and-down strokes. After swelling in the hypotonic medium for 15mins., the cells were disrupted by a further 20 strokes in the Dounce homogeniser, and the tonicity restored by the addition of 1/9th vol. of 10 fold concentrated medium K.

The homogenate was centrifuged at 30,000g for 10mins. and the supernatant removed by pipette. Care was taken to avoid the pellet of cell debris, and the thin film of fat. Ribosomes were prepared by centrifuging the supernatant at 150,000g in a Spinco Ti50 rotor for 2.5h. The pelleted ribosomes were resuspended in medium K, and centrifuged as before through a 5ml cushion of 1M sucrose in medium K.

In initial experiments, the ribosomes from the first ultracentrifugation step were layered on the sucrose cushions to give two ribosome pellets after the second

ultracentrifugation. However, this appears to have resulted in the preparation of impure ribosomes (see Fig. 3.1.4). Therefore in subsequent experiments if, say, eight pellets were obtained from the first ultracentrifugation step, these were layered on the sucrose so as to give eight purified ribosome pellets after the second ultracentrifugation step. The purified ribosome pellets were rinsed with medium K, and stored at -70°C until required.

Typical experiments started with 400ml of medium containing 10^7 cells/ml (i.e. a total of 4×10^9 cells), and normally afforded 8 ribosome pellets containing a total of about 750 E_{260} of ribosomes.

2.5.2 Preparation of Ribosomes from BHK Cells.

Ribosomes were prepared by a modification of the method of Ascione and Arlinghaus (1970). BHK cells were removed from bottles into ice-cold PBS using a rubber scraper, and pelleted by centrifugation at 1,000g for 10mins. The pelleted cells were resuspended in a hypotonic medium containing 20mM Tris-HCl (pH 7.5), 50mM KCl, 5mM $MgCl_2$, and 5mM $CaCl_2$, and allowed to swell on ice for 10mins. Then, an equal volume of 1% (v/v) Triton X-100 containing 40mM Tris-HCl (pH 7.5), 0.5M sucrose, 0.55M KCl, 10mM $MgCl_2$, 5mM $CaCl_2$ and 1mM EDTA was added. The ribonuclease inhibitor, dextran sulphate, was added to a final concentration of 50µg/ml, and after standing on ice for 5mins., the cellular slurry was lysed by 3 strokes of a Dounce homogeniser. The nuclear fraction was then prepared by centrifugation at

1,000g for 10mins. To increase the yield of polysomes, the nuclei were washed by resuspending twice, using the Dounce homogeniser, in 10ml of a buffer containing 40mM Tris-HCl (pH 7.5), 0.275M sucrose, 0.1M KCl, 50mM NaCl, 5mM $MgCl_2$, 5mM $CaCl_2$, 0.5% Triton X-100, and 0.05% sodium deoxycholate, and an extra 20µg/ml dextran sulphate was also added. The washed nuclei were recentrifuged as before, and the cytoplasmic supernatants were combined. Cytoplasmic membranes were dispersed using 0.25% sodium deoxycholate. The detergent-treated cytoplasm was centrifuged in a Spinco Ti50 rotor at 150,000g for 2h, and translucent polysome pellets were obtained. To further purify the polysomes, these pellets were gently resuspended in a Teflon/glass homogeniser in a buffer containing 10mM Tris-HCl (pH 7.5), 10mM KCl, 1mM $MgCl_2$, and 0.1mM EDTA, and pelleted by centrifugation through a 2M sucrose solution (similarly buffered) at 65,000g for 18h in the Ti50 rotor. 24 roller bottles containing a total of approx. 5×10^9 cells were the starting material. Such a number of cells would normally afford about 300 E_{260} units of ribosomes, which were stored at -70°C until required.

2.6 Sucrose Density Gradient Analysis of Polysomes.

Polysome pellets were suspended at 0°C in a buffer containing 10mM Tris-HCl (pH 7.6), 80mM KCl, and 5mM $MgCl_2$. Approximately 0.1-0.2ml of suspension containing 1 E_{260} of ribosomes was then layered on to 4.4ml of a linear 15-30% (w/v) sucrose gradient containing 10mM Tris-HCl (pH 7.6), 0.5M KCl, 5mM $MgCl_2$ and 20mM 2-mercaptoethanol.

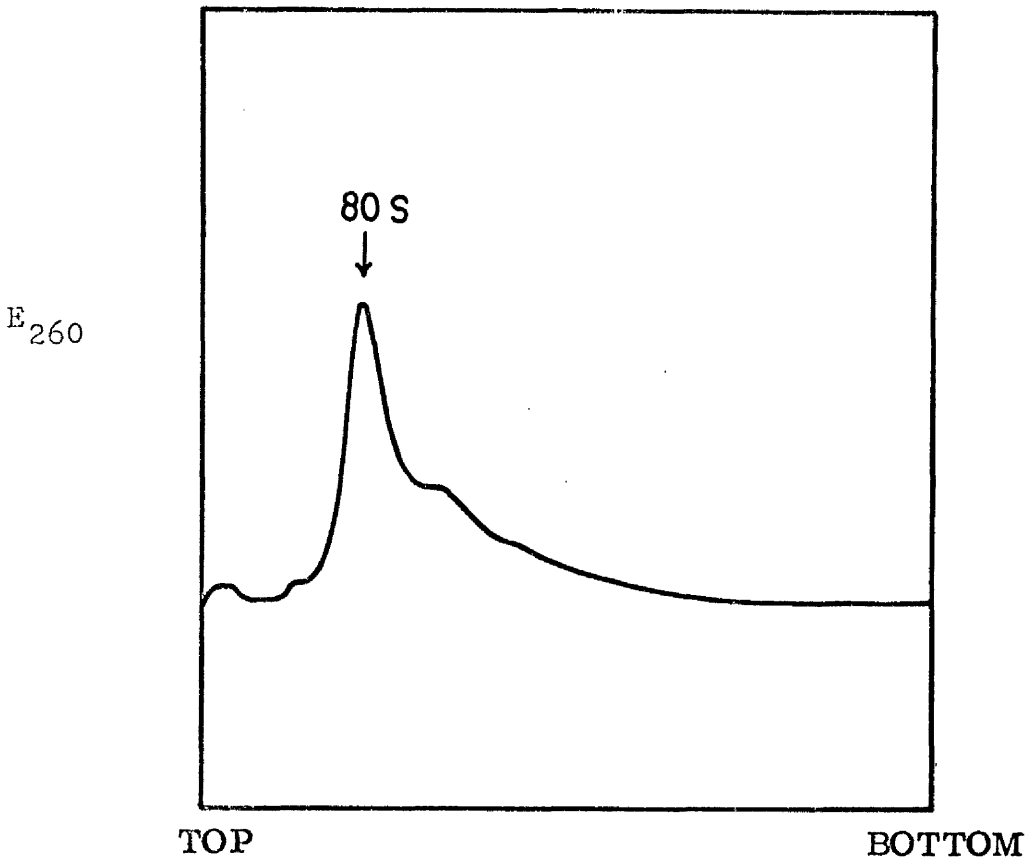


Fig. 2.6.1 Sucrose Density Gradient Analysis of Polysomes from Ascites Cells.

For details see Section 2.6.

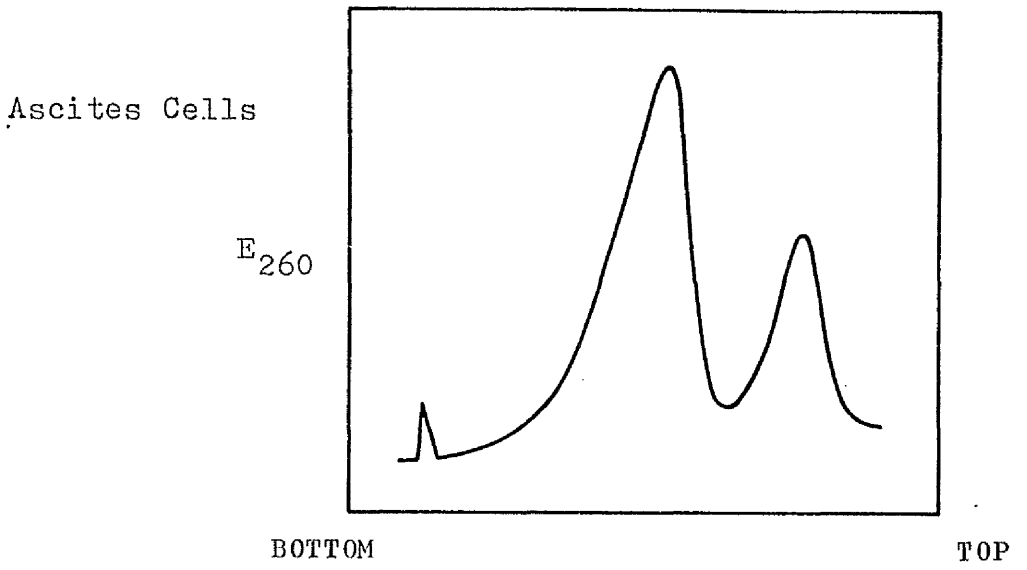
The gradients were centrifuged at 234,000g for 1h at 20°C in a Spinco SW50.1 rotor. After centrifugation, the gradients were analysed at 254nm. on an ISCO Model D gradient fractionator (Instrumentation Specialties Co., Lincoln, Nebraska, U.S.A.) The separation of polysomes from Ascites cells is shown in Fig. 2.6.1.

2.7 Preparation and Concentration of Ribosomal Subunits.

2.7.1 Preparation of Ribosomal Subunits.

Ribosomal subunits were prepared from both BHK and Ascites cells by the method of Leader and Wool (1972). Ribosomes were suspended at 0°C in 10mM Tris-HCl (pH 7.6), 80mM KCl and 5mM $MgCl_2$. The concentration of KCl was adjusted to 910mM by addition of 0.5 vols. of 2.5M KCl, and the suspension clarified by centrifugation at 1,000g for 5mins. To remove nascent peptide and promote separation of the subunits, the clarified ribosome suspension was incubated at 37°C for 15mins. with 0.1mM puromycin and 20mM 2-mercaptoethanol. Without cooling, aliquots of the suspension containing 75 E_{260} units of ribosomes were layered on to 37ml of a linear 10-30% (w/v) sucrose gradient containing 10mM Tris-HCl (pH 7.6), 830mM KCl, 5mM $MgCl_2$ and 20mM 2-mercaptoethanol.

After centrifugation at 105,000g for 5h at 20°C in a Spinco 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 collected. These fractions were pooled and dialysed overnight at 2°C against 2l of



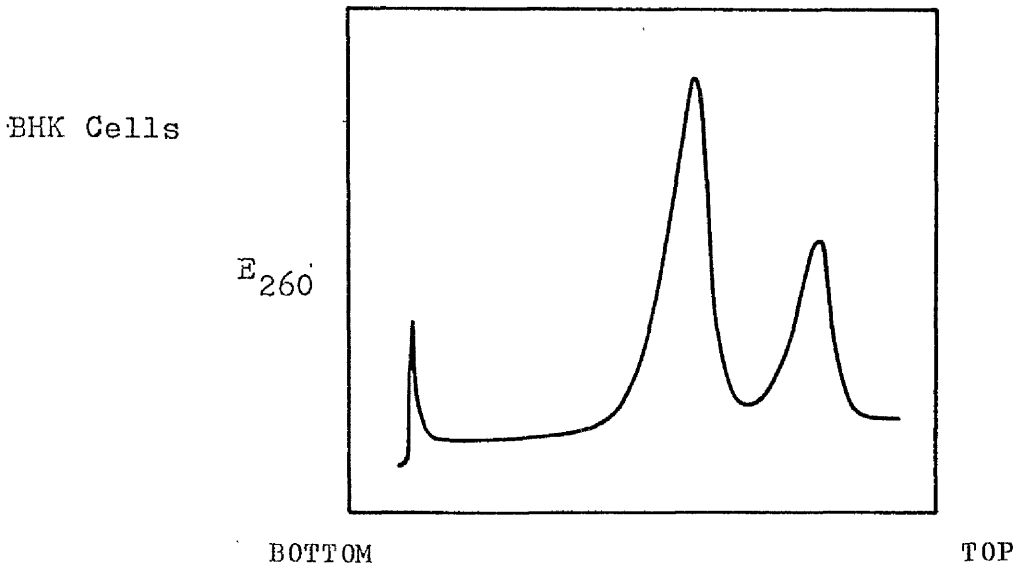


Fig. 2.7.1.1 Separation of Ribosomal Subunits from Ascites Cells and BHK Cells.

For details see Section 2.7.1

buffer containing 50mM Tris-HCl (pH 7.6), 80mM KCl, 12.5mM $MgCl_2$ and 10mM 2-mercaptoethanol. Densitometric traces of typical separations of ribosomal subunits from Ascites and BHK cells are shown in Fig. 2.7.1.1. Yields varied considerably from 50% for subunits of ribosomes from Ascites cells, to more than 80% for subunits of ribosomes from BHK cells.

2.7.2 Concentration of Ribosomal Subunits.

Ribosomal subunits were precipitated by adding to them 0.2 vols. of absolute ethanol and 0.02 vols. of buffer containing 0.5M Tris-HCl (pH 7.6), 0.8M KCl, and 125mM $MgCl_2$. The subunits were kept at $-10^{o}C$ for 2h, and pelleted by centrifugation at 30,000g for 20mins. The supernatant was then recentrifuged to ensure maximum recovery of the subunits, which was usually greater than 80%.

2.8 Extraction of Protein from Ribosomes and Ribosomal Subunits.

Protein was extracted from ribosomes and ribosomal subunits as described by Sherton and Wool (1974a). Ribosomes or ribosomal subunits were suspended at a concentration of 75 E_{260} units/ml in a buffer containing 10mM Tris-HCl (pH 7.7) and 100mM magnesium acetate. To this was added 2 vols. glacial acetic acid, and the mixture stirred at $0^{o}C$ for 1h. Ribosomal RNA was removed by centrifugation at 15,000g for 10mins. The supernatant was decanted and the pellet extracted with acetic acid/Tris-magnesium acetate as before. The supernatants were pooled and dialysed for 48h against 1 litre

of 1M acetic acid with 5 changes, and then lyophilised.

To ensure maximum retention of protein, the dialysis tubing was acetylated by the method of Craig (1967) before use.

Overall yields of protein were quite variable, but routinely more than 1mg of protein from both subunits could be obtained from Ascites cells, and 0.5mg of protein of both subunits from BHK cells. It was dissolved at a concentration of 1mg/ml in H_2O, and the radioactivity of the extracted protein was determined by collecting 50µl of protein solution on Whatman GF/C glassfibre filters, and the radioactivity estimated in 10ml of a scintillant containing (per litre) 666ml toluene, 333ml Triton X-100, 4g PPO, and 100mg POPOP with an efficiency of about 70% for ^{32}P.

2.9 One-Dimensional Gel Electrophoresis.

2.9.1 Electrophoresis in SDS Gels.

SDS gels were prepared by the method of Laemmli (1970). The separation gel was cast the day before it was to be used, and contained 10% (w/v) acrylamide, (2.6% (w/w) bisacrylamide) or 15% (w/v) acrylamide (6% (w/w) bisacrylamide), 0.1% SDS, 0.03% TEMED, 375 40mM Tris-HCl (pH 8.5) and 0.05% ammonium persulphate.

Stacking gels were cast on the day they were to be used, and contained 2.8% (w/v) acrylamide (2.1% (w/w) bisacrylamide), 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 10cm. and 2cm. respectively; and they were 6mm. in

diameter.

Immediately prior to electrophoresis, lyophilised ribosomal protein was heated at 100°C for 5mins. in a reducing mixture containing 50mM Tris-HCl (pH 8.5), 2% SDS, 5% (v/v) 2-mercaptoethanol and 0.001% Bromophenol Blue tracker.

The running buffer contained 25mM Tris-HCl (pH 8.5), 0.19M glycine and 0.1% SDS. The gels were electrophoresed at 4 mA/gel and required 2-2.5h for the tracker to reach 0.5cm. from the bottom of the gel. The gels were then removed from their tubes and processed as described in Section 2.11.

2.9.2 Electrophoresis in Urea Gels.

Urea gels were prepared according to Low et al. (1969). Separation gels were prepared the day before they were to be used, and contained 10% (w/v) acrylamide (1.5% (w/w) bisacrylamide), 7M urea, 0.5% TEMED, 0.125M KOH, 3M acetic acid (pH 4.5) and 0.04% ammonium persulphate. Stacking gels were cast just prior to use and contained 2.5% (w/v) acrylamide (25% (w/w) bisacrylamide), 7M urea, 0.06% TEMED, 0.125M KOH, 0.25M acetic acid and 0.0025% riboflavin. The dimensions of the gels were the same as those of SDS gels.

Prior to electrophoresis, proteins, in solution at a concentration of 1mg/ml, were adjusted to 60mM 2-mercapto-ethanol, and the pH adjusted to 8.5 by addition of solid Tris. The proteins were then exhaustively reduced by incubation at 37°C for 3h.

Pyronin Y was used as a tracker, and in a running

buffer containing 88mM β-alanine and 35mM acetic acid (pH 4.5) electrophoresis required about 3h at 4 mA/gel.

2.10 Two-Dimensional Gel Electrophoresis.

2.10.1 The Method of Kaltschmidt and Wittmann (1970b).

The first dimension gel contained 4% (w/v) acrylamide (3.3% (w/w) bisacrylamide), 6M urea, 20mM EDTA, 0.52M boric acid, 0.4M Tris-HCl (pH 8.7) and 0.045% TEMED (modification of Howard and Traut, 1973). Polymerisation was catalysed by 50µl of 10% (w/v) ammonium persulphate per 10ml gel solution.

In preparation for electrophoresis, lyophilised ribosomal protein was dissolved at a concentration of 1-2mg/ml in a solution containing 8M urea, 10mM sodium bicarbonate (pH 8.3) and 1mM 2-mercaptoethanol. Generally, 125-200µg of protein were analysed in each gel. Electrophoresis was towards the cathode at 6 mA/gel for 3h, using Pyronin Y to check that the direction of electrophoresis was correct, and the running buffer was 6mM EDTA, 77mM boric acid, and 60mM Tris-HCl (pH 8.2).

The gels were carefully removed from the gel tubes and equilibrated with 100ml of 8M urea, 13mM acetic acid and 12mM KOH (pH 5.2) for a total of 1h with two changes of buffer.

The frame in which the second-dimension slab gel was cast comprised two plain glass plates (8.2cm. x 8.2cm.) sandwiched together with glass spacers held by waterproof electrical insulating tape, allowing a gel sheet of 4mm. thickness to be formed.

