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The Phosphorylation of Eukaryotic Ribosomal Proteins

by

Michael J. McGarvey

A thesis submitted for the Degree of Ph.D.

Department of Biochemistry,
University of Glasgow.

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Abbreviations

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

BHK	:	baby hamster kidney fibroblasts
BSA	:	bovine serum albumin
PPO	:	2,5 diphenyloxazole
POPOP	:	p-bis- 2-(5-Phenyloxazolyl) -benzene
TEMED	:	N,N,N'N'-tetramethylethylene diamine
SDS	:	sodium dodecyl sulphate

The system of nomenclature for ribosomal proteins proposed by McConkey et al., (1979) was used in this thesis.

One unit of protein kinase is the amount of enzyme that will catalyse the transfer of one picomole of phosphate from ATP to a protein substrate in one minute.

1 A_{260} Unit : The quantity of material contained in 1 ml of a solution which has an absorbance of 1 at 260 nm, when measured in a cell with a 1 cm light path. This is taken to be equivalent to:

100 μg of ribosomes, or

50 μg of ribosomal RNA

SUMMARY

Summary

Two different casein kinase (CKI and CKII) and one histone kinase (HK) activities were resolved from the ribosome-free cytoplasm of Krebs II ascites cells by chromatography on DEAE-cellulose and phosphocellulose. One of these, CKII was able to use either ATP or GTP to phosphorylate the acidic 60S subunit protein L_γ, but phosphorylated no other ribosomal proteins. A second casein kinase, CKI could use ATP but not GTP, to phosphorylate L_γ. CKI was less specific than CKII and apart from L_γ was able to phosphorylate a number of proteins which were phosphorylated *in vivo* (S6, Sa and Sb) as well as a protein which was not phosphorylated *in vivo* (S7). The other kinase, HK was able to use ATP to phosphorylate S6 as well as other proteins (S7, S10, S14, S20 and L35) which were not phosphorylated *in vivo*.

None of these three protein kinases was stimulated by cyclic AMP or inhibited by the heat stable cyclic AMP-dependent protein kinase inhibitor protein. CKI and CKII were both inhibited, to different extents, by heparin whereas HK activity was stimulated by the presence of heparin. Neither CKI nor CKII was affected by the presence of calmodulin but HK was stimulated four-fold. CKII, but not CKI or HK, was selectively inhibited by protein kinase inhibitor protein 'CKGI' (Job *et al.*, 1979). Krebs II ascites ribosomal proteins S3, S2 and L14 (which became phosphorylated when ascites cell incubation medium contained glucose and amino acids) were not phosphorylated by any of these three protein kinases. Ascites cells incubated in medium containing glucose and amino acids

however contained a labile protein kinase activity not seen previously.

Protein kinases from both Krebs II ascites cells and other tissues phosphorylated proteins of similar molecular weight to S6 and L_γ in a preparation of extracted ribosomal protein.

Protein kinases from the cytosol of BHK cells were resolved on DEAE-cellulose. In cytosol from cells infected by pseudorabies virus a novel histone kinase activity was detected. Analysis on one-dimensional SDS gel electrophoresis showed that an as yet unidentified protein of the 40S subunit of molecular weight 22,000 was specifically phosphorylated by this new protein kinase activity.

1. INTRODUCTION

1.1 The Eukaryotic Ribosome

The ribosome is central in the translation of genetic information into proteins required for the structure and function of the cell.

The first evidence for the existence of ribosomes came from Claude (1941) who observed microscopic particles within chick and mammalian embryo cells, and also from Luria, Delbruck and Anderson (1943) who similarly observed granular particles in electron micrographs of phage lysed *E. coli*. Studies by electron microscopy (Palade and Siekevitz, 1956) and electrophoresis (Petermann, Hamilton and Mizen, 1954) confirmed the existence of these structures, and their nucleoprotein composition. Subsequently the term 'ribosome' was first used by Roberts (1958) to describe this ribonucleoprotein particle. At about this time a direct relationship between the RNA content of a cell and the rate of protein synthesis was shown by Brachet (1941) and Casperson (1941).

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

Sedimentation analysis of the ribosome of the prokaryote *E. coli* showed it to have a sedimentation coefficient of 70S, and that it could be dissociated to give a 30S and a 50S

subunit (Tissieres et al., 1959) at low magnesium concentrations (Chao, 1957). Similarly the 80S eukaryotic ribosome was shown to consist of a 40S and a 60S subunit, (Petermann, 1964). Evidence from starch gel electrophoresis and carboxymethyl-cellulose chromatography, demonstrated that ribosomes contain a large number of different proteins (Waller, 1964). This was confirmed by analysis on polyacrylamide gel electrophoresis, peptide mapping, and further chromatographic resolution of the proteins (Traut, et al., 1967; Kaltschmidt et al., 1967; Hardy et al., 1969). The E. coli ribosome was eventually shown to contain 21 unique proteins in the 30S subunit, and 34 in the 50S subunit (Wittmann, et al., 1980). Eukaryotic ribosomes have been shown to contain between 70 and 85 different proteins (reviewed by Wool, 1979). This variation in a number might reflect genuine differences in number in different species of eukaryotes (Wool and Stöffler, 1974) or alternatively it could result from the difficulty in deciding whether or not a protein is a ribosomal structural protein (Warner et al., 1973).

The molecular weight of mammalian ribosomal subunits have been estimated to be approximately 1.5×10^6 for the 40S and 2.9×10^6 for the 60S (Hamilton, Pavlovec and Petermann, 1971). The term '80S ribosome' is commonly used to refer to eukaryotic ribosomes, although this term is somewhat misleading since the mass of the'80 ribosome' can vary from 3.9×10^6 in plants to 4.55×10^6 in mammals (Cammarano et al., 1972).

Four species of RNA have been found in the eukaryotic ribosome, 18S in the 40S subunit: and 28S, 5S and 5.85 in the

60S subunit (reviewed by Maden, 1971). This compares to three RNA molecules in the E. coli ribosome: 16S (Kurland, 1960) in the 30S subunit, and 23S (Stanley and Block, 1965) and 5S (Rosset and Monier, 1963) in the 50S subunit.

Ribosomes have been observed within all eukaryotes (reviewed by Wool and Stoffler, 1974; Bielka and Stahl, 1978; Wool, 1979; and Wool, 1980) and prokaryotes (reviewed by Jaskumas, Nomura and Davies 1974; Brimacombe, Stöffler and Wittmann, 1978; Osawa and Hori, 1980). In addition it has been found that ribosomes exist within the mitochondria (Borst and Grivell, 1971; Kroon, Agsteribbe and De Vries, 1972) and chloroplast (Lyttleton, 1962) but are different from those in the cytoplasm. Chloroplast ribosomes have a sedimentation coefficient of approximately 70S and contain similar ribosomal RNA species to bacteria i.e. 16S, 23S and 5S (reviewed by Chua and Luck, 1974; Boynton, Gillham and Lambowitz, 1980). Mitochondrial ribosomes from fungi and higher plants have a sedimentation coefficient of 70-75S and contain two large species of rRNA and a 5S RNA whereas those from animal cells have a sedimentation coefficient of 55S-60S and contain only the two larger species of rRNA (reviewed by Chua and Luck, 1974; Boynton, Gillham and Lambowitz, 1980). Mitochondrial and chloroplast ribosomal proteins are different from those found in cytoplasmic ribosomes, although most of these organelle ribosomal proteins are encoded by nuclear DNA and are synthesised on cytoplasmic ribosomes (Chua and Luck, 1974). Only cytoplasmic eukaryotic ribosomes will be considered further.

1.1.1 Ribosomal Proteins

Since there are no covalent bonds between the components of a ribosome, it is necessary to define what exactly is a ribosomal protein. This is especially important since many proteins can bind to ribosomes both specifically (e.g. initation factors in both prokaryotes and eukaryotes: Weissbach and Ochoa, 1976) and non-specifically (e.g. E. coli RNase: Waller, 1964). For most purposes proteins are usually regarded as ribosomal if they remain with the ribosome after repeated washing with high concentrations of salt (Wool and Stöffler, 1974). However, this can lead to difficulties since the high salt concentrations needed to remove contaminating proteins can sometimes remove ribosomal proteins (Hardy, 1975).

Zinker and Warner (1976) have expanded the definition of a ribosomal protein to include three classes of protein. Class one proteins are assembled with a ribosomal RNA molecule in the nucleus, and remain with that RNA molecule. Class two proteins undergo exchange between the ribosome and the soluble cytoplasmic protein in vivo as shown by their appearance on ribosomes when no ribosome synthesis is taking place (Warner et al., 1973). These proteins remain part of the ribosome under washing conditions of high ionic strength. Class three proteins can be removed by washing with high ionic strength salt and may either be essential ribosomal proteins (e.g. protein S1 in E. coli : Van Dieijen et al., 1975) or adventitiously adsorbed proteins.

Ribosomes can be dissociated into 40S and 60S subunits under conditions of low magnesium concentration and high ionic

strength (Martin and Wool, 1968) and these subunits can be separated on the basis of their different sedimentation coefficients (e.g. by sucrose density gradient centrifugation). Ribosomal proteins can then be extracted from the purified subunits under conditions which will solubilize the proteins, but will at the same time precipitate ribosomal RNA (e.g. Lithium Chloride - Urea : Huvos and Cox, 1975; HCl : Welfle, Stahl and Bielka, 1972; or guanidine - HCl : Huvos and Cox, 1975). However the method of Sherton and Wool (1972), which uses acetic acid and magnesium chloride (c.f. 2.3.8) has been described as the most efficient (Bielka and Stahl, 1978; Wool, 1979).

Ribosomal proteins can be partially resolved by one-dimensional sodium dodecyl sulphate gel electrophoresis into a number of protein bands. However the molecular weights of many proteins are similar which means that the number of discrete bands seen on one-dimensional gel electrophoresis (approximately 20 for 40S subunit protein and 21 for 60S subunit protein) is far fewer than the actual number of proteins (Collatz et al., 1977; Tsurugi et al., 1977). Better resolution and estimates of the number of proteins have been obtained by two-dimensional polyacrylamide gel electrophoresis. Kaltschmidt and Wittmann(1970) devised a system for the resolution of E. coli ribosomal proteins in which proteins were separated on the basis of net charge at pH 8.7 at low acrylamide concentration in the first dimension and mainly on the basis of size at high acrylamide concentrations in the second dimension. This system was used with eukaryotic ribosomal proteins by Welfle, Stahl and Bielka (1971); Huynh

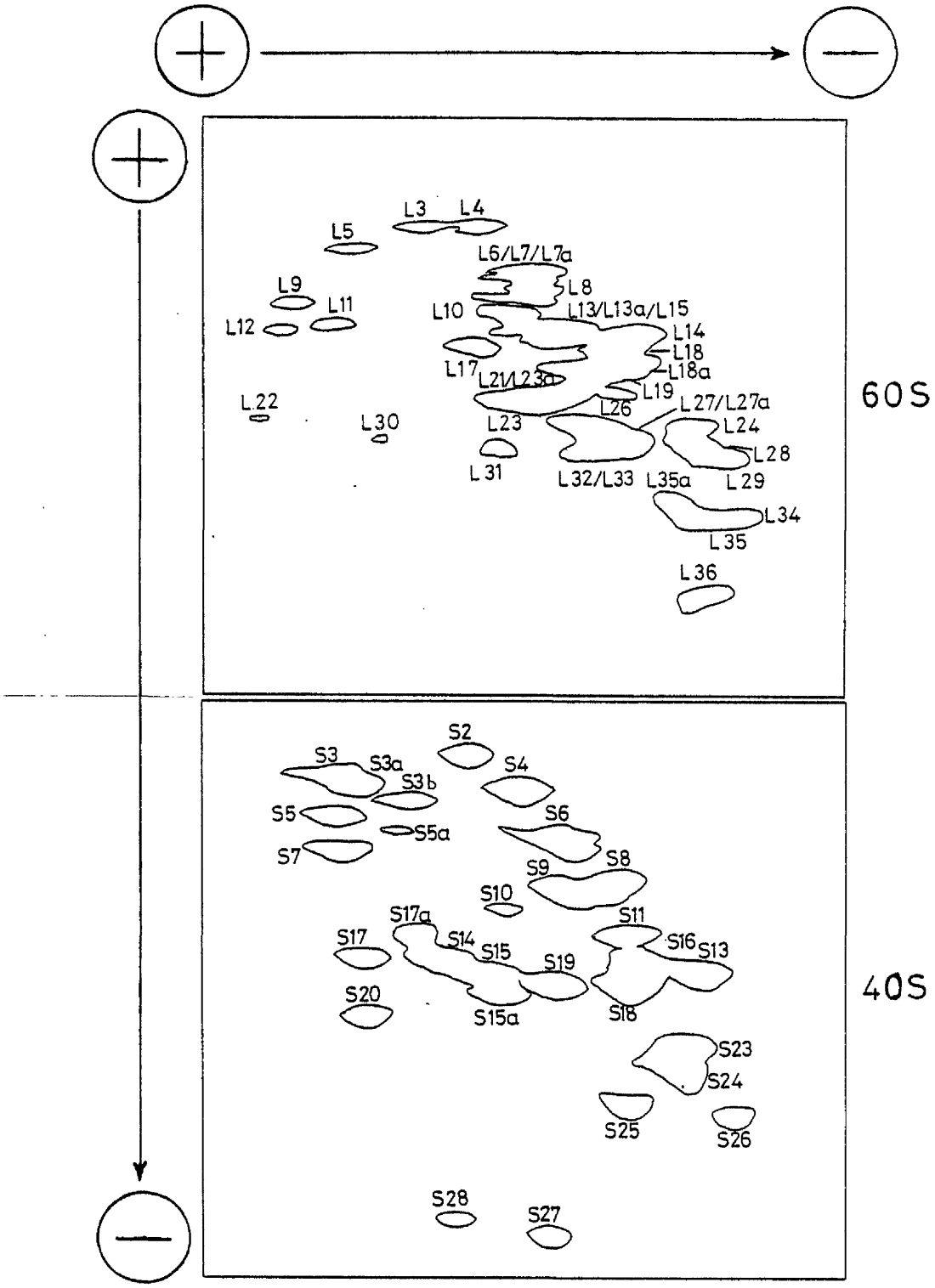
Delauny and Schapira (1971) and Sherton and Wool (1972) to enumerate the proteins in rat liver ribosomes.

Other two-dimensional gel electrophoresis systems have been devised in which the first-dimensional pH differs from that used by Kaltschmidt and Wittmann(1970) and SDS has been included in the second dimension (e.g. Martini and Gould, 1975; Mets and Bogorad, 1974). A modified form of the Kaltschmidt-Wittmanngel electrophoresis system has been introduced by Lastick and McConkey (1976), and it results in the separation of most ribosomal proteins into discrete spots (c.f. 2.3.19).

The positions of separate protein spots on two-dimensional gels forms the basis of nomenclature of ribosomal proteins (Fig 1.1). A recent attempt to standardise the nomenclature of basic ribosomal proteins has been undertaken by McConkey et al., (1979), and this is the system of nomenclature which has been used in the present study (Fig 1.1). One problem with the Kaltschmidt and Wittmann two-dimensional gel electrophoresis system is that acidic proteins of isoelectric point less than 8.6 will migrate in the opposite direction to the more basic proteins. This makes analysis of all the ribosomal proteins on a single gel difficult. To overcome this problem Knopf et al., (1975) devised a two-dimensional gel system in which the first dimensional pH was 5.0, with a conventional Kaltschmidt-Wittmannsecond dimension. However, a better system for the resolution of very acidic proteins was derived from that of Knopf et al., (1975) by Leader and Coia (1978a) described in 2.3.20. In this gel electrophoresis system, acidic proteins of isoelectric points between pH 3.55 and 5.5 are 'swept' into the first dimensional gel in a concentrated band. In the

Fig 1.1 Two-Dimensional Gel Electrophoresis and Nomenclature of Ribosomal Proteins (McConkey et al., 1979)

This figure is a schematic representation of 40S and 60S rat liver ribosomal proteins separated by two-dimensional gel electrophoresis (Lastick and McConkey, 1976). The proteins are numbered from left to right in descending tiers (i.e. lowest number is top left; highest number is bottom right).


L3
L4
L5
L6/L7/L7a
L8
L9
L11
L12
L10
L13/L13a/L15
L14
L18
L17
L18a
L21/L23a
L19
L22
L26
L27/L27a
L30
L23
L24
L28
L31
L32/L33
L35a
L29
L34
L35
L36
60S
S2
S3
S3a
S4
S3b
S5
S6
S5a
S7
S8
S9
S10
S11
S17a
S17
S14
S15
S16
S13
S19
S20
S15a
S18
S23
S24
S25
S26
S28
S27
40S

second dimension this band is resolved to give a corresponding 'sweep-spot' of acidic proteins. It should be mentioned however that the resolution of basic proteins is poorer in the 'Sweep' gel system than in the system of Lastick and McConkey (1976).

Eukaryotic ribosomal proteins have been purified by chromatographic procedures (Wool, 1979). Tsurugi et al.,(1977), Collatz et al., (1977) and Tsurugi et al., (1978) have separated rat liver ribosomal proteins by ion-exchange chromatography on carboxymethycellulose, phosphocellulose and DEAE-cellulose, as well as by gel filtration. The number of proteins identified by these procedures was 84 (Wool, 1980), considerably more than were estimated previously by two-dimensional polyacrylamide gel electrophoresis (Wool and Stoffler, 1974). The identities of these proteins were determined by two-dimensional gel electrophoresis and their molecular weights were estimated by one-dimensional SDS gel electrophoresis (Table 1.1). A number of proteins were found which had not been seen previously. Some of these proteins (e.g. the acidic proteins Sa, Sb, P1 and P2) might have been overlooked in earlier studies since they did not migrate in the same direction as basic proteins on two-dimensional gel electrophoresis.

Some caution must be used however before accepting that all of these proteins purified from rat liver are in fact unique. Madjar and Traut (1980) have compared the proteins from rat liver ribosomes prepared in the presence or absence of the protease inhibitor phenylmethysulphonylfluoride (PMSF) on the 'four-corner' two-dimensional gel electrophoresis system (Madjar et al., 1979). In the presence of PMSF several

Legend to Table 1.1

In the 40S subunit the ΣM_r = 746,000 ; number of proteins = 34; average M_r = 22,000. In the 60S subunit the ΣM_r = 1,049,000; number of proteins = 50 average M_r = 21,000.

Table 1.1 Molecular Weights of Rat Liver Ribosomal Proteins (Wool, 1980).

40S Subunit		60S Subunit			
Proteins	Mr($\times 10^{-3}$)	Proteins	Mr($\times 10^{-3}$)	Proteins	Mr($\times 10^{-3}$)
Sa	41.5	La	37.9	L27a	18.0
Sb	33.0	Lb	29.8	L28	17.8
S1	39.0	Lf	14.6	L29	20.5
S2	33.1	P1	16.1	L30	14.5
S3	30.4	P2	15.2	L31	15.6
S3a	32.0	P3	13.0	L32	17.2
S3b	30.4	L3	37.8	L33	15.6
S4	29.5	L4	41.8	L34	15.8
S5	22.8	L5	32.5	L35	17.5
S5a	21.5	L6	33.0	L35a	13.7
S6	31.0	L7	29.2	L36	14.3
S7	22.2	L7a	28.7	L36a	16.2
S8	26.8	L8	28.4	L37	15.4
S9	24.3	L9	24.7	L37a	12.8
S10	20.1	L10	24.2	L38	11.5
S11	20.7	L11	21.3	L39	11.6
S12	14.9	L12	18.7		
S13	18.6	L13	26.3		
S14	17.3	L13a	24.6		
S15	19.6	L14	25.8		
S15a	15.7	L15	24.5		
S16	17.1	L16	18.7		
S17	18.0	L17	22.1		
S18	18.5	L18	24.5		
S19	17.1	L18a	21.3		
S20	16.5	L19	25.3		
S21	12.3	L20	16.2		
S23/24	18.8	L21	20.3		
S25	17.0	L22	16.1		
S26	16.5	L23	15.6		
S27	14.5	L23a	18.0		
S27a	12.8	L25	17.5		
S28	11.3	L26	18.6		
S29	11.2	L27	17.8		

rat liver ribosomal proteins disappeared, and some double protein spots became single spots. This suggests that some of the rat liver ribosomal proteins purified by Tsurugi et al., (1977), Collatz et al., (1977) and Tsurugi et al., (1978) could be derived from others by proteolysis.

The molecular weight number average for the 34 rat liver ribosomal proteins from 40S subunit is approximately 22,000 with the range 11,200 to 39,000 and for the 50 proteins from the 60S subunit the number average is 21,000 with the range 11,500 to 41,800.

Rat liver ribosomal proteins contain large proportions of both basic and acidic amino acids (between 15-30% and 14-24% of the total respectively : Wool and Stöffler, 1974; Bielka and Stahl, 1978). Many of the acidic amino acids are amidated thus adding to the basic nature of most ribosomal proteins. The amino acid composition of many basic ribosomal proteins is very similar and this has been taken as evidence for a possible evolutionary relationship between them (Wool, 1979).

Sequence analysis of eukaryotic ribosomal proteins unlike prokaryotic ribosomal proteins (Wittmann,Littlechild and Wittmann-Liebold, 1980) is mostlylimited to the amino-terminus of several rat liver ribosomal proteins (Wittmann-Liebold et al., 1979). One eukaryotic ribosomal protein which has been completely sequenced is eL 12 from Artemia salina (Amons, Pluijms and Moller, 1979). A considerable amount of evidence indicates that this protein is the eukaryotic equivalent of E. coli protein L7/L12 (Kischa, Möller and Stöffler, 1971) and most eukaryotic species studied contain similar acidic proteins. The most interesting feature of this protein (in

relation to the present study) is that it can be phosphorylated, and this will be considered in more detail in 1.3.3.

Two-dimensional gel analysis of ribosomal proteins from different tissues of the same organism gives essentially the same pattern (Martini and Gould, 1975; Delaunay et al., 1973; Fujisawa and Elicein, 1975). There are some differences in the pattern of ribosomal proteins from different species of eukaryotes(e.g. chick and rat liver : Ramjoue and Gordon, 1977; HeLa and Krebs II ascites cells : Issinger and Beier, 1978; rat liver and rabbit reticulocytes : Madjar and Traut, 1980) suggesting evolutionary divergence between the ribosomes of different species.

In contrast to the wealth of information concerning the genes for ribosomal proteins in E. coli (Nomura, Morgan and Jaskunas, 1977; Isono, 1980; Nomura and Post, 1980) much less is known about the genes for eukaryotic ribosomal proteins. Some recent evidence indicates that the genes for individual ribosomal proteins in higher eukaryotes are encoded by multigene families with an average of ten members (Monk et al., 1981), and that these gene families are widely dispersed throughout the genome (D'Eustachio et al., 1981).

1.1.2. Synthesis and Assembly of Eukaryotic Ribosomes

Ribosome synthesis in eukaryotes requires the coordinate expression of genes encoding between 70-85 different proteins as well as the genes specifying 18S, 28S, 5.8S and 5S rRNA (Perry, 1972). The synthesis of 18S, 28S and 5.8S RNA is coordinate since the genes for all these RNA molecules belong

to the same transcription unit (Maden et al., 1977; Long and Dawid, 1980). However the different genome locations of the genes for 5S RNA (Long and Dawid, 1980) and for ribosomal proteins (D'Eustachio et al., 1981) indicates that more complex mechanisms, perhaps involving feedback regulation (Pelham and Brown, 1980) are also involved. Further complexity is added since the 45S pre-rRNA transcription unit is transcribed by RNA polymerase I, whereas the 5S RNA genes are transcribed by RNA polymerase III, and ribosomal protein genes are transcribed by RNA polymerase II.

Eukaryotic ribosomal proteins are synthesised in the cytoplasm (Craig and Perry, 1971; Maisel and McConkey, 1971) and migrate rapidly to the nucleolus (Warner and Soeiro, 1967; Maisel and McConkey, 1971; Wu and Warner, 1971). Little is known about the way in which ribosomal proteins migrate to the nucleus. The nuclear envelope is studded with polyribosomes, but these do not appear to be enriched with mRNA for ribosomal proteins (Craig and Perry, 1971). The complex structure of the nuclear envelope, with a double layer of membranes and a proteinaceous lamina (Kirschner, Rusli and Martin, 1977) makes direct extrusion of newly formed proteins into the nucleus seem unlikely. There is also little evidence to determine whether the passage of proteins into the nucleus occurs by passive diffusion or some form of facilitated transport (Warner et al., 1980).

Warner (1979) has shown that most newly formed proteins are concentrated 10 to 15 fold in the nucleolus and two to five fold in the nucleoplasm. Pre-treatment of cells with

actinomycin D, to deplete pre-rRNA, had no effect on the concentration of newly formed ribosomal proteins in the nucleus, but did lead to an increased amount in the nucleoplasm at the expense of the nucleolus. This suggests that ribosomal proteins concentrate in the nucleus independently of the presence of pre-rRNA. However, subsequent binding to RNA may account for the very high concentration found in the nucleolus. This suggestion is supported by the finding that most ribosomal proteins are present in equimolar amounts in the nucleolus.

Inhibition of rRNA synthesis by low doses of actinomycin D (this treatment specifically inhibits RNA polymerase I) shows that ribosomal protein synthesis continues for more than 24 hours after the transcription of 45S rRNA has been suppressed (Warner, 1977). However ribosomal proteins made in the absence of 45S RNA transcription are unstable and are degraded with half-lives of 30-90 minutes. Yet under normal conditions they are very stable, presumably because their assembly into a ribosome provides a long-lived structure in which the components are much less susceptible to degradation (Warner, 1977). This finding suggests that the synthesis of pre-mRNA for ribosomal proteins is independent of the synthesis of ribosomal precursor RNA. In contrast to the continued synthesis of ribosomal proteins in the absence of ribosomal RNA, it appears that rRNA synthesis decreases in the absence of ribosomal proteins (Warner *et al.*, 1980).