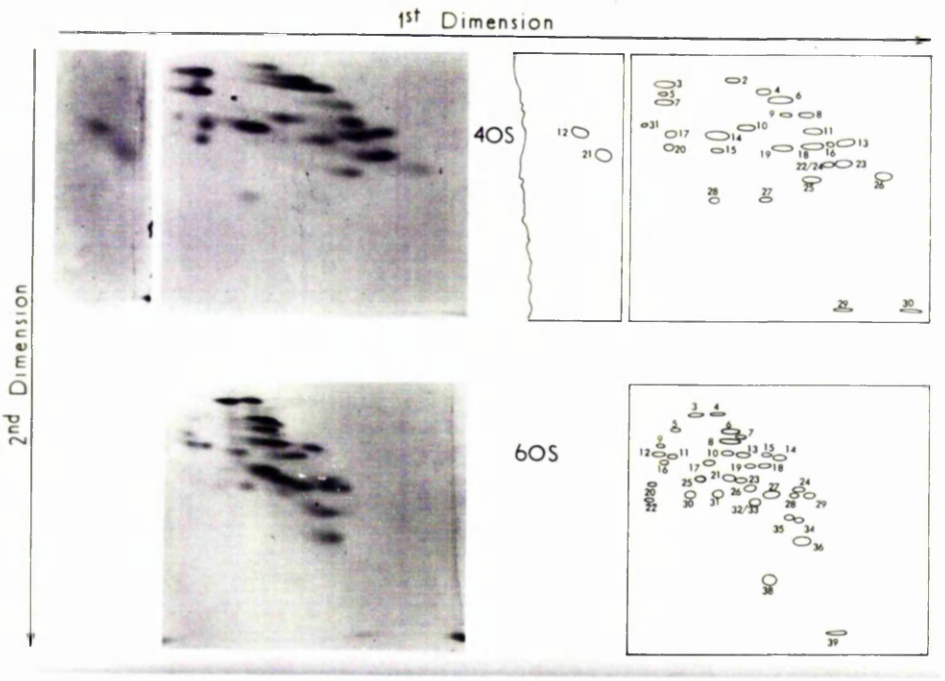


Fig. 2.10.1.1 Two-Dimensional Gel Electrophoresis of 40S and 60S Ribosomal Subunit Proteins from Rat Liver by the Method of Kaltschmidt and Wittmann.

For details see Section 2.10.1. The panels on the left-hand side of the figure are photographs of stained gels, while the panels on the right-hand side are schematic representations of those gels, giving the nomenclature of the proteins used in this thesis. The two proteins of the 40S subunit, S12 and S21, were detected by reversing the electrodes in the first dimension.

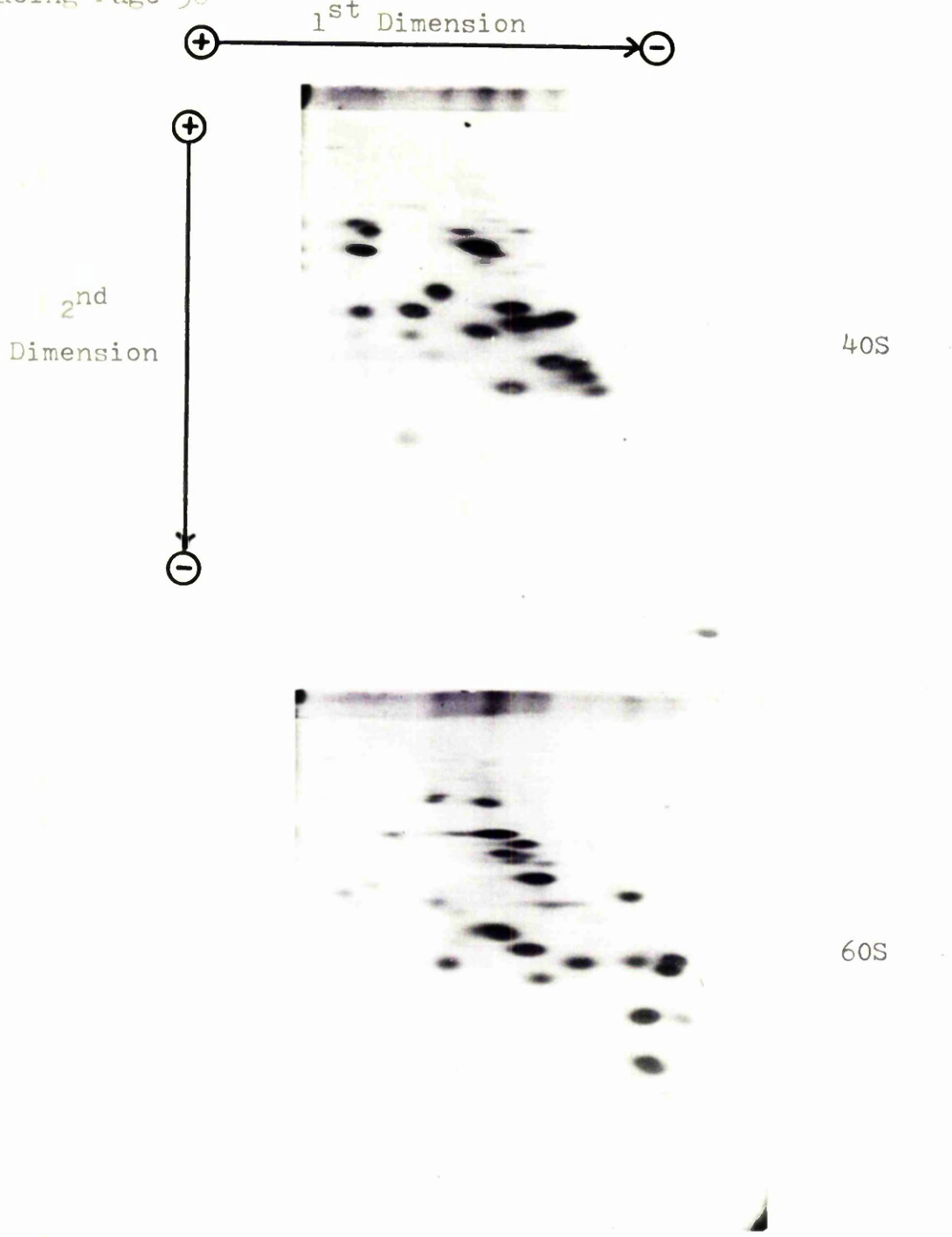


Fig. 2.10.1.2 Two-Dimensional Gel Electrophoresis of the 40S and 60S Ribosomal Subunit Protein from Ascites Cells by the Method of Kaltschmidt and Wittmann.

For details see Section 2.10.1.

The second-dimension gel contained 18% (w/v) acrylamide (1.4%(w/w) bisacrylamide), 6M urea, 0.93M acetic acid, 58mM KOH (pH 4.5) and 0.58% TEMED (Howard and Traut, 1973). Polymerisation was catalysed by 3ml of 10% (w/v) ammonium persulphate per 100ml of gel solution.

Once the first dimension gel had been equilibrated, it was annealed to the top of the slab gel by means of more second dimension gel solution. Electrophoresis in the second dimension was towards the cathode at 9 mA/gel for 18h. Fig. 2.10.1.1 shows the separation of the ribosomal proteins of rat liver in this system, and gives the nomenclature of the proteins, as described by Shorton and Wool (1972, 1974b). Fig. 2.10.1.2 shows the separation of the ribosomal proteins of Ascites cells in this system.

2.10.2 <u>The Method of Martini and Gould (1971).</u>

Electrophoresis in the first dimension was in a separation gel containing 6.5% (w/v) acrylamide (3.3% (w/w) bisacrylamide), 6M urea, 60mMpotassium acetate buffer (pH 4.3), 0.5% TEMED, and 1.15% ammonium persulphate. A stacking gel was employed, and this contained 2.5% (w/v) acrylamide (25% (w/w) bisacrylamide), 6M urea, 60mM potassium acetate buffer (pH 4.3), 0.05% TEMED and 0.001% riboflavin.

The sample (approx. 200µg in 0.1ml. H_2O) was dialysed against 12mM potassium acetate buffer (pH 5.5), 10mM 2-mercaptoethanol and 6M urea overnight at 0°C. The running buffer was 70mM β-alanine acetate (pH 4.5). Electrophoresis was at 2.5 mA/gel towards the cathode for

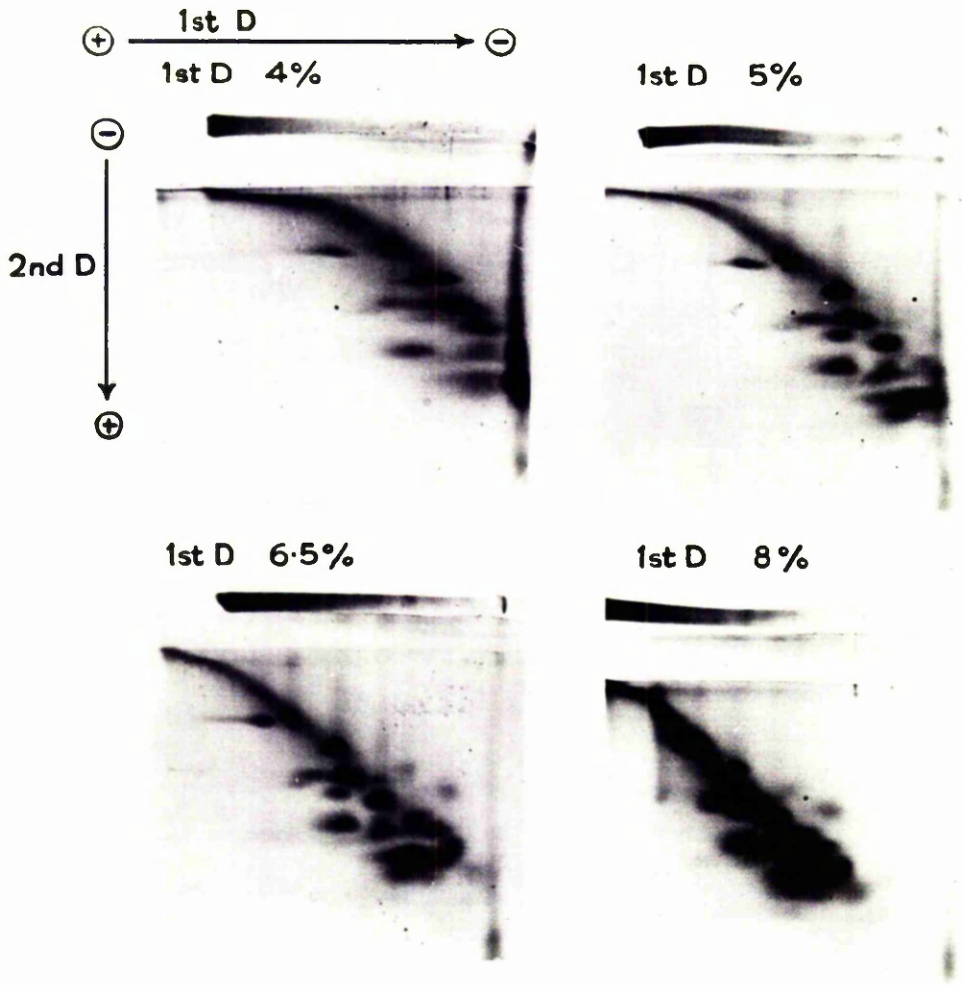


Fig. 2.10.2.1 Two-Dimensional Gel Electrophoresis of 80S Ribosomal Protein of Ascites Cells by the Method of Martini and Gould: Effect of Varying the Concentration of Acrylamide in the First Dimension.

For details see Section 2.10.2.

1-1.5h with Pyronin Y as a tracker. The gels were next equilibrated for second dimension electrophoresis. The incubation began in a buffer containing 1% SDS, 0.1M sodium phosphate buffer (pH 7.1), 5M urea and 10mM 2-mercapto-ethanol, continued in a buffer containing 1% SDS, 0.01M sodium phosphate buffer (pH 7.1), 5M urea and 10mM 2-mercaptoethanol, and ended in 0.1% SDS, 0.01M sodium phosphate buffer (pH 7.1), 5M urea and 10mM 2-mercapto-ethanol. Incubation was at 40°C for 25mins. in each buffer with vigorous stirring.

The second dimension slab gel contained 10% (w/v) acrylamide (3.3% (w/w) bisacrylamide), 0.1% SDS, 0.1M sodium phosphate buffer (pH 7.1), 6M urea, 0.1% TEMED and 0.05% ammonium persulphate. A stacking gel was also used in the second dimension, the same gel being used to anneal the tube gel to the slab gel. This contained 3% (w/v) acrylamide (20% (w/w) bisacrylamide), 0.1% SDS, 0.1M sodium phosphate buffer (pH 7.1), 6M urea, 0.1% TEMED and 0.0005% riboflavin. The running buffer was 0.1M sodium phosphate buffer (pH 7.1), 0.1% SDS and 5mM thioglycollic acid. Electrophoresis was towards the anode at 1/4 mA/gel for 24h.

In initial experiments, a first dimension acrylamide concentration of 4% (w/v) was used, as recommended by Martini and Gould (1971). However, it was found that at this concentration of acrylamide many proteins electrophoresed with the tracker (Fig. 2.10.2.1). Therefore a number of different first dimension acrylamide concentrations were tried, to determine which would give the best resolution. By inspection of Fig. 2.10.2.1., this is seen

to be at 6.5% (w/v) acrylamide. This was therefore the concentration used in subsequent experiments.

2.11 Processing of Gels.

2.11.1 Staining of Gels.

Urea gels were stained for 1h in 1% (w/v) Amido Black (Naphthalene Black 10B) in 7.5% acetic acid. SDS gels and two-dimensional gels were stained for 2-3h in 0.1% Coomassie Brilliant Blue R-250 in 50% methanol and 10% acetic acid.

2.11.2 Destaining of Gels.

Cylindrical gels were destained overnight by diffusion in 7.5% acetic acid and 50% methanol at 37°C. Two-dimensional gels were destained electrophoretically in 7.5% acetic acid in a UDS 1 apparatus supplied by Universal Scientific Ltd., 231, Plashet Rd., London, E.13.

2.11.3 Autoradiography of Gels.

10% gels were prepared for autoradiography by slicing longitudinally in a device similar to that described by Fairbanks et al. (1965). Quartered gels could then be dried on to Whatman No.1 Filter Paper by a combination of heat from an infra-red lamp and suction from an electrical vacuum pump (Speedivac - W. Edwards & Co., London, Ltd.). 15% gels were halved longitudinally and sealed in thin polythene bags using an electric heat sealer (Bosch. Model FG16 - supplied by Gallenkamp Ltd., Nerston, East Kilbride). Autoradiography was on Kodirex KD 54T film, generally for

2-3 weeks for cylindrical gels, and 3-4 weeks for two-dimensional gels.

2.11.4 Densitometry of Gels.

Densitometric records of gels were obtained from either whole or halved gels using the linear transport accessory to the Gilford 240 Spectrophotometer. Gels stained with Amido Black were scanned at 600nm., and gels stained with Coomassie Brilliant Blue were scanned at 572nm.

2.11.5 Measurement of Radioactivity in Gels.

Gels were sliced transversely into 1mm. sections on an automatic gel slicer (Mickle Laboratory Eng. Co., Gernshall, Surrey), dissolved overnight at 37°C in 0.3ml. 100 vols. hydrogen peroxide, and the radioactivity determined in 10ml. of scintillant containing per litre, 660ml toluene, 330ml methoxyethanol, 4g PPO, and 100mg POPOP, with an efficiency of about 70% for ^{32}P.

2.12 Extraction of Whole Cell Protein from Ascites Cells.

Whole cell protein of Ascites cells was extracted essentially as described by Mayne et al., (1966). 5ml. ice-cold 10% trichloroacetic acid was added to 5×10^6 cells and the mixture kept on ice for 30mins. Protein was then precipitated by centrifugation at 1,000g for 10mins. The protein precipitate was washed once in acetone and once in light petroleum-diethyl ether (3:1 v/v) with reprecipitation by centrifugation at each step. The pelleted protein was resuspended in 2ml 1M NaOH and reprecipitated by addition

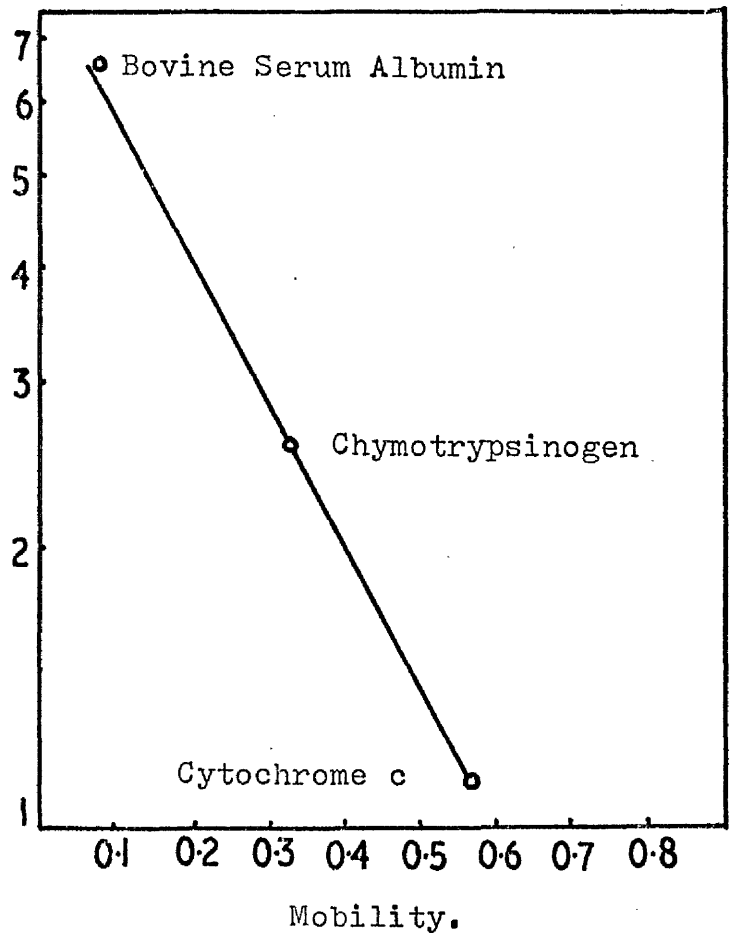


Fig. 2.13.1. Determination of Molecular Weights of Proteins in SDS Gels: Mobility of Standard Proteins.
For details see Section 2.13.

of 2ml 20% trichloroacetic acid containing 4mM leucine. After standing for 30mins., the protein was removed by filtration on Whatman GF/C glassfibre discs. 0.7ml concentrated formic acid was added to each disc in a scintillation vial and the radioactivity determined in 10ml of scintillation fluid containing (per litre) 500ml toluene, 500ml methoxyethanol, and 5g BBOT with an efficiency of 5% for ^{3}H.

2.13 Determination of Molecular Weights of Proteins in SDS Gels.

Molecular weights of the proteins were estimated by running parallel to the gels with ribosomal protein, gels of molecular weight standards which were cytochrome c (molecular weight 12,400), chymotrypsinogen A (molecular weight 25,750) and bovine serum albumin (Fraction V) (molecular weight 66,000). The molecular weights of ribosomal proteins could then be estimated by the method of Weber and Osborn (1969) (Fig. 2.13.1).

2.14 Determination of Protein.

Protein was measured by the method of Lowry et al., (1951) using bovine serum albumin (Fraction V) as a standard.

2.15 Detection of O-Phosphoserine and O-Phosphothreonine.

Ribosomal protein labelled with ^{32}P was dissolved at 1mg/ml in 2M HCl and hydrolysed by heating at 105°C for 16h in a sealed evacuated glass ampoule (Langan 1968b).

The hydrolysates were dried on a rotary evaporator and resuspended in 100µl of electrophoresis buffer (2.5% (v/v) formic acid, 7.8% (v/v) acetic acid, pH 1.85). 50µl of each sample was applied to Whatman No.3MM Chromatography paper, together with 25µl samples of o-phosphoserine (1mg/ml), o-phosphothreonine (1mg/ml) and NaH_2PO_4 (100mM).

The paper was wetted with buffer and electrophoresed at 2,500V for 2h in a high-voltage electrophoresis apparatus. After electrophoresis, the paper was allowed to dry thoroughly and then cut up for analysis. The portion containing the phosphate marker was stained with acidic ammonium molybdate (Hanes and Isherwood, 1949); the portion containing the phosphoserine and phosphothreonine markers was stained with ninhydrin/cadmium acetate (Dreyer and Bynum, 1967); and the portion containing the labelled samples was autoradiographed on Kodirex KD 5HT film for 25d.