One suggested regulatory mechanism for the synthesis of eukaryotic ribosomal proteins is that each protein could limit its own synthesis (Warner *et al.*, 1980). Although there is no

evidence for such a mechanism in eukaryotes, certain ribosomal proteins in E. coli do have the capacity to prevent the further translation of their own mRNA by feedback repression (Dean et. al., 1981).

Assembly of ribosomes occurs in the nucleolus (Maden, 1968) and an 80S ribonucleoprotein particle, thought to be the precursor for 40S and 60S subunits, can be isolated from the nuclei of cells engaged in ribosome synthesis (Warner and Soeiro, 1967). This 80S particle contains the 45S pre-rRNA transcript together with many 40S and 60S ribosomal proteins, and some non-ribosomal 'assembly' proteins (Auger-Buendia and Longuet, 1978; Auger-Buendia et al., 1979). This 80S particle undergoes maturation to give the 40S ribosomal subunit together with a 55S (HeLa cells : Warner and Soeiro, 1967; Kumar and Subramanian, 1975) or 60S (rat liver : Higashinakagawa and Muramatsu, 1974; mouse leukaemia cells : Auger-Buendia and Longuet, 1978) ribonucleoprotein particle.

During the maturation of the 80S precursor particle, the 45S pre-rRNA transcript is cleaved to give 18S rRNA and 32S pre-rRNA in a process which requires the presence of ribosomal proteins (Warner and Udem, 1972). The 55-60S precursor particle contains the 32S pre-rRNA, the 5S RNA and approximately 60 proteins at least half of which are found in the mature 60S ribosomal subunit (Kumar and Subramanian, 1975). Many of the non-ribosomal proteins found in the 55-60S precursor particle are similar to those found in the 80S precursor particle (Auger-Buendia and Longuet, 1978). These non-ribosomal or 'assembly' proteins appear to be used

repeatedly as scaffolding for ribosome assembly in the nucleolus (Warner et al., 1980). The 55-60S precursor particle matures to give the 60S ribosomal subunit by further processing of the 32S pre-rRNA to 28S and 5.8S RNAs and a change in the complement of associated proteins. The processes involved in transporting mature ribosomal subunits from the nucleus to the cytoplasm are largely unknown (Warner et al., 1980).

The question as to whether cytoplasmic ribosomes undergo an exchange of proteins with free ribosomal proteins present in cytoplasmic pools has been controversial. Dice and Schimke (1972) reported that up to 17% of the total amount of ribosomal proteins in rat liver were present in free cytoplasmic pools, and that there was a rapid exchange between the free proteins and those in ribosomes. Subsequent attempts (Wool and Stöffler, 1976 ; Warner 1977) have repeatedly failed to demonstrate significant pools of most ribosomal proteins in the cytoplasm. Some exchange of ribosomal proteins does however take place between the ribosome and the cytoplasm (Warner and Gornstein, 1978), but it is likely that this exchange is restricted to a relatively small number of proteins.

Ribosomes are relatively stable with a half-life of approximately five days (Hirsch and Hiatt, 1966) and they appear to turnover as whole units with the degradation of both proteins and rRNA occurring simultaneously (Warner et al., 1980

1.1.3. The Structure of the Ribosome

The supramolecular structure of ribosomes has been investigated by a wide range of chemical and physical techniques. Most investigations have concentrated on *E. coli* ribosomes since the identities of all the protein moieities, and the primary structure of most of these, have been determined (Wittmann, Littlechild and Wittmann-Liebold, 1980). Reconstitution of *E. coli* ribosomes from purified rRNAs and ribosomal proteins has been useful both in the study of assembly intermediates and also the protein topography of the ribosome (reviewed by Nierhaus, 1980). Reconstitution studies of eukaryotic ribosomes have been limited by a lack of clear identification of all the structural ribosomal proteins, and it is possible that the eukaryotic ribosome undergoes irreversible steps in the assembly process (e.g. the removal of transcribed spacers from the 45S pre-rRNA could allow conformational changes to occur in the nascent ribosome precursor particle : Warner, 1974). One further advantage in using *E. coli* to study ribosome structure is that the ribosomal components are much more amenable to genetic analysis in *E. coli* than in eukaryotes.

However, many of the chemical labelling techniques used to investigate *E. Coli* ribosome structure have been used with eukaryotes. Enzymatic radio-iodination with lactoperoxidase (Leader, 1975) has shown that most eukaryotic ribosomal proteins have at least some part of their structure on the surface of the ribosomal subunits. Similar results were obtained when reductive methylation was used to label proteins with ^{3}H (Reboud *et al.*, 1977; Kisilevsky, Weiler and Treloar, 1978).

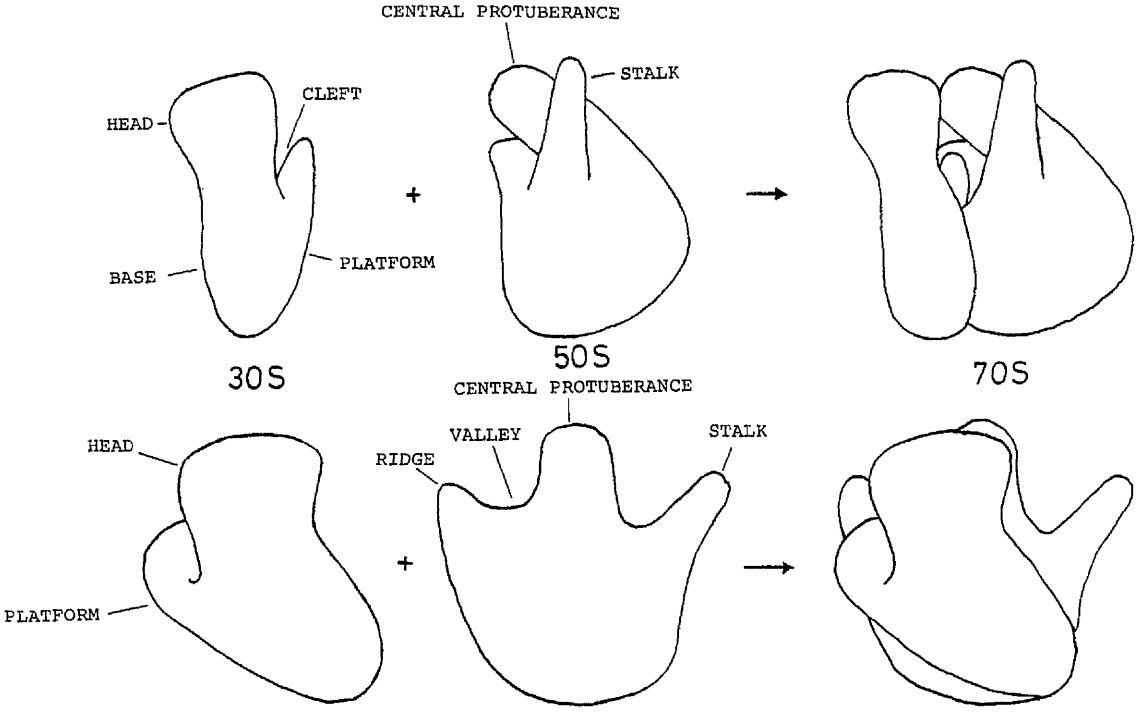
Crosslinking of E. coli ribosomal proteins with bifunctional agents (reviewed by Traut, 1980) and affinity labelling of specific sites in the ribosome (reviewed by Pellegrini and Cantor, 1977) have been used to build maps of ribosomal protein topography, and the identification of proteins involved at specific functional sites. Cross-linking of rat liver ribosomal proteins has recently been performed in both the 40S (Terao et al. 1980) and 60S (Uchiumi, Terao and Ogata, 1980) subunits. Rat liver 40S subunit proteins have also been crosslinked to initiation factor eIF-2 (Westermann et al., 1979), and proteins S and S6 in particular have also been cross-linked to the ternary initiation complex (Westermann, Nygard and Bielka, 1981). Proteins involved in the peptidyl transferase centre of rat liver and yeast 60S subunits have been labelled with analogues of puromycin (Bohm, Stahl and Bielka, 1979), and tRNA (Stahl et al., 1979; Perez-Gosalboz, Vazquez and Ballestra, 1978). Proteins within ribosomal subunits have also been cross-linked to ribosomal RNA and poly U either chemically (Svoboda and McConkey, 1978; Terao and Ogata, 1979a) or by ultra-violet irradiation (Reboud et al., 1978; Buisson et al., 1979; Reboud et al., 1980; Terao and Ogata, 1979b). Crosslinking and affinity labelling studies might be useful in showing the proximity of various ribosomal components, but these studies on their own cannot do more than suggest the function of ribosomal proteins.

Protein-RNA interactions have been studied by non-covalent binding of isolated E. coli ribosomal proteins to rRNA molecules (Zimmermann, 1980). The specificity of binding of particular proteins to defined regions of RNA has also been examined by

the degree of protection afforded against ribonuclease (Douthwaite et al., 1979) or ketoxal (Garrett and Noller, 1979). RNA-protein interaction studies have been carried out with eukaryotic ribosomal components by binding isolated proteins to immobilized RNA molecules. 5S RNA (Metspalu et al., 1978; Ulbrich and Wool, 1978), 5.8S RNA (Metspalu et al., 1978; Toots et al., 1979) and tRNA (Metspalu et al., 1978) have all been used in this manner. Among the findings of these studies are that proteins L6 and L19 bind to both 5S and 5.8S RNA suggesting the close proximity of these two RNA molecules, and that 5.8S RNA could also bind a small number of 40S subunit proteins (including S6 : Toots et al., 1979) suggesting that it is located at the subunit interface. It is however difficult to make any definite conclusions on the basis of those studies since the binding of proteins to RNA could in some instances be artifactual. Electron microscopy has been used to directly visualise the supramolecular structure of E. coli ribosomal subunits (Stöffler et al., 1980; Lake, 1980). Used in conjunction with divalent antibodies raised against purified E. coli proteins, electron microscopy has made possible the location of individual ribosomal proteins on the surface of subunits. Three-dimensional models of E. coli ribosomal subunits have been proposed based largely on electron microscopy studies by various workers including Stöffler et al., (1980) and Lake (1980). The models of E. coli ribosomal subunits described by Stöffler et al., (1980) and Lake (1980) are in many respects the most detailed models available. Fig 1.2 shows some features of the Lake (1980) model of the E. coli ribosomal subunits. The 30S subunit has been divided into a main body comprising

Fig 1.2 Model of *E. coli* Ribosomal Subunits, Lake (1980)

This model is based on electron microscopy studies of 30S and 50S *E. coli* ribosomal subunits described in 1.1.3.


CENTRAL PROTUBERANCE
STALK
CLEFT
HEAD
PLATFORM
BASE
30S
50S
70S
CENTRAL PROTUBERANCE
VALLEY
RIDGE
STALK
HEAD
PLATFORM

of a head and a base, and a platform, with a cleft dividing the main body from the platform. The 50S subunit has been divided into a rounded main body, a central protuberance, a ridge, a valley and a stalk (Fig 1.4). The stalk consists mainly of proteins L7/L12 (Strycharz, Nomura and Lake, 1978), and a very stable complex of proteins. L7/L12 and L10 can be isolated from 50S subunits (Dijk, Littlechild and Garrett, 1977) as a complex.

In addition to the recognised amino acyl (A) and peptidyl (P) sites within the ribosome, Lake (1979) has proposed the existence of a third tRNA binding site, the 'recognition' or 'R' - site. It has been speculated that such a site could be employed to proof-read amino acyl tRNAs and thereby help maintain a high fidelity of translation.

Electron-microscopy of eukaryotic ribosomal subunits indicate that they have essentially the same morphology as those from E. coli despite their larger size (Boublik and Hellmann, 1978), though some differences (protrusions from the head of the 40S subunit, and the bottom of the 60S subunit) were observed. Immune electron microscopy has been used to determine the position of proteins S3 and S6 in rat liver 40S subunits (Noll et al., 1978; Bommer et al., 1980), and together with data indicating that antibodies to these proteins can block the binding of the ternary initiation complex, it has been suggested that these proteins are involved in the P site organisation.

Neutron scattering has also been used to generate information on the relative position of ribosomal proteins within

the subunits (reviewed by Moore, 1980). In this technique, E. coli ribosomal subunits are reconstituted with purified proteins, two of which have had most of their hydrogen atoms replaced by deuterium. The scattering of neutrons by these two 'deutrated' proteins can be used to determine the distance between them. In this way the relative positions of various proteins be determined, and a corresponding model of ribosomal subunits built up.

1.1.4. Protein Synthesis

Protein synthesis in both prokaryotes and eukaryotes can be divided into three stages : initiation, elongation and termination, each of which is composed of a number of steps. (There are some differences between prokaryotes and eukaryotes, mainly at the stage of initiation.) Each step requires specific interactions between two or more components.

Eukaryotic initiation involves at least eight initiation factors (Schreier, Erni and Staehlin, 1979; Trachsel et al., 1977; Thomas et al., 1979), designated eIF-1 etc. (Anderson et al., 1977) compared to three in E. coli (IF-1 etc: Stanley et al., 1966; Revel et al., 1968). The association of 40S and 60S subunits to form inactive 80S monosomes, which would otherwise occur readily under physiological conditions, is prevented by the binding of the anti-association factor eIF-3 to 40S subunits (Trachsel and Staehlin, 1979; Thompson et al., 1977). Eukaryotic cells do contain a sizeable pool of 80S monosomes (Hogan and Korner, 1968; Kaempfer 1969), but these do not participate in protein synthesis unless they can

dissociate into subunits.

The first step in eukaryotic initiation is the binding of Met-tRNA (the initiator tRNA) to native 40S ribosomal subunits, which have previously bound eIF-3, in a reaction that requires eIF-2 and GTP (Schreier and Staehlin, 1973; Chen et al., 1972; Benne et al., 1979).

The 40S -Met-tRNA-eIF-2 - eIF-3 complex then binds additional factors eIF-1, eIF-4A, -4B, and -4C together with mRNA, in a reaction that requires the hydrolysis of ATP.

In prokaryotes 16S rRNA plays a central role in location of the initiation site on mRNA by the 40S subunit. Binding between 16S rRNA and the leader sequences of mRNA was suggested on the basis of sequence analysis by Shine and Dalgarno (1974). Sequence analysis of eukaryotic mRNAs and 18S rRNA has not demonstrated a corresponding complementarily (Hagenbuchle et al., 1978). Eukaryotic ribosomes appear to start initiation at the first AUG codon, unlike prokaryotic ribosomes which can initiate at internal codons or even on closed circular mRNA (Kozak, 1979). There is growing evidence that the cap structure (7-methyl guanosine: m^7pG) found at the 5' end of most mRNAs acts as a guide in eukaryotic initiation (Both et al., 1975). Evidence for this suggestion comes from the findings that improperly capped mRNAs initiate at a much lower frequency (Both et al., 1975) and that competitive inhibitors (e.g. m^7pG : Roman et al., 1976) strongly inhibit translation. A 24,000 molecular weight protein which is not one of the established initiation factors can be cross-linked to mRNA bound in initiation complexes, and it has been suggested that this protein

may play a role in the recognition of the cap structure (Sonenberg *et al.*, 1979). There are however uncapped mRNAs e.g. histone or viral mRNAs which can be translated at high efficiencies (Hewlett, Rose and Baltimore, 1976; Nomoto, Lee and Wimmer, 1976) *in vitro*.

The 60S subunit is likely to join the 40S mRNA Met-tRNA complex when the initiator tRNA has bound to the AUG codon, and eIF-5 is required for this joining step. The bound GTP is then hydrolysed to GDP and P_i and all the initiation factors are released (Grunberg-Manago, 1980).

Elongation begins by amino acyl tRNA binding to the A-site in the form of a ternary complex with GTP and elongation factor - 1α (EF-1α : Weissbach, 1980). After the binding of amino acyl tRNA, EF-1α and GDP are released by EF-1β binding to EF-1α in a similar manner to that which occurs in prokaryotic elongation (McKeehan and Hardesty, 1969). A peptide bond is then formed between the nascent peptide or initiator methionine at the P-site and the amino acyl tRNA at the A-site, thus the length of the nascent peptide is increased by one amino acid, and it is transferred from the P-site to the A-site. Peptide bond formation is catalysed by the peptidyl transferase centre which is an inherent catalytic activity of the large subunit in both prokaryotes and eukaryotes (Neth *et al.*, 1970).

Translocation of peptidyl tRNA from the A-site to the P-site requires EF-2 (Galasinski and Moldave, 1969; Raeburn *et al.*, 1975) and GTP. As well as movement of the peptidyl tRNA, there is also movement of mRNA relative to the ribosome, and the ejection of deacylated tRNA (Weissbach, 1980). Elongation of

the polypeptide chain continues by cycles of the events just described until a termination codon is reacted.

In eukaryotes (unlike prokaryotes) a single release factor (RF) recognises all three termination codons (Beudet and Caskey, 1971). RF is thought to interact with the peptidyl transferase centre thereby allowing it to hydrolyse the peptide-tRNA bond (Caskey, 1977). RF has a requirement for GTP, and this may be necessary for its dissocation from the ribosome (Weissbach, 1980). In prokaryotes the mRNA and deacylated tRNA are removed in a reaction which requires GTP, EF-G and a ribosome release factor RRF. The situation in eukaryotes is less clear, though an RRF factor has been described (Hirashima and Kaji, 1972).

It has been shown that when ribosomal proteins L7/L12 are removed from E. coli 50S subunits, none of the GTP requiring reactions take place. These proteins have been implicated in the binding of the factors associated with GTP hydrolysis (IF-2, EF-Tu, EF-G and RF : Weissbach, 1980). Although less is known about the binding of equivalent factors in eukaryotes, there is considerable evidence that proteins equivalent to L7/L12 are present in the eukaryotic 60S subunit, and that they can be phosphorylated in vivo (c.f. 1.3.1).

At least three mechanisms have been suggested to account for the observation that virus infection often decreases host cell protein synthesis but at the same time viral mRNAs are translated efficiently.

In poliovirus infected cells initiation with 'capped' host cell mRNA is inhibited, whereas poliovirus mRNA, which is

not capped, is translated efficiently. One possible explanation is, that virus infection inactivates the 'cap-binding' protein, thereby host cell mRNA initiation is reduced and that poliovirus mRNA is not dependent on 'cap-binding' proteins for efficient translation (Rose et al., 1978; Sonenberg et al., 1978).

Another mechanism could be that viral mRNAs are initiated at a higher efficiency than cellular mRNAs at high monovalent cation concentrations (Carrasco and Smith, 1976). Some indirect evidence for this mechanism has come from studies in which newly infected cells can be made to synthesize proteins that are normally found late after infection with virus, merely by transferring these cells to a hypertonic medium (Kramer, 1980).

A third possible mechanism for the regulation of protein synthesis by virus infection is that it involves ribosomal protein phosphorylation. This is considered in more detail in 1.3.

There is considerable evidence that under certain cell conditions, protein synthesis can be inhibited by the phosphorylation of eIF-2. This is discussed in 1.2.2.

1.2. Protein Phosphorylation

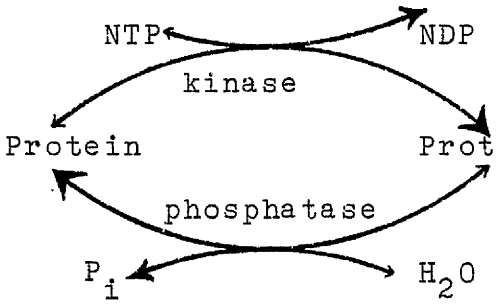
Protein phosphorylation was first shown to be important in regulating the activity of a protein by Krebs and Fischer (1956). They discovered that glycogen phosphorylase could be converted from a dephosphorylated form whose activity was dependent on 5'-AMP to a phosphorylated form whose activity was largely independent of 5'-AMP. Subsequently phosphorylase kinase (Krebs, Graves and Fischer, 1959) and glycogen synthase (Friedman and Larner, 1963) were also shown to be interconvertible between two forms by phosphorylation.

More than one hundred proteins have now been identified as being phosphorylated though far fewer of these have actually been shown to be regulated by phosphorylation (approximately 25: Cohen, 1980a). Almost all proteins shown to be regulated by reversible phosphorylation are enzymes (reviewed by Krebs and Beavo, 1979).

Some phosphorylation events may not in fact be part of a dynamic phosphorylation-dephosphorylation regulatory mechanism (e.g. casein phosphorylation might be a means of maintaining a high phosphorus content in milk: Weller, 1979).

Protein phosphorylation can either increase or decrease the activity of a protein. Cohen (1980a) has suggested that in general enzymes which are activated by phosphorylation tend to be involved in biodegradative pathways whereas those which are inactivated by phosphorylation are involved in biosynthetic pathways. This could allow different metabolic pathways to be regulated by the same protein kinases and phosphatases.

Two classes of enzymes; protein kinases and phosphoprotein phosphatases, are involved in the phosphorylation and dephosphorylation of proteins (as depicted in equation 1.1).



(equation 1.1)

1.2.1. Protein Kinases and Phosphoprotein Phosphatases

Protein kinases catalyse the transfer of the terminal phosphate from a triphosphate nucleotide donor to a substrate protein (equation 1.1). NTP is usually ATP, but GTP can be used by some protein kinases.

Protein kinases have been divided into two classes on the basis of whether or not they are regulated by specific interaction with molecules which usually serve as messengers in the mediation of signals from outside the cell (Krebs and Beavo, 1979). The first protein kinase shown to be regulated in a specific manner was rabbit skeletal muscle cAMP-dependent protein kinase. (Walsh, Perkins and Krebs, 1968). Regulation of protein kinases by cGMP was shown in lobster muscle (Kuo and Greengard, 1970) and later in several mammalian tissues. Two calcium dependent protein kinases have been described; phosphorylase kinase (Osawa, Hosoi and Ebashi, 1967) and myosin light chain kinase (Pires, Perry and Thomas, 1974). The most recent protein kinase shown to be regulated by a specific molecule is the double stranded RNA dependent protein kinase from interferon treated cells (Lebleu et al., 1976) and reticulocytes (Ernst, et al., 1976).

The second class of protein kinases are those which appear to be messenger-independent (though they may eventually be shown to be regulated by specific messengers) and are usually named according to the substrate they phosphorylate (e.g. histone kinase, casein kinase, phosvitin kinase).

There are two types of cyclic-AMP dependent protein kinases, peak I and peak II, which contain identical catalytic (C) subunits but different regulatory (R) subunits (reviewed by Nimmo and Cohen, 1977). The subunit structure of the inactive holoenzyme form of both types of cAMP-dependent protein kinase is believed to be $R_2 \cdot C_2$. Stimulation by binding cAMP causes dissociation of the regulatory and catalytic subunits forming regulatory subunit dimers and active catalytic subunit monomers as shown in equation 1.2.

$$R_2C_2 \text{ (inactive)} + 2\text{cAMP} \rightleftharpoons R_2 \cdot \text{cAMP} + 2C \text{ (active)} \qquad \text{(equation 1.2)}$$

Peak I regulatory subunit has a molecular weight of 49,000 and is not phosphorylated, whereas peak II regulatory subunit has a molecular weight of 55,000 and can be phosphorylated (Nimmo and Cohen, 1977). Holoenzymes of peak I and peak II protein kinases can also be distinguished by their elution from DEAE-cellulose at different conductivities (2.5mmho and 6mmho respectively at pH 6.5 : Hofmann et al., 1975). The relative proportions of the peak I and peak II forms of cAMP-dependent protein kinase varies considerably between different tissues (Nimmo and Cohen, 1977). Extreme examples would be rabbit psoas (skeletal) muscle, in which 90% of the cAMP-dependent

protein kinase is peak I and 10% peak II (Burchell, Cohen and Cohen, 1976) and pig brain, in which 100% of cAMP-dependent protein kinase is peak II (Nesterova et al., 1975). The reason for the existence of two types of cAMP-dependent protein kinases and the variation in their relative proportions in different tissues is not clear (Krebs and Beavo, 1979) but it may reflect differences in their responses to cAMP or their sub-cellular locations.

Cyclic AMP-dependent protein kinases can be inhibited by heat stable inhibitor proteins found in most tissues. The most extensively studied inhibitor protein is inhibitor -1 from rabbit skeletal muscle (Walsh et al., 1971), though two other types of inhibitor protein have been reported, one in rat testis (Beale, Dedman and Means, 1977) the other in brain (Szmigielski, Guidotti and Costa, 1977). Inhibitor protein -1 binds to the catalytic subunit of cAMP-dependent protein kinase, and its inhibition is competitive with respect to protein substrates. The exact physiological function of this inhibitor is not clear since even in tissues where it is relatively abundant (e.g. rabbit skeletal muscle), its concentration is such that only 20% of the cAMP-dependent protein kinase could be blocked (Walsh and Ashby, 1973). One suggested role for the inhibitor protein is that it inactivates any free catalytic subunits present at basal levels of cAMP, thereby preventing a response to cAMP except when present at high concentrations (Krebs and Beavo, 1979).

Cyclic GMP dependent protein kinase from beef lung is composed of two identical subunits of molecular weight 74,000

(Gill et al., 1976) or 81,000 (Lincoln, Dills and Corbin, 1977). The cGMP protein kinase does not contain separate regulatory and catalytic subunits and, unlike cAMP-dependent protein kinase, does not dissociate in the presence of cGMP (Gill, Walton and Sperry, 1977;) as summarised in equation 1.3.

$$(RC)_2 \text{ (inactive)} + 2cGMP \rightleftharpoons (RC)_2 \cdot cGMP_2 \text{(active)}$$

(equation 1.3)

A modulator protein has been shown to stimulate cGMP-dependent protein kinase in vitro (Shoji et al., 1978; Yamamoto et al., 1977), but its role within the cell is not known. It has been suggested that the differences in tissue distribution and protein substrates phosphorylated by cAMP and cGMP dependent protein kinases indicate that these two types of protein kinase are likely to be involved in different cellular processes. (Nimmo and Cohen, 1977).

Two examples of calcium regulated protein kinases are known, phosphorylase kinase (Osawa, Hosoi and Ebashi, 1967) and myosin light chain kinase (Pires, Perry and Thomas, 1974). Phosphorylase kinase is a multisubunit enzyme of the structure $(\alpha\ \beta\ \gamma\ \delta)_4$ where δ is the calcium binding protein calmodulin (Cohen et al., 1978). This molecule of calmodulin is tightly bound to phosphorylase kinase and in the presence of calcium will stimulate phosphorylase kinase (Cohen, 1980). A second calcium binding protein can also bind to phosphorylase kinase (though less tightly than the δ subunit) and in the presence of calcium will further stimulate phosphorylase kinase actively five-fold (Shenolikar et al., 1979). However it is not clear whether

this second calcium binding protein is calmodulin or troponin C (which also binds Ca^{2+} and shares up to 50% sequence homology with calmodulin : Cohen, 1980a).

Active myosin light chain kinases also contain a bound molecule of calmodulin which allows them to be stimulated by calcium (smooth muscle myosin kinase : Dabrowska et al., 1978; and skeletal muscle myosin kinase : Yagi et al., 1978). In the absence of calmodulin however, myosin kinases have no basal activity (Adelstein and Klee, 1980). Apart from the stimulation of these two types of calcium - dependent protein kinases, calmodulin stimulates many other types of enzyme (reviewed by Cheung, 1980; and Means and Dedman, 1980) and it is likely to be the major intracellular receptor for calcium. Almost all protein kinases phosphorylate either a serine or threonine residu though tyrosine has recently been shown to be phosphorylated by RNA tumour virus protein kinases (Sefton et al., 1980).

The susceptibility of a particular amino acid to phosphorylation appears to be largely determined by the amino acid sequence surrounding the phosphorylated residue. In general, cAMP-dependent protein kinases phosphorylate serine residues which are contained in either of the sequences :

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

where X can be most amino acids (Cohen, 1980a).

The dephosphorylation of phosphorylated proteins is catalysed by phosphatases which, unlike protein kinases, do not appear to be regulated by specific messenger molecules (Krebs and Beavo, 1979). Instead they are regulated by

competition between various protein substrates, interaction with inhibitor proteins and substrate directed effects (e.g. the specific interaction of ligands with phosphoprotein substrates).

A single phosphatase capable of dephosphorylating a number of different protein substrates (including phosphorylase a, histones, glycogen synthase D and phosphorylase kinase) has been obtained from a variety of animal tissues (England, Stull and Krebs, 1972; Kato and Bishop, 1972; Zieve and Glinsmann, 1973). This phosphatase has been purified and has a molecular weight of 35,000 (Killilea, et al., 1976; Chou, Alfano and Rosen, 1977). However Brandt, Capulong and Lee (1975) have shown that a less active high molecular weight form of this enzyme can be converted to the more active 35,000 molecular weight form. It has been suggested that the enzyme has two forms; a less active holoenzyme which consists of two catalytic subunits complexed with one inhibitor subunit and the fully active catalytic subunits released by dissociation from the holoenzyme (Krebs and Beavo, 1979).

There are two heat stable inhibitor proteins of this phosphatase and inhibitor -1 is of particular interest since it has virtually no activity when unphosphorylated (Huang and Glinsmann, 1975). The phosphorylation of inhibitor -1 by cAMP-dependent protein kinase provides a mechanism for amplifying the effects of cAMP since phosphorylation of this inhibitor leads to the inactivation of the phosphatase (Cohen, 1980a).

Other phosphatases which are specific for a particular substrate have been described (Krebs and Beavo, 1979).

1.2.2. The Regulation of Protein Activity by Phosphorylation

The best understood system demonstrating the regulation of protein activities by reversible phosphorylation are the enzymes concerned with glycogen metabolism (reviewed by Nimmo and Cohen, 1977; Cohen, 1980a). Among the features of this system are that the enzymes involved are regulated in a highly coordinated manner by phosphorylation (i.e. the biodegradative enzymes are activated by phosphorylation whereas the biosynthetic enzymes are inactivated). Other molecules can also enhance specificity in response to cAMP (i.e. in the absence of cAMP, kinase inhibitor proteins and active phosphatases can maintain essentially a zero level of kinase activity, whereas in the presence of cAMP phosphatase levels can be greatly reduced by the activation of phosphatase inhibitor). Some of the enzymes of glycogen metabolism can be phosphorylated at more than one site, glycogen synthase in particular can be phosphorylated at several sites. Additional phosphorylations can alter the kinetic parameters in ways that either enhance the original phosphorylation or produce some different effect (Cohen, 1980a). Multisite phosphorylation also allows the possibility that different kinases can phosphorylate the differen sites (glycogen synthase is phosphorylated by three different kinases) thereby confering sensitivity to more than one set of stimuli.

In some instances, protein phosphorylation appears to be an essential step in the functioning of certain proteins. When smooth muscle myosin light chains are phosphorylated the interaction of myosin with actin increases and the muscle

contracts. Conversely upon dephosphorylation this interaction decreases and the muscle relaxes (Adelstein, 1978; England, 1980).

In other situations the role of protein phosphorylation has not been demonstrated directly but circumstantial evidence does suggest that it has important functions. Histone H1 shows a large increase in phosphate content between prophase and S phase of the cell cycle (Bradbury et al., 1973). Experiments performed in vitro show that the more highly phosphorylated prophase H1 is more effective in aggregating DNA than the less phosphorylated S phase H1 (Mathews, 1980). This suggests that H1 might have a role in chromosome condensation.

It has been established that the transforming genes of RNA tumour viruses code for protein kinases and consequently protein phosphorylation is likely to play an important role in transformation (Collett and Erikson, 1978; Maness et al., 1979). However the native substrate(s) for these kinases and the mechanism of transformation have yet to be elucidated.

One example of protein phosphorylation which is of particular interest in relation to the present study is the inhibition of protein synthesis by the phosphorylation of eIF-2.

It was first observed in reticulocyte lysates that in the absence of sufficient quantities of haemin the rate of protein synthesis rapidly decreased (Bruns and London, 1965). Following this there was a corresponding disaggregation of polysomes and the disappearance of the initiation complex

Met-$tRNA_f$· GTP · eIF-2 although normal concentrations of eIF-2, Met-$tRNA_f$ and GTP were present (Legon, Jackson and Hunt, 1973). The cessation of protein synthesis was accompanied by the formation of a translational inhibitor (haemin controlled repressor or HCR : Maxwell and Rabinovitz, 1969) and activation of a protein kinase which specifically phosphorylates the α subunit of eIF-2 (Farrell, Hunt and Jackson, 1978).

Most of the evidence relating eIF-2 phosphorylation to the inhibition of protein synthesis has been indirect. The HCR and the protein kinase activity which phosphorylates eIF-2 co-purify during all steps in their purification (Farrell et al., 1977). The inhibitory and protein kinase activities have the same heat inactivation kinetics. Antibodies against HCR abolish both its kinase and inhibitory activities (Kramer, Cimadevilla and Hardesty, 1976). Compounds (e.g. certain purines) which relieve inhibition inhibit the protein kinase (Farrell, et al., 1977). Although there is a strong correlation between eIF-2 phosphorylation and the inhibition of protein synthesis in crude reticulocyte lysates, in more purified systems there is no decrease in ternary initiation complex formation following phosphorylation by HCR. Neither is there any inhibition of the subsequent binding of this complex to the 40S ribosomal subunits. (Trachsel and Staehlin, 1978; Safer and Anderson, 1978). One possible explanation is that other factors are required to interact with eIF-2 to give a stable initiation complex in vivo and that in purified systems these factors might be missing (de Haro and Ochoa 1978; Das et al., 1979).

The formation of stoichometric complexes of eIF-2, Met-tRNA$_f$ and GTP is difficult to achieve with the purified components (presumably due to the absence of these extra factors) and it might not be possible to distinguish between phosphorylated and unphosphorylated eIF-2 under these conditions (Hunt, 1980).

The mechanism of inhibition of eIF-2 is not known, but the α-subunit has been reported to bind GTP and phosphorylation might interfere with this binding (Hunt, 1980).

HCR is inactivated by haemin binding as well as by other conditions (e.g. high hydrostatic pressure and oxidized glutathione) and it is thought to undergo a conformation change upon activation (Hunt, 1980). HCR appears to be highly specific for a single site in the α subunit of eIF-2 and will phosphorylate no other substrates.

A second kind of protein kinase, which is activated by double stranded RNA, can also inactivate eIF-2 by phosphorylation. This protein kinase is present in reticulocyte lysates (Farrell, et al., 1977) and cell-free systems prepared from cells which have been exposed to physiological doses of interferon (Zilberstein et. al., 1976). This double stranded RNA activated inhibitor phosphorylates the same site on the α-subunit of eIF-2 as HCR. There is however considerable evidence to show that the double stranded RNA activated protein kinase and HCR are different enzymes (Hunt, 1980).

Apart from erythroid cells and cells pre-treated with interferon there have been few reports of translational regulation in other cell types by eIF-2 phosphorylation. It may be therefore that this is a relatively specific mechanism for translational regulation.

1.3. Ribosomal Protein Phosphorylation

The first indication that certain ribosomal proteins were phosphorylated in intact cells came from Kabat (1970), who described several ribosomal phosphoproteins in rabbit reticulocytes, and Loeb and Blat (1970) who described a single phosphoprotein in rat liver. Phosphorylation of ribosomal proteins in cell-free systems was reported by Kabat (1971), and Li and Amos (1971) using endogenous protein kinases. Phosphorylation by exogenous protein kinases was reported by Eil and Wool (1971), and Walton *et al.* (1971).

Ribosomal phosphoproteins have been detected in most eukaryotic cells and tissues. They have been found in primitive eukaryotes; yeast (Grankowski and Gasior, 1975; Becker-Ursic and Davies, 1976; Zinker and Warner, 1976) and *Artemia salina* (Van Agthoven *et al.*, 1977); in plants (Trewavas, 1973); and higher animals such as mouse (Bitte and Kabat, 1972), hamster (Leader *et al.*, 1976), rabbit (Kabat, 1970), rat (Loeb and Blat, 1970; Gressner and Wool, 1974) and human (Kaerlein and Horak, 1976; Lastick, Nielsen and McConkey, 1977). They are also present in various tissues; liver (Loeb and Blat, 1970), kidney (Hill and Trachewsky, 1974), brain (Ashby and Roberts, 1975), reticulocytes (Kabat, 1970) adrenal cortex (Roos, 1973), mammary gland (Majumder and Turkington, 1972), pituitary (Barden and Labrie, 1973) and various tumour cells (Bitte and Kabat, 1972; Rankine and Leader, 1975; Stahl, Bohm and Bielka, 1974; Jolicoeur *et al.*, 1974).

Although there was onereport of ribosomal phosphoproteins from intact *E. coli* (Kurek, Grankowski and Gasior, 1972a) and

one of ribosomal protein kinase (Kurek, Grankowski and Gasior, 1972b), others have been unable to confirm these findings (Rahmsdorf et al., 1973 ; Rahmsdorf et al., 1974).

1.3.1. Phosphorylation in Intact Cells

Estimates of the number of phosphorylated ribosomal proteins found in intact cells labelled with ^{32}P-orthophosphate showed considerable variation between early reports. Thus Majumder and Turkington (1972) described eight different proteins whereas Stahl, Bohm and Bielka (1974), and Gressner and Wool (1974) described only one. Other workers have reported five (Kabat, 1972; Ashby and Roberts, 1975), four (Rankine, Leader and Coia, 1977) or two (Pierre, Creuzet and Loeb, 1974) ribosomal phosphoproteins.

This variation in number of ribosomal phosphoproteins could in some cases be due to contamination with non-ribosomal phosphoproteins and the lack of a clear identification of each ribosomal protein from one-dimensional SDS gel electrophoresis. Using two-dimensional gel electrophoresis it became possible to resolve and unambiguously identify most ribosomal proteins. Other difficulties in identifying ribosomal phosphoproteins were, the possible loss of acidic ribosomal proteins from ribosomes prepared under conditions of high ionic strength, or failure to detect these proteins on certain gel electrophoresis systems (1.1.1). Individual ribosomal phosphoproteins may also be distinguished (on the basis of their stoichometry of phosphorylation or their relative radioactive labelling) as being either major or minor phosphoproteins (Leader, 1980a).

However, there can be problems when making such distinctions. For example in Krebs II ascites cells proteins S3 and L14 (Leader and Coia, 1978b) are normally unphosphorylated or contain very little phosphate (less than 0.1 moles per mole of protein) but can become highly phosphorylated under different cellular conditions (Leader and Coia, 1978b).

1.3.1.1. Ribosomal Protein S6

Protein S6 was the first ribosomal protein to be identified as phosphorylated by analysis on two-dimensional gel electrophoresis. S6 phosphorylation was first shown in rat liver (Gressner and Wool, 1974) and subsequently in a number of mammalian cells including hepatoma (Stahl et al., 1974), Krebs II ascites cells (Rankine and Leader, 1975), HeLa cells (Kaerlin and Horak, 1976), reticulocytes (Traugh and Porter, 1976), L-cells (Marvaldi and Lucas-Lenard 1977) cerebral cortex (Roberts and Ashby, 1978), and Ehrlich ascites cells (Rosnitschek, Traub and Traub 1978). The phosphorylation of S6 has also been observed in more primitive eukaryotes e.g. yeast (Zinker and Warner, 1976; Hebert, Pierre and Loeb, 1977) Physarum polycephalum (Belanger, Bellemarie and Lemieux 1979) and Tetrahymena pyriformis (Kristiansen, Plesner and Kruger, 1978).

S6, like most ribosomal proteins (c.f. 1.1.1), is chemically basic with an approximate molecular weight of 31,000 (Collatz et al., 1977) though estimates have varied between 30,000 and 35,000. There are approximately five different phosphorylation sites in S6 and this accounts for the 'anodic tail' (corresponding to the various phosphorylated derivatives) observed on two-

dimensional gel electrophoresis when S6 is highly phosphorylated (Gressner and Wool, 1974). However in most tissues S6 phosphorylation is normally much lower than the maximum possible (e.g. in Krebs II ascites cells S6 has been estimated to contain only 0.1 moles of phosphate per mole of protein : Rankine, Leader and Coia, 1977).

Enhanced phosphorylation of S6 has been correlated to an increase in cellular cAMP concentration in many tissues. Such an increase in phosphorylation can be obtained by treating cells with cAMP or a derivative such as dibutryl cAMP; rat liver (Gressner and Wool, 1976), rabbit reticulocytes (Cawthorn et al., 1974), cerebral cortex (Roberts and Ashby, 1978) and pancreatic islet tumour cells (Schubart et al., 1977). Phosphorylation of S6 can also be enhanced by treating cells with hormones that increase cellular cAMP concentration e.g. glucagon (rat liver: Blat and Loeb, 1971; pancreatic islet tumour cells : Schubart et al., 1977) or ACTH (mouse adrenal cells : Roos, 1973). However, other cells do not show a cAMP related increase in S6 phosphorylation, e.g. ascites cells (Rankine, 1976), glioma cells (Horak and Koschel, 1977) and BHK cells (Leader and Coia, 1978a).

The phosphorylation of S6 has been correlated to an increase in protein synthesis in several instances. Thus enhanced phosphorylation of S6 has been observed during cellular proliferation in BHK cells, whereas the extent of phosphorylation decreases when cells have reached confluence (Leader, Rankine and Coia, 1976). Addition of fresh medium to HeLa cells (Lastick, Nielsen and McConkey, 1977) or chick embryo fibroblasts (Haselbacher, Humbel and Thomas, 1979) also increases

S6 phosphorylation. Regenerating rat liver is another situation in which there is an increase in protein synthesis and also enhanced S6 phosphorylation (Gressner and Wool, 1974; Anderson, Grundholm and Sells, 1975; Scheinbuks, Sypherd and Moldave, 1974; Tas and Sells, 1978). Polysomes have been shown to contain more highly phosphorylated S6 than monosomes in many cell types. Rabbit reticulocytes (Kabat, 1971; Li and Amos, 1971), sarcoma cells (Bitte and Kabat, 1972), BHK cells (Leader and Coia, 1978) and myeloma cells (Kruppa and Martini, 1978) all show such a difference. It could be speculated then that S6 phosphorylation might somehow increase the rate of protein synthesis (although the evidence described does not rule out the possibility that S6 phosphorylation is an effect of increased protein synthesis).

The main argument against the role of S6 in increasing the rate of protein synthesis is that many inhibitors of protein biosynthesis can also enhance the phosphorylation of S6. However, it might still be possible to reconcile a role for S6 phosphorylation in the regulation of protein synthesis if one considers the mode of action of these protein synthesis inhibitors. A number of agents which damage liver cells (D-galactosamine : Gressner and Greiling, 1977; thioacetamide : Gressner and Greiling, 1978; dimethylnitrosamine : Gressner and Greiling, 1979) also stimulate an increase in S6 phosphorylation. It could be argued that the cellular response to this damage would be to attempt to increase the rate of protein synthesis.

Virus infection often decreases the rate of cellular protein synthesis and in a number of instances S6 phosphorylation

is also enhanced; HeLa cells infected with vaccinia virus (Kaerlein and Horak, 1976), HeLa cells infected with adenovirus (Blair and Horak, 1977) Ehrlich ascites cells infected with mengovirus (Rosnitschek, Traub and Traub, 1978). More recently, Kennedy, Stevely and Leader (1981) have demonstrated that pseudorabies virus infection of BHK cells greatly increases the phosphorylation of S6 from an average of one phosphate per protein molecule to between 4 and 5. However, it is possible that viral infection could selectively inhibit host cell protein synthesis, this could be achieved by one of the mechanisms described in 1.1.4 or some process such as phosphorylation of other ribosomal proteins (e.g. S2 and S16 : Kaerlin and Horak, 1976; S16 or S18 : Kennedy, Stevely and Leader, 1981), while simultaneously enhancing viral protein synthesis by S6 phosphorylation.

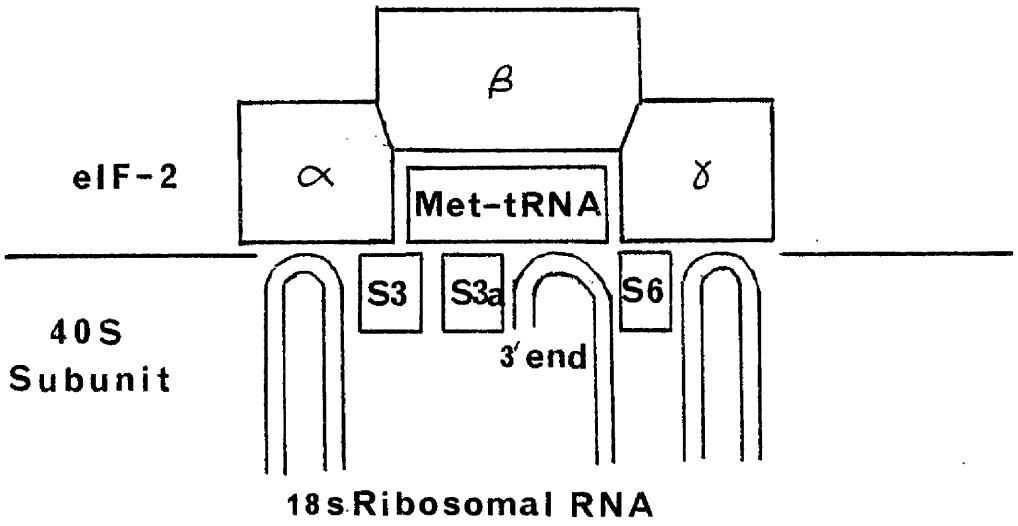
More difficult to explain is the stimulation of S6 phosphorylation by compounds such as sodium fluoride (Kabat, 1971), puromycin (Gressner and Wool, 1974) cycloheximide (Gressner and Wool, 1974; Kaerlin and Horak, 1976), fusidic acid (Ziv and Stratman, 1976) and aurintricarboxylic acid (Werenne, Laurel and Laurel, 1973). These compounds could effect S6 phosphorylation by mechanisms unrelated to the inhibition of protein synthesis such as effect on cAMP metabolism (Appleman and Kemp, 1966; Wititsuwannakul and Kim, 1977; Ziv, Wagner and Stratman, 1978; Rall and Sutherland, 1962) or in the case of sodium fluoride by inhibiting phosphoprotein phosphatases.

It is possible however that, if S6 phosphorylation increases the rate of protein synthesis, protein synthesis inhibitors

might indirectly cause the stimulation of S6 phosphorylation in a compensating reaction. Leader (1980a) has suggested that the phosphorylation of S6 could alter the K_m for binding of another molecule(s) involved in protein synthesis. A decrease in the concentration of this molecule(s) could be compensated for by phosphorylating S6 thereby increasing the affinity of the ribosome for this molecule(s). As mentioned previously (1.1.3) S6 has been cross-linked to Met-$tRNA_f$ within the initiation complex (eIF-2· GTP· Met-$tRNA_f$· 40S subunit) and a model for the binding site of the ternary initiation complex to the 40S subunit based on this information has been suggested by Westermann, Nygard and Bielka(1981: Fig. 1.3). It could be speculated therefore that S6 phosphorylation might enhance the binding of the initiation complex, and that this could lead to an increased rate of protein synthesis. Three of the proteins involved in this proposed binding site, eIF-2, S6 and S3 can be phosphorylated. It is possible then that the rate of initiation of protein synthesis is decreased by eIF-2 phosphorylation (c.f. 1.1.4) whereas S6 phosphorylation increases initiation.

Relatively few experiments have been performed to examine the effects of phosphorylation of S6 on ribosome function (and even fewer for the other ribosomal phosphoproteins) and most of these studies have been strongly criticised (Leader, 1980a). What is often considered to be one of the most careful studies of the effect of S6 phosphorylation on protein synthesis (Eil and Wool, 1973) has also been criticised, on the grounds that exogenous cAMP-dependent protein kinase was used to phosphorylate the ribosomes and a number of proteins in addition to S6

Fig 1.3 : Model of the Interaction of the Ternary Initiation Complex with the Small Ribosomal Subunit (Westermann, Nygard and Bielka, 1981).



This schematic representation shows the interaction of the ribosomal phosphoproteins S6 and S3 with the initiator tRNA and initiation factor eIF-2 also a phosphoprotein (shown as being composed of three subunits, α, β and γ). This model is based on the cross-linking data of Westermann, Nygard and Bielka (1981). The possible significance of this model with respect to ribosomal protein phosphorylation is discussed in 1.3.1.1.

were also phosphorylated.

1.3.1.2. The Acidic Phosphoprotein of the 60S Subunit

From early studies on ribosomal protein phosphorylation a relatively low molecular weight protein was observed as phosphorylated in the 60S subunit of reticulocyte or sarcoma cells (Cawthorn et al., 1974). A protein of similar molecular weight was later observed in the 60S subunit of many mammalian tissues; ascites cells (Rankine and Leader, 1976; Rankine, Leader and Coia, 1977) BHK cells (Rankine and Leader, 1976) HeLa cells (Horak and Schiffman , 1977) mouse myeloma (Martini and Kruppa, 1979) and cerebral cortex (Roberts and Ashby, 1978).

There was however disagreement for some time over the existence of such a protein since it proved difficult to find a similar phosphoprotein in rat liver (Gressner and Wool, 1974). It was eventually shown by alkaline phosphatase treatment and electrophoretic analysis that rat liver does indeed contain a low molecular weight 60S subunit phosphoprotein. In lower eukaryotes too, a low molecular weight 60S subunit phosphoprotein has been found (Artemia salima : Van Agthoven, Maasen and Moller, 1977; yeast : Zinker and Warner, 1976) indicating that eukaryotic cells normally contain at least two ribosomal phosphoproteins (the other being S6).

Another reason why the large subunit proteins were not always observed is that they are acidic (Leader and Coia, 1977; Zinker and Warner, 1976) and migrate in the opposite direction to the basic ribosomal proteins on most two-dimensional gel electrophoresis systems. As mentioned previously (1.1.1) methods

of gel electrophoresis such as the 'Sweep-Gel' system of Leader and Coia (1977) allow better detection and analysis of this protein.

On two-dimensional gel electrophoresis most workers have observed either two (Zinker and Warner, 1976; Van Agthoven, Maasen and Moller, 1977; Otaka and Kobata, 1978; Houston, 1978) or three (Leader and Coia, 1978c; Horak and Schiffman, 1977; Arpin, Madjar and Reboud 1978) closely migrating stained spots not more than two of which are phosphorylated. In Artemia salina the acidic 60S subunit protein eL12 has been sequenced and this has shown that only a single site is phosphorylated (Amons, Pluijms and Moller, 1979). Sweep gel analysis of protein L_γ from Krebs II ascites 60S subunits can resolve three stained spots, two of which are derivatives containing either one or two phosphates since prior treatment of 60S subunits with alkaline phosphatase results in a single stained spot (Leader and Coia, 1978c).

Evidence now suggests that there are in fact two acidic 60S subunit phosphoproteins with similar molecular weights (Zinker and Warner, 1976; Rankine and Leader, 1976; Van Agthoven, Maasen and Moller, 1977; Tsurugi et al., 1978). The second acidic protein is slightly larger and more acidic than the one first described and it was originally thought to be only a minor species. This now appears to be incorrect since it can be recovered in yields of up to 50% that of the smaller protein in Artemia salina (Van Agthoven et al., 1978). It was suggested that the smaller acidic phosphoprotein is derived from the larger by proteolysis, but Van Agthoven et al., (1978) have shown, by amino acid sequence analysis, that eL12 and the

larger acidic protein eL12' from *Artemia salina* are in fact unique proteins.

The relative ease with which the acidic 60S subunit proteins can be extracted (compared to most ribosomal proteins) and the existence of a cytoplasmic pool of free acidic protein in yeast (Zinker, 1980; Sanchez-Madrid and Ballesta, 1979) suggests that this protein can undergo an exchange reaction between the ribosome and cytoplasm *in vivo* (c.f. 1.1.2). Other evidence for such an exchange reaction has been obtained by injecting ^{3}H-labelled protein eL12 from *Artemia salina* into *Xenopus laevis* oocytes and observing the association of the injected protein with the ribosomes (Kalthoff and Richter, 1979). Another interesting feature of such an exchange reaction is suggested by the lack of phosphorylation of free acidic protein in the cytoplasmic pool whereas this protein is phosphorylated when it is associated with the ribosome (Zinker, 1980).