SECTION 3.

RESULTS.

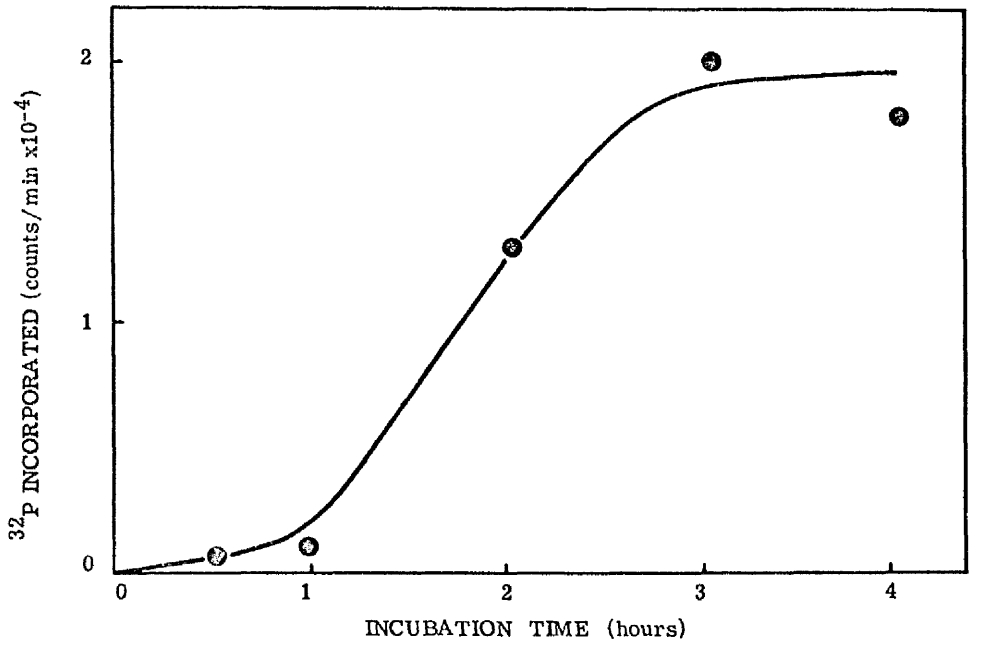


Fig. 3.1.1 Time Course of the Incorporation of [^{32}P]-Orthophosphate into the Ribosomal Protein of Ascites Cells.

Suspensions of Ascites cells (100 ml) were incubated in duplicate with 1 mCi [^{32}P]-orthophosphate (Section 2.4.1), for the times indicated. Ribosomes and ribosomal protein were prepared (Sections 2.5.2 and 2.8). The protein was resuspended in 0.5ml H_2O, dried on to Whatman GF/C glassfibre filters, and radioactivity determined in Toluene/Triton/PPO/POPOP scintillant (see Section 2.8).

Phosphate Concentration. (mM)	Counts/Min ^{32}P incorporated. (+ Serum)	Counts/Min ^{32}P incorporated. (- Serum)
0	37200	31889
0.09	29270	24198
0.9	19856	14834

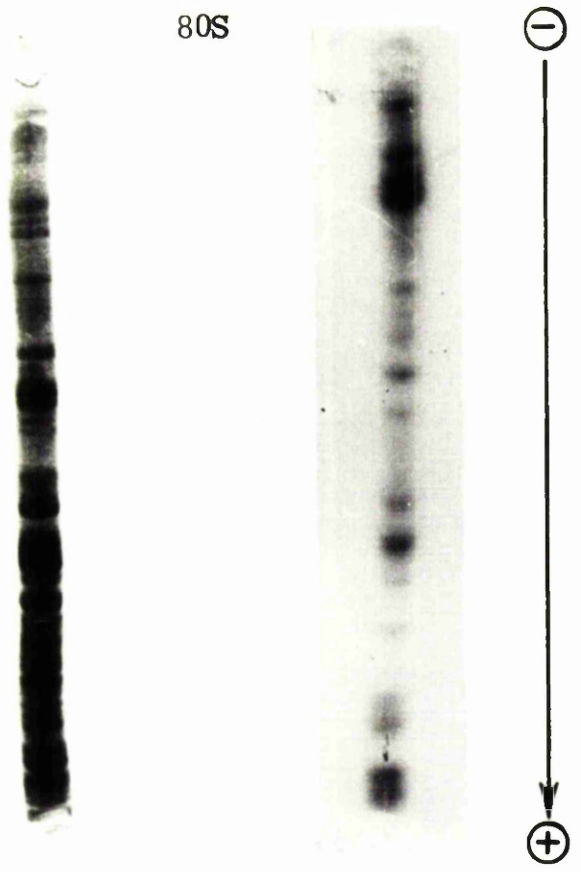
Table 3.1.1 Effect of Inorganic Phosphate and Calf Serum on the Incorporation of [^{32}P]-Orthophosphate into Ribosomal Protein of Ascites Cells.

Suspensions of Ascites cells (70 ml) were incubated in duplicate with 250 µCi [^{32}P]-orthophosphate (Section 2.4.1) for 3h in the presence or absence of 10% (v/v) calf serum and the amount of orthophosphate indicated. Ribosomes and ribosomal protein were prepared (Sections 2.5.2 and 2.8). The protein was resuspended in 0.5ml H_2O, dried on to Whatman GF/C glassfibre filters, and radioactivity determined in Toluene/Triton/PPO/POPOP scintillant (see Section 2.8).

3.1 Characterisation of the Incorporation of ^{32}P into Ribosomes of Ascites Cells.

The first objective of this work was to characterise the phosphorylation of ribosomal proteins. Initial experiments showed that ribosomal proteins were labelled when Ascites cells were incubated with [^{32}P]-orthophosphate. A number of further experiments were therefore carried out to try to determine the optimum conditions for labelling the cells. In the first of these experiments, Ascites cells were incubated for various times with [^{32}P]-orthophosphate as described in Section 2.4.1. Ribosomes were prepared, their protein extracted, and its radioactivity determined. It can be seen from Fig. 3.1.1. that maximum labelling was achieved after 3h and this incubation time was used in subsequent experiments.

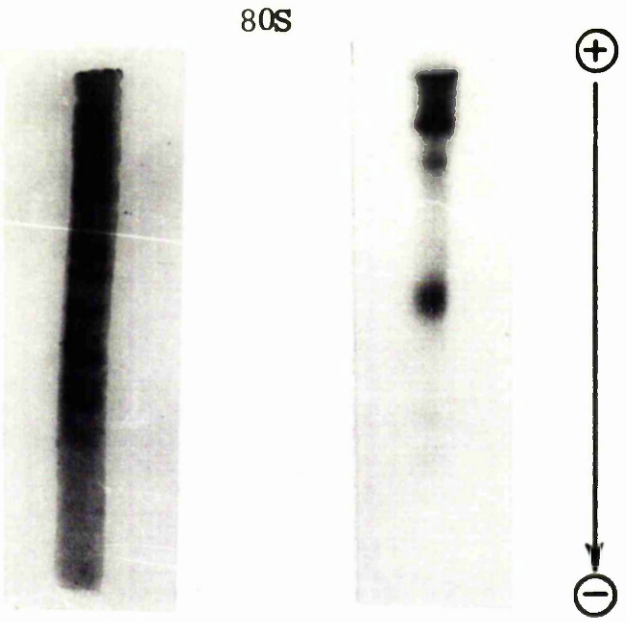
To obtain the maximal labelling of ribosomal proteins, it was necessary to find the effect of unlabelled orthophosphate present in the medium on the incorporation of [^{32}P]-orthophosphate. Ascites cells were therefore incubated for 3h in medium containing the normal level (0.9mM), 0.09mM, or no orthophosphate. Table 3.1.1 indicates that the inclusion of orthophosphate reduced the incorporation of ^{32}P by up to 50%. In subsequent experiments, Ascites cells were therefore labelled in a medium containing no phosphate except added [^{32}P]-orthophosphate. The table also shows that supplementing the medium with 10% (v/v) calf serum resulted in the cells incorporating approximately 20% more ^{32}P into ribosomal protein, and serum was therefore included in subsequent



(a) Stained Gel (b) Autoradiograph

Fig. 3.1.2 SDS Gel Electrophoresis of Phosphorylated Ribosomal Protein from Ascites Cells.

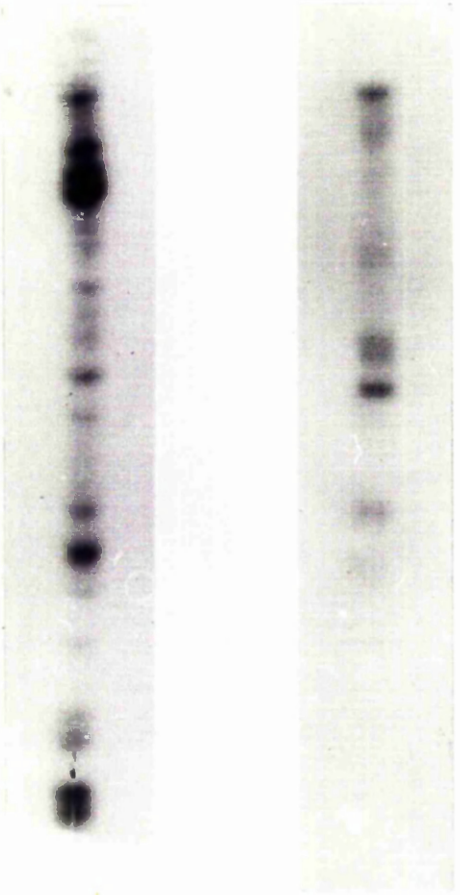
Ribosomal Protein from Ascites cells, labelled with [^{32}P]-orthophosphate was electrophoresed (Section 2.9.1), stained (Section 2.11.1), and subjected to autoradiography (Section 2.11.3).



(a) Stained Gel (b) Autoradiograph

Fig. 3.1.3 Urea Gel Electrophoresis of Phosphorylated Ribosomal Protein from Ascites Cells.

Ribosomal protein from Ascites cells, labelled with [^{32}P]-orthophosphate was electrophoresed (Section 2.9.2), stained (Section 2.11.1), and subjected to autoradiography (Section 2.11.3).



(a) (b)

Fig. 3.1.4 Effect of Method of Preparation of Ribosomes on the Extent of Phosphorylation of Ribosomal Protein from Ascites Cells.

For detailed description see Section 3.1. Ascites cells were labelled with [^{32}P]-orthophosphate and ribosomes prepared (Section 2.4.1) by slightly differing methods. Ribosomal protein was prepared (Section 2.8), electrophoresed (Section 2.9.1), and subjected to autoradiography (Section 2.11.3). The frames show autoradiographs of the SDS gels of protein from ribosomes prepared either before, (a), or after, (b), the modifications in the preparation of ribosomes were made.

experiments.

Having established suitable conditions for the labelling of ribosomal protein, the next step was to examine the phosphorylated ribosomal proteins on SDS gels. Fig. 3.1.2a shows one such gel, and it can be seen that the ribosomal protein is resolved into about 40 bands. The autoradiograph (Fig. 3.1.2b) shows that about 18 proteins were labelled with $[^{32}P]$-orthophosphate. It was, however, unclear whether all of these were true ribosomal proteins. This is because SDS gel electrophoresis does not resolve ribosomal protein from non-ribosomal protein contaminants, which may also be labelled with $[^{32}P]$-orthophosphate. This problem is less acute in urea gel electrophoresis at pH 4.5, where the basic ribosomal proteins are separated from most of the acidic components. In this system, many protein bands were again resolved (Fig. 3.1.3), although not as many as were found on SDS gels. However, autoradiography of these gels revealed only three or four radioactive bands. This result suggests that many of the bands found on the autoradiograph shown in Fig. 3.1.3b might have been due to non-ribosomal phosphoproteins loosely bound to the ribosomes.

Other results were consistent with this interpretation, for it was observed that following certain minor modifications in the preparation of the ribosomes, fewer labelled bands were seen on SDS gel electrophoresis (Fig. 3.1.4). These modifications consisted of reducing the amounts of material layered on the sucrose gradients in the last stage of the preparation of ribosomes, and

Fraction	Radioactivity Extracted (counts/min)	Percentage of total
1. Hot TCA Extract (20 min @ 90° with 10% Trichloroacetic acid)	499	13.1
2. Organic Solvent Extract (acetone, ethanol, chloroform, ether)	389	10.2
3. Residue	2920	76.7

Table 3.1.2 Effect of Extraction with Trichloroacetic Acid and Organic Solvents on the Amount of ^{32}P Associated with Ribosomal Protein from Ascites Cells.

Ribosomal protein from Ascites cells, labelled with [^{32}P]-orthophosphate was extracted in triplicate with 10% trichloroacetic acid (Kabat, 1970). Extraction with ice-cold trichloroacetic acid solubilized negligible (0.4%) amounts of radioactivity. The precipitate from the hot acid extraction was then extracted with acetone, ethanol, chloroform, and diethyl ether. The final residue was resuspended in H_2O and the radioactivity estimated in Toluene/Methoxyethanol/PPO/POPOP scintillant (see Section 2.11.5).

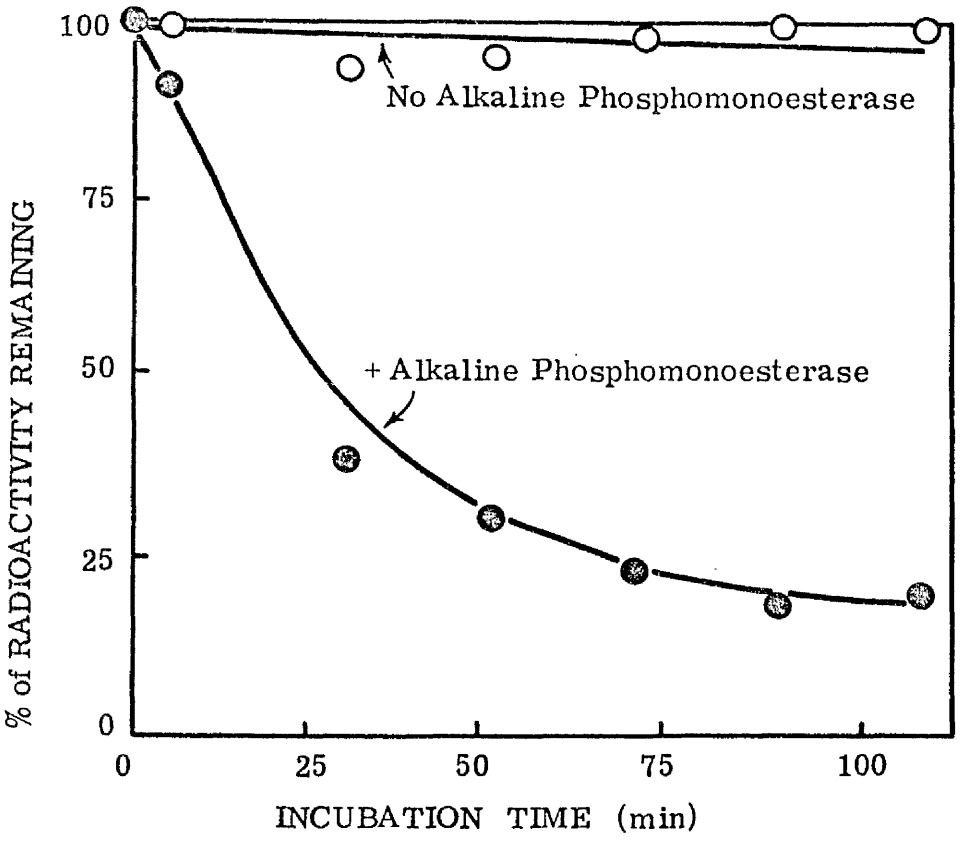


Fig. 3.1.5 Effect of Alkaline Phosphomonoesterase on the Amount of ^{32}P Associated with Ribosomal Protein from Ascites Cells.

Ribosomal protein labelled with [^{32}P]-orthophosphate was suspended at a concentration of 0.5 mg/ml in buffer containing 0.1M glycine (pH 10.4), 1mM $ZnCl_2$ and 1mM $MgCl_2$. The reaction was started by addition of 50µl of 40µg/ml alkaline phosphomonoesterase in the above buffer to 0.95ml protein suspension. Control samples received 50µl buffer. The samples were incubated at 37°C for the times indicated, then 100µl aliquots were removed and added to 2ml ice-cold 10% trichloroacetic acid and heated to 90°C for 2mins. The protein precipitate was collected on Whatman GF/C glassfibre filters and radioactivity determined in Toluene/Methoxyethanol/PPO/POPOP scintillant (see Section 2.11.5). "100% radioactivity remaining" represents about 750 counts/min.

this probably yielded purer ribosomes.

In view of this, it was decided to work with more highly purified ribosomes. Most reproducible results were obtained with purified ribosomal subunits, presumably because of the high ionic strength and temperature used in their preparation (see Section 2.7). Ribosomal subunits were therefore used in subsequent experiments.

Having obtained purified ribosomal proteins labelled with $[^{32}P]$-orthophosphate, it was possible to proceed with their characterisation and identification. Ribosomes labelled with $[^{32}P]$-orthophosphate were subjected to a series of extractions designed to remove RNA and phospholipids. Ribosomes were extracted for 20mins. at 90°C. with 10% trichloroacetic acid. This procedure solubilizes RNA, but converted only 13% of the ^{32}P-labelled preparation to soluble material (Table 3.1.2). The precipitate from this extraction was next extracted with a series of solvents for lipids, but again only a small amount (10%) of the radioactivity was converted to soluble material by this procedure. About three-quarters of the radioactivity incorporated into ribosomes is thus associated with the protein residue.

The next step in the characterisation of the ribosomal phosphoproteins was to determine whether radioactive phosphate could be released from the protein by incubation with alkaline phosphomonoesterase. As can be seen from Fig. 3.1.5., after incubation for 1h, about 75% of the ^{32}P was released by the enzyme, indicating that the phosphate was originally in ester linkage with the protein.