There is considerable evidence that the acidic proteins in eukaryotic 60S subunits are equivalents of *E. coli* ribosomal protein L7/L12. Antibodies to L7/L12 will cross-react with 60S subunits from rat liver (Wool and Stoffler, 1974; Stoffler *et al.*,1974) chicken liver (Howard, Smith and Gordon, 1976) and yeast (Wool and Stoffler, 1974) and can also block polyphenylalanine synthesis (Grasmuk, Nolan and Drews, 1977). Replacement of the acidic proteins in *Artemia salina* (Moller *et al.*, 1975) or yeast (Richter and Moller, 1974) by *E. coli* L7/L12 resulted in active ribosomes suggesting the functional equivalence of these proteins from different species. Similarly antibodies to *Artemia salina* protein eL12 inhibits the EF-2 dependent

GTPase reaction of *Artemia salina* ribosomes indicating that it has a similar function to L7/L12 (c.f. 1.1.4). Both L7/L12 and the eukaryotic acidic proteins (Tischendorf, Zeichardt and Stoffler, 1975; Rankine and Leader, 1976; Horak and Schiffman, 1977) appear to be located at the subunit interface, and as is the case for L7/L12 the eukaryotic acidic ribosomal proteins are present in multiple copies (Van Agthoven *et al.*, 1978; Kruiswijk De Hey and Planta, 1978). In Table 1.2 some features of eukaryotic large subunit phosphoproteins are compared to L7/L12.

Despite the observed immunochemical cross-reactivity of L7/L12 with eukaryotic acidic ribosomal proteins there are some anomalies. Stoffler *et al.*, (1974) were originally able to show a cross-reaction between rat liver proteins L40 and L41 and *E. coli* L7/L12. However subsequent analyses have failed to detect the existence of these proteins, though a pair of phosphorylated acidic proteins (different from L40 and L41) have been purified but these do not cross-react with antibodies to L7/L12 (Tsurugi *et al.*, 1978). Protein eL12 from *Artemia salina* appears to be functionally equivalent to *E. coli* protein L7/L12, but does not show any immunochemical cross-reactivity to L7/L12 (Van Agthoven, Maasen and Moller, 1977).

The primary sequence of *Artemia salina* eL12 has little homology with *E. coli* L7/L12, however both of these proteins have some homology with certain regions of protein HL20 from the archeobacteria *Halobacterium cutirubrum* (Amons, Pluijms and Moller, 1979). Thus it appears that there is some evidence (albeit indirect) for an evolutionary relationship between the eukaryotic 60S subunit acidic phosphoprotein and protein

Table 1.2 Eukaryotic Ribosomal Proteins Analogous to Protein L7/L12 from *E. coli*

Species	Protein	Molecular Weight	Phosphorylated
Artemia salina[1]	eL12	13,000	Yes
	eL12'	>13,000	Yes
Yeast[2]	P5/P5'	14,000	Yes
Physarum polycephalum[3]	L20/L24	15,000-15,500	Yes
Krebs II Ascites Cells[4]	L_γ	13,500-14,000	Yes
HeLa Cells[5]	L40(a,b,c)	13,700	Yes
Rat Liver[6]	P1/P2	15,200-16,000	Yes
Mouse Myeloma[7]	L28	14,000	Yes
E. coli[8]	L7/L12	12,200	No

1. Van Agthoven *et al.*, (1978)
2. Zinker (1980)
3. Belanger, Bellemare and Lemieux (1979)
4. Rankine, Leader and Coia (1977)
5. Horak and Schiffmann (1977)
6. Tsurugi *et al.* (1978)
7. Martini and Kruppa (1979)
8. Terhorst *et al.*, (1973)

L7/L12 from E. coli.

In contrast to protein S6 there has been little indication that the phosphorylation state of the acidic phosphoprotein of the 60S subunit can be altered in intact cells. One notable exception is the dephosphorylation of protein L_γ in isolated Krebs II ascites cells which are incubated in a medium containing glucose (Leader and Coia, 1978b).

As is the case for protein S6, the function of phosphorylation of ascites L_γ and the equivalent protein in other eukaryotes is at present a subject for speculation. Kabat (1970) described the presence of a ^{32}P-labelled 60S subunit acidic protein only in monosomes but not polysomes when rabbit reticulocytes were labelled with ^{32}P-orthophosphate. This suggested that phosphorylation of L_γ could maintain 60S subunits in the form of inactive monosomes. However, subsequent analysis in sarcoma cells (Bitte and Kabat, 1972) and in comparison between normal and diabetic rat skeletal muscle (where there is a large decrease in the proportion of polysomes : Leader, Coia and Fahmy, 1978) have not demonstrated such a difference in L_γ phosphorylation between monosomes and polysomes.

The exchange reaction of the acidic protein between the ribosome and cytoplasm (Zinker, 1980) could act as a key step in the final stages of ribosome assembly. The presence of L_γ (or its equivalents) could be essential for the formation of a fully functional ribosome, possibly by forming a binding site for factors involved in the GTPase reactions (Weissbach, 1980). Phosphorylation might increase the affinity of L_γ for the 60S subunit either after it has become bound or just before it does so. An increase in affinity for the 60S

subunit following phosphorylation could account for the lack of phosphorylated acidic protein in the free cytoplasmic pool in yeast (Zinker, 1980) despite the presence of protein kinases capable of phosphorylating this protein in the cytoplasm (Kudlicki, Grankowski and Gasior, 1976).

The primary structure of eL12 from Artemia salina has some sequence homology with the contractile protein myosin, i.e. both proteins contain an alanine rich region of amino acids (Amons et al., 1978). This structural homology could reflect similar functions for these two proteins namely that both might have a motile function. Phosphorylation of eL12 (and its equivalent in other cells) might effect its interaction with other proteins involved in the translocation process (e.g. elongation factors) and therby either increase or decrease its efficiency in performing such a function.

A role for the phosphorylation of L_γ could be inferred from the position of L7/L12 in the Lake model of the E. coli ribosome (c.f. 1.1.3). Protein L7/L12 has been suggested as stabilizing amino acyl tRNA·EF-Tu to the proposed 'R-site' (Lake, 1980). If this process were to occur in the eukaryotic ribosome, then modification of the structure of L_γ by phosphorylation might effect the ability of the ribosome to bind amino acyl tRNA at the 'R-site'.

1.3.1.3 Other Ribosomal Phosphoproteins

Proteins S2, S3 and L14 which were normally unphosphorylated in ascites cells became phosphorylated after incubation of cells in medium containing glucose and amino acids (Leader and Coia, 1978b). Simultaneously, as these proteins became phosphorylated proteins L_γ and S6 were dephosphorylated. This altered phosphorylation pattern also occurred if cells were incubated in medium which was lacking glucose but contained amino acids. Under these conditions S2 did not however become phosphorylated. The rapid rate of glycolysis in ascites cells means that incubation medium originally containing glucose and amino acids becomes glucose depleted during the labelling of cells. It is possible that the stimulus for the phosphorylation of S3 and L14 is the absence of glucose and that amino acids are required for this to occur, possibly by the synthesis of a new protein kinase(s). The situation may however be more complicated since glucose deprivation in other cell types does not cause a similar altered phosphorylation pattern (Landschutz ascites cells : Jolicoeur 1974; BHK cells : Leader, 1980a).

Others have reported the phosphorylation of S3 (Traugh and Porter, 1976; Marvaldi and Lucas-Lenard, 1977; Roberts and Ashby, 1978) and L14 (Lastick Nielsen and McConkey, 1977; Traugh and Porter, 1976; Houston, 1978) but there was much less phosphate in the proteins in these studies. The function(s) of proteins L14 and S3 are not yet known (as is the case for L_γ and S6). There is some circumstantial evidence that S3 may be functionally important. Antibodies to S3 can block

initiation of protein synthesis (Noll et al., 1978) and Westermann, Nygard and Bielka (1981) have shown that S3 can be cross-linked to eIF-2 present in the ternary initiation complex (Fig 1.3; also see 1.3.1.1).

Protein S2 is phosphorylated in ascites cells which have produced large quantities of lactic acid by anaerobic glycolysis (thereby reducing the pH) and it is possible that S2 phosphorylation is a response to these abnormal cellular conditions. S2 has also been reported as phosphorylated in HeLa cells infected with vaccinia virus (also an abnormal cellular condition; Kaerlein and Horak, 1976).

Protein S16 was also reported to be phosphorylated in HeLa cells infected with vaccinia virus. More recently, Kennedy, Stevely and Leader (1981) have reported that S16 (or S18) is highly phosphorylated in BHK cells (as well as a large increase in S6 phosphorylation), following infection with pseudorabies virus. However, S16/S18 phosphorylation does not seem to be a general response to virus infection since no phosphorylation of S16/S18 was observed after infection of BHK cells with herpes simplex virus I (although S6 became highly phosphorylated under these conditions; Kennedy, Stevely and Leader, 1981).

1.3.2 Phosphorylation of Ribosomal Proteins by Protein Kinases

Prior to the start of the present study there had been a large number of reports that ribosomes from many different eukaryotes could be phosphorylated by protein kinases in cell-free systems (Leader, 1980).

Protein kinases capable of phorphorylating ribosomal proteins have been found in the cytosol fraction of many cells; rat liver (Eil and Wool, 1971), reticulocytes (Traugh and Porter, 1976), yeast (Becker-Ursic and Davis, 1976), adrenal cortex (Walton and Gill, 1973), corpus luteum (Azhar and Menon, 1975). Protein kinases have also been found associated with the ribosome; reticulocytes (Kabat, 1971; Martini and Gould, 1973) trout-testis (Jergil, 1972) thyroid (Pavlovic-Hournac *et al.*, 1973).

Many of these studies did not characterize which ribosomal proteins were phosphorylated and in some cases the proteins phosphorylated could be non-ribosomal (Quirin-Stricker *et al*, 1976). Others have shown that the pattern of phosphorylation in cell-free phosphorylation systems can be different to that observed in the intact cells from which the cell-free systems are derived (Traugh, Mumby and Traut, 1973; Walton and Gill, 1973; Ventamiglia and Wool, 1974). This indicates that it is necessary to fully characterize proteins phosphorylated in cell-free systems, and to demonstrate their phosphorylation in intact cells before they can be regarded as functionally significant.

Protein S6 can be phosphorylated by cAMP-dependent protein kinase (Traugh and Porter, 1976) but only at a maximum of two of the possible phosphorylation sites (Du Vernay and Traugh,

1978). However, this protein kinase can also phosphorylate a number of other ribosomal proteins and it is unclear whether it is responsible for S6 phosphorylation *in vivo*. Some evidence that S6 might be phosphorylated by more than one type of protein kinase has been given by Wettenhall and Howlett (1979). They showed that in thymocytes prostaglandin E1 stimulates phosphorylation of S6 by elevation of cAMP concentration, whereas concanvalin A acts through a cyclic nucleotide-independent mechanism to phosphorylate S6.

Some indication that acidic proteins analogous to L_γ can be phosphorylated by a casein kinase has been shown by Kudlicki, Grankowski and Gasior (1976) in yeast and by Issinger (1977) in rabbit reticulocytes.

To fully understand the control and extent of ribosomal protein phosphorylation it is necessary to characterize the protein kinase(s) responsible.

The objective of the present study was to investigate the protein kinase(s) responsible for the phosphorylation of ribosomal proteins in Krebs II ascites cells, especially the 60S ribosomal subunit acidic protein L_γ. Also to examine for possible alterations in the activity or pattern of protein kinases from Krebs II ascites cells incubated under conditions which alter the pattern of ribosomal protein phosphorylation.

2. MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Biological:

BHK/C13 cells are an established line of hamster fibroblasts, (Macpherson and Stoker, 1962).

Krebs II ascites cells are derived from the solid Krebs II Carcinoma and are grown in the peritonea of mice as a homogeneous suspension (Klein and Klein, 1951). Pseudorabies virus (Pig Herpesvirus 1) was derived from a stock preparation (Kaplan and Vatter, 1959) and has subsequently been plaque purified.

2.1.2 Radiochemicals:

All radiochemicals were obtained from, The Radiochemical Centre, Amersham, England.

[γ-^{32}P] ATP specific activity approximately 20 Ci/mmole

[γ-^{32}P] GTP " " " 20 Ci/mmole

^{32}P-Orthophosphoric acid, carrier free at concentration of 10mCi/ml.

^{14}C-Methylated Protein Mixture, containing 0.833μCi/ml of each of the following proteins.

^{14}C	Methylated	myosin	specific activity 35μCi/mg
"	"	phosphorylase-b	" " 39μCi/mg
"	"	bovine serum albumin	" 60μCi/mg
"	"	carbonic anhydrase	" 18μCi/mg
"	"	lysozyme	" " 17μCi/mg

2.1.3 Enzymes and Other Proteins:

Calmodulin was a gift from Dr. P. Cohen, Department of Biochemistry, University of Dundee, Scotland.

Protein Kinase Inhibitor Protein 'PKGI' was a gift from Dr. E.M. Chambez, Centre d'Etudes et de Recherches sur les Macromolecules Organisees, Grenoble, France.

Beef Heart cAMP dependent protein kinase.	Sigma Chemical Company, Poole, England.
Casein (partially hydrolysed, and dephosphorylated).	
Histone IIA (Calf thymus).	
Cytochrome C (Horse heart).	
Bovine Serum Albumin (BSA).	Armour Pharmaceutical Co.Ltd., Eastbourne, England.

2.1.4 Chemicals:

Most chemicals were Analar grade obtained from, BDH Chemicals Ltd., Poole, England, except the following :

Acrylamide	Bio-Rad Laboratories
Bis-Acrylamide	Richmond, California
Bis-Tris	U.S.A.
Ammonium Persulphate	
N,N,N',N',-Tetramethylethylene-diamine (TEMED)	Eastman Kodak Company, Rochester,
Kodak No-Screen Film NS-2T	New York,
DX80 Photographic Developer	U.S.A.
FX40 Photographic Fixer	

Heparin B.P.	Evans Medical Ltd., Liverpool, England.
Eagle's MEM medium (Glasgow Modification)	Gibco Bio-cult, Paisley, Scotland.
MEM Vitamins	
MEM Amino Acids	
Calf Serum	
Penicillin	Glaxo Pharmaceuticals Ltd., London, England.
Streptomycin	
Pyronine Y	G.T. Gurr Ltd., London, England.
p-bis- 2-(5-Phenyloxazolyl) - benzene,(POPOP).	Koch-Light Laboratories Ltd., Colnbrook, England.
2,5-diphenyloxazole, (PPO)	
PEI Cellulose sheets	Macherey, Nagel and Co., Duren, Federal German Republic.
Tryptose Phosphate	Oxoid Ltd., Basingstoke, England.
ATP	P-L Biochemicals Inc., Milwaukee, Wisconsin, U.S.A.
GTP	
cAMP	
Tris	Sigma Chemical Company, Poole, England.
Coomassie Brilliant Blue R	
" " " G250	
Agarose Type IV	

DEAE-cellulose (DE52)	M. Reeve-Angel and Co. Ltd.,
Phosphocellulose (P11)	London, England.
3MM chromatography paper	
Spectrophor 3 dialysis tubing	Spectrum Medical Industries Inc., Los Angeles, U.S.A.

2.2 Standard Solutions:

Tris Buffered Saline (TBS)

35mM Tris-HCl pH 7.5, 146mM NaCl.

Reticulocyte Standard Buffer (RSB) (Penman, 1966)

10mM Tris-HCl pH 7.5, 10mM KCl, 1.5mM Magnesium acetate.

Medium K

25mM Tris-HCl pH 7.5, 125mM KCl, 5mM Magnesium acetate.

Balanced Salt Solution minus Phosphate (BSS-P) (Earle, 1943)

116mM NaCl, 5.4mM KCl, 1mM $MgSO_4$, 1.8mM $CaCl_2$, and 0.002% (w/v) phenol red. The pH of the solution was adjusted to 7.0 with 8.4% (w/v) $NaHCO_3$.

ETC

Eagle's MEM containing 10% (w/v) Tryptose phosphate, 5% (v/v) Calf serum, 0.22% (w/v) $NaHCO_3$, 0.004% (w/v) phenol red, 10^5 units/L penicillin, 100mg/L streptomycin pH 7.0.

Eagle's MEM minus glucose

Is composed of a solution of MEM amino acids and MEM vitamins.

Tris-Magnesium-Citrate (TMC) (Hogan and Korner, 1968)

16mM tris, 10mM $MgSO_4$, 5mM citric acid.

Formol Saline

80mM NaCl, 100mM Na_2SO_4, 3.6% (v/v) Formaldehyde.

Scintillation Fluid

66% (v/v) toluene, 33% (v/v) 2-methoxyethanol, 0.4% (w/v) 2,5-diphenyloxazole, 0.01% (w/v) p-bis-2-(5-Phenyloxazolyl) -benzene.

2.3 METHODS

2.3.1 Growth and Harvesting of Krebs II Ascites Cells.

Growth of Cells :

Ascites cells were propagated in Porton mice by intraperitoneal injection of 0.2ml of ascitic fluid (approximately 2×10^6 cells) at seven day intervals.

Harvesting :

Eight days after inoculation the mice (17-20 in number) were killed. The skin covering the peritoneum was cut away, and an incision made in the peritoneal membrane. The ascitic fluid was collected in a 250ml centrifuge bottle containing 50-100ml of ice-cold TBS. All subsequent operations were carried out at 0-4°C. To remove contaminating blood cells, the ascites cells were washed three times by centrifugation at 375g following by resuspension in 150-200ml TBS. After the final wash, cells were resuspended in the minimum volume of TBS, and centrifuged in a 50ml graduated tube for 10 minutes at 730g. The packed volume of cells was recorded at this stage (typically 20ml).

2.3.2 Growth of BHK Cells

BHK-21/C13 cells were grown as monolayers on 80oz roller bottles. The bottles contained 180ml of ETC medium in an atmosphere of 5% (v/v) CO_2 in air. Cells were grown for three days before harvesting or inoculation with virus, (Macpherson and Stoker, 1962).

Contamination Checks.

Cells were regularly checked for contaminating micro-organisms. Aliquots were grown on blood agar plates at 37°C to detect bacterial contamination. Yeast and fungal contamination were monitored by incubating aliquots of media in Sabouraud's medium at 32°C. Infection by mycoplasma was checked by using PPLO agar plates seeded with cells and incubated at 37°C.

2.3.3 ^{32}P-Labelling of Ascites Cells

It has been shown that when ascites cells are labelled in an incubation medium, the particular ribosomal proteins which become ^{32}P-labelled depends on the composition of the medium. For this purpose, two different incubation media were used.

Medium 1 (Rankine, Leader, and Coia, 1977)

This medium contained no glucose so that cells became labelled with ^{32}P but the medium did not become acidic, (since the glycolytic production of large quantities of lactic acid from glucose could not take place). The medium contained 10% (v/v) Eagle's MEM minus glucose, BSS-P, 10% (v/v) calf serum minus phosphate (dialysed vs BSS-P), 10^5 units/L penicillin, 100mg/L streptomycin, buffered with TMC adjusted to pH 7.6 with NaOH at 37°C.

Medium 2 (Leader and Coia, 1978)

Medium 2 was the same as Medium 1 except that it contained 10% (v/v) Eagle's MEM (complete Eagle's medium containing glucose). Lactic acid was produced by glycolysis of the

glucose and this lowered the pH of the medium to approximately 6.5 after 1.5 hours of incubation.

Labelling of Cells

Washed cells were resuspended at a concentration of 10^7 cells/ml in 200ml of either incubation medium. Carrier free ^{32}P -orthophosphate was then added to a final concentration of 0.05 mCi/ml and the cells were incubated at 37°C for three hours in a 2 litre conical flask with constant stirring. After labelling, the cells were collected and washed as described above (2.3.1).

2.3.4 Infection of BHK Cells with Pseudorabies Virus

BHK cells were grown for three days in 80 oz roller bottles as described (2.3.2). The 'used' medium was removed, and the cells were then infected with pseudorabies virus in 20ml of 'used' medium at a multiplicity of infection of one plaque forming unit/300 cells. The virus was allowed to adsorb to cells for one hour at 37°C. Next 50ml of 'used' ETC were added and the infected cells were incubated for a further five hours. The post-ribosomal supernatant of infected cells were prepared as described (2.3.6).

2.3.5 Pseudorabies Virus Plaque Assay

Serial dilutions of the virus were made in Eagle's medium minus serum in the range 10^{-5} - 10^{-9}. BHK cells were grown for 24 hours at 37°C in 5cm plastic petri dishes in ETC medium in an atmosphere of 5% CO_2 in air. After confluence was

reached, the medium was removed and 0.2ml of virus dilution was used to inoculate the cell monolayer. Virus was allowed to adsorb to cells for one hour at 37°C. ETC medium, (5ml), was added and the cells were incubated for a further two hours at 37°C. Heparin, (250 units in a volume of 25µl) was added, and incubation at 37°C was continued for a further 28 hours. The medium was removed, and the cell monolayer was fixed with 10% (v/v) formol saline for 20 minutes. The formol saline was removed, and the monolayer stained with 1ml of a 0.15% (w/v) Giemsa dye solution for 20 minutes. The stain was washed off with H_2O, and the number of plaques was counted using a plate microscope.

2.3.6 Preparation of Ribosomes from Ascites Cells

This was based on the method of Mathews and Korner, (1970). Washed cells were resuspended in 1.5 volumes of ice-cold hypotonic RSB, by 2 up-and-down strokes in a glass-teflon homogenizer. All subsequent operations were carried out at 0-4°C. The cells were homogenized by 20 strokes of the same homogenizer. Then 0.11 volumes of 10 x Medium K were added to restore the salt concentration.

The cell homogenate was then centrifuged for 10 minutes at 30,000g to remove the cell nuclei, mitochondria, and other membranous fractions. The supernatant was carefully removed avoiding the fat surface layer, and pellet, and centrifuged for 2.5 hours at 164,000g in a Beckman Ti50 rotor.

The post-ribosomal supernatant was filtered through glass wool to remove any fat, and was either frozen at -70°C or dialysed against DEAE-cellulose chromatography buffer (2.3.10).

The ribosomal pellets were either kept at this stage, for use in endogenous phosphorylation experiments by freezing at -70°C, or were further purified.

The ribosomal pellets were resuspended in 6ml of Medium K using a glass-teflon homogenizer. Then 7ml of homogenate was layered over 5ml of 1M sucrose in Medium K, and centrifuged for 2.5 hrs at 164,000g. The supernatant contained non-ribosomal protein contaminants and was discarded. The final ribosomal pellets were stored at -70°C.

2.3.7 Preparation of Ribosomal Subunits

This was a minor modification of the method of Leader and Wool,(1972). Ribosomal pellets were resuspended in buffer containing 10mM Tris-HCl pH 7.5, 500mM KCl, 5mM $MgCl_2$ by 5 strokes of a glass-teflon homogenizer. The ribosome suspension was centrifuged for 5 minutes at 760g to pellet any aggregated material.

Then 2-mercaptoethanol and puromycin were added to the homogenate to give final concentrations of 20mM and 0.1mM respectively. The ribosome suspension was incubated for 15 minutes at 37°C.

Without cooling, 75-100A_{260} (approx. 1.8ml) were layered on 18.5ml of a 10-30% linear sucrose density gradient in a solution containing 10mM Tris-HCl pH 7.5, 500mM KCl, 5mM $MgCl_2$, and 20mM 2-mercaptoethanol. The loaded density gradients were centrifuged for 4 hours at 96,000g in a Beckman SW27 centrifuge rotor at 28°C. After centrifugation, the gradients were analysed at 260nm wavelength with a Gilford model 240

recording spectrophotometer using a flow cell. All of the 40S subunit peak was collected, but only the 'heavy' side of the 60S subunit peak (Fig 2.1).

The subunits were pelleted from the pooled sucrose gradient fractions by centrifugation for 16 hours at 176,000g in a Beckman Ti60 rotor at 4°C. Subunit pellets were stored at -70°C.

2.3.8 Extraction of Protein from Ribosomal Subunits

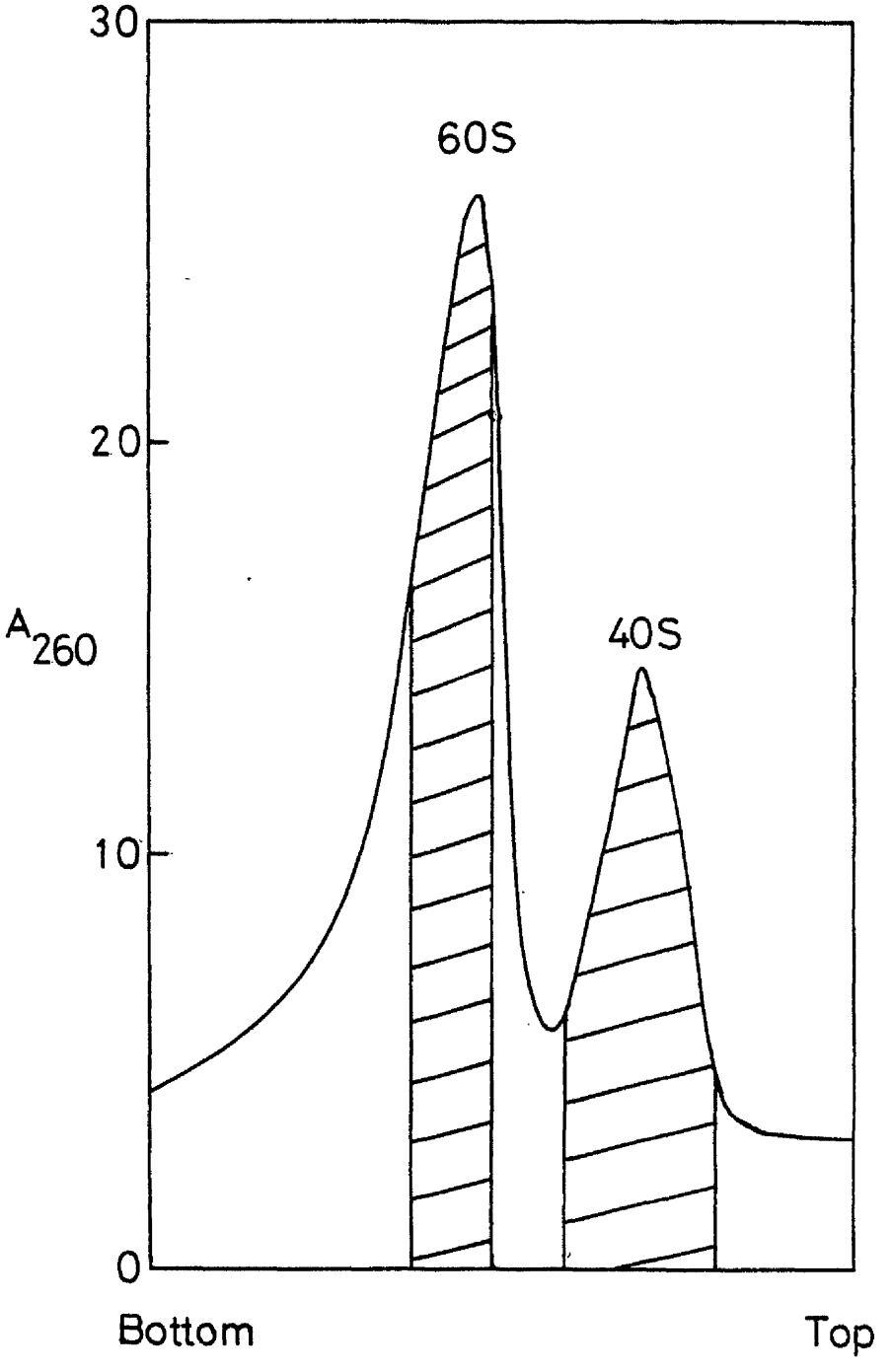
This was based on the method of Sherton and Wool,(1974). Both 40S and 60S ribosomal subunits were suspended at a concentration of approximately $1000A_{260}$ units/ml in a solution containing 10mM Tris-HCl pH 7.7, 600mM Magnesium Acetate. Next 2 volumes of glacial acetic acid were added, and the solution was stirred for one hour at 0°C. The subunit suspension was then centrifuged for 10 minutes at 15,000g to sediment the ribosomal RNA. The supernatant containing the ribosomal proteins was kept, and the pellet was re-extracted. The supernatants from both extractions were pooled, placed in dialysis tubing, and dialysed for 48 hours against 500 volumes of 1M acetic acid with two or three changes of dialysis solution. Dialysed protein samples were lyophilised.

2.3.9 Estimation of Protein Concentration

The dye binding method of Bradford (1976) for estimation of protein concentration was used. The reagent was prepared by dissolving 100mg of Coomassie Brilliant Blue G250 in 50ml 95% (v/v) ethanol, adding 100ml 85% (w/v) phosphoric acid,

Figure 2.1 : Separation of Ascites Ribosomal Subunits

Ascites ribosomal subunits were separated on 10-30% (w/v) sucrose density gradients (2.3.7). The shaded regions below the subunit peaks indicate the portions of each gradient collected as 60S and 40S subunits.


30
20
10
0
A260
60S
40S
Bottom
Top

diluting to 1 litre with distilled water, and filtering. The reagent finally contained 0.01% (w/v) Coomassie Brilliant Blue, 4.7% (v/v) ethanol 8.5% (w/v) phosphoric acid.

Reagent, (5ml) was mixed with 0.1ml of protein sample and the absorbance at 595nm was measured after 2 minutes. Bovine serum albumin standards were prepared in the same buffer as the sample (Fig. 2.2).

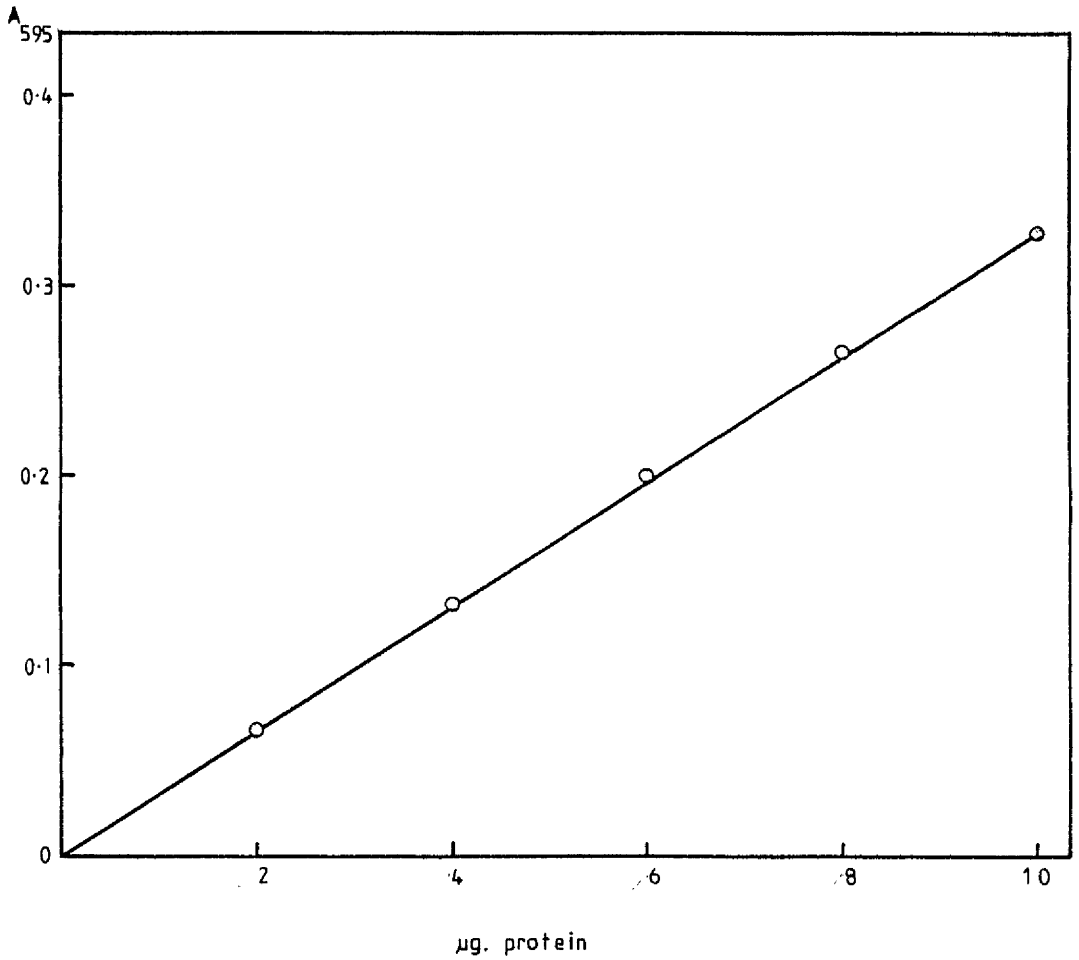
2.3.10 DEAE-cellulose Chromatography

DEAE-cellulose was equilibrated with chromatography buffer (20mM Tris-HCl pH 7.5, 1mM EDTA, 10mM 2-mercaptoethanol, 10% (v/v) glycerol, conductivity 0.45mmho) at 4°C. This and all subsequent operations were carried out at 0-4°C. A column of 12ml volume and 4.5cm length was poured in a 20ml plastic syringe of 1.8cm internal diameter. A syringe barrel containing the column inlet tube was lowered to within 1-3mm of the gel surface (Fig 2.3). After connection to a LKB Broma 2120 Variopex II pump set at a pump speed of 30ml/hr, the column was ready for operation.

Approximately 20ml of post-ribosomal supernatant (2.3.6) from Krebs II ascites, or BHK cells was dialysed extensively against DEAE-cellulose chromatography buffer. Equilibrated post-ribosomal supernatant was then adsorbed to the column. The loaded column was washed with 150ml of chromatography buffer to remove non-adsorbed material. A 500ml linear gradient of 0-400mM KCl in chromatography buffer was then applied to the column. Samples (7.5ml) were collected using a Uniscil UFC 120 fraction collector. The absorbance of each sample at

Figure 2.2 : Protein Estimation Standard Curve

The figure shows the standard curve obtained when the method of Bradford (1976) (2.3.9) was used to estimate protein. Bovine serum albumin was used as protein standard.


A595
0·4
0·3
0·2
0·1
0
2
4
6
8
10
µg. protein

260nm was measured on a Unicam SP500 spectrophotometer. The conductivity of each sample was measured using a Radiometer Type CDM 2c conductivity meter.

2.3.11 Concentration of Protein Kinase Fraction from DEAE-cellulose.

Protein kinase containing fractions from DEAE-cellulose chromatography (2.3.10) were combined to give a volume of 150-200ml. This combined fraction was poured into an Amicon stirred ultrafiltration cell, Model 202, containing a Diaflo UM10 ultrafiltration membrane (molecular weight cut off approximately 10,000). The apparatus was sealed, and a pressure of 25-30 p.s.i. applied with nitrogen gas. Throughout the operation the sample was gently stirred with a magnetic stirrer. The entire procedure was carried out at 0-4^{o}C. The protein kinase sample was removed when the volume was reduced to 10-15ml. This sample was dialysed against phosphocellulose chromatography buffer (2.3.12) until equilibrated.

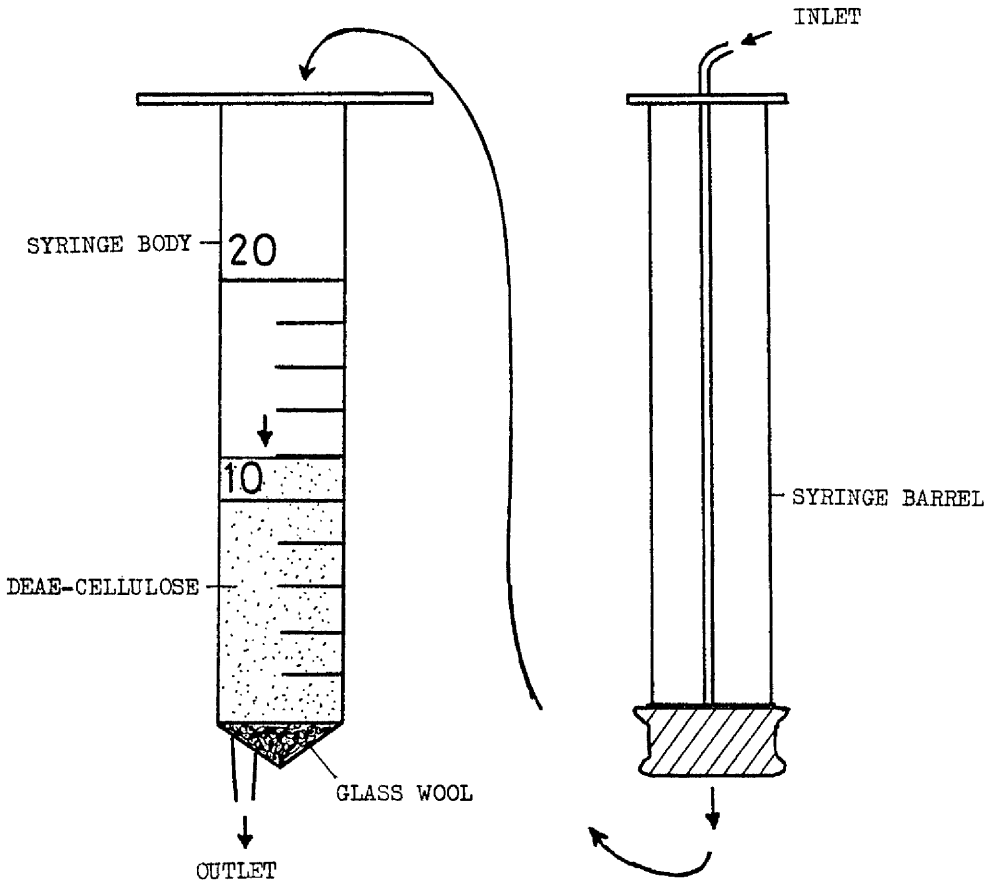
2.3.12 Phosphocellulose Chromatography

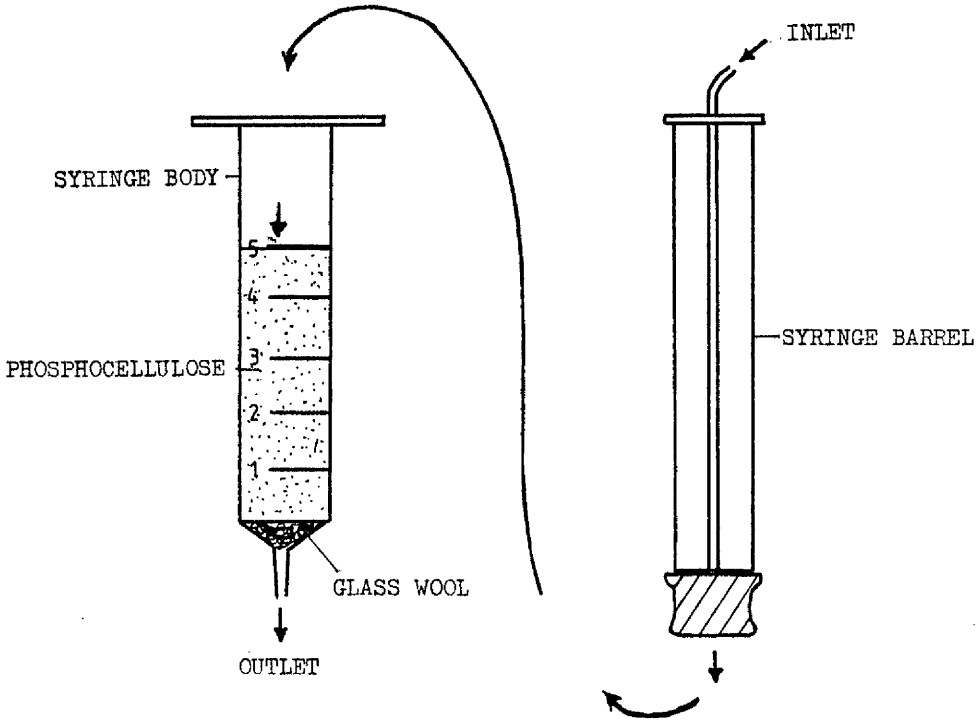
All procedures were essentially the same as for DEAE-cellulose chromatography, with the following exceptions.

1. The chromatography buffer contained 50mM Tris-HCl pH 7.5, 1mM EDTA, 250mM NaCl, 10mM 2-mercaptoethanol of conductivity 7.0mmho.
2. Equilibrated phosphocellulose was poured to give a column of 4ml volume, and 4.5cm length in a 5ml syringe of internal diameter 1.2cm. (Fig 2.3).

Figure 2.3 : Chromatography Columns

This figure shows the apparatus used for DEAE-cellulose (Fig 2.3.1) and phosphocellulose (Fig 2.3.2) chromatography. For clarity the syringe barrel and body are shown separated. It should be noted however, that during operation, the lower surface of the syringe barrel rubber washer was within 1mm of the surface of the DEAE-cellulose or phosphocellulose.


INLET
SYRINGE BODY
20
10
DEAE-CELLULOSE
SYRINGE BARREL
GLASS WOOL
OUTLET


INLET
SYRINGE BODY
5
4
3
2
1
PHOSPHOCELLULOSE
SYRINGE BARREL
GLASS WOOL
OUTLET

3. The column was washed with 20ml of chromatography buffer prior to elution by 150ml of a linear gradient of 0.25-1M NaCl in the same buffer.
4. Samples of 2.5ml volume were collected.

2.3.13 Preparation of Rabbit Skeletal Muscle cAMP Dependent Protein Kinases.

Preparation of Crude Protein Kinase Fraction

Approximately 500g of rabbit skeletal muscle were minced, and then homogenised in 2.5 volumes of 4mM EDTA for 30 seconds in an Atomix blender at 0-4°C. The homogenate was then centrifuged at 4000g for 45 minutes. The supernatant was removed, adjusted to pH 5.5 with acetic acid, and centrifuged as before. The second supernatant was removed, and the pH adjusted to 7.0 by adding EDTA to 2mM, K_2HPO_4 to 10mM, and NH_4OH until pH 7.0 was reached. The supernatant was stirred for 30 minutes at 0°C after the slow addition of $(NH_4)_2SO_4$ (to avoid frothing) to 45% (w/v) saturation. Precipitated protein was sedimented by centrifugation at 4000g for 45 minutes. The protein pellet was redissolved in approximately 20ml of buffer A containing 0.2mM EDTA, 5mM sodium β-glycerophosphate pH 7.0, and dialysed extensively against approximately 100 volumes of this buffer with four changes of solution.

DEAE-cellulose Chromatography

Approximately 60ml of DEAE-cellulose equilibrated with buffer A, was poured into a Wright Chromatography column to give a gel bed 4cm in diameter, and 5cm in height. Approximately

20ml of crude protein kinase fraction was loaded onto the column and washed with buffer A. Fractions were collected as described (2.3.10), and the absorbance at 280nm of each fraction was measured. When the A_{280} reading was less than 0.1, the elution buffer was changed, and a buffer which was twice the concentration of A (i.e. buffer 5 x A) was used. This procedure was repeated twice more when the A_{280} value fell below 0.1. The buffers used were buffer 10 x A, and buffer 10 x A which contained 100mM NaCl. Fractions eluted by each buffer were pooled to give four large fractions each one corresponding to one of the elution buffers. Elution fraction two contained isoenzyme 1, and fraction four contained isoenzyme 2. Protein kinase activities were determined as described (2.3.15).

2.3.14 Preparation of cAMP Dependent Protein Kinase Inhibitor Protein

This was a minor modification of the method of Walsh et al., (1971). The first stages in the preparation of rabbit skeletal muscle cAMP dependent protein kinase inhibitor protein were the same as for the preparation of cAMP dependent protein kinase, (2.3.13). The supernatant from the first 4,000g centrifugation step after homogenisation of the tissue was heated by slowly raising the temperature to 95°C over a 30 minute period. The resulting precipitate of denatured proteins was removed by filtering through cheese cloth and filter paper. The filtrate was adjusted to 15% (w/v) trichloroacetic acid, and the precipitate which contained the

inhibitor protein was sedimented by centrifugation at 10,000g for 30 minutes. This precipitate was resuspended in 25ml of buffer containing 5mM glycerophosphate pH 7.0, and 2mM EDTA. This suspension was dialysed against 2 litres of the same buffer with two changes of buffer. The dialysed solution containing the inhibitor protein was stored at -20°C.

2.3.15 Cell Free Phosphorylation of Proteins

Various protein substrates were phosphorylated by protein kinase mediated enzymic transfer of ^{32}P-orthophosphate from [γ-^{32}P] ATP or [γ-^{32}P] GTP.

2.3.15.1 Assay for Protein Kinase Activity

Casein kinase assays were carried out using a buffer-substrate assay mixture which contained the following ingredients :

200μg of casein, 0.10mM [γ-^{32}P] ATP or 0.10mM [γ-^{32}P] GTP at specific activity, 4mCi/mmol, 20mM Tris-HCl pH 7.5, 50mM KCl, 10mM $MgCl_2$, 10mM 2-mercaptoethanol. Buffer-substrate mixtures were pre-incubated at 30°C prior to addition of enzyme fractions. Enzyme fractions were mixed with the buffer-substrate mixture, giving a final volume of 250μl, and the reaction was allowed to proceed for 20 minutes. The reaction was terminated by removing 100μl samples of the reaction mixture, absorbing them to 2.5cm diameter circles of Whatman 3MM chromatography paper, and dropping these into 10% trichloroacetic acid at 0-4°C. Approximately 10ml of trichloroacetic acid was used for every assay sample. To remove any radioactivity which was not

covalently bound to the protein substrate, the assay samples were washed with trichloroacetic acid. They were washed in batches in a porous plastic container, by constant magnetic stirring (Fig. 2.4). Each batch of samples was washed twice for 15 minutes in 10% (w/v) trichloroacetic acid, followed by 4 washes of 15 minutes in 5% (w/v) trichloroacetic acid. They were finally given a 10 minute wash in 95% (v/v) ethanol, and dried using a heat-lamp. To each sample, 5ml of scintillation fluid was added and radioactive incorporation was determined in a Packard Tri-Carb Model 3255 liquid Scintillation Counter.

Histone kinase assays were carried out in a similar way with the following exceptions :
200μg of histone IIA was used as protein substrate. Assay buffer contained 20mM MES-NaOH pH 6.5 instead of Tris-HCl. Assays were terminated in 20% trichloroacetic acid, followed by 2 washes in 20% trichloroacetic acid and 4 washes in 10% trichloroacetic acid.

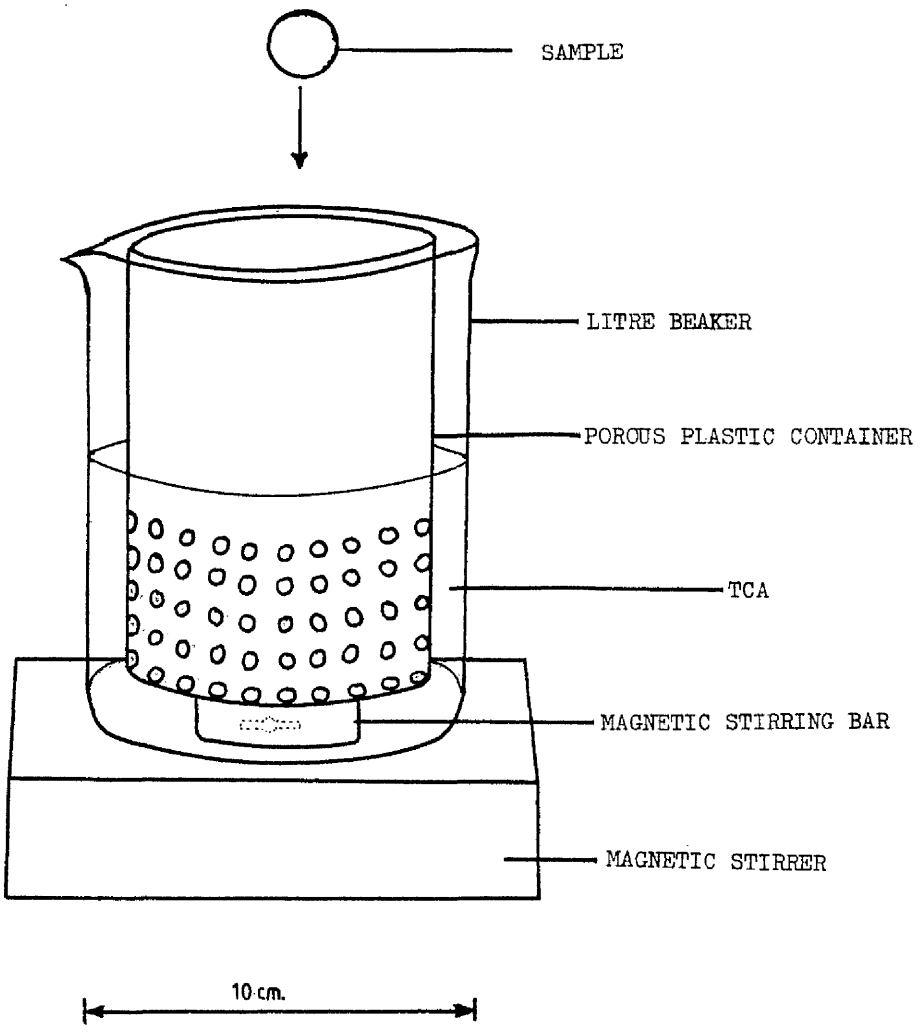
2.3.15.2 Preparative Phosphorylation of Ribosomal Proteins

Ribosomal protein substrates were phosphorylated using either of the two assay conditions described (2.3.15.1), depending on the protein kinase used. A variety of ribosomal protein substrates were used. These included 80S ribosomes, either salt-washed or unwashed (2.3.6), 60S and 40S ribosomal subunits, and 60S and 40S extracted ribosomal protein.

For one-dimensional SDS slab gels, 100μl of ribosomal protein phosphorylation mixture was heated in an SDS,

Figure 2.4 : Protein Kinase Assay Wash Apparatus

This figure shows the arrangement used for the termination and washing of protein kinase assay samples.


SAMPLE
LITRE BEAKER
POROUS PLASTIC CONTAINER
TCA
MAGNETIC STIRRING BAR
MAGNETIC STIRRER
10 cm.

2-mercaptoethanol solution (2.3.17), loaded directly onto gels, and electrophoresed. Radioactively labelled ribosomal protein (250µl) which was to be electrophoresed on two-dimensional gels was mixed with a five-fold excess of ribosomal carrier and the proteins extracted from the mixture (2.3.8) and electrophoresed.

2.3.16 PEI-Cellulose Chromatography of Nucleotides

PEI-cellulose sheets 0.1mm thick were cut into strips 20cm long and of varying widths. Solutions of 5-10µl containing ^{32}P-labelled or unlabelled nucleotides were applied 1.5cm from the bottom edge of the strips. The standards were either 10mM ATP or 10mM GTP. After the application of samples, the strips were dried using a hot air blower. The strips were then placed in a vertical glass chromatography tank containing a solution of 0.85M KH_2PO_4 pH 3.4, 1cm deep. The lid of the tank was replaced, and the chromatography strips were left for 2-3 hours until the ascending solvent front was approximately 1cm from the top. The strips were then removed and dried by hot air. The positions of ATP and GTP standard nucleotides was determined by u.v. fluorescence at 340nm wavelength using a Mineralight Lamp, Model UVSL-58. The chromatograms were autoradiographed as described (2.3.18).

2.3.17 One-Dimensional SDS Polyacrylamide Gel Electrophoresis

This was based on the method of Laemmli,(1970).