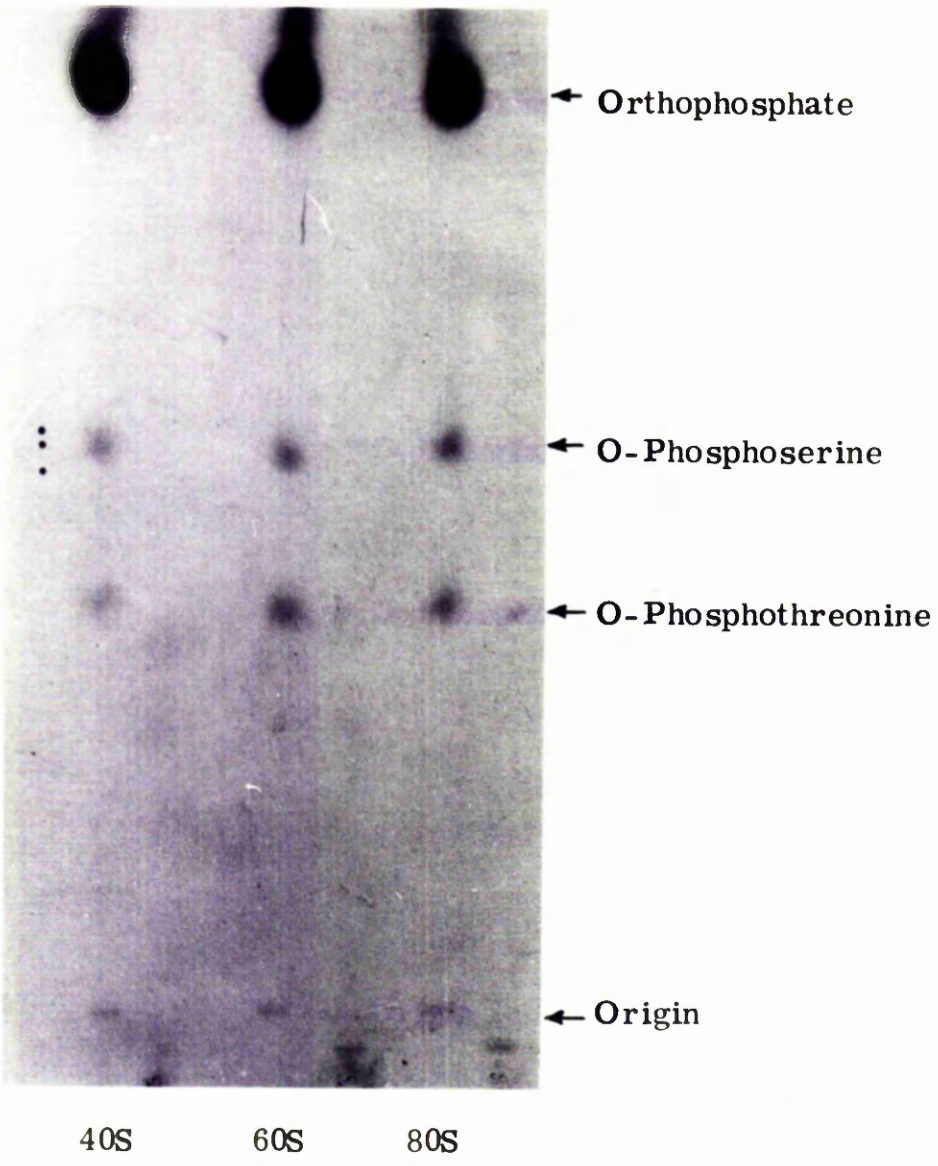
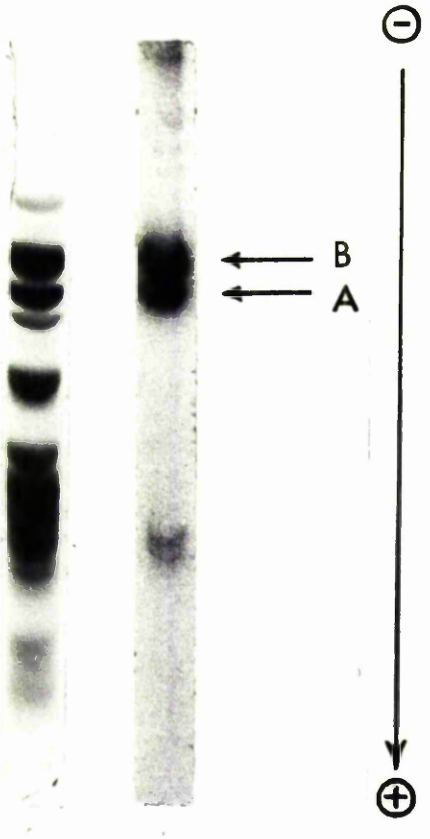


Fig. 3.1.6 Detection of ^{32}P-labelled o-Phosphoserine and o-Phosphothreonine in ^{32}P-labelled Ribosomal Protein from Ascites Cells.

Ribosomal protein from Ascites cells was hydrolysed, electrophoresed and subjected to autoradiography as described in Section 2.15.



(a) Stained Gel (b) Autoradiograph

Fig. 3.2.1 SDS Gel Electrophoresis of ^{32}P-labelled Ribosomal Protein from the 40S Subunit of Ascites Cells.

Ribosomal protein from the 40S subunit of Ascites cells, labelled with [^{32}P]-orthophosphate was separated on 15% SDS gels (Section 2.9.1) and subjected to autoradiography (Section 2.11.3). The faint unlabelled bands on (b) represent minor cross-contaminants of phosphoproteins from the 60S ribosomal subunit.

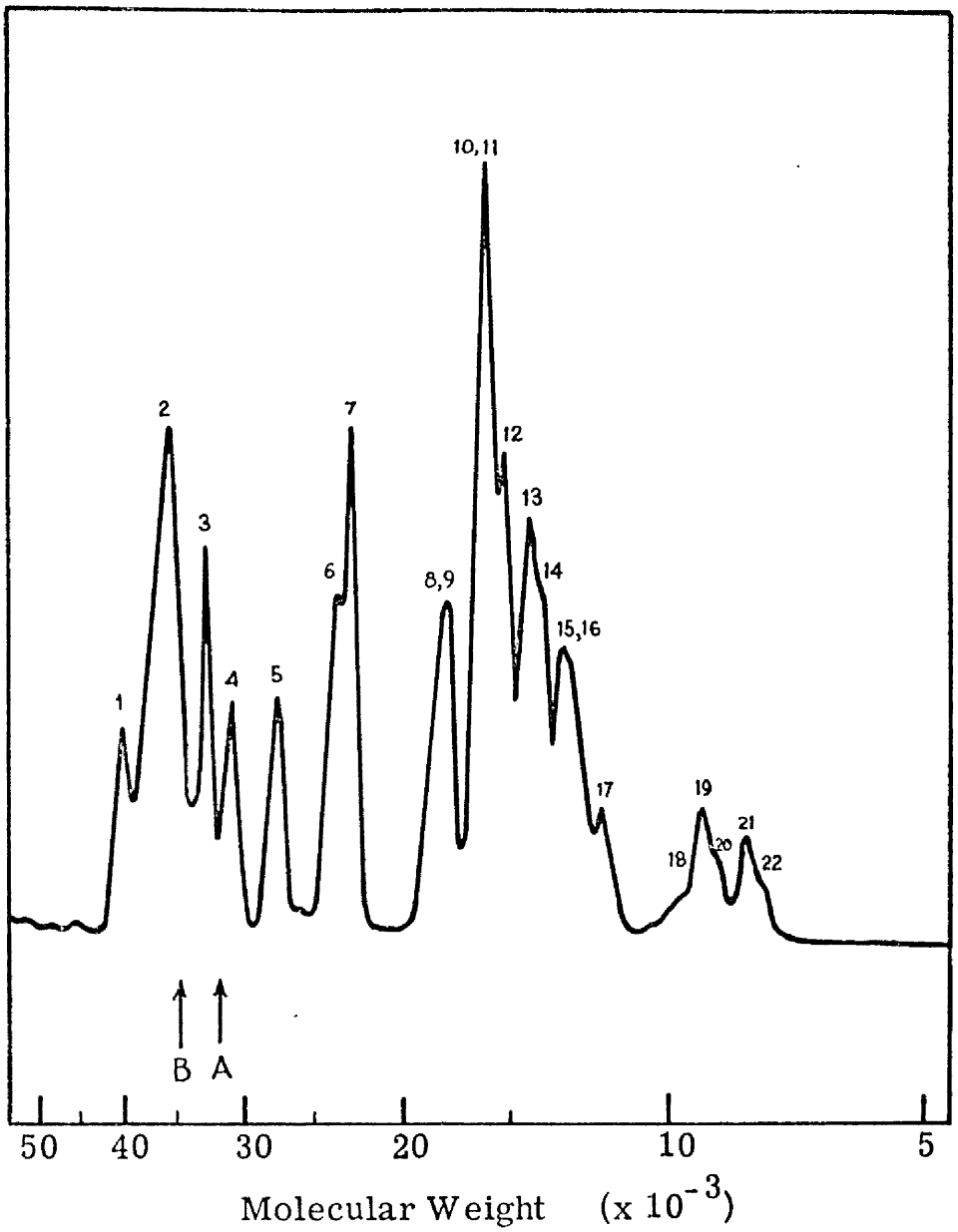


Fig. 3.2.2 SDS Gel Electrophoresis of Ribosomal Protein from the 40S Subunit of Ascites Cells: Densitometric Trace and Molecular Weights.

15% SDS gels of ribosomal protein from the 40S subunit of Ascites cells were scanned densitometrically (Section 2.11.4) and molecular weights estimated (Section 2.13). The molecular weights are the average of determinations on 12 different gels. The positions corresponding to the radioactive bands A and B of Fig. 3.2.1 are indicated.

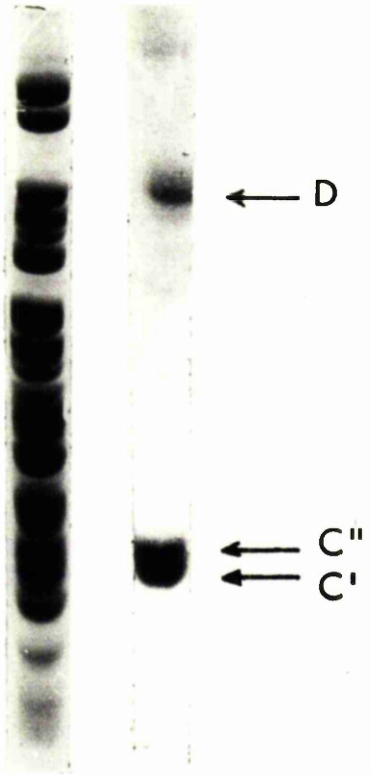
The final characterisation of ribosomal phosphoproteins was the demonstration of the presence of o-phosphoserine and o-phosphothreonine in a hydrolysate of ^{32}P-labelled ribosomal protein. Labelled ribosomal protein was hydrolysed by hydrochloric acid and electrophoresed as described in Section 2.15. An autoradiograph of the paper electropherogram was prepared, and this shows the presence of o-phosphoserine and o-phosphothreonine in ribosomal protein from Ascites cells (Fig. 3.1.6).

These experiments together demonstrate the presence of phosphorylated proteins in ribosomes and ribosomal subunits from Ascites cells.

3.2 Analysis of Phosphorylated Protein of Ribosomal Subunits from Ascites Cells.

Having obtained a purified preparation of ribosomal proteins labelled with $[^{32}P]$-orthophosphate, it was then possible to analyse and characterise them on polyacrylamide gels.

Ascites cells were labelled with $[^{32}P]$-orthophosphate as described in Section 2.4.1., and ^{32}P-labelled ribosomal protein prepared. Ribosomal protein from the 40S subunit was analysed on SDS gels, and autoradiographs prepared (Fig. 3.2.1). The autoradiograph indicates the presence of two phosphoproteins, designated A and B. These two phosphoproteins were, however, not always resolved, and on many autoradiographs appeared as a single broad band. Fig. 3.2.2. shows a densitometric trace of an SDS gel of ribosomal protein from the 40S subunit. From an



(a) Stained Gel (b) Autoradiograph

Fig. 3.2.3 SDS Gel Electrophoresis of ^{32}P-labelled Ribosomal Protein from the 60S Subunit of Ascites Cells.

Ribosomal protein from the 60S subunit of Ascites cells, labelled with [^{32}P]-orthophosphate was separated on 15% SDS gels (Section 2.9.1) and subjected to autoradiography (Section 2.11.3).

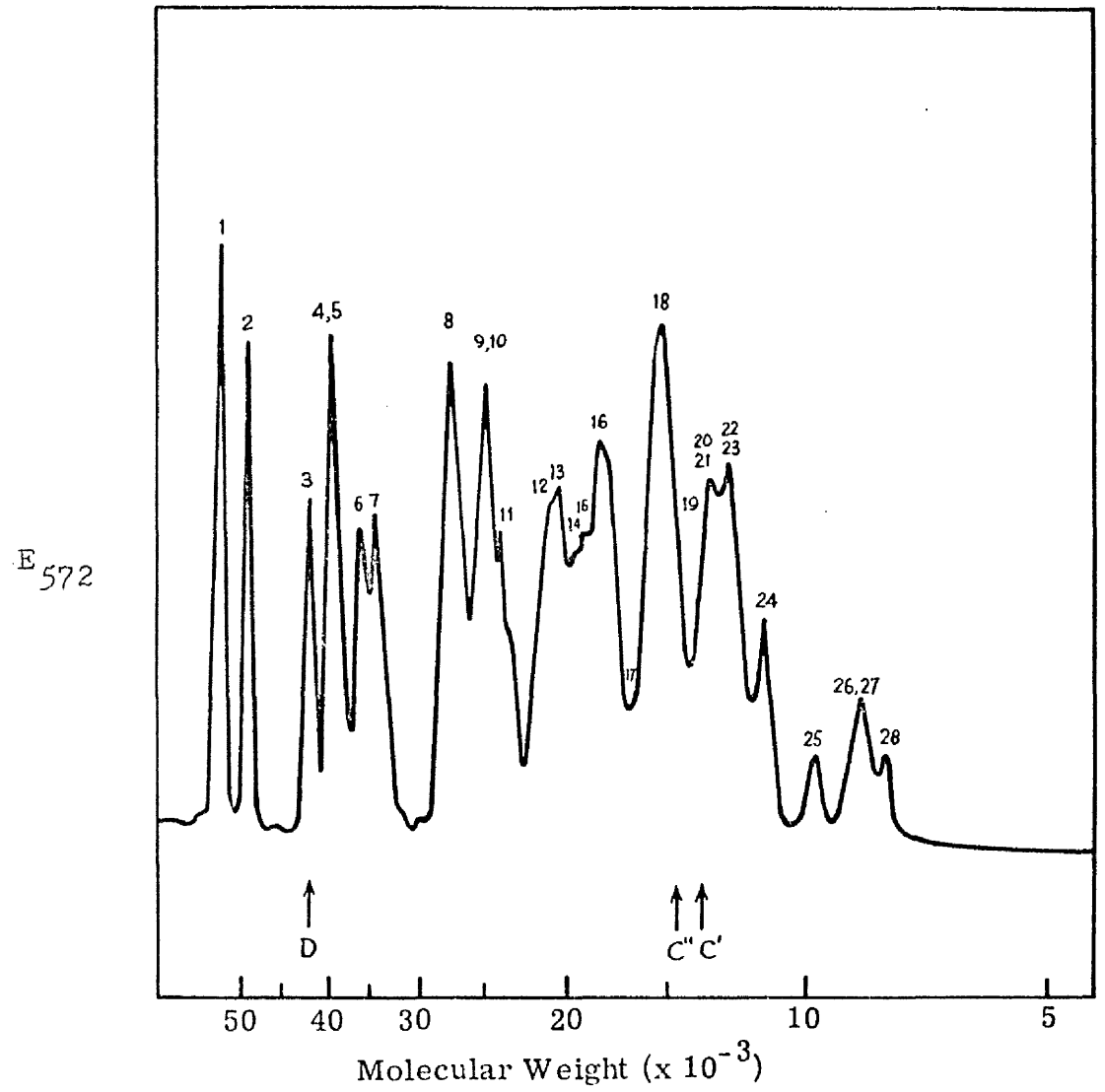


Fig. 3.2.4 SDS Gel Electrophoresis of Ribosomal Protein from the 60S Subunit of Ascites Cells: Densitometric Trace and Molecular Weights.

15% SDS gels of ribosomal protein from the 60S subunit of Ascites cells were scanned densitometrically (Section 2.11.4), and molecular weights estimated (Section 2.13). The molecular weights are the average of determinations on 12 different gels. The positions corresponding to radioactive bands C', C" and D of Fig. 3.2.3 are indicated.

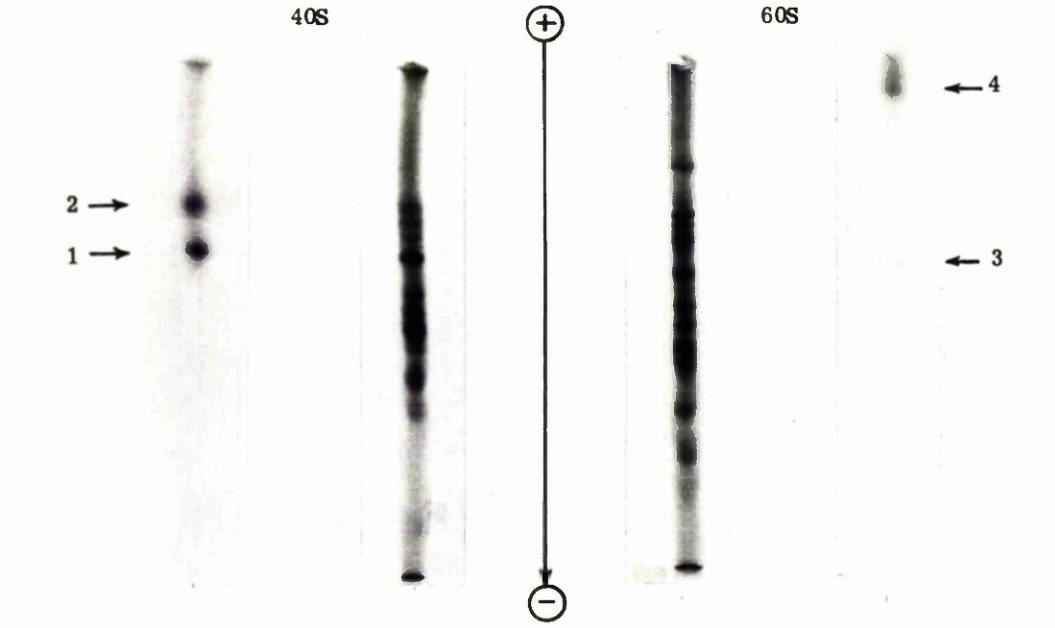


Fig. 3.2.5 Urea Gel Electrophoresis of ^{32}P-labelled Ribosomal Protein from the 40S and 60S Subunits of Ascites Cells.

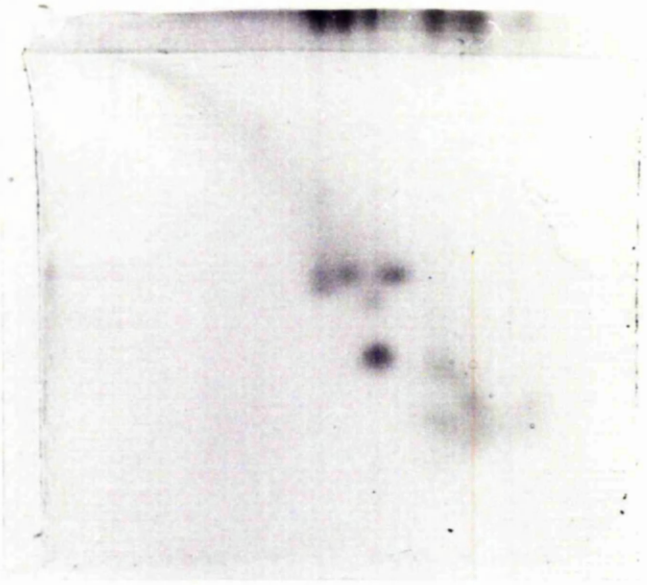
Ribosomal protein from the 40S and 60S subunits of Ascites cells, labelled with [^{32}P]-orthophosphate was separated on urea gels (Section 2.9.2) and subjected to autoradiography (Section 2.11.3).

examination of many gels, including some which were sliced transversely into 1mm. sections for direct quantitation of radioactivity. A and B were identified as peaks 3 and 2 of Fig. 3.2.2. which correspond to proteins of molecular weight 31,000 and 34,000 respectively. The proteins of the 60S ribosomal subunit were also analysed on SDS gels, and a photograph of the stained gel and its autoradiograph appears in Fig. 3.2.3. The autoradiograph indicates the presence of three phosphoproteins designated C', C" (which were not always resolved) and D. Again by examination of a number of gels, these phosphoproteins were identified as corresponding to peaks 20, 19 and 3 of the densitometric trace shown in Fig. 3.2.4. and have molecular weights 13,500, 14,500 and 41,000 respectively.