Separation Gel :

Separation gels contained 12.5% (w/v) acrylamide, 0.33%

(w/v) bis-acrylamide, 0.1% SDS, 0.375M Tris-HCl pH 8.8, 0.03% (v/v) N,N,N',N',-Tetramethylethylenediamine. Polymerization was achieved by addition of ammonium persulphate to 0.05% (w/v). A flat upper gel surface was made by overlaying the gels with H_2O prior to polymerization. Gels were allowed to polymerize for at least one hour before use.

Stacker Gel :

Stacker gels contained 3% (w/v) acrylamide, 0.08% (w/v) bis-acrylamide, 0.1% SDS, 0.12M Tris-HCl pH 7.0, 0.03% (v/v) N,N,N',N',-Tetramethylethylenediamine. Ammonium persulphate was added to 0.1% (w/v) and polymerization was allowed to occur for at least one hour.

Cylindrical Gels :

Separation gels of 8cm length were poured in siliconized glass tubes 15cm long of internal diameter 6mm. Stacker gels 1.5cm long were poured on top of the separation gel.

Slab Gels :

Separation gels 20.5cm length and 18cm width were poured between glass plates 1.5mm apart. Stacker gels were poured 18cm wide and 3cm long, with the comb forming the wells protruding 1cm below the surface. Thus there was 2cm of stacking gel between the sample wells and the separation gels.

Protein Sample Preparation :

Protein samples of approximately 100µg were dissolved in 100µl of 50mM Tris-HCl pH 7.0, 2% (w/v) SDS, 5% (v/v)

2-mercaptoethanol, and 0.001% (w/v) Bromophenol Blue. They were then heated to 100°C for two minutes to form SDS-protein complexes. After they had cooled, 50µl of glycerol was added. The samples were then loaded onto the gels. Up to 150µl of protein sample was loaded onto cylindrical gels, and approximately 40µl was applied to each well in slab gels. The protein samples were either extracted ribosomal protein, protein standards of known molecular weight, protein kinase fractions, ribosomal subunits, or ribosomes.

Electrophoresis Conditions :

Electrophoresis was performed in buffer containing 25mM Tris-HCl pH 8.5, 192mM glycine, 0.1% (w/v) SDS. Cylindrical gels were maintained at 4mA/gel until the dye front was within 0.5cm of the bottom of the separation gel (normally 1.5 hours). The conditions for slab gels were normally 16mA/gel for 16 hours. Before staining, the position of the dye front was marked for both types of gel. In both cases the positive electrode was connected to the lower gel compartment.

Staining and De-staining of Gels :

Gels were stained in a solution containing, 45% (v/v) methanol, 9% (v/v) acetic acid, 0.1% (w/v) Coomassie Brilliant Blue R250 dye, for 2 hrs at 37°C. Gels were destained by diffusion in 7.5% (v/v) acetic acid, with several changes of solution.

Scanning of Cylindrical Gels :

Gels were scanned at 586nm wavelength using a Gilford 240 Spectrophotometer and linear transport accessory Model 2410. (Figs 2.5, 2.6).

Molecular Weight Standard Proteins (Weber and Osborn, 1969)

For cylindrical gels an estimate of the molecular weights of sample proteins was obtained by comparison with proteins of known molecular weight, electrophoresed in parallel. The proteins used were bovine serum (69,000), chymotrypsinogen (25,700), and cytochrome C (11,700). The molecular weights of proteins on slab gels were estimated from standard proteins on the same gel. These standards were a mixture of ^{14}C-methylated proteins consisting of myosin (200,000), phosphorylase-b (92,000), bovine serum albumin (69,000), ovalbumin (46,000), carbonic anhydrase (30,000), and lysozyme (14,300).

2.3.18 Autoradiography of Gels

Drying of Gels

Cylindrical gels and two-dimensional slab gels were sliced longitudinally. Cylindrical gels were cut into two or three slices, and two-dimensional slab gels were halved. One-dimensional slab gels were dried intact. Gel slices or whole gels were laid on sheets of moistened Whatman 3mm chromatography paper, and dried under vacuum on a Pharmacia GSD-4 slab gel drier.

Fig. 2.5 Scan of 40S Ribosomal Subunit Protein Gel

The figure shows a densiometric tracing at 585nm of a typical one-dimensional SDS gel after electrophoresis of 40S ribosomal subunit protein.

Molecular Weight Markers :-

BSA (bovine serum albumin)	- 69,000
Chym (chymotrypsinogen)	- 25,700
cyt C (cytochrome C)	- 11,700

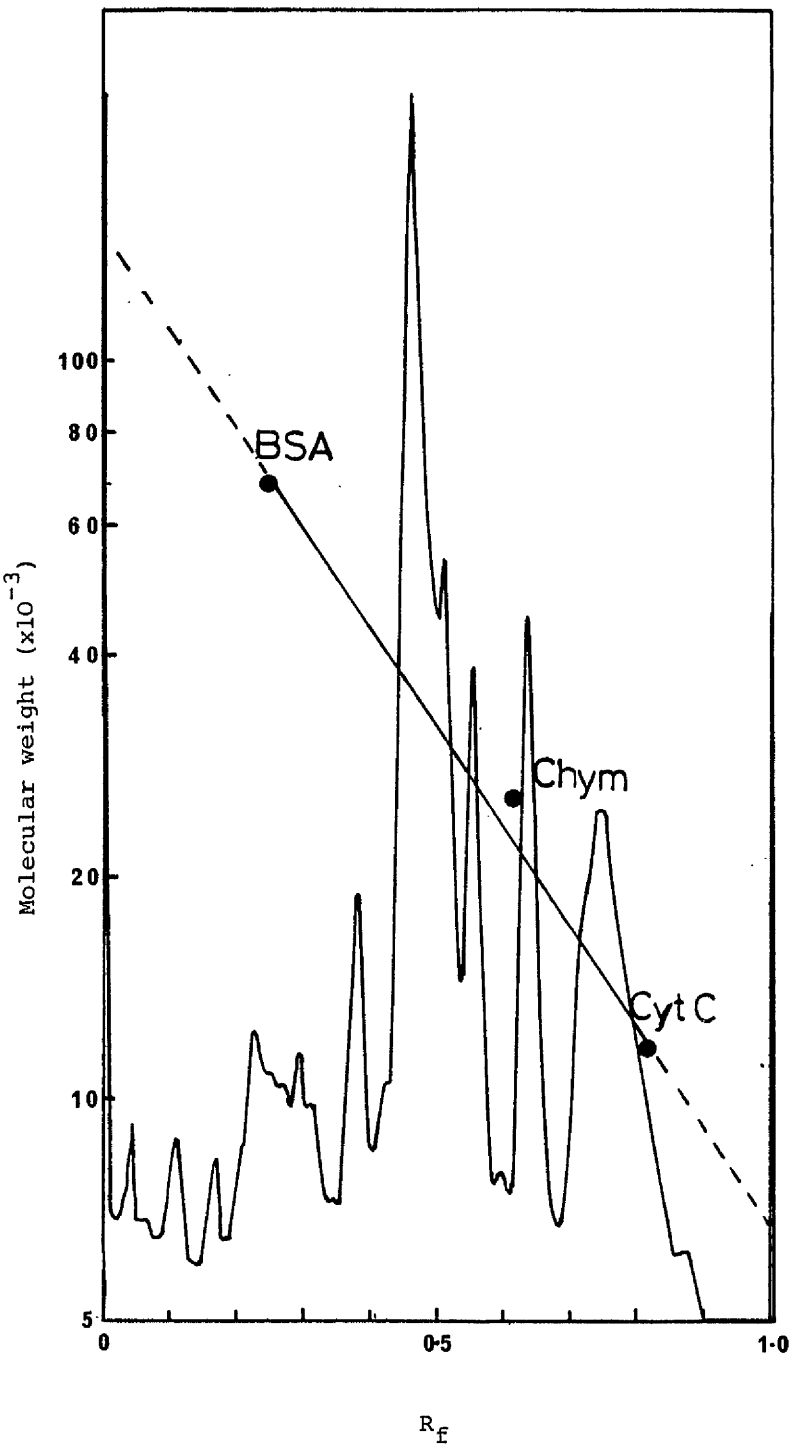
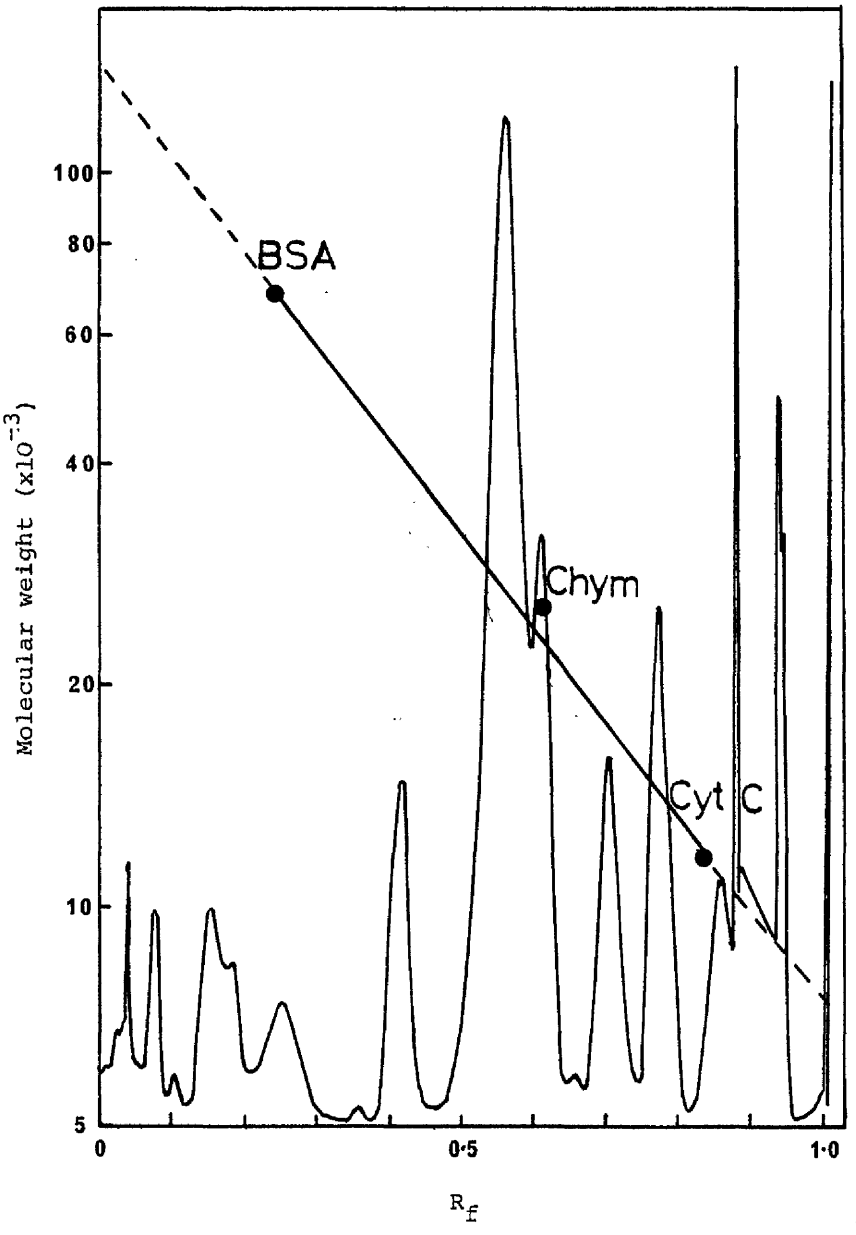

BSA
Chym
Cyt C
100
80
60
40
20
10
5
0
0·5
1·0
Molecular weight (x10^{-3})
R_f

Fig. 2.6 Scan of 60S Ribosomal Subunit Protein Gel

The figure shows a densiometric tracing at 585nm of a typical one-dimensional SDS gel after electrophoresis of 60S ribosomal subunit protein.

Molecular Weight Markers :-

BSA	(bovine serum albumin	- 69,000
Chym	(chymotrypsinogen)	- 25,700
cyt C	(cytochrome C)	- 11,700


BSA
Chym
Cyt C
100
80
60
40
20
10
5
0
0·5
1·0
Molecular weight (x10^{-3})
R_f

Autoradiography :

Most dried gels were sandwiched between 2-4 sheets of Kodak No-Screen X-ray film and exposed for 2-4 weeks. One-dimensional SDS slab gels were sandwiched between 2 sheets of Kodak X-Omat H X-ray film and placed in a cassette containing two Dupont Cronex autoradiography intensifying screens for 1-2 weeks at -70°C. Exposed autoradiographs were developed for 5 minutes in 20% v/v Kodak DX 80 developer, rinsed in H_2O, fixed for 5-10 minutes in 20% (v/v) Kodak FX 40 fixer, washed for 1 hour in H_2O, and finally air dried.

2.3.19 Two-Dimensional Basic Polyacrylamide Gel Electrophoresis

This was based on the method of Lastick and McConkey (1976).

First-Dimension :

The separation gel consisted of 4% (w/v) acrylamide, 0.13% (w/v) bis-acrylamide, 6M urea, 0.2M Tris, 0.26M Boric Acid pH 8.7, 10mM EDTA, 0.2% (v/v) N,N,N',N',Tetramethylethylene-diamine. Ammonium persulphate was added to 0.05% (w/v) and 6cm gels were poured in siliconized glass tubes, 14cm in length and 4mm in internal diameter. Gels were overlaid with H_2O (2.3.17) and allowed to polymerize prior to use. Ribosomal protein was dissolved in sample buffer consisting of 8M urea, 0.02M Tris, 0.026M Boric Acid pH 8.0, 1mM EDTA, 5% (v/v) 2-mercaptoethanol at a concentration of 10mg/ml. Protein samples, 50-100μg were heated for 3 minutes at 100°C before layering on top of the gels. Electrophoresis was carried out in buffer containing 0.06M Tris, 0.075M Boric Acid, 3mM EDTA pH 8.6. The current was maintained at 3mA/gel for about 3 hours at 20°C. The positive electrode was connected to the top

compartment of the gel apparatus, and a sample of cytochrome C run on a parallel gel was used to determine the exact time for electrophoresis, (Leader, 1980a). After electrophoresis, gels were removed from the tubes, and frozen in hexane at -70°C. Gels were equilibrated by thawing for 5 minutes in a soaking solution consisting of 6M urea, 0.35M acetic acid, 5% (v/v) 2-mercaptoethanol.

Second-Dimension

Gels of 7cm length, 7cm width, and 4mm thickness were cast between 8cm square glass plates separated by 4mm thick vertical glass spacers. The gels contained, 15% (w/v) acrylamide, 0.47% bis-acrylamide, 6M urea, 0.44M acetic acid, 0.025M KOH, 0.5% (v/v) N,N,N',N',Tetramethylethylenediamine pH 4.5. Polymerization was achieved by addition of ammonium persulphate to 0.3% (w/v). Equilibrated first-dimensional gels were laid on the top surface of the second-dimension gels. They were annealed together by the addition of a solution containing, 1% (w/v) agarose, 6M urea, 0.35M acetic acid, 5% 2-mercaptoethanol followed by cooling for 20 minutes at -20°C. Electrophoresis was carried out at 20°C for 16 hours at a current of 6mA/gel with the positive electrode connected to the top of the gel apparatus. Electrophoresis buffer consisted of 0.093M glycine, 0.03M acetic acid pH 4.05. Gels were stained and destained as described (2.3.17).

2.3.20 Two-Dimensional 'Sweep' Polyacrylamide Gel Electrophoresis (Leader and Coia, 1978a).

First-Dimension

Gels 6cm long and 4mm diameter containing 4% (w/v) acrylamide, 0.066% (w/v) bis-acrylamide, 6M urea, 0.045% (v/v) N,N,N',N',Tetramethylethylenediamine, 0.038mM bis-Tris adjusted to pH 5.5 with acetic acid were poured, polymerized, and layered with protein samples as described (2.3.19). The lower buffer chamber (cathodic) contained 0.01M bis-Tris pH 7, and the upper chamber buffer was 0.01M bis-Tris, 0.212M acetic acid pH 3.55. The principle of this electrophoresis system depends on a pH discontinuity between the upper electrode buffer, the separation gel, and the lower electrode buffer. Electrophoresis was carried out towards the cathode at a current of 3mA/gel at 20°C for 1 hour. At this time, the first refractile band (the bis-tris ion front) was 1-0.5cm from the bottom of the gel, whereas the second refractile band (the H^+ ion front containing acidic ribosomal proteins) had travelled approximately 1cm into the gel. Pyronine Y (0.01% (w/v)) was included in the sample to aid the location of the first front. After electrophoresis, the first-dimension gels were annealed directly to the second-dimension without equilibration. The second-dimension was as described (2.3.19).

2.3.21 Two-Dimensional Acidic Polyacrylamide Gel Electrophoresis

This was based on gel electrophoresis 'system IV' described by Madjar *et al.*, (1979b). The first dimension of this gel system was identical to the 'Sweep Gel' system except that the separation gel contained 8M urea, the upper buffer chamber was adjusted to pH 3.8 and the lower chamber buffer adjusted to pH 6. The second dimension was as described (2.3.19).

3. RESULTS

3.1 The Phosphorylated Ribosomal Proteins of Intact Ascites Cells

Before examining enzymes responsible for the phosphorylation of ribosomal proteins, some studies were carried out to identify which ribosomal proteins were phosphorylated *in vivo*.

3.1.1 Incubation of Ascites Cells in the absence of Glucose

It had been shown by Rankine, Leader and Coia (1977) that isolated ascites cells radioactively labelled with ^{32}P-orthophosphate in a medium lacking glucose, had a similar pattern of phosphorylation of ribosomal proteins to those labelled in mouse peritonea. It was more convenient, and relevant to the present study to label isolated cells.

Ascites cells were harvested (2.3.1), and resuspended in incubation medium which contained no glucose, (2.3.3). Next, ^{32}P-orthophosphate was added, and the cells were stirred for three hours. After the period of labelling, ribosomes were isolated from the cells (2.3.6), and dissociated into their constituent 40S and 60S subunits (2.3.7). The extracted protein was then analysed for incorporation of ^{32}P by one-dimensional SDS gel-electrophoresis. Autoradiography of 60S ribosomal protein showed that one major band of approximate molecular weight 14,000, and two minor bands of 40,000 and 32,000 were phosphorylated (Fig 3.1). The 40S protein was weakly labelled, with only one distinguishable band of molecular weight 32,000.

Fig 3.1 : One-Dimensional SDS Gel Electrophoresis of ^{32}P-Labelled Ribosomal Protein from Intact Cells.

100μg of 60S and 40S protein of approximate specific activities 5 x 10^4 cpm/mg and 2 x 10^4 cpm/mg, respectively was subjected to electrophoresis (2.3.17) and autoradiography (2.3.18).

a. Stain of 60S protein

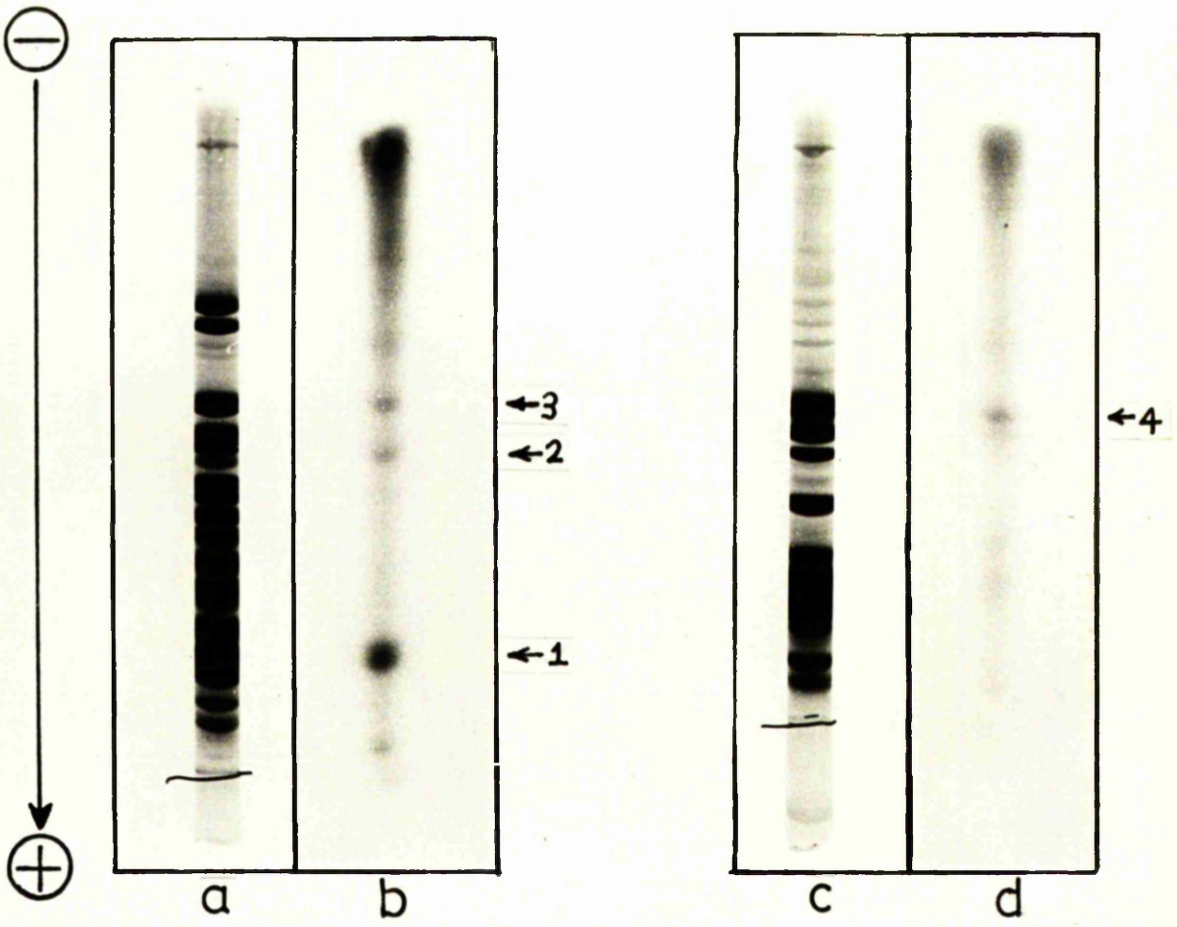
b. Autoradiograph of 60S protein

c. Stain of 40S protein

d. Autoradiograph of 40S protein.

Approximate molecular weights of ^{32}P-labelled protein bands.

Band No.	Mol. wt.
1	14,000
2	32,000
3	40,000
4	32,000


⊖
⊕
←3
←2
←1
←4
a
b
c
d

The identities of these radioactively-labelled proteins cannot be accurately determined from one-dimensional gel electrophoresis, since many of the protein bands consist of more than one ribosomal protein. A better identification of these proteins was obtained by two-dimensional gel electrophoresis. 'Sweep' gel electrophoresis (2.3.20) of 60S subunit protein clearly identified one of the phosphorylated proteins as that previously designated Lγ (Fig 3.2). The other 60S subunit proteins were not apparent from this gel. Similar analysis of 40S subunit protein indicated that protein S3 was phosphorylated. Two-dimensional 'Acidic-Acidic' gel electrophoresis (2.3.21) was also used (Fig 3.3). Again only Lγ and S3 were found to be labelled although protein Lγ appears less highly labelled in Fig 3.3 than Fig 3.2. The lack of phosphorylation of S6, and the phosphorylation of S3, were in contrast to the results obtained by Rankine, Leader and Coia (1977). Using a similar incubation medium, they found that S6 was radioactively labelled, whereas S3 was not labelled. One possible reason for the absence of phosphorylated S6 is that the amount of S6 phosphorylated in ascites cells was generally found to be much lower than in other cell types e.g. BHK cells (Leader and Coia, 1978d). Rankine, Leader and Coia (1977) did show the presence of a second phosphorylated protein in the 40S subunit, and although they suggested that it might be protein S2 the possibility remains that it could be S3. For the 60S subunit protein, they found that Lγ was the major phosphoprotein, and the results presented here are consistent with that finding.

Fig 3.2 : Two-Dimensional 'Sweep' Gel Electrophoresis of ^{32}P-Labelled Ribosomal Protein from Intact Cells.

100μg of 60S and 40S protein of approximate specific activities 5 x 10^4 cpm/mg and 2 x 10^4 cpm/mg respectively were subjected to electrophoresis (2.3.20) and autoradiography (2.3.18).

a. Stain of 60S protein

b. Autoradiograph of 60S protein

c. Stain of 40S protein

d. Autoradiograph of 40S protein

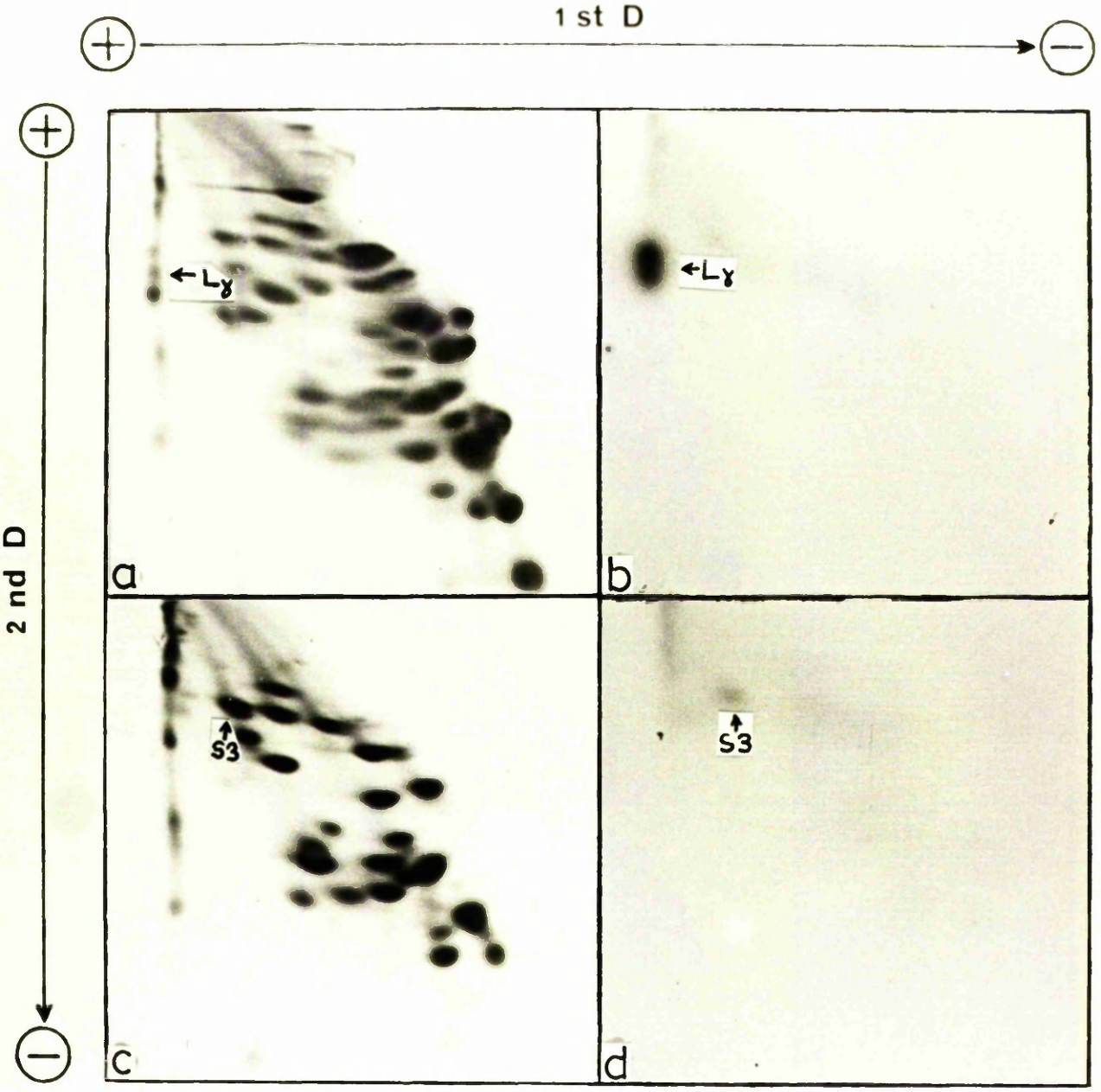

1 st D
2 nd D
+
−
+
−
←Lγ
←Lγ
S3
S3
a
b
c
d

Fig 3.3 : Two-Dimensional 'Acidic-Acidic' Gel Electrophoresis of ^{32}P-Labelled Ribosomal Protein from Intact Cells.

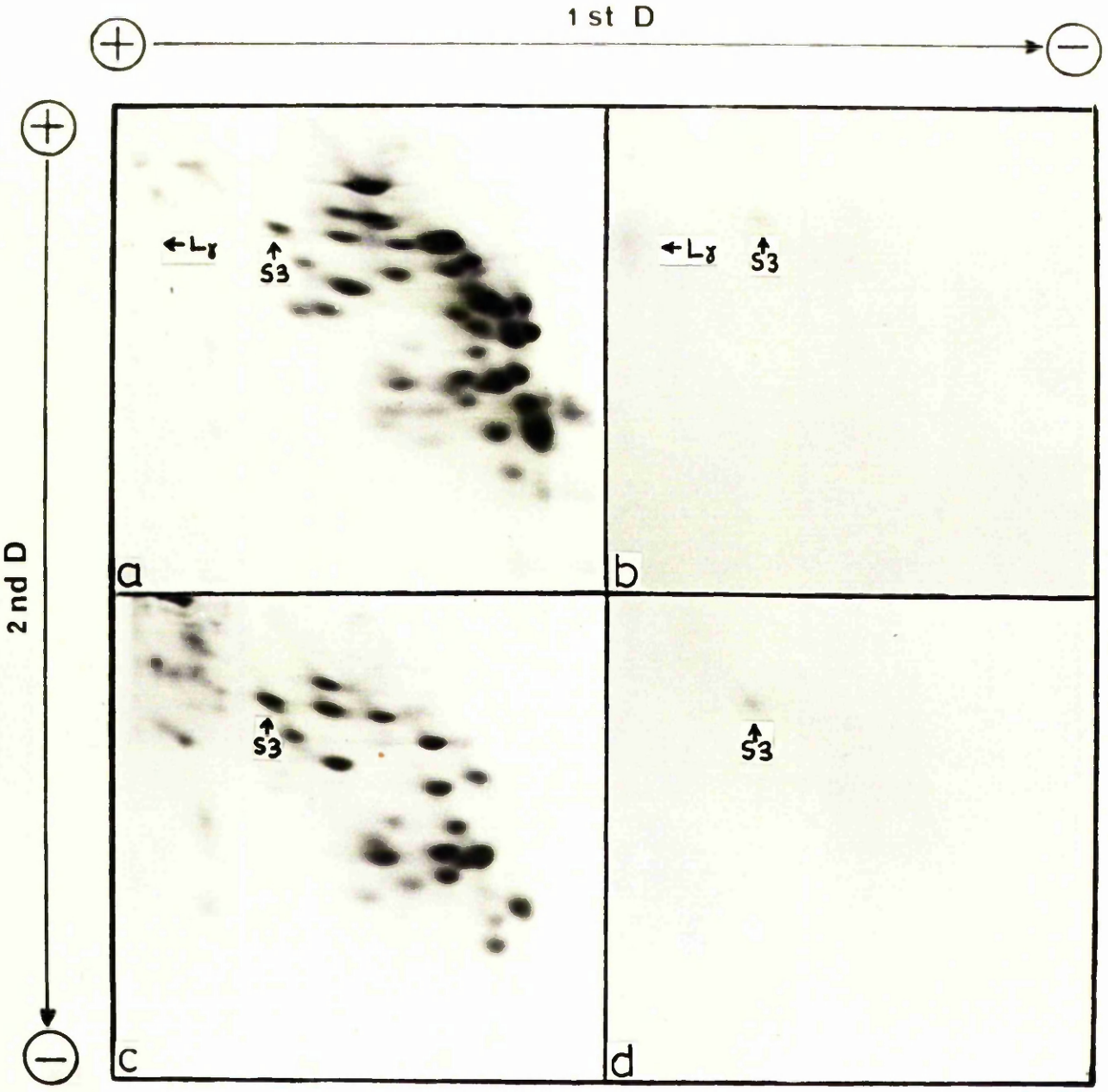
100μg of 60S and 40S protein of approximate specific activities 5 x 10^4 cpm/mg and 2 x 10^4 cpm/mg respectively were subjected to electrophoresis (2.3.21) and autoradiography (2.3.18).

a. Stain of 60S protein

b. Autoradiograph of 60S protein

c. Stain of 40S protein

d. Autoradiograph of 40S protein


1 st D
2 nd D
+
−
←Lγ
S3
a
b
c
d

3.1.2 Incubation of Ascites Cells in the presence of Glucose

It had been shown by Leader and Coia (1978b) that the pattern of phosphorylation of ascites cell ribosomes could be altered by incubating cells in a medium containing glucose. It was necessary first to see whether the altered phosphorylation pattern of ribosomes from cells incubated under these conditions could be confirmed.

Ascites cells were incubated in medium which contained 2.5mM glucose (2.3.3) and ^{32}P-orthophosphate was added to the cell suspension, as before. After three hours the pH of the cell incubation medium had fallen from 7.6 to 6.5 due to the glycolytic production of lactic acid from the glucose in the medium. Cells were then fractionated (3.1.1), and 40S and 60S ribosomal subunit protein isolated. Analysis of the ribosomal protein on two-dimensional 'Acidic-Acidic' gels (2.3.21) revealed that the pattern of phosphorylation (Fig 3.4) was different from that seen previously (3.1.1). In the 60S subunit L14 was now phosphorylated, whereas Lγ appeared to be unphosphorylated. In the small subunit, proteins S6, S3 and S2 were phosphorylated together with two other proteins Sa and Sb. These phosphorylated proteins correspond to those identified by Leader and Coia (1978b) except that the acidic proteins Sa and Sb (Collatz et al., 1977) were not observed in the gel system they used. Indeed, in the present study it was also found that using this two-dimensional gel electrophoresis system (Fig 3.5) only S2, S3 and S6 were seen as phosphoproteins in the 40S subunit, and no stained spots

Fig 3.4 : Two-Dimensional 'Acidic-Acidic' Gel Electrophoresis of ^{32}P-Labelled Ribosomal Subunit Protein from Cells Incubated in Medium Containing Glucose (2.3.3).

100μg of 60S and 40S protein of approximate specific activities 3.5×10^4 cpm/mg and 3×10^4 cpm/mg respectively were subjected to electrophoresis (2.3.21) and autoradiography (2.3.18).

a. Stain of 60S protein

b. Autoradiograph of 60S protein

c. Stain of 40S protein

d. Autoradiograph of 40S protein

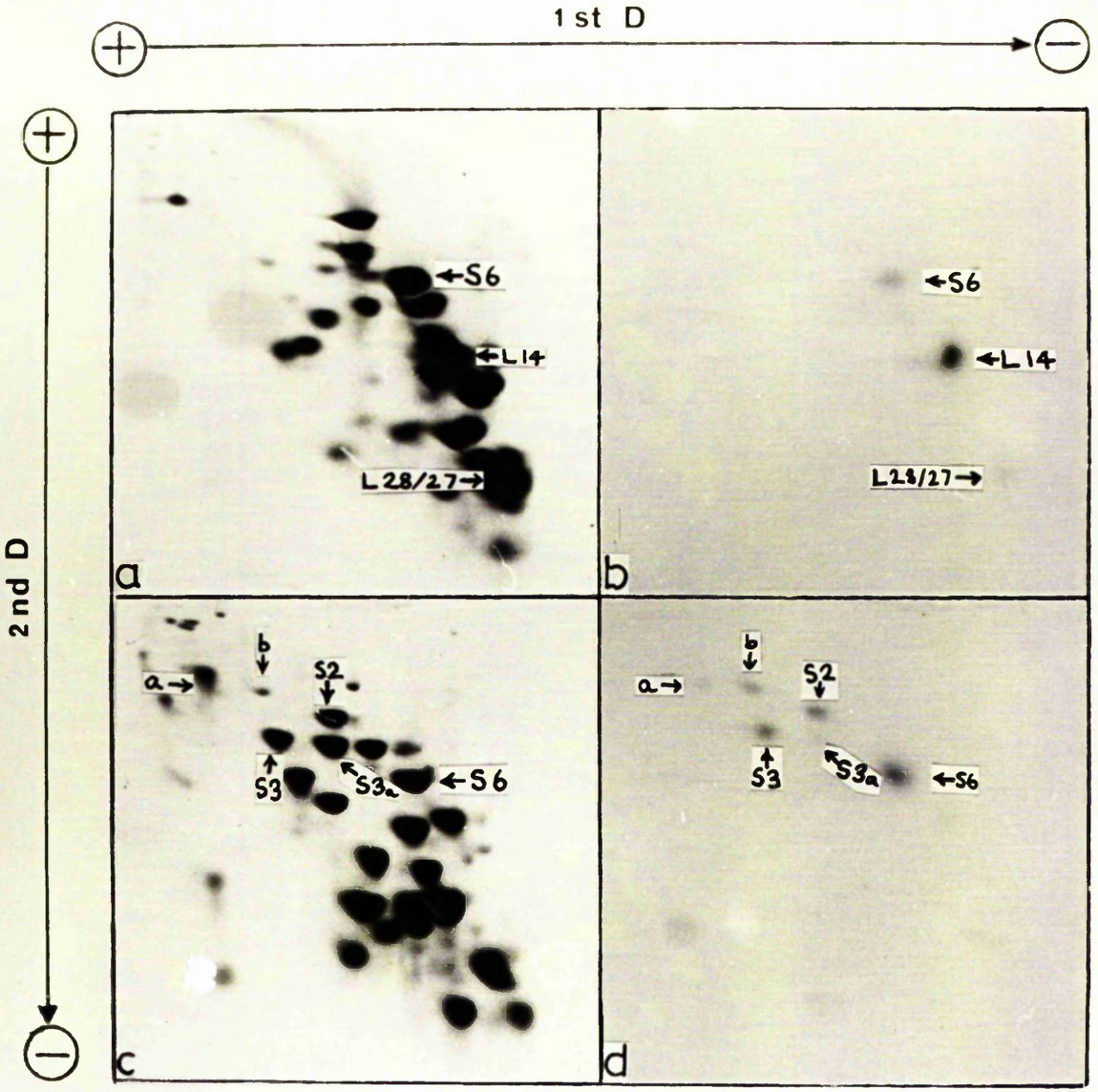

1 st D
2 nd D
+
−
←S6
←L14
L28/27→
a
b
c
d
a→
b
S2
S3
S3a
←S6

Fig 3.5 : Two-Dimensional 'Basic-Acidic' Gel Electrophoresis of ^{32}P-Labelled Ribosomal Subunit Protein from Cells Incubated in Medium Containing Glucose (2.3.3).

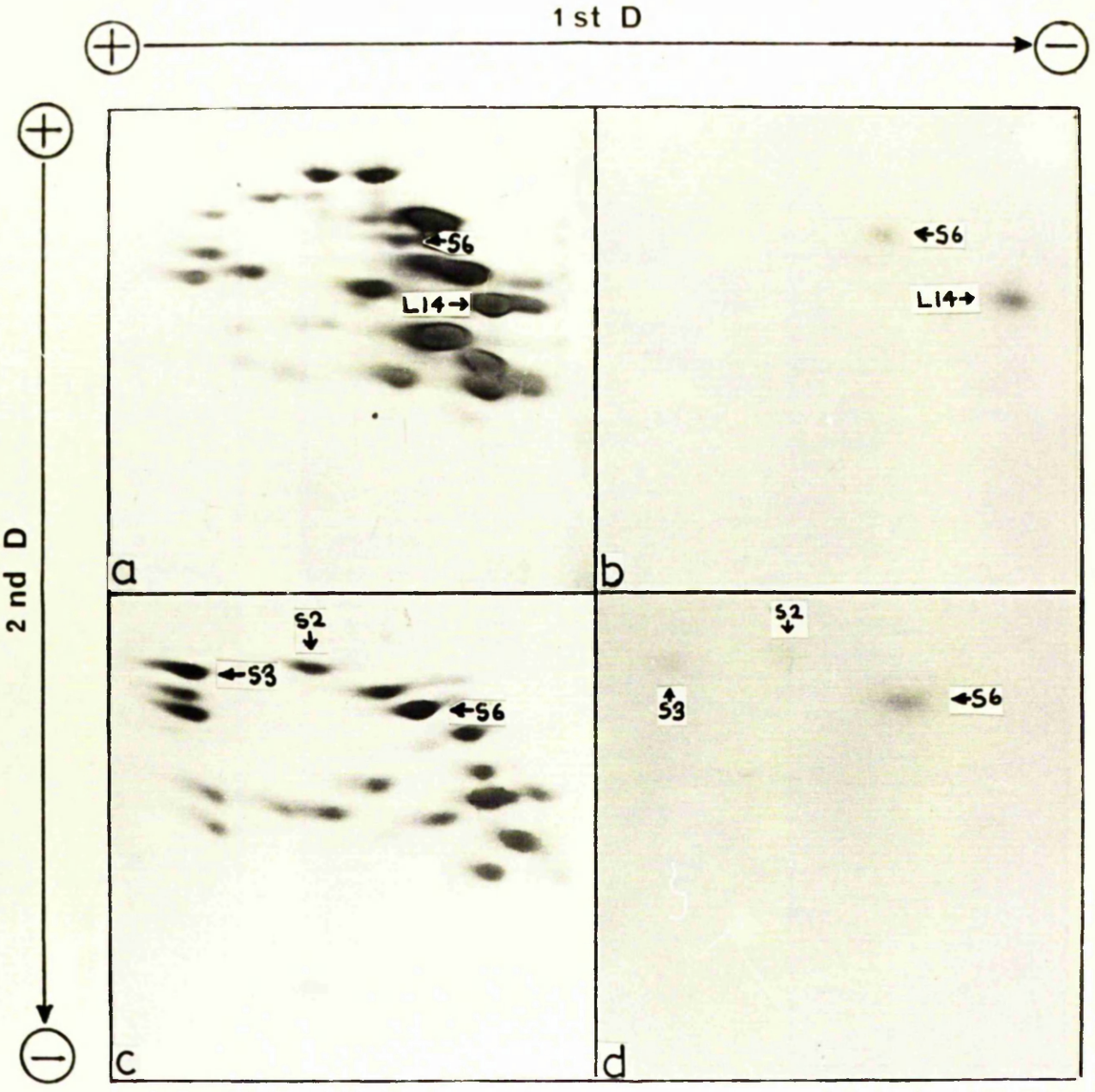
100μg of 40S and 60S protein of approximate specific activities 3.4 x 10^4 cpm/mg and 3 x 10^4 cpm/mg respectively were subjected to electrophoresis (2.3.19) and autoradiography (2.3.18).

a. Stain of 60S protein

b. Autoradiograph of 60S protein

c. Stain of 40S protein

d. Autoradiograph of 40S protein


1 st D
2 nd D
S6
L14
a
S6
L14
b
S2
S3
S6
c
S2
S3
S6
d

corresponding to Sa and Sb were observed.

In both Fig 3.4b and Fig 3.5b, the 60S subunit protein autoradiographs showed that phosphorylated S6 was present as a contaminant. This can also be seen from the stained pattern, (Fig 3.5a) where minor stained spots corresponding to 40S subunit protein can be seen. One other faintly-labelled spot corresponding to protein L27 or L28 is seen in Fig 3.4.

The resolution of S3 from S3a by two-dimensional 'Acidic-Acidic' gel electrophoresis (Fig 3.4) shows that S3 is phosphorylated and S3a is unphosphorylated. This resolution is not obtained using the 'Basic-Acidic' two-dimensional gel system (Fig 3.5).

3.2 Endogenous Phosphorylation of Ascites Ribosomes

Initial attempts to identify the protein kinases responsible for phosphorylating ribosomal proteins concentrated on the possibility that these would be associated with isolated undissociated ribosomes as had been found in other cell types (e.g. rabbit reticulocytes : Kabat, 1971; Martini and Gould, 1973; and bovine adrenal cortex: Walton and Gill, 1973). The reason for not initially looking in the cytosol fraction was that this fraction in other cells contains kinases that phosphorylate *in vitro* ribosomal proteins that are not phosphorylated *in vivo*. By looking at the ribosomes it was hoped to avoid this problem and to simplify purification of the enzymes.

Ribosomes were isolated from ascites cells (2.3.6), resuspended in buffer (2.3.15.1), and [γ-^{32}P] ATP was added to allow radioactive labelling of ribosomal proteins (2.3.15.2). Reactions were terminated by cooling to 0^{o}C and adding a 10-fold excess of unlabelled ascites ribosomes. The ribosomes were then dissociated into their subunits, (2.3.7) and protein was extracted (2.3.8). The preparation of subunits removes non-ribosomal phosphoproteins associated with the ribosomes (Issinger and Reichert, 1979). Table 3.1 shows that no incorporation of ^{32}P into ribosomal proteins could be detected under these conditions. It was possible that the conditions under which the ribosomes were prepared led to the loss of associated protein kinases. The procedure for the isolation of the ribosomes was modified by omitting the second 150,000g centrifugation step of the ribosome preparation used to

increase the purity of the preparation. Another modification employed to try to prevent removal of associated proteins from ribosomes was to lower the concentration of salt in the ribosome preparation buffer from 146mM to 20mM NaCl. However, neither of these modifications resulted in any incorporation of ^{32}P into ribosomal protein (Table 3.1).

Other possible reasons for the lack of phosphorylation of 80S ribosomal proteins were then tested. Cell-free phosphorylation of 80S ribosomes was carried out immediately after fractionation of the ribosomes from cells, since freezing and thawing of stored ribosomes could denature labile enzymes (Quirin-Stricker et al., 1976). The specific activity of the [γ-^{32}P] ATP was increased from 8 cpm/pmole to 80 cpm/pmole. The phosphorylation reaction was performed in the presence of 10mM sodium fluoride, a known inhibitor of phosphoprotein phosphatases. However, none of these modifications resulted in any significant incorporation of ^{32}P into ribosomal protein (Table 3.1). The possibility was examined that the lack of labelling of ribosomal protein was because the [γ-^{32}P] ATP was degraded by high concentrations of ATPase. A sample was removed from the reaction mixture at the end of the reaction time, and was subjected to chromatography on PEI-cellulose (2.3.16). Most of the radioactive material migrated to the position of ATP, with little ^{32}P-orthophosphate being present (Fig 3.6) indicating little ATPase activity.

Table 3.1 : Endogenous Phosphorylation of Ribosomes

Conditions	Intention	^{32}P incorporated into ribosomal protein (cpm)	
		40S	60S
80S ribosomes	-	14	10
'Dirty' 80S ribosomes (no second 150,000g centrifugation step)	Prevent loss of kinase	15	14
Low salt prepared 80S ribosomes	" "	12	11
Unfrozen 80S	Prevent kinase inactivation	11	14
Increased specific activity of γ-^{32}P to 80 cpm/pmole	Increase incorporation of ^{32}P	12	15
10mM NaF + 80S	Inhibit Phosphatase	13	14
Control: No addition to scintillation fluid	-	12	

Incorporation of ^{32}P into ribosomal protein was measured after incubation of 80S ribosomes with γ-^{32}P ATP (2.3.15.2). A 10μl aliquot of extracted 40S or 60S protein at 1mg/ml concentration was examined for incorporation of ^{32}P by scintillation counting.

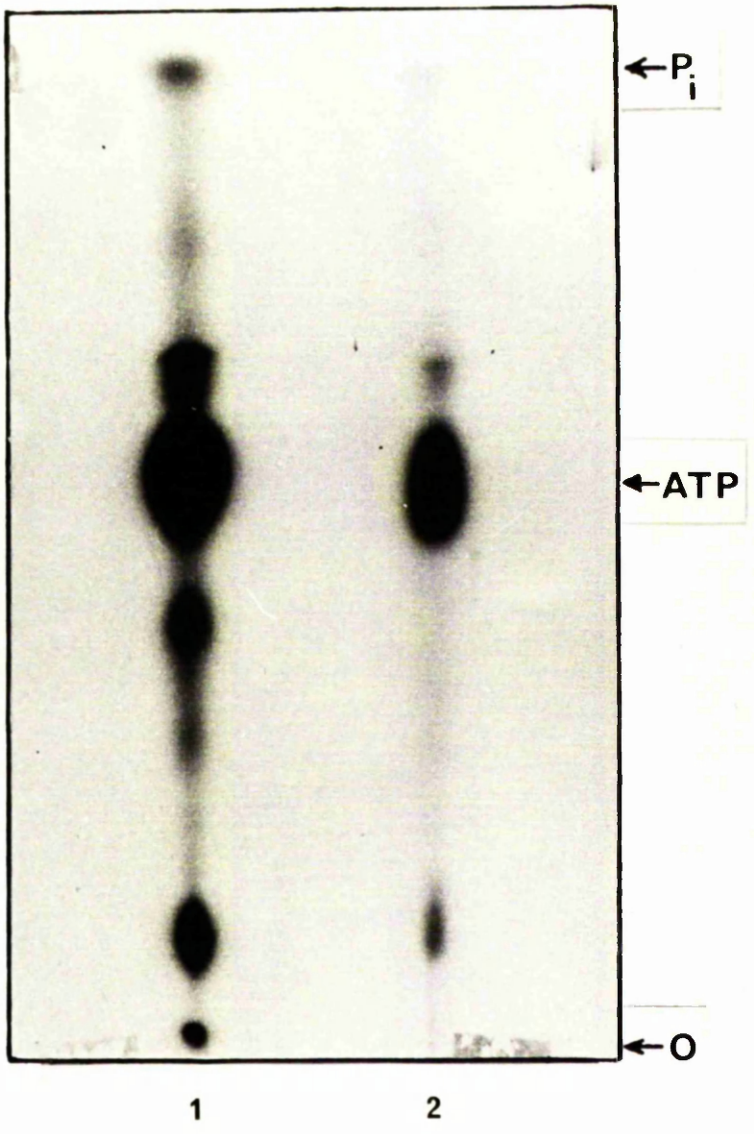
Fig 3.6 : PEI-Cellulose Chromatography of ATP After Incubation with Ascites 80S Ribosomes.

A 10µl sample of reaction mixture (2.3.15.2) was chromatographed on PEI-cellulose as described in (2.3.16). The position of ATP was determined by using [γ-^{32}P] ATP and unlabelled ATP.

Track 1 Standard [γ-^{32}P] ATP (10µl of specific activity 0.1mCi/ml)

Track 2 Sample (10µl) from endogenous phosphorylation reaction.

The origin (O), and positions of ATP and orthophosphate (P_i) are shown.


←P_i
←ATP
←O
1
2

3.3 Phosphorylation of Ascites Ribosomes by Exogenous Cytoplasmic Protein Kinases

Because of the inability to obtain endogenous phosphorylation of ascites ribosomal proteins, attention was focused on the cytoplasmic protein kinases of ascites cells, despite the possible disadvantages of this approach mentioned in section 3.2. The 'S-150' supernatant fraction remaining after the first 150,000g sedimentation of the ribosomes (2.3.6) was examined for protein kinase activity. This is a cytosol containing most of the soluble molecules and enzymes of the cytoplasm. Ascites 'S-150' fraction was used to phosphorylate 40S or 60S ribosomal subunits in the presence of [γ-^{32}P] ATP. Reactions were terminated by cooling and addition of 10-fold excess of non-radioactive 40S or 60S subunits. The phosphorylated subunits were then dialysed extensively to remove [γ-^{32}P] ATP prior to measuring incorporation of ^{32}P. Phosphorylated ribosomal protein was analysed by one-dimensional gel electrophoresis (2.3.17). Fig 3.7 shows that two phosphoprotein bands of approximate molecular weights 14,000 and 40,000 were present in the 60S subunit protein. Only one 40S subunit protein band could be identified as being phosphorylated, having a molecular weight of approximately 32,000. The 40,000 molecular weight band from the 60S subunit was similar to that phosphorylated in intact cells Fig 3.1.