Phosphoproteins A and B were frequently unresolved on SDS gels. However, on urea gels at pH 4.5 they were completely resolved into bands 1 and 2 (Fig. 3.2.5a). Autoradiographs of urea gels of ribosomal protein from the 60S subunit show only two phosphorylated bands (Fig. 3.2.5d). However, band 4 did not appear to correspond to a stained protein, implying that band 4 on urea gels, and hence possibly one of the phosphoproteins on SDS gels, may not be a ribosomal protein but a trace contaminant.

Through the use of one-dimensional electrophoresis it therefore seems that the ribosomes of the Ascites cell have between three and five phosphoproteins, which although more than was originally observed by Kabat (1970) or Loeb and Blat (1970), is fewer than the 12 reported to be phosphorylated <u>in vitro</u> (Eil and Wool, 1971).

Stained Gel

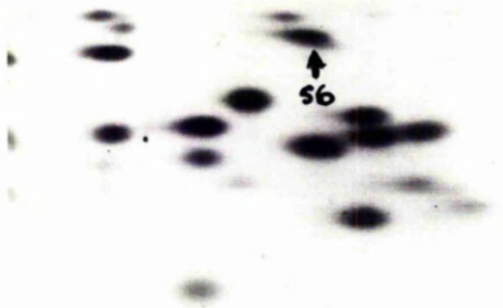


Autoradiograph

Fig. 3.2.6 Two-Dimensional Gel Electrophoresis of Ribosomal Protein from the 40S Subunit of Ascites Cells by the Method of Martini and Gould.

Ribosomal protein from the 40S subunit of Ascites cells labelled with [^{32}P]-orthophosphate was analysed by two-dimensional gel electrophoresis (Section 2.10.2) and subjected to autoradiography (Section 2.11.3). Note that the darkening on the autoradiograph does not appear to correspond to an identifiable protein spot.

(a)



(b)

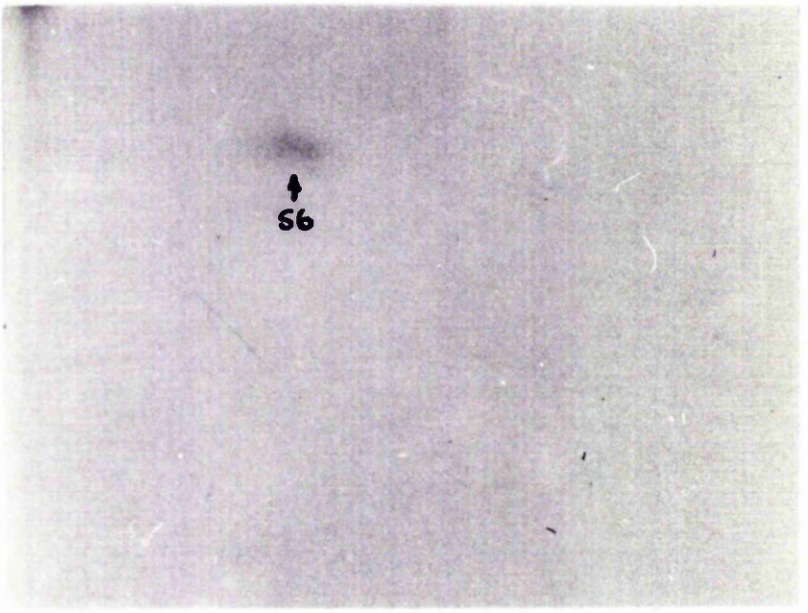


Fig. 3.2.7 Two-Dimensional Gel Electrophoresis of Ribosomal Protein from the 40S Subunit of Ascites Cells by the Method of Kaltschmidt and Wittmann.

Ribosomal protein from the 40S subunit of Ascites cells labelled with [^{32}P]-orthophosphate was analysed by two-dimensional gel electrophoresis (Section 2.10.1) and subjected to autoradiography (Section 2.11.3). The upper frame shows the stained gel of the ribosomal protein, and the lower frame shows the corresponding autoradiograph.

One-dimensional gel electrophoresis can resolve up to 22 proteins of the 40S ribosomal subunit (Fig. 3.2.2) and 28 proteins of the 60S subunit (Fig. 3.2.4). These figures are however, much less than the number of proteins known to be present in the subunits (see Section 1.3). It was thus not possible to resolve unequivocally all the ribosomal phosphoproteins by this method. For this reason, it was decided to analyse the proteins of the ribosomal subunit by two-dimensional gel electrophoresis in order to identify them more precisely, and to determine whether further resolution of the phosphoproteins might be possible.

Initially, the two-dimensional gel electrophoresis system of Martini and Gould was used. A two-dimensional gel of ^{32}P-labelled ribosomal protein of the 40S subunit by this method is shown in Fig. 3.2.6. The one spot on the autoradiograph shown beside it does not seem to correspond to any of the stained ribosomal proteins. Moreover, this gel system does not afford optimum resolution of the protein spots, because of the low pH of 4.3 used in the first dimension. For these reasons, rather than use it to characterise the ribosomal phosphoproteins further, this gel system was abandoned in favour of the system of Kaltschmidt and Wittmann.

Because of the higher pH of 8.7 in the first dimension, this system provides superior separation of the basic ribosomal proteins. Fig. 3.2.7a shows such a two-dimensional gel of ^{32}P-labelled 40S ribosomal protein. Each stained protein spot was carefully excised by means of an opthalmic micro-scalpel and its radioactivity quantitated

Protein	Counts/ min. ^{32}P
S2	2
S3	4
S4	7
S6	40
S7	10
S8	5
S9	2
S10	0
S11	0
S13	3
S14	2
S15	3
S17	4
S18	1
S19	7
S20	1
S22	2
S23	5
S25	2
S26	1
S27	4
S28	4

40S Subunit Proteins.

Protein	Counts/ min. ^{32}P
L3	1
L5	0
L6	0
L7	1
L8	0
L9	0
L10	0
L11	1
L12	2
L13	1
L14	0
L15	1
L16	1
L17	4
L18	3
L20	9
L21	8
L22	3
L23	5
L24	3
L25	4
L26	6
L27	2
L28	3
L29	4
L30	5
L31	4
L32	9
L35	9
L36	1
L38	1
L39	2

60S Subunit Proteins.

Table 3.2.1 Two-dimensional Gel Electrophoresis of Ribosomal Protein from the 40S and 60S Subunits of Ascites Cells: Direct Quantitation of Radioactivity.

Protein spots from two-dimensional gels of ribosomal protein from the 40S and 60S subunits of Ascites cells were excised by means of an opthalmic micro-scalpel and their radioactivity determined as described in Section 2.11.5. The background was 11 counts/min.

directly (Table 3.2.1). This shows that only protein S6 (in the nomenclature of Sherton and Wool, 1972, 1974b) contained an amount of radioactivity which was significantly above background. So far, S6 is the only phosphoprotein of the 40S subunit which has been identified in the two-dimensional gel system. Fig. 3.2.7b shows the autoradiograph corresponding to the two-dimensional gel shown in Fig. 3.2.7a. As can be seen, the darkening of the autoradiographic film corresponds to protein S6, or, more exactly to the 'tail' of S6, to its left. As the first dimension of the Kaltschmidt-Wittmann gel system is a separation on the basis of charge, the tail of a protein spot is more negatively charged than the main body of the spot. It would thus be expected that a phosphorylated derivative of S6 would run in this region. Indeed, while preparing this finding for publication (Rankine and Leader, 1975), Gressner and Wool (1974a,b) reported that several phosphorylated derivatives of S6 of the ribosomes of rat liver did electrophorese in this position. This finding is important inasmuch as it indicates that the stoichiometry of a phosphoprotein in a two-dimensional gel can be estimated from the size of its tail, independently of radioactive labelling and the various difficulties this entails.

The possibility was examined that the second phosphoprotein of the 40S subunit, which had not been observed on the two-dimensional gels, might be relatively acidic, and would therefore move towards the anode in the first dimension. Although two acidic proteins were detected by staining a gel in which the electrodes of the

first dimension were reversed, neither of these contained radioactivity.

When two-dimensional gels of ^{32}P-labelled 60S ribosomal protein were subjected to autoradiography, no darkening of the film was detected. After excision of the spots and direct quantitation of radioactivity, one gel was found to have an almost double background level of radioactivity in protein L35. However, this observation has not yet been repeated.

The use of two-dimensional gels has allowed the identification of the major phosphoprotein of the 40S ribosomal subunit. However, the method did not result in the resolution and detection of more phosphoproteins. Indeed, fewer were observed, probably because of the greater area occupied by a given amount of protein in a two-dimensional gel, than in a one-dimensional gel, and the consequent reduction in radioactive intensity during autoradiography.

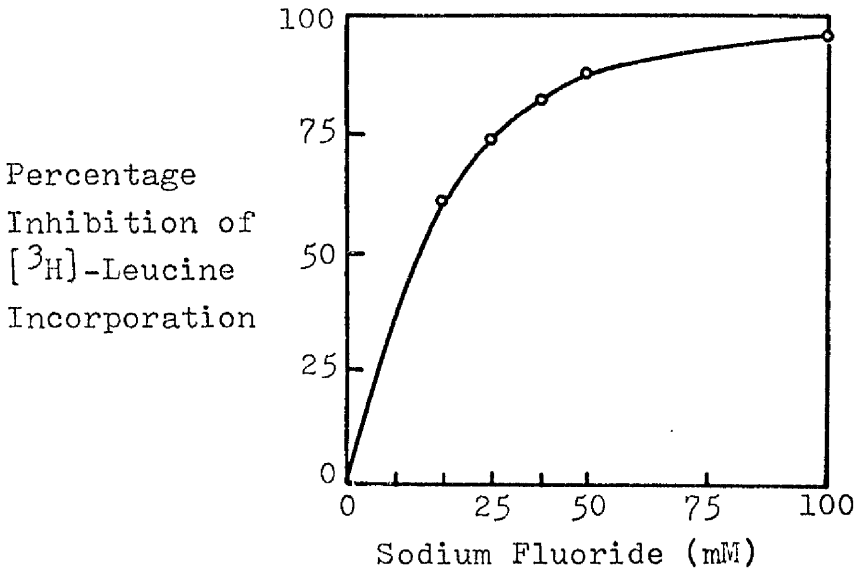
3.3 Effect of Inhibitors of Protein Synthesis on the Phosphorylation of Ribosomal Protein from Ascites Cells.

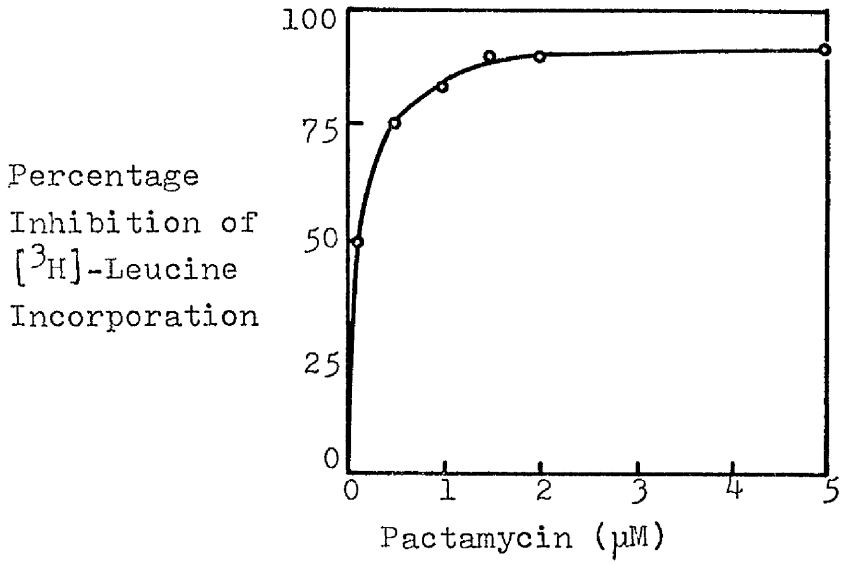
Having characterised the ribosomal phosphoproteins of Krebs II Ascites cells, it was decided to examine possible ways in which the phosphorylation might be controlled. Kabat (1970) had found that sodium fluoride specifically stimulated the incorporation of [^{32}P]-orthophosphate into one protein of the 40S ribosomal subunit of the rabbit reticulocyte. He therefore suggested that this stimulation of phosphorylation was related directly to the

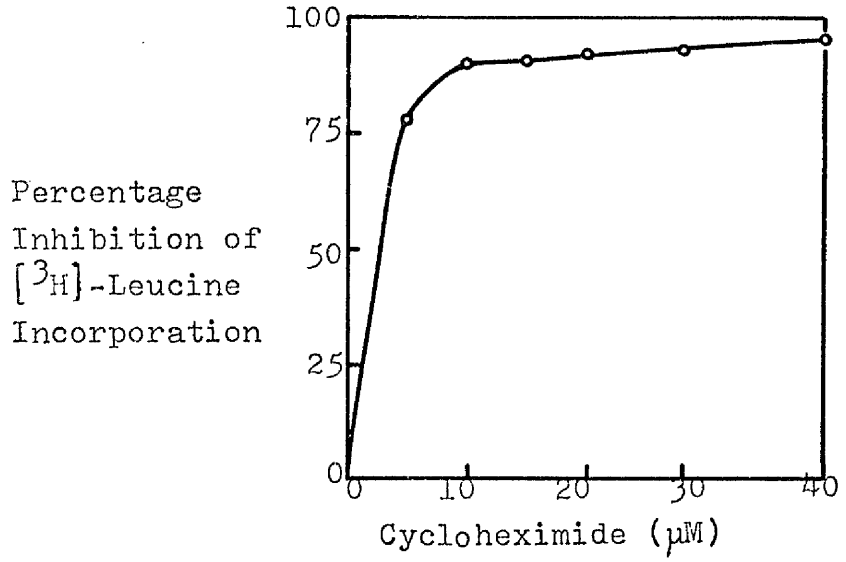
Fig. 3.3.1 Effect of Inhibitors of Protein Synthesis on the Incorporation of $[^3H]$-Leucine into Whole Cell Protein of Ascites Cells.

Suspensions of Ascites cells (10 ml) were incubated (Section 2.4.1) in Eagle's Medium from which amino acids had been omitted. Incubation was for 15mins. at 37°C with 10 μCi $[^3H]$-leucine, and the amount of inhibitor indicated. 0.5ml aliquots were then removed and added to 5ml ice-cold 10% trichloroacetic acid. After 30mins. whole cell protein was extracted and its radioactivity determined (Section 2.12).

Fig. 3.3.1


Percentage Inhibition of [3H]-Leucine Incorporation
100
75
50
25
0
0
25
50
75
100
Sodium Fluoride (mM)


Percentage Inhibition of [3H]-Leucine Incorporation
100
75
50
25
0
0
1
2
3
4
5
Pactamycin (μM)


Percentage Inhibition of [3H]-Leucine Incorporation
100
75
50
25
0
0
10
20
30
40
Cycloheximide (μM)

Inhibitor	Specific activity of ribosomal protein counts/min/mg.	
	40S	60S
None (Experiment 1)	16,300	26,900
None (Experiment 2)	10,000	9,800
Sodium Fluoride (75 mM)	10,300	9,500
Pactamycin (2μM)	11,000	24,200
Cycloheximide (20μM)	8,200	4,500

Table 3.3.1 Effect of Inhibitors of Protein Synthesis on the Specific Activity of Ribosomal Protein from Ascites Cells Labelled with [^{32}P]-Orthophosphate.

Ascites cells were labelled with [^{32}P]-orthophosphate (Section 2.4.1) in the presence of the amount of inhibitor indicated, and protein from ribosomal subunits prepared (Sections 2.5.1, 2.7, and 2.8). Ribosomal protein was resuspended at 1 mg/ml in H_2O and 50μg aliquots taken, which were dried on to Whatman GF/C glassfibre filters and radioactivity determined in Toluene/Triton/PPO/POPOP scintillant (see Section 2.8).

inhibition of protein synthesis. However, fluoride has many metabolic effects other than the inhibition of protein synthesis, and for this reason, it was decided to study the effects of other inhibitors of protein synthesis on the phosphorylation of ribosomal proteins. Pactamycin and cycloheximide were chosen because they inhibit different phases of protein synthesis from sodium fluoride.

It was first necessary to determine the amount of inhibitor required to inhibit protein synthesis. The results of such experiments are shown in Fig. 3.3.1. For studies on phosphorylation, it was decided to use a concentration of inhibitor sufficient to cause about 80% inhibition of protein synthesis. This concentration, while causing a significant inhibition of protein synthesis, would be less likely to have toxic side-effects than a concentration sufficient for 100% inhibition. The concentrations used were 75mM for sodium fluoride, 2μM for pactamycin and 20μM for cycloheximide.

Ribosomal protein labelled with [^{32}P]-orthophosphate was prepared from cells incubated with these inhibitors, and the specific radioactivity of the protein determined (Table 3.3.1). As can be seen from the table, there was no overall effect of the inhibitors on the specific activity of the ribosomal protein from the 40S subunit, in contrast to the threefold stimulation of phosphorylation observed by Kabat (1970) with sodium fluoride. The figures for the specific activity of the proteins of the 60S ribosomal subunit are more difficult to interpret because of a wide variation in specific activity (c.f. control

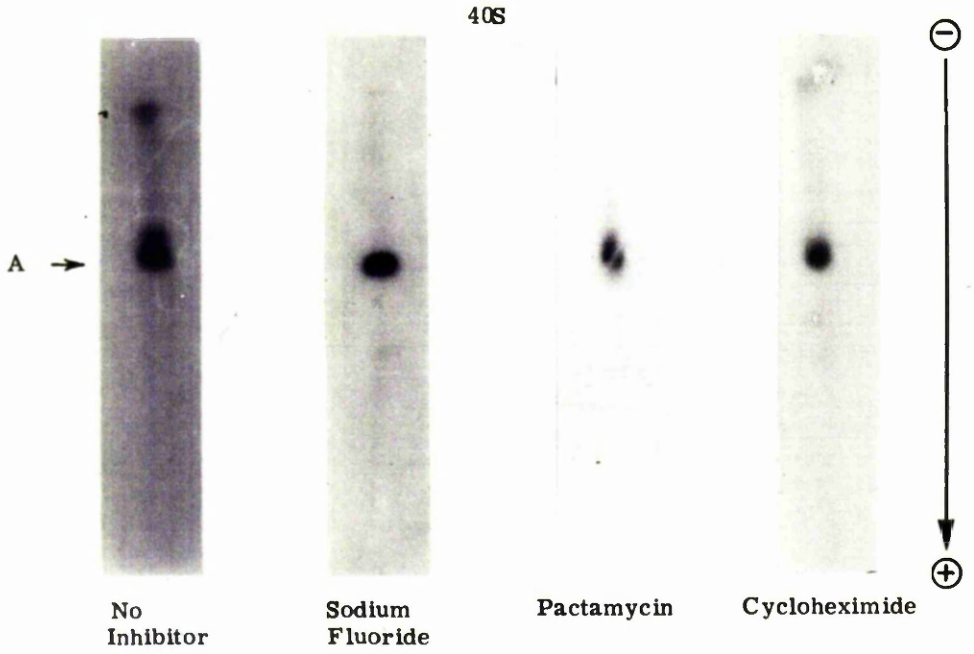


Fig. 3.3.2 Effect of Inhibitors of Protein Synthesis on the Phosphorylation of the Ribosomal Protein from the 40S Subunit of Ascites Cells.

Ascites cells were incubated with [^{32}P]-orthophosphate (Section 2.4.1) in the presence of the amount of inhibitor indicated in Table 3.3.1 and protein from the 40S ribosomal subunits prepared (Sections 2.5.1, 2.7, and 2.8). This protein was then analysed on SDS gels (Section 2.9.1) and subjected to autoradiography (Section 2.11.3). The photographs show autoradiographs of the SDS gels.