To try to obtain a more precise identification of the phosphorylated proteins, two-dimensional gel electrophoresis was performed. Because urea, unlike sodium dodecyl sulphate,

Fig 3.7 : One-Dimensional Sodium Dodecyl Sulphate Gel Electrophoresis of Ribosomal Protein Phosphorylated by 'S-150' Cytosol Fraction.

Ribosomal subunits were phosphorylated (2.3.15.2), in a 500µl reaction mixture which contained approximately 150µg of 'S-150' cytosol and 20 A_{260} units of 40S or 60S ribosomal subunits. Approximately 5 A_{260} units of these subunits were subjected to electrophoresis after addition of a 10-fold excess of unlabelled subunits.

a. Stain of 60S protein

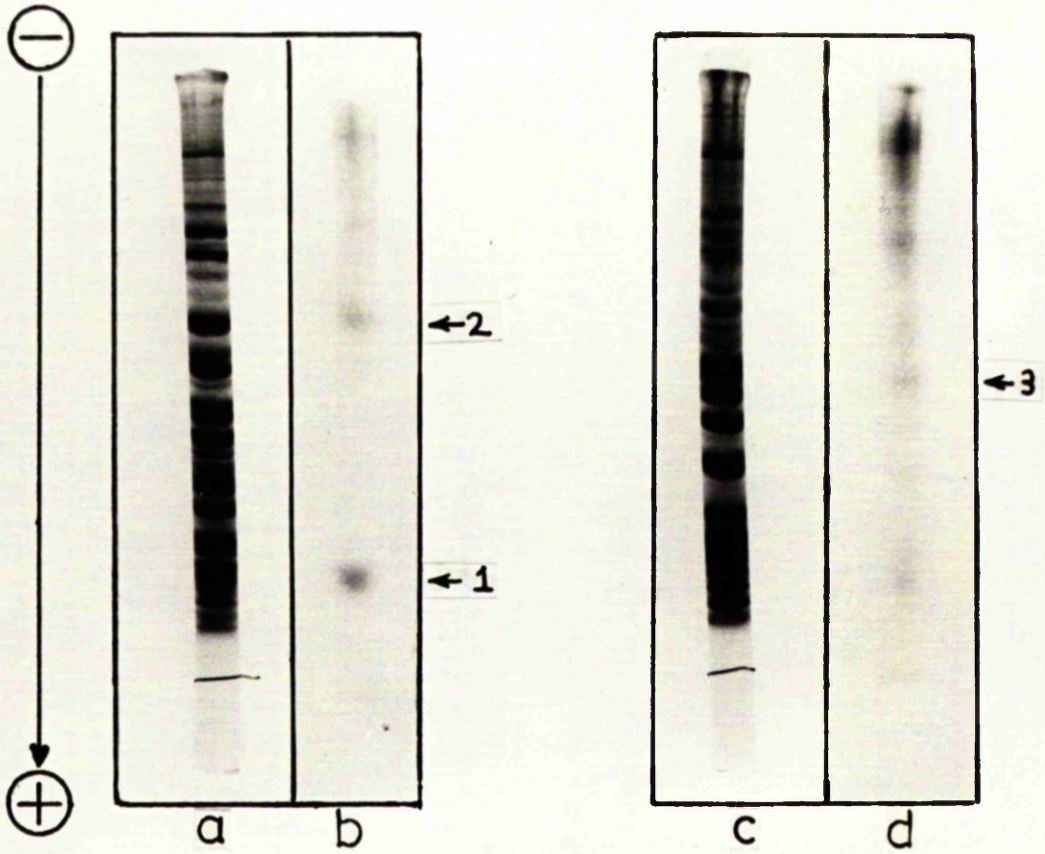
b. Autoradiograph of 60S protein

c. Stain of 40S protein

d. Autoradiograph of 40S protein

Approximate molecular weights of ^{32}P-labelled protein bands.

Band No.	Mol. wt.
1	14,000
2	40,000
3	32,000


⊖
⊕
←2
←1
←3
a
b
c
d

does not completely disrupt the RNA-protein interactions, it is necessary to extract the protein from subunits before two-dimensional gel electrophoresis (2.3.19). Extracted 60S and 40S protein were electrophoresed on two-dimensional 'Sweep' gels (2.3.20). Fig 3.8 shows that a weakly-labelled protein spot is present on the 60S gel, and its position is the same as that expected for Lγ. There is little indication of phosphorylated ribosomal proteins on the 40S gel. The 40,000 molecular weight phosphoprotein observed on one-dimensional SDS gels of 60S protein was not identified on 'Sweep' gels. From its position on one-dimensional gels this protein could be either L5 or L6, however, the possibility that it is a non-ribosomal contaminant cannot be excluded.

The difficulty in identifying the ribosomal proteins phosphorylated using the 'S-150' fraction was the result of low incorporation of radioactivity. This could well have been due to the presence of various inhibitors (both specific and non-specific) in this relatively crude fraction. As the main interest in this work was the protein kinases that phosphorylated the proteins of the 60S subunit under various conditions, and as the S-150 seemed to contain a protein kinase activity capable of phosphorylating protein Lγ, it was decided to proceed with the further fractionation of the S-150 fraction.

Fig 3.8 : Two-Dimensional 'Sweep' Gel Electrophoresis of Ribosomal Protein Phosphorylated by 'S-150' Cytosol Fraction.

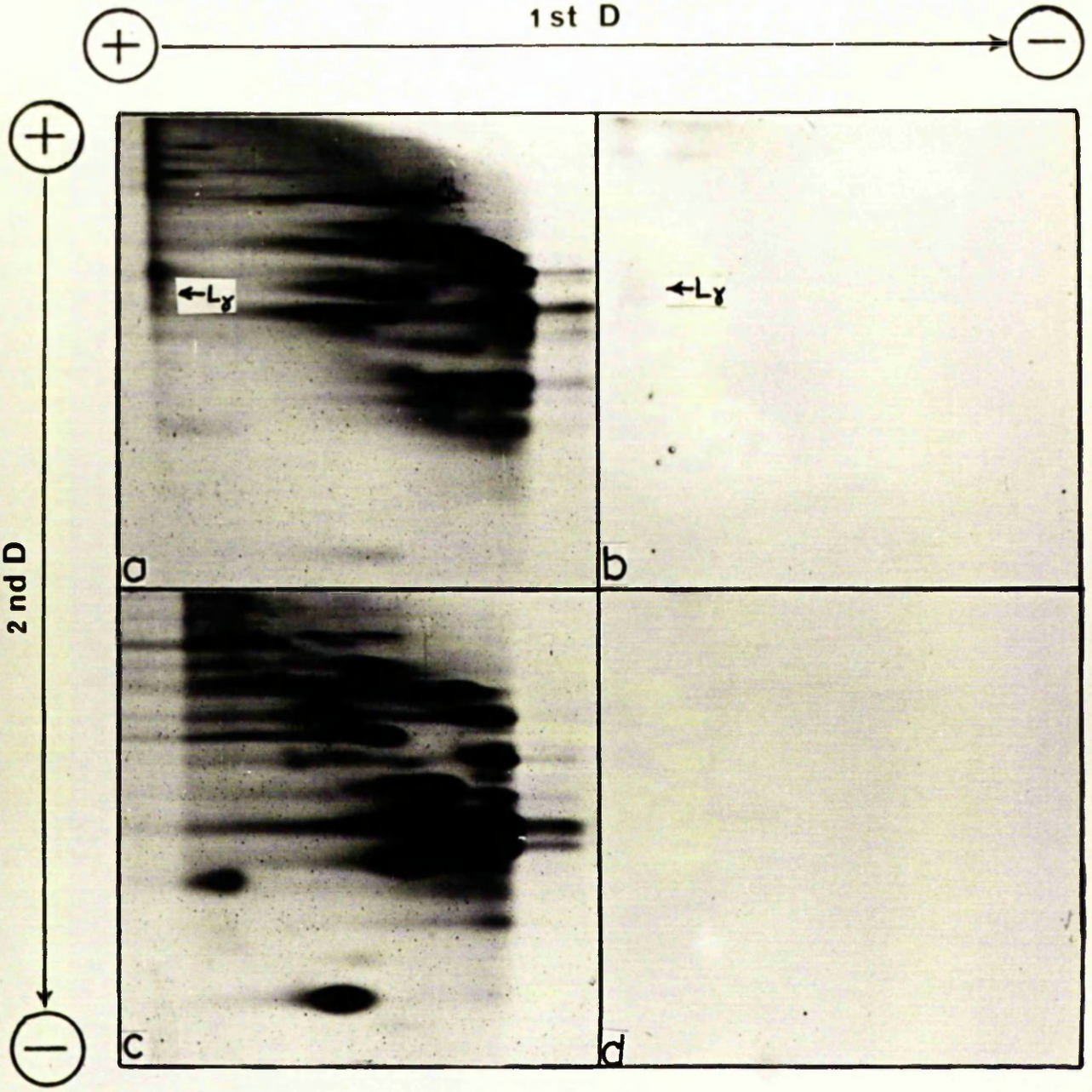
Ribosomal subunits were phosphorylated, as described in Fig 3.7, and samples of approximately 200μg of 40S and 60S extracted ribosomal protein were resolved by two-dimensional 'Sweep' gel electrophoresis (2.3.20), and subjected to autoradiography. The specific activity of the protein was approximately 1.9×10^4 cpm/mg for 60S, and 0.5×10^4 cpm/mg for 40S.

a. Stain of 60S protein

b. Autoradiograph of 60S protein

c. Stain of 40S protein

d. Autoradiograph of 40S protein


1 st D
2 nd D
+
−
←Lγ
←Lγ
a
b
c
d

3.4 Purification of 'S-150' Protein Kinases

It was more convenient in the initial stages of this work to use the commercially available substrates, casein and histone, rather than ribosomal subunits. Thus only 3mg of casein or 7.25mg of histone are required to incorporate 1μmole of phosphate (assuming 8 phosphorylation sites for each molecule of casein and a molecular weight of 24,000; and 2 phosphorylation sites for each molecule of histone and a molecular weight of 14,500), whereas 1,500mg of 40S or 3,000mg of 60S subunits would be required (assuming 1 phosphorylation site per subunit). Casein appears to be a good model substrate for protein kinases that phosphorylate acidic proteins (including ribosomal proteins; Kudlicki, Grankowski and Gasior, 1978) and histone for protein kinases that phosphorylate basic proteins (Traugh, Mumby and Traut, 1973). However, in adopting this approach, the possibility of specific ribosomal protein kinases that do not phosphorylate these substrates had to be considered.

3.4.1 DEAE-Cellulose Chromatography of 'S-150' Fraction

The 'S-150' fraction was dialysed against DEAE-cellulose chromatography buffer until equilibrium was reached. Chromatography was then carried out as described (2.3.12). The pattern of a sharp peak of unadsorbed protein, followed by a peak eluted by the KCl gradient (Fig 3.9) was found reproducibly in all DEAE-cellulose chromatography of ascites 'S-150' fraction. In some cases (e.g. Fig 3.9) the broad peak of protein eluted by the KCl gradient appeared to resolve into a number of 'sub-peaks', but this was less evident in

Fig 3.9 : DEAE-Cellulose Chromatography of Ascites 'S-150' Protein Kinases.

Approximately 375mg of 'S-150' protein was subjected to chromatography on DEAE-cellulose (2.3.10) and assayed for protein kinase activity with,

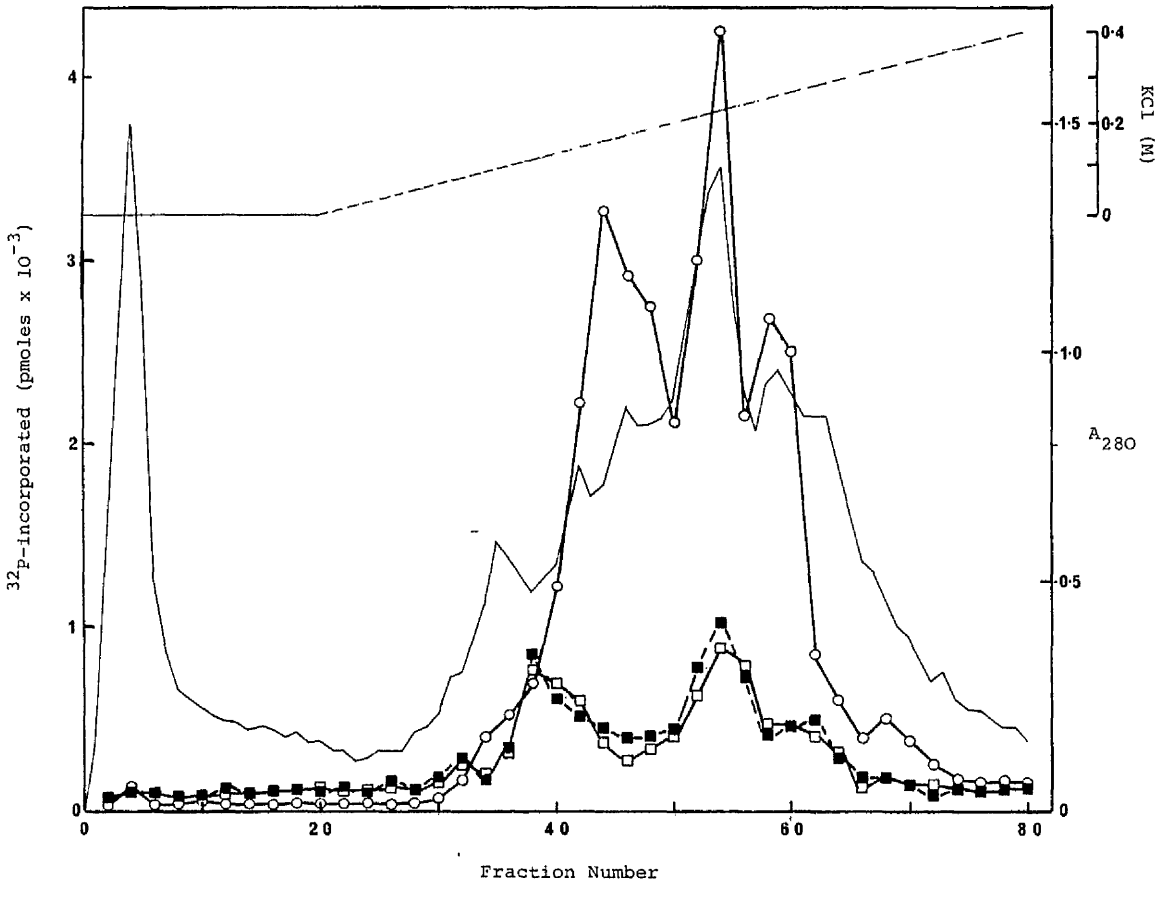
casein and ATP ○——○

histone and ATP □——□

histone and ATP plus 10μM cAMP ■- - - -■

Protein concentration was monitored by absorbance at 280nm ————

Concentration of KCl - - - - - - (estimated)


^{32}P-incorporated (pmoles x 10^{-3})
0
1
2
3
4
0
20
40
60
80
Fraction Number
0·4
0·2
0
KCl (M)
1·5
1·0
0·5
0
A_{280}

most other experiments (e.g. Fig 3.12).

Protein kinase assays were then carried out using casein or histone as substrate (2.3.15.1). Casein kinase activity was observed in the fractions eluted from the column by the KCl gradient (Fig 3.9). Histone kinase activity was also found in similar regions of the gradient, but with a lower activity than that of casein kinase. In the experiment shown in Fig 3.9, the kinases appeared to resolve into multiple peaks of activity. However this resolution into distinct peaks was not observed reproducibly, although 'shoulders' were sometimes observed (Fig 3.12). The presence of 10μM cyclic AMP did not appear to stimulate the histone kinase activity (Fig 3.9).

3.4.2 Phosphocellulose Chromatography of DEAE-Cellulose Fractions Containing Protein Kinase Activity

The fractions from DEAE-cellulose columns which had protein kinase activity were pooled and re-chromatographed on phosphocellulose as described (2.3.12). Initially the different kinase peaks resolved on DEAE-cellulose (Fig 3.9) were applied separately to different phosphocellulose columns. The DEAE-cellulose fractions used were in tubes 40-49 and 50-62 of Fig 3.9. Fractions eluted from phosphocellulose by NaCl were assayed for kinase activity using histone as a substrate with [γ-^{32}P] ATP as phosphoryl donor, and with casein as a substrate and either [γ-^{32}P] ATP or [γ-^{32}P] GTP as phosphoryl donor. The results of re-chromatography on phosphocellulose of the first peak of protein kinase eluted from

DEAE-cellulose is shown in Fig 3.10. Histone kinase activity was eluted in the 'flow-through' fractions of the column, together with some casein kinase activity. When a linear salt gradient of 0.25-1M NaCl was applied, casein kinase using ATP as phosphoryl donor was eluted at approximately 0.3-0.4M NaCl. This activity appeared to be in two poorly-resolved peaks. Casein kinase using GTP as phosphoryl donor was only found in the second peak (Fig 3.10).

The results of re-chromatography on phosphocellulose of the 'second peak' of kinase activity eluted from DEAE-cellulose is shown in Fig 3.11. As for the first peak from DEAE-cellulose, histone kinase activity appeared in the 'flow-through' fractions, and casein kinase was eluted by the NaCl gradient. This time however, the two peaks of casein kinase activity were much more clearly resolved. The first peak of casein kinase activity (designated CKI) was eluted from the column at 0.4 - 0.475M NaCl, and could use ATP but not GTP as phosphoryl donor. The second casein kinase activity (designated CKII) was eluted at 0.575 - 0.725M NaCl, and could use either ATP or GTP as phosphoryl donor. Comparison of the re-chromatography on phosphocellulose of the two 'peaks' of kinase activity eluted from DEAE-cellulose (Figs 3.10 and 3.11) indicates that they are essentially similar, differing only in the relative proportions of the different kinase activities. Thus the resolution of kinases on DEAE-cellulose was not complete, and it was decided to pool all the active fractions from DEAE-cellulose for rechromatography on phosphocellulose. Fig 3.12 shows a DEAE-cellulose fraction from which fractions 26 - 48 were pooled, concentrated (2.3.11),

Fig 3.10: Phosphocellulose Chromatography of the First Peak of Protein Kinase Activity from DEAE-Cellulose.

Pooled fractions (40 - 49 Fig 3.9) containing approximately 68mg of protein were concentrated (2.3.11), equilibrated and subjected to chromatography on phosphocellulose (2.3.12). Protein kinase assays were carried out (2.3.15.1) with,

casein and ATP ○——○

casein and GTP ●- - - - -●

histone and ATP □——□

Protein concentration was monitored by absorbance at 280nm ————

Concentration of NaCl - - - - - - - - (estimated)

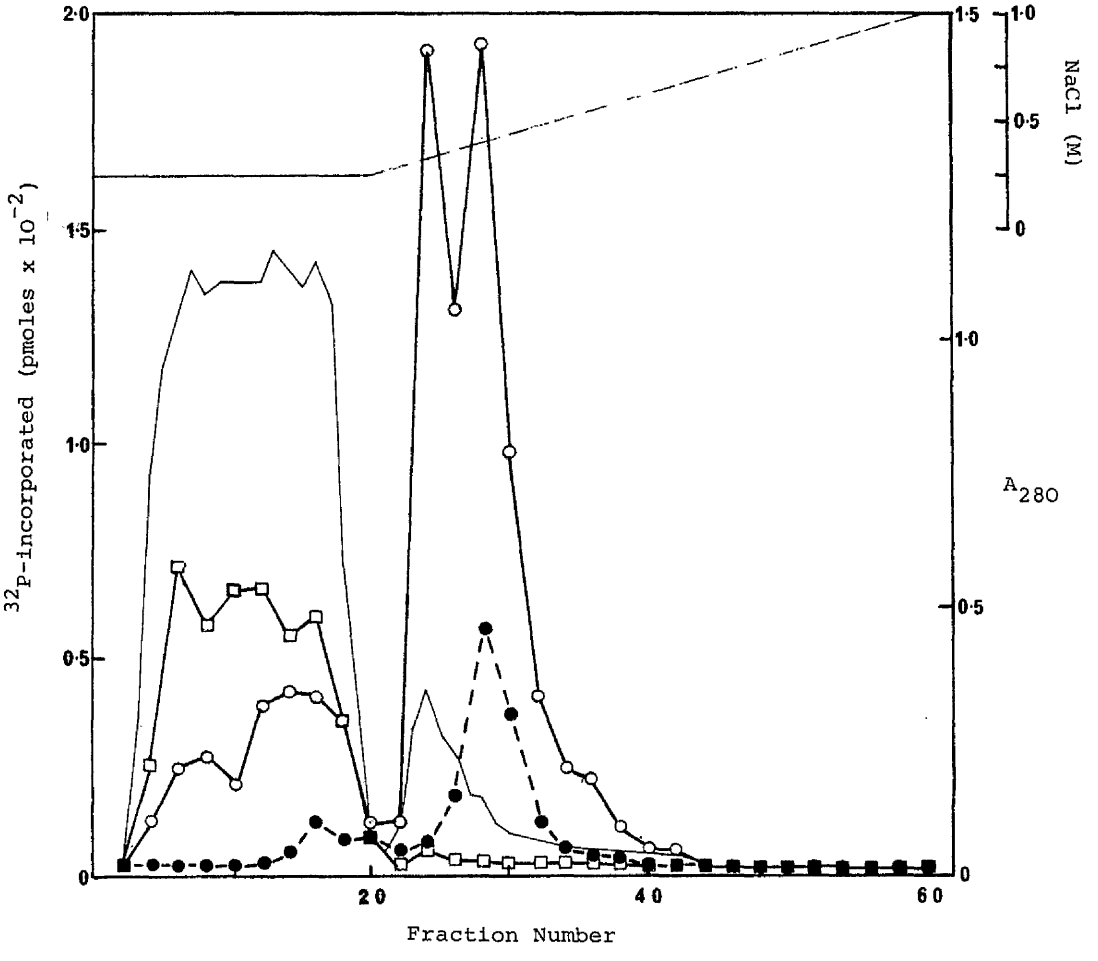

2·0
1·5
1·0
0·5
0
^{32}P-incorporated (pmoles x 10^{-2})
1·5
1·0
0·5
0
A_{280}
1·0
0·5
0
NaCl (M)
20
40
60
Fraction Number

Fig 3.11 : Phosphocellulose Chromatography of the Second Peak of Protein Kinase Activity from DEAE-Cellulose.

Pooled fractions (50 - 62 Fig 3.9) containing approximately 42mg of protein were concentrated (2.3.11), equilibrated and subjected to chromatography on phosphocellulose (2.3.12). Protein kinase assays were carried out (2.3.15.1) with,

casein and ATP ○——○

casein and GTP ●- - - - -●

histone and ATP □——□

Protein concentration was monitored by absorbance at 280nm ——·——

Concentration of NaCl ·—·—·—·— (estimated)

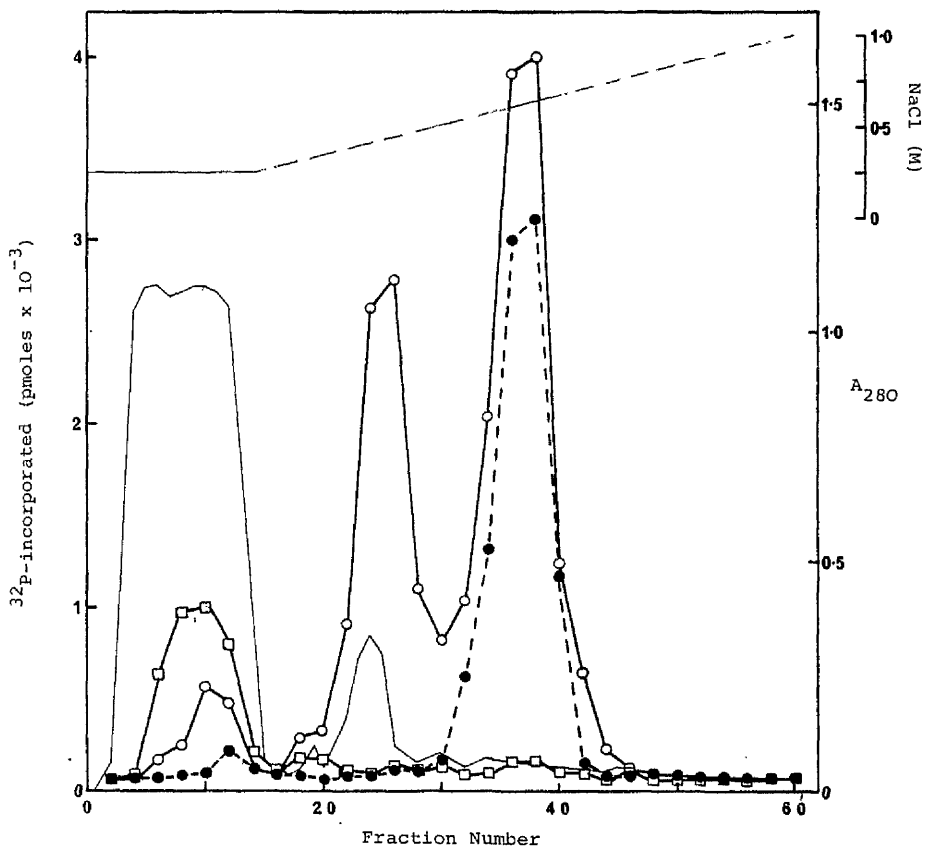
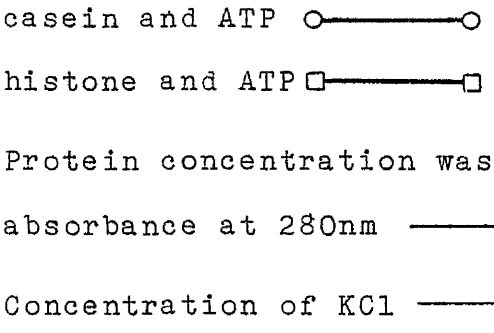