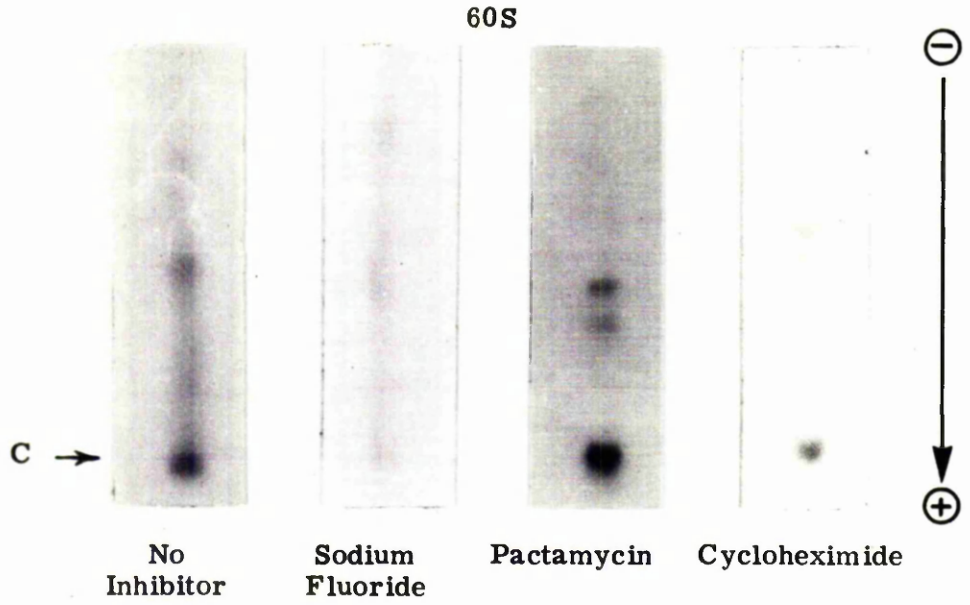


Fig. 3.3.3 Effect of Inhibitors of Protein Synthesis on the Phosphorylation of the Ribosomal Protein from the 60S Subunit of Ascites Cells.

Ascites cells were incubated with [^{32}P]-orthophosphate (Section 2.4.1) in the presence of the amount of inhibitor indicated in Table 3.3.1 and protein from the 60S ribosomal subunits prepared (Sections 2.5.1, 2.7 and 2.8). This protein was then analysed on SDS gels (Section 2.9.1) and subjected to autoradiography (Section 2.11.3). The photographs show autoradiographs of the SDS gels.

experiments). It is therefore not possible to say whether the inhibitors had any effect on phosphorylation or not. Fortunately, the possible effects of the inhibitors of protein synthesis on the phosphorylation of the 40S subunit were of greater importance to this study, as it was the phosphorylation of a protein of this subunit which had been reported to be stimulated by sodium fluoride (Kabat, 1970).

Despite the lack of effect of inhibitors of protein synthesis on the specific activity of phosphoprotein from the 40S subunit, it was possible that there were changes in the phosphorylation of some proteins which were obscured by there being no change (or an opposite change) in the phosphorylation of other proteins. Moreover, although Kabat (1970) found no extra proteins phosphorylated after inhibition by sodium fluoride, this was the only inhibitor he studied, so the possibility remained that extra proteins might be phosphorylated in the presence of pactamycin or cycloheximide. For these reasons, the ^{32}P-labelled proteins were analysed on polyacrylamide gels. Fig. 3.3.2 shows autoradiographs of SDS gels of 40S ribosomal protein from a control experiment and experiments in which inhibitors were present. It is evident from an examination of these figures that no extra proteins were phosphorylated in the presence of the inhibitors of protein synthesis. A similar conclusion can be reached by an examination of Fig. 3.3.3 which shows similar comparisons between control and inhibitor experiments for the proteins of the 60S ribosomal subunit. On some gels, only phosphoprotein C is seen, while others show D as well as other

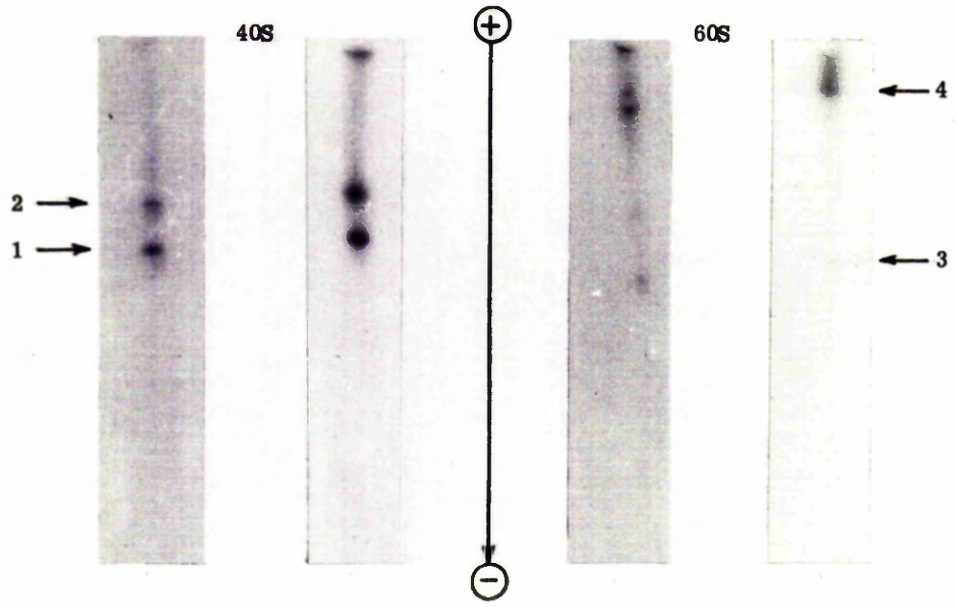


Fig. 3.3.4 Effect of the Inhibition of Protein Synthesis on the Phosphorylation of Ribosomal Protein from Ascites Cells.

Ascites cells were incubated with [^{32}P]-orthophosphate (Section 2.4.1) in the presence of 75mM sodium fluoride and protein prepared from the ribosomal subunits (Sections 2.5.1, 2.7, and 2.8). This protein was then analysed on urea gels (Section 2.9.2) and subjected to autoradiography (Section 2.11.3). The photographs show autoradiographs of the urea gels.

trace contaminants. Differences in the intensity of phosphorylated bands in both Fig. 3.3.2 and Fig. 3.3.3 can be accounted for by different amounts of protein applied to individual gels, and by different exposure times of the autoradiographs. The changes in intensity of phosphorylated bands, do to some extent, parallel the specific activity of the protein used. However, this aspect of the work was not pursued in any great detail.

One minor disadvantage of the SDS gel system used in these experiments was that phosphoproteins A and B were not always resolved. Electrophoresis on urea gels, however, does allow resolution of these proteins. Urea gels of protein from Ascites cells incubated with sodium fluoride are shown in Fig. 3.3.4 where it can be seen that the fluoride treatment has not altered the relative intensity of the two phosphoproteins 1 and 2. The fact that the autoradiograph of the gel of ribosomal protein from cells incubated with sodium fluoride is much darker than the autoradiograph of the control is due to the gel corresponding to the former being much more heavily loaded with protein. It can also be seen from Fig. 3.3.4 that sodium fluoride has no effect on the relative intensity of the phosphoproteins of the 60S subunit.

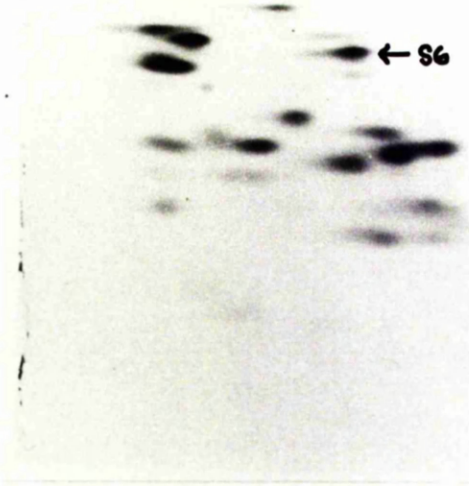
The use of urea gels thus showed that inhibitors of protein synthesis had no apparent effect on the phosphorylation of ribosomal proteins in Krebs II Ascites cells. Rather than pursue this further using one-dimensional gels, use was made of the fact that the 'tail' of protein S6 on two-dimensional gels of the Kaltschmidt/Wittmann system

Fig. 3.3.5 Effect of Inhibition of Protein Synthesis on the Phosphorylation of Ribosomal Protein of the 40S Subunit from Ascites Cells.

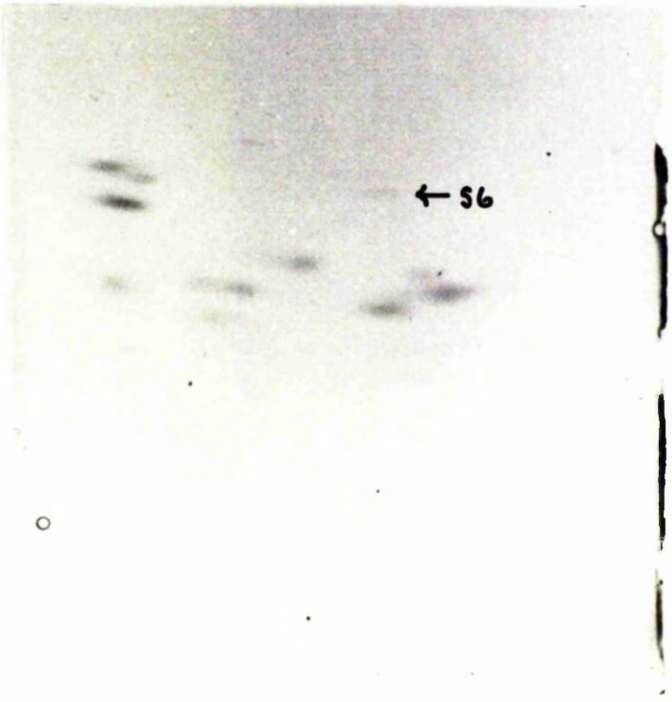
Ascites cells were labelled with [^{32}P]-orthophosphate (Section 2.4.1) in the presence of the amount of inhibitor indicated in Table 3.3.1. and protein from ribosomal subunits prepared (Sections 2.5.1, 2.7, and 2.8). This protein was then analysed by the two-dimensional electrophoretic system of Kaltschmidt and Wittmann (Section 2.10.1).

Fig. 3.3.5

(a)
Sodium
Fluoride



(b)
Pactamycin



(c)
Cycloheximide

represents the phosphorylated derivative of this protein (see Fig. 3.2.7). Therefore, it was felt that using this system of two-dimensional gel electrophoresis, any changes in the extent of phosphorylation of S6 due to the inhibitors of protein synthesis could be clearly detected by the size of its phosphorylated 'tail'. The results of such experiments with inhibitors of protein synthesis are shown in Fig. 3.3.5. It is evident that none of the inhibitors tested had any effect on the size of the phosphorylated 'tail' of protein S6. Therefore, under no circumstances has an effect been observed of any inhibitor of protein synthesis (including sodium fluoride) on the phosphorylation of ribosomal protein of the 40S subunit of Ascites cells in this work. These results are in contrast to the observations made by Kabat (1970) in rabbit reticulocytes, using a one-dimensional system of gel electrophoresis.

3.4 Analysis of Phosphorylated Protein of Ribosomal Subunits of BHK Cells.

As the work described in Section 3.3 did not reveal any effect of inhibitors of protein synthesis on the phosphorylation of ribosomal proteins, it was decided to examine the phosphorylation in different physiological conditions where changes in the protein synthetic activity of the ribosomes might be expected to occur. For these studies, Ascites cells were unsuitable because of their limited viability outside the mouse peritoneum. The use of cells growing in tissue culture under controlled conditions seemed more appropriate and for these reasons non-neoplastic

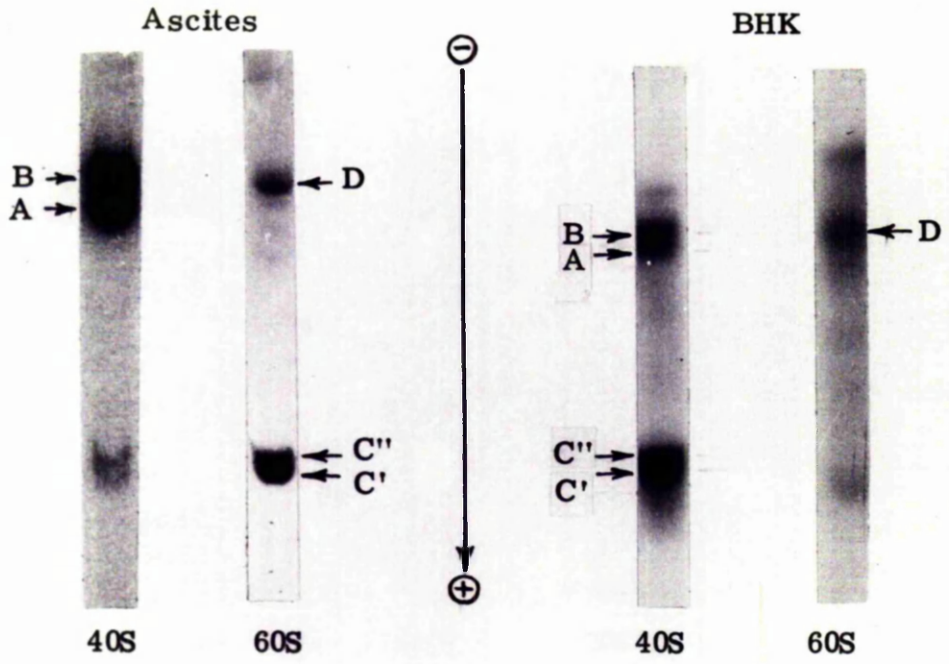
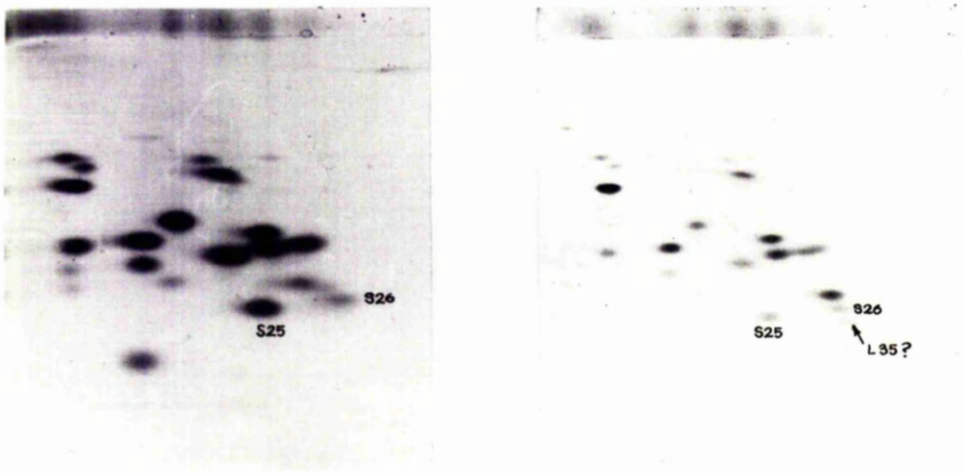


Fig. 3.4.1 Subunit Location of Ribosomal Phosphoproteins: Comparison between Autoradiographs of SDS Gels of Ribosomal Protein from Ascites and BHK Cells.

BHK cells were incubated with [^{32}P]-orthophosphate (Section 2.4.2). Ribosomal proteins were prepared from ribosomal subunits (Sections 2.5.2, 2.7, and 2.8), were analysed on SDS gels and subjected to autoradiography (Sections 2.9.1 and 2.11.3). The photographs show autoradiographs of SDS gels of ribosomal protein from both BHK and Ascites cells.

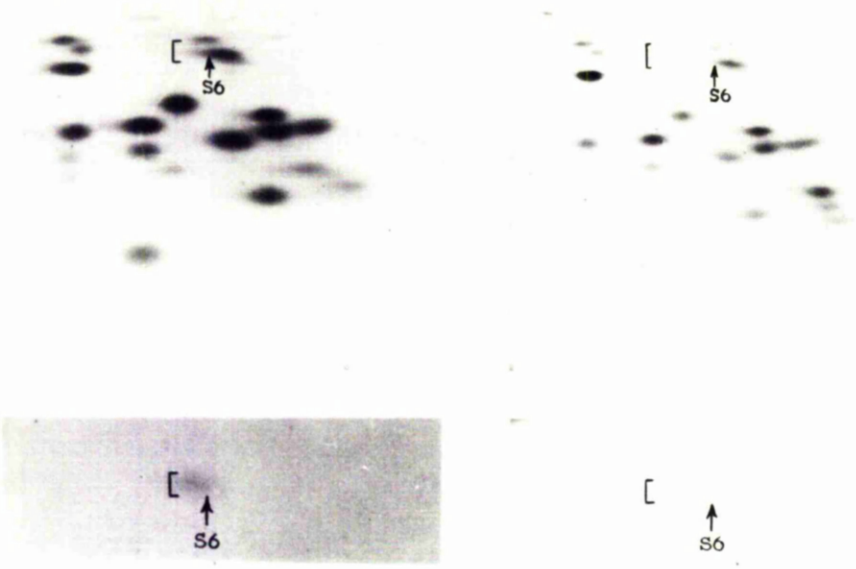
baby hamster kidney fibroblasts (BHK cells) were chosen. These had not been used previously because of the number of cells required to give a sufficient yield of ribosomal protein. It was first necessary to characterise the phosphoproteins of the ribosome of the BHK cell, therefore, BHK cells were grown and labelled with [^{32}P]-orthophosphate as described in Sections 2.3.2 and 2.4.2 and their ribosomal proteins isolated. These proteins were analysed on SDS gels and autoradiographs prepared. The results of this experiment are shown in Fig. 3.4.1. Autoradiographs of SDS gels of ribosomal protein from Ascites cells are included for comparison. The major phosphoproteins A and B are still found on the 40S subunit, and the minor phosphoprotein D is still found on the 60S subunit. However, it can be seen that C' and C" have almost entirely disappeared from the 60S subunit, being weaker in intensity than D. In the 40S subunit two new bands have appeared in addition to A and B. These have similar molecular weights to C' and C" (average molecular weight 13,500 as compared with 14,000) and it is most likely that they are identical to these, and have therefore been designated accordingly. This observation suggests that there has been a transfer of labelled C' and C" from one subunit to the other. Sherton and Wool (1974b), using two-dimensional electrophoresis, have found a similar transfer of certain unlabelled ribosomal proteins between subunits where different methods had been used to prepare the ribosomes. Indeed, the considerable differences in the methods used to prepare ribosomes from BHK and Ascites cells could explain the transfer of C' and C" observed in this work.



Ascites BHK

Fig. 3.4.2 Two-Dimensional Gel Electrophoresis of Ribosomal Protein from the 40S Subunits of Ascites and BHK Cells.

See Section 3.4 for a detailed description. The photographs show stained gels of ribosomal proteins separated by the Kaltschmidt-Wittmann two-dimensional gel system (Section 2.10.1).



(a) Ascites (b) BHK

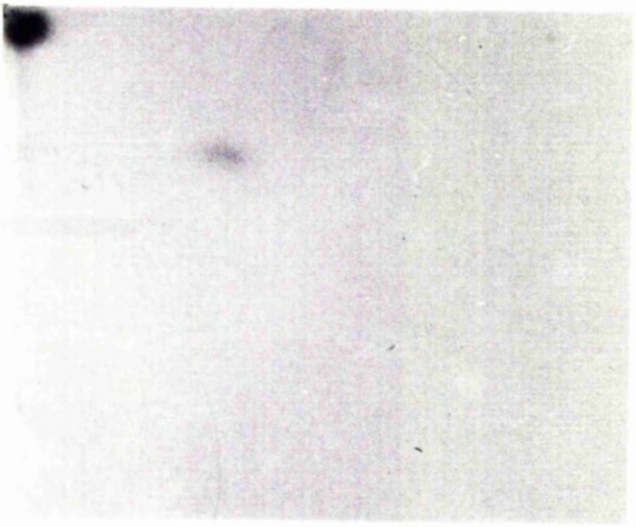
Fig. 3.4.3 Identification of Protein S6 as the Major Phosphoprotein of the 40S Ribosomal Subunit from BHK Cells.

See Section 3.4 for a detailed description. The upper frames show stained gels of ribosomal proteins separated by the Kaltschmidt-Wittmann two-dimensional gel system (Section 2.10.1), and the lower frames show the relevant area of the corresponding autoradiograph. The arrow and parenthesis marks indicate the beginning and the end of the 'tail' of S6.

Two-dimensional gels of ribosomal protein from BHK and Ascites cells were examined to see if there was also transfer of stained protein spots. In gels of 40S ribosomal protein from BHK cells an extra spot below S26 was observed (Fig. 3.4.2.) This appears to correspond in position with L35 of the 60S subunit on gels of 40S and 60S proteins together and suggests that L35 might represent one or both of C' and C". This result was interesting as in one previous experiment, L35 had been found to be labelled in Ascites cells (Section 3.2.8). However, there was no apparent reduction in the intensity of staining of L35 on the 60S subunit of BHK cells, although it should be mentioned that this protein always stains rather weakly on two-dimensional gels. Because of the low intensity of the protein spots on a two-dimensional gel it was not possible to obtain autoradiographs of phosphoproteins from the 60S ribosomal subunit of either Ascites or BHK cells to confirm this designation.

Autoradiographs of two-dimensional gels show that in BHK cells, as well as in Ascites cells, protein S6 is the major phosphoprotein of the 40S ribosomal subunit (Fig. 3.4.3). However, an examination of both the gel and its autoradiograph shown in Fig. 3.4.3 indicates that S6 was much more extensively phosphorylated in BHK cells than in Ascites cells. The next Section includes experiments which were designed to discover the cause of this increased phosphorylation.

Stained Gel



Autoradiograph

Fig. 3.5.1 Effect of Dibutyryl Cyclic AMP on the Phosphorylation of the Ribosomal Protein of the 40S Subunit from Ascites Cells.

Ascites cells were labelled with [^{32}P]-orthophosphate (Section 2.4.1) in the presence of 0.1mM dibutyryl cyclic AMP and 0.1mM 3-isobutyl-1-methylxanthine. Ribosomal proteins were prepared from the 40S subunit (Sections 2.5.1, 2.7, and 2.8). The protein was analysed (Section 2.10.1) and subjected to autoradiography (Section 2.11.3).

3.5 Examination of the Phosphorylation of Ribosomal Protein in Cells under different Physiological Conditions.

Inhibitors of protein synthesis had been found to have no effect on the phosphorylation of ribosomal proteins in Ascites cells. It was thus necessary to consider other possible ways in which phosphorylation of ribosomal proteins might be regulated. Blat and Loeb (1971) had reported that glucagon stimulated the incorporation of [^{32}P]-orthophosphate into the ribosomal proteins of rat liver, and it was suggested that the stimulation was due to an increase in the concentration of cyclic AMP. It was decided that as well as studying the effects of inhibitors of protein synthesis it was important to examine the possible effects of this nucleotide in Ascites cells. However, dibutyryl cyclic AMP was found to have no effect on the stoichiometry of the phosphorylation of S6 (Fig. 3.5.1.), nor was there any effect on the specific radioactivity of the protein, or the one-dimensional gel autoradiograph (results not shown).

To study the phosphorylation of ribosomal proteins under more physiological conditions, BHK cells were again used, and it was decided to perform studies on growing and resting cells. This was because the state of cellular growth can be correlated directly with protein synthesis, and also with the cellular concentration of cyclic AMP, which is low when cells are growing rapidly, but much higher when the cells have grown to confluence (Rudland *et al*, 1974).

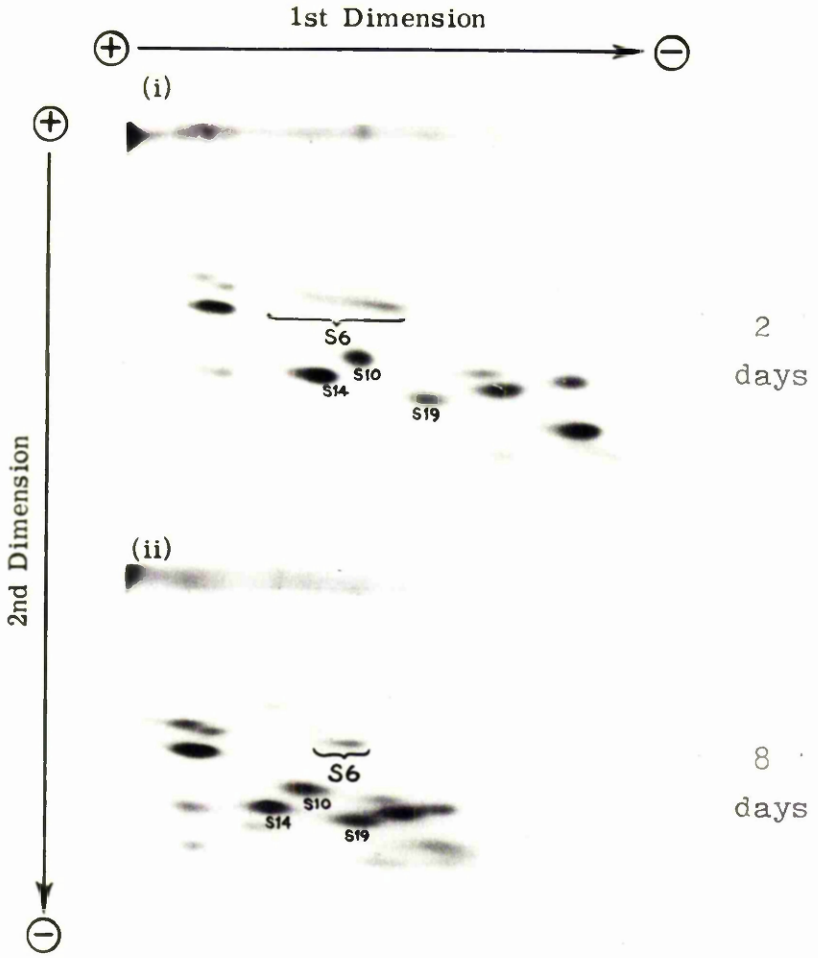


Fig. 3.5.2 Effect of Growth Period on the Phosphorylation of Ribosomal Protein from BHK Cells.

BHK cells were grown for 2 or 8 days (Section 2.3.2), and ribosomal proteins prepared (Sections 2.5.2, 2.7, and 2.8). The frames show stained gels of protein from the 40S ribosomal subunit separated by the Kaltschmidt-Wittmann two-dimensional gel system (Section 2.10.1). The parentheses delineate the visible extent of protein S6 and its derivatives, and the positions of proteins S14, S10 and S19 are indicated as reference points. In this experiment, no radioactivity was added, but in analogous experiments, where [^{32}P]-orthophosphate was added, similar patterns were seen, and the darkened areas on the autoradiographs corresponded to the 'tail' of S6. The length of the 'tail' of S6 was similar after 2 or 3 days' growth. After confluence was reached (approx. 4 days) the 'tail' of S6 was similar in length to that found after 8 days' growth.

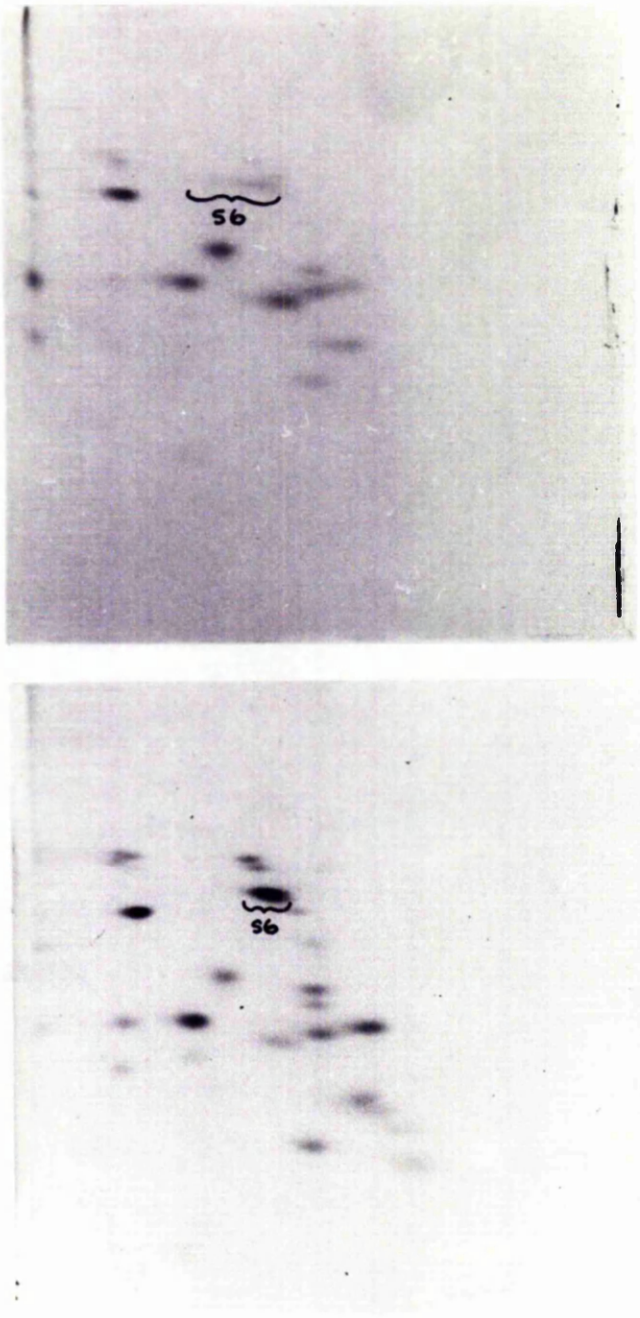


Fig. 3.5.3 Effect of Nutrition on the Phosphorylation of Ribosomal Protein S6 of BHK Cells after Different Growth Periods.

For details see Section 3.5. The frames show stained gels of ribosomal protein of the 40S subunit analysed by the two-dimensional gel system of Kaltschmidt and Wittmann (Section 2.10.1). The parenthesis marks delineate the visible extent of protein S6.

Initially the phosphorylation of protein S6 was examined before and after the cells had reached confluence. It can be seen that when the cells had reached confluence the phosphorylated 'tail' of S6 had almost disappeared, whereas in rapidly growing cells it was considerably extended (Fig. 3.5.2.). Rapidly growing cells are characterised by high rates of protein synthesis and low concentrations of cyclic AMP, as compared with resting cells. Thus, the physiological conditions which favour the phosphorylation of S6 are the exact opposite to those suggested by the results of Kabat (1970) with studies on the effect of sodium fluoride, and the results of Blat and Loeb (1971) with glucagon.

These results did suggest, however, that there might be some correlation between cell growth and the phosphorylation of ribosomal proteins. It was therefore decided to see if there was an alteration in phosphorylation on stimulating cell growth by the addition of fresh medium to BHK cells before and after confluence had been reached. Resting and confluent cells were taken as usual, and 1h before harvesting, the medium was replaced by fresh medium. The results of these experiments are shown in Fig. 3.5.3. and indicate that even stimulating growth after 8 days had no effect on the size of the 'tail' of S6. The correlation therefore seems to be more with the growth history of the cell rather than its nutritional state immediately prior to harvesting.

These results also suggested a possible explanation for previous inconsistent observations on the size of

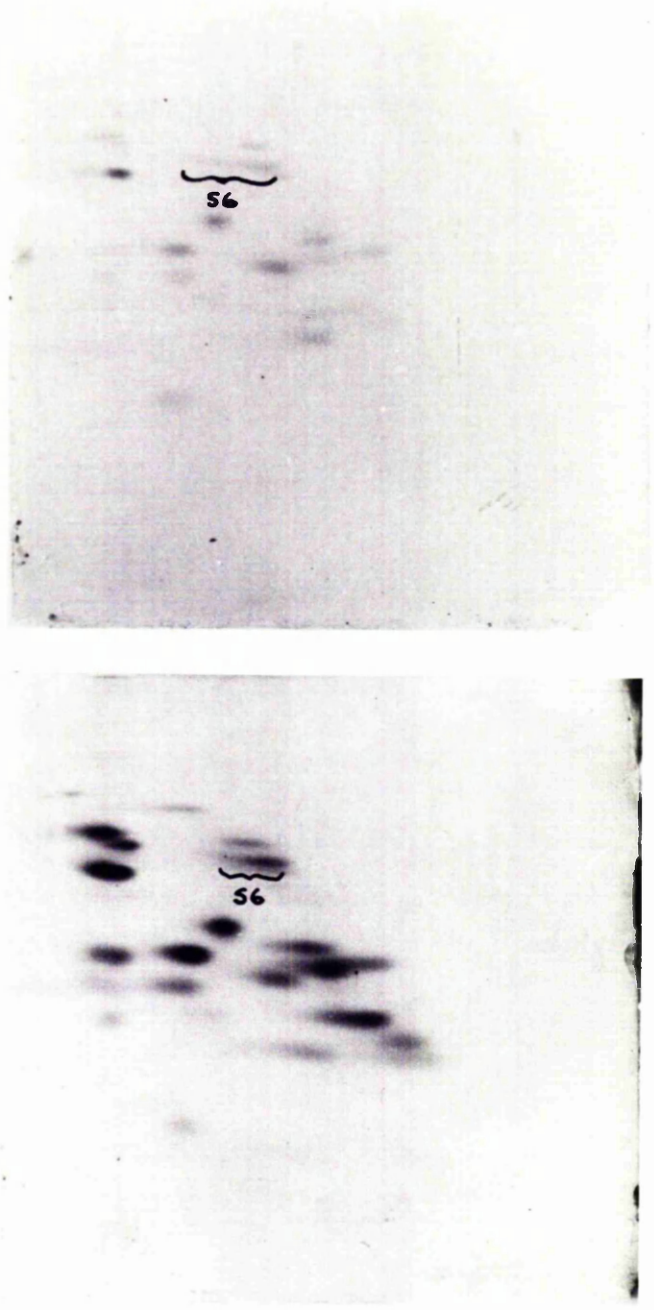


Fig. 3.5.4 Effect of Age on the Phosphorylation of Ribosomal Protein S6 from Mouse Liver.

Ribosomes were prepared from the livers of mice, 3 weeks or 10 weeks old, by the method of Florini and Breuer (1966). Ribosomal protein was prepared from 40S subunits (Sections 2.7 and 2.8). This protein was then analysed by the two-dimensional gel system of Kaltschmidt and Wittmann (Section 2.10.1). The parenthesis marks delineate the visible extent of protein S6.

the 'tail' of S6 in the liver of normal mice. This might have been due to the fact that different experiments on mouse livers were with animals of different ages. Consequently, the size of the 'tail' of S6 was compared in 3 week old mice, whose livers were still growing, and mice which had attained constant weight and had livers which were not growing. It was found that in liver ribosomes from young mice, the 'tail' of S6 was considerably longer than in adult mice. (Fig. 3.5.4).

Thus, in two different circumstances, there appears to be a correlation between the phosphorylation of ribosomal protein S6 and the rate of cellular growth.

SECTION 4.

DISCUSSION.

Author	Tissue	Type of Gel	Phospho-proteins on 40S Subunit	Phospho-proteins on 60S Subunit	Phospho-proteins on 80S Ribosomes
Rankine - present work	Krebs Ascites Cell BHK/C13	SDS	2 4	3 1	-
Gavthon et al (1974)	Rabbit Reticulo-cyte	SDS	2	3	-
Ashby and Roberts (1975)	Rat Cerebral Cortex	SDS	1	4	-
Pierre et al (1974)	Rat Liver	Urea	2	3-4	-
Majumder and Turkington (1972)	Mouse Mammary Gland	Urea or SDS	-	-	8
Trewavas (1973)	Lemna Minor	Urea or SDS	1	0	-
Grankowski and Gaslor (1975)	Yeast	Urea	-	-	5

Table 4.1 Estimates of the Number of Phosphoproteins found on Ribosomes of various Eukaryotic Species.

Data on the number of phosphoproteins on 80S ribosomes are only given where none on phosphoproteins from subunits was available.

4.1 The Number of Ribosomal Proteins Phosphorylated *in vivo*.

The first objective of the work presented in this thesis was to determine the number of ribosomal proteins which are phosphorylated in animal cells *in vivo*. I have detected 5 phosphoproteins on the purified ribosomal subunits of Ascites and BHK cells analysed by SDS gel electrophoresis, and, in the case of Ascites cells, 3 on urea gels. This former figure is more than was originally reported by Kabat (1970) or Loeb and Blat (1970), although both these groups have detected more ribosomal phosphoproteins while this work was in progress (Cawthon *et al*.1974; Pierre *et al*., 1974). Table 4.1 shows the estimates obtained by these and other workers, of the numbers of phosphoproteins on ribosomes from several eukaryotic species. It can be seen that the total number of phosphoproteins I have found is similar to that obtained by most of these groups. One exception is the report that there are 8 phosphoproteins on the ribosomes of mouse mammary gland (Majumder and Turkington, 1972). However, these workers only analysed the proteins of 80S ribosomes, which I have found may be easily contaminated by non-ribosomal phosphoproteins (Fig. 3.1.4). Trewavas (1973), on the other hand, found only one phosphoprotein on ribosomes from the plant *Lemna Minor*. The reason for this discrepancy is unknown at present, although it may conceivably be related to the evolutionary distance between plants and other eukaryotes.

It seems most profitable to make detailed comparisons between my results and those of Kabat and co-workers, which are the most extensive of those cited in Table 4.1. In an examination of several tissues, including rabbit reticulocytes, chick embryo heart, liver and skeletal muscle, and sarcoma cells, they reproducibly find 5 ribosomal phosphoproteins. The estimated molecular weights of these proteins and their designations are:- On the 60S ribosomal subunit: Ia - 18,500, Ib - 19,500, III - 33,000; on the 40S ribosomal subunit: II - 27,500 and IV - 53,000. Although there are differences between the absolute values of these molecular weights and those I have determined (Figs. 3.2.2 and 3.2.4), these differences may be more apparent than real, because of the lower percentage of acrylamide (8%) in the polyacrylamide gels used by Kabat. Thus it is likely that the low molecular weight doublet Ia and Ib (estimated average molecular weight 19,000) they have detected is identical to the doublet C' and C" (estimated average molecular weight 14,000) which I have found.

More serious is the discrepancy between the subunit locations of the various proteins. Thus Kabat's largest phosphoprotein, IV, is generally found on the 40S subunit, whereas the largest I have found, phosphoprotein D, is on the 60S subunit. However, it is still possible that these are identical, as phosphoprotein IV has been detected on both ribosomal subunits (Krystosek *et al.*, 1974). This recalls the behaviour of C' and C"

in this work, for I have found that these latter proteins, though located predominantly on the 60S ribosomal subunit from Ascites cells, are located predominantly on the 40S ribosomal subunit in BHK cells (Section 3.4). Variable partition of certain (non-phosphorylated) proteins of both prokaryotes (Stoffler, 1974) and eukaryotes (Sherton and Wool, 1974b) between the two ribosomal subunits has also been observed, and in the latter study this was shown to be due to the different methods used to isolate ribosomes from different tissues. This has been interpreted to suggest that <u>in situ</u> these proteins are located at the interface between the subunits, and in one case this has been supported by chemical evidence (Stoffler, 1974).

Thus it seems likely that C' and C" may be located at the interface of the subunits, and possibly phosphoprotein D also. If phosphoprotein D and IV are equivalent, then there remains the difficulty that, although Ia and Ib clearly correspond to C' and C", my remaining phosphoproteins are both on the 40S subunit, while Kabat's phosphoprotein II is on the 40S subunit, but phosphoprotein III is on the 60S subunit. However, it is possible that phosphoprotein III may correspond to either proteins A or B, if this former protein is also located at the interface of the ribosomal subunits. It is not inconceivable that all but one of these phosphoproteins are at the subunit interface, as proteins I, III and IV all appear functionally similar in being more heavily phosphorylated in monosomes than in polysomes (Krystosek <u>et al.</u>, 1974). This might also reconcile my results, and

the results of Kabat with those of Ashby and Roberts (1975) who found only one major ribosomal phosphoprotein on the 40S subunit of rat cerebral cortex, but 4 on the 60S subunit.

The results presented in this thesis suggest that the ribosomes of the Krebs II Ascites cells contain between three and five phosphoproteins. The uncertainty concerns the number of ribosomal phosphoproteins on the 60S subunit. The fact that only two bands (3 and 4) were seen on urea gels at pH 4.5 (Fig. 3.2.5) compared with three on SDS gels (Fig. 3.2.3b) could be due, in part, to proteins C' and C" not being resolved in the former gel system. However, the fact that band 4 (Fig. 3.2.5) did not correspond to a stained protein suggests that it might be a trace non-ribosomal contaminant, a serious possibility considering the original contamination of 80S ribosomal proteins (Fig. 3.1.4). Clearly, this work emphasizes the need to have rigorously pure ribosomes, and to characterise the phosphoproteins from them with more than one electrophoretic system in studies of this type.

Elucidation of the actual number of ribosomal phosphoproteins will require their identification in the standard two-dimensional gel electrophoresis system (Kaltschmidt and Wittmann, 1970b). So far this has only been done for one of the phosphoproteins of the 40S subunit, which has been shown to be ribosomal protein S6 (Rankine and Leader, 1975; Gressner and Wool, 1974a). It is possible that the second phosphoprotein on the

40S subunit seen on SDS gels has not yet been identified on two-dimensional gels because of insolubility, or low mobility in the first dimension. Systematic alterations of the pH in the first dimension might allow this protein to be identified. However, the possibility cannot be ruled out that it is, in fact, a non-ribosomal protein such as a phosphorylated initiation factor (see below).

It did not prove possible to identify the phosphoproteins of the 60S ribosomal subunit on two-dimensional gels. This is probably because the transfer of protein from the first to the second dimension results in a considerable increase in the area occupied by the protein and a consequent decrease in the effective concentration of radioactivity available for autoradiography. Direct quantitation of the radioactivity in each spot revealed, on one occasion, a small amount of radioactivity in protein L35. This protein may be one or both of C' and C", and certainly the high mobility of L35 in the second dimension of the electrophoresis system would be consistent with the difficulty in detecting its radioactivity. It is necessary, however, to note that although Gressner and Wool (1974b) were able to obtain more counts in S6 from rat liver ribosomes than I have been able to achieve in Ascites cells, they were unable to detect any other phosphoproteins on two-dimensional gels. However, this may be because the ribosomes in normal rat liver are present predominantly as polysomes with few monosomes (Wettstein, 1963), in contrast to Ascites cells (Fig. 2.6.1). Thus if, as

reported by Kabat (1970), the other ribosomal phosphoproteins are only phosphorylated on monosomes, one would not expect to detect them in normal rat liver.

The identity of the contaminating material in protein extracted from unpurified 80S ribosomes (Fig. 3.1.2) remains obscure. One possibility is that this might include an initiation factor. Kabat (1970) suggested this for a phosphoprotein (of molecular weight about 70,000) which he found only on free 40S ribosomal subunits, but which was removed from the ribosome by washing in buffer of high ionic strength. Indeed, there is recent evidence that following depletion of haem, an initiation factor from reticulocytes does become phosphorylated (Balkow et al., 1975; T. Hunt, personal communication).

4.2 The Difference between the Number of Ribosomal Proteins Phosphorylated in vivo and in vitro.

The number of ribosomal phosphoproteins reported in this thesis, though greater than originally found by Kabat (1970) and Loeb and Blat (1970) in vivo, is still less than Eil and Wool (1971) obtained in vitro. Eil and Wool (1973a) isolated two cyclic AMP - dependent protein kinases from rat liver cytosol and found that these would phosphorylate 4 proteins from the 40S subunit, and 10 proteins from the 60S subunit of rat liver ribosomes. Similarly, Walton and Gill (1973) found that 2 proteins from the 40S subunit, and 10 from the 60S subunit of bovine adrenal cortical ribosomes

could be phosphorylated by a cyclic AMP - dependent protein kinase which was isolated either from the cytosol, or by washing the ribosomes in buffers of high ionic strength. Most workers find that whatever the source of the ribosomes or protein kinase, protein S6 is always phosphorylated *in vitro*, the number of other proteins phosphorylated being variable (Traugh *et al.*, 1973; Traugh and Traut, 1974; Ventimiglia and Wool, 1974).

There are several possible explanations for these differences between phosphorylation *in vivo* and *in vitro*. The protein kinase preparations used to catalyse the latter phosphorylation may differ in their specificity from those responsible for phosphorylation *in vivo*. Kinases with different specificities for ribosomal proteins do exist : many more proteins being phosphorylated *in vitro* by a protein kinase using ATP as the phosphate donor than when a protein kinase requiring GTP was used (Traugh and Traut, 1974; Ventimiglia and Wool, 1974). In this respect it is important to note that Kabat (1971), in contrast to others, found that the same proteins of the reticulocyte ribosome were phosphorylated *in vivo* and *in vitro*, using an endogenous protein kinase eluted from the ribosomes by washing in buffers of high ionic strength. It is possible that this enzyme is, in fact, the one responsible for phosphorylating ribosomes *in vivo*.

Another possible explanation of the discrepancies between the phosphorylation *in vivo* and *in vitro*

is that *in vivo* many ribosomal proteins may be masked by the substrates of the ribosome (mRNA, tRNA etc.), or indeed the complementary ribosomal subunit, and thus be inaccessible to protein kinase. Consistent with this possibility are the findings that fewer of the ribosomal proteins of polysomes (Stahl *et al.*, 1972), or 80S ribosomes (Stahl *et al.*, 1974) are phosphorylated *in vitro* than those of individual isolated ribosomal subunits.

4.2(a) The Quantitative Extent of the Phosphorylation of Ribosomal Protein S6.

In the following sections, the possible significance of the observed variations in the phosphorylation of ribosomal protein S6 is discussed. The method used to detect these changes in phosphorylation has been to examine the size of the anodic 'tail' of S6, assuming it to represent the increasingly phosphorylated derivatives of the protein. Although convenient, this approach has a limitation which should be emphasised at this juncture. This is that it provides no quantitative information on the actual proportion of ribosomes in which S6 is phosphorylated. Thus it is uncertain whether a larger anodic 'tail' to S6 indicates an actual increase in the number of phosphoryl groups per ribosome or a redistribution of the phosphate, say, from being mainly in the form of monophosphorylated S6 to being in the polyphosphorylated state, though involving

fewer ribosomes. Measurement of the radioactivity in S6 (with correction for the specific radioactivity of the cellular ATP pool) would be required to resolve this question.

Whether or not there is a net increase in the number of phosphoryl groups per ribosome, there is evidently a difference in the molecular nature of the phosphorylation under those conditions where the anodic 'tail' of S6 is elongated. However, the physiological significance of this difference clearly depends on what proportion of ribosomes it involves. The position of the darkened area of the autoradiograph of S6 in Fig 3.4.3(b) suggests that this proportion is, in fact, quite small (not more than about 10%) even where there is an anodic 'tail' to S6. In this situation (pre-confluent BHK cells) it would therefore be unwise to ascribe major changes in growth behaviour and ribosome metabolism to the phosphorylation (even if proportionately far greater than in post-confluent cells) because of the small number of ribosomes involved. Indeed, the possibility arises that the change in phosphorylation has no function in this case, and perhaps merely reflects the general level of cellular protein kinase and phosphatase activities. The ideas expressed in Section 4.5 regarding the possible function of the

phosphorylation of S6 will be seen to be more in accord with this possibility than those criticised in Section 4.3 below.

4.3 The Relationship between the Phosphorylation of Ribosomal Protein S6 and Protein Synthesis.

Another objective of the present work was to determine whether inhibitors of protein synthesis in general, (rather than merely sodium fluoride, as used by Kabat in reticulocytes) stimulated the phosphorylation of ribosomal proteins. It is evident, however, that neither sodium fluoride, pactamycin, nor cycloheximide had any effect on the phosphorylation of ribosomal protein in Ascites cells (Section 3.3). As sodium fluoride was the only inhibitor which Kabat (1970) examined, it might have been argued that the stimulatory effects he observed were due to some of the other inhibitory metabolic effects of sodium fluoride. However, Gressner and Wool (1974a) found that cycloheximide and puromycin stimulated the phosphorylation of protein S6 in rat liver, suggesting more strongly that inhibition of protein synthesis was involved. It thus seems that there is a difference in the way that rabbit

reticulocytes (Kabat, 1970), or rat liver (Gressner and Wool, 1974a) respond to inhibitors of protein synthesis, as compared with Ascites cells. One possible explanation (Gressner and Wool, 1974a) for these apparently discrepant results is that the inhibitors might act by preventing the synthesis of a phosphatase, which is turning over rapidly, and which has as its substrate the phosphorylated derivatives of S6 (Lightfoot et al., 1975). If the level of phosphatase (or its half-life) were much higher in Ascites cells, then the inhibition of protein synthesis might not completely eliminate the phosphatase activity in this tissue. However, this raises the disquieting alternative possibility that the level of phosphatase might be so high in Ascites cells that dephosphorylation of the ribosomes occurs during their isolation, obscuring possible direct effects of inhibitors of protein synthesis on the phosphorylation of ribosomal proteins. Although sodium fluoride would be expected to inhibit phosphoprotein phosphatase, one cannot be sure of this. However, other evidence argues against a necessary link between protein synthesis and phosphorylation of ribosomal protein. Thus I have found that in growing BHK cells, where protein synthesis is rapid, there is extensive phosphorylation of protein S6 (Section 3.4). Clearly, under physiological conditions, there is no requirement that protein synthesis be inhibited for phosphorylation of S6 to occur. Nor, indeed, is there a need for a high rate of protein synthesis, as protein S6 is not extensively phosphorylated in the livers of adult mice (Fig. 3.5.4)

where protein synthesis is rapid. Certainly, the slow rate of turn-over of ribosomal phosphoproteins would preclude phosphorylation and dephosphorylation occurring at each round of protein synthesis (Kabat, 1972).

Studies *in vitro* also do not support a correlation between phosphorylation of S6 and protein synthesis. Thus, Eil and Wool (1973b) examined several functions of rat liver ribosomes before and after phosphorylation by protein kinase, but could find no appreciable and consistent difference in the activity of phosphorylated and non-phosphorylated ribosomes. Similar results were obtained by Krystosek *et al.* (1974) who found that the addition of alkaline phosphatase to rabbit reticulocyte ribosomes had no effect on their ability to synthesise protein using exogenous mRNA. There is one report that phosphorylation of rat liver ribosomes *in vitro* decreased their ability to synthesise polyphenylalanine (Monier *et al.*, 1972). These results have not so far been substantiated, and may have been due to non-specific inhibitors present in the crude protein kinase preparation.

4.4 Cyclic AMP and the Phosphorylation of Ribosomal Protein S6.

Although there are no consistent data relating the phosphorylation of protein S6 to protein synthesis, there is a body of evidence relating its phosphorylation to the cellular concentration of cyclic AMP. Several workers have reported that the phosphorylation of ribosomal proteins can be stimulated by the direct

administration of cyclic AMP or dibutyryl cyclic AMP to rabbit reticulocytes (Cawthon et al., 1974) or rat liver (Gressner and Wool, 1974a) and in the latter case, the phosphorylated protein was identified as S6. Moreover, the phosphorylation of ribosomal proteins is also increased in the livers of rats under conditions in which increased concentrations of cyclic AMP have been observed. These conditions include treatment with glucagon (Blat and Loeb, 1971) and diabetes, where the protein phosphorylated is again known to be S6 (Gressner and Wool, 1976). There are also reports that thyroid hormones (Correze et al., 1972) and ACTH (Roos, 1973) can stimulate the phosphorylation of proteins associated with ribosomes. In these studies, 80S ribosomes rather than ribosomal subunits were examined, and were not analysed on polyacrylamide gels. Nevertheless, the possibility remains that cyclic AMP was stimulating the phosphorylation of ribosomal protein S6 in these situations.

In apparent contrast to these results, I was unable to detect any effect of dibutyryl cyclic AMP on the phosphorylation of ribosomal protein from Ascites cells (Fig. 3.5.1). This might again be due to high levels of phosphoprotein phosphatase, or perhaps high levels of phosphodiesterase, (although in this experiment the inhibitor of phosphodiesterase, 3-isobutyl-1-methylxanthine, was present). However, in my studies on growing and resting BHK cells (Section 3.5), I have found the greatest phosphorylation of S6 in growing cells, where cyclic AMP is relatively low (Rudland et al., 1974).

My results, therefore, indicate that in normal cells, growing under physiological conditions, the phosphorylation of S6 may be controlled independently of the concentration of cyclic AMP. In the next Section I shall discuss whether this type of phosphorylation may be more significant than that which can be induced by cyclic AMP.

4.5 Possible Functions for the Phosphorylation of Protein S6.

If, as I have argued, the phosphorylation of S6 need not be related to protein synthesis or the cellular concentration of cyclic AMP, then the questions still remain - how is this phosphorylation controlled and what is its function *in vivo*? I have found that in BHK cells there seems to be a correlation between the phosphorylation of S6 and the overall growth history of the cell. The metabolic process occurring during rapid cellular growth and division that seems to me to be most likely to be functionally correlated to the phosphorylation of ribosomes is, in fact, the synthesis of ribosomes *de novo*. Thus ribosomal protein S6 may normally be phosphorylated in the nucleolus and gradually dephosphorylated in the cytoplasm. Hence, in growing cells the extensive phosphorylation of S6 would reflect the large proportion of newly synthesised ribosomes. Moreover, the short-term stimulation of the growth of BHK cells by the addition of fresh medium (Fig. 3.5.3) would not be expected to result in the synthesis of sufficient new ribosomes to significantly alter the overall phosphorylation of S6. The idea that S6

is initially phosphorylated in the nucleolus might also explain the greater phosphorylation of this protein in regenerating rat liver (Gressner and Wool, 1974b) where the synthesis of ribosomes is stimulated. However, in this situation the transient early rise in cyclic AMP (MacManus et al., 1972) is a complicating factor. A nucleolar role for the phosphorylation of S6 is attractive as it would rationalise the finding that prokaryotes - which lack nucleoli - also lack phosphorylated ribosomal proteins (Gordon, 1971).

This idea, which suggests that the phosphorylation of S6 might be important for the formation of ribosomes in the nucleolus rather than for their function in the cytoplasm, was first considered by Gressner and Wool (1974b). It was discarded by them because of their subsequent findings of the effects of cyclic AMP and inhibitors of protein synthesis on the phosphorylation of this protein (Gressner and Wool, 1974a, 1976). However, their results can be rationalised if it is assumed that although ribosomal protein S6 is normally slowly dephosphorylated after leaving the nucleolus, it is susceptible to rephosphorylation under the unphysiological conditions they and others have used (Kabat, 1970; Cawthon et al., 1974; Gressner and Wool, 1974a). However, I would suggest that such rephosphorylation is probably non-functional.

A possible role for the putative phosphorylation of S6 in the nucleolus is suggested by observations that there is normally 'wastage' of ribosomal precursor

RNA (Cooper and Gibson, 1971; Chaudhuri and Lieberman, 1968). This has led Warner and co-workers to postulate that ribosomal protein rather than ribosomal precursor RNA is limiting for the assembly of the precursor ribonucleoprotein particle (Warner et al., 1973). A regulatory role for the phosphorylation, rather than the availability of ribosomal protein in this process seems an attractive possibility, in view of the well-documented increase in phosphorylation of nuclear proteins during cell growth (Rubin and Rosen, 1975). Indeed, the phosphorylation of a variety of nuclear proteins - histones, non-histone proteins, and ribosomal proteins - might be a co-ordinated response to the demands of such growth. Clearly, it would be important to establish whether S6 is phosphorylated on pre-ribosomal particles in the nucleolus. The question of whether this putative nucleolar phosphorylation of S6 is required for ribosome assembly, or for some other function, such as extra-nuclear transport, is clearly a suitable subject for future studies.

There remains one observation in this work which is somewhat difficult to incorporate into this scheme, namely, that S6 from the liver ribosomes of young mice is more extensively phosphorylated than S6 from liver ribosomes of adult mice (Fig. 3.5.4). The more rapid rate of cell growth in this case would not be likely to produce a substantial proportion of new ribosomes. It has been reported that in rapidly growing cells there is a slower turnover of ribosomes (Green, 1974), and there thus exists the possibility that the phosphorylation of S6 might play

an additional role in determining the overall lifespan of the ribosome in the cytoplasm. Alternatively, the presumed low level of phosphoprotein phosphatase in the livers of young mice and the consequent increased phosphorylation of S6 may have no functional significance.

4.6 Possible Functions of the Other Ribosomal Phosphoproteins.

What of the possible functions of ribosomal phosphoproteins other than protein S6? It was found that these proteins are extensively phosphorylated on monosomes, but not on polysomes (Kabat, 1970, 1972). Thus it is possible that the phosphorylation of these proteins might play a role in the recruitment of ribosomal subunits into the pool of inactive monosomes, which is apparently unique to eukaryotes (Kabat, 1970). The probable position of C' and C" (and perhaps the other phosphoproteins) between the two ribosomal subunits, suggested here, is of interest because such a location would be most appropriate to a role in holding the subunits together as inactive monosomes.

However, it is necessary to try to reconcile such a possible role with the lack of effect of phosphorylation *in vitro* on protein synthesis (Eil and Wool, 1973b; Krystosek *et al.*, 1974). Eil and Wool (1973b) did not, in fact, show that proteins C' and C" were phosphorylated in rat liver, and the cell-free system used by Krystosek *et al.* (1974) may have been contaminated by phosphoprotein phosphatases. In any case, the approaches these groups have used may not have been the most appropriate

to the suggested role of the phosphoproteins. A more relevant approach would be to first establish some functional difference between monosomes and native ribosomal subunits or polysomes, and then determine whether the phosphorylation or dephosphorylation of ribosomal proteins affected such a function. Until such experiments are performed, it remains a possibility that the role of these ribosomal phosphoproteins is in the recruitment of ribosomes for monosomes.

Thus it seems that eukaryotic cells may have evolved the phosphorylation of ribosomal proteins so as to control two separate processes, in both of which they differ appreciably from prokaryotes.

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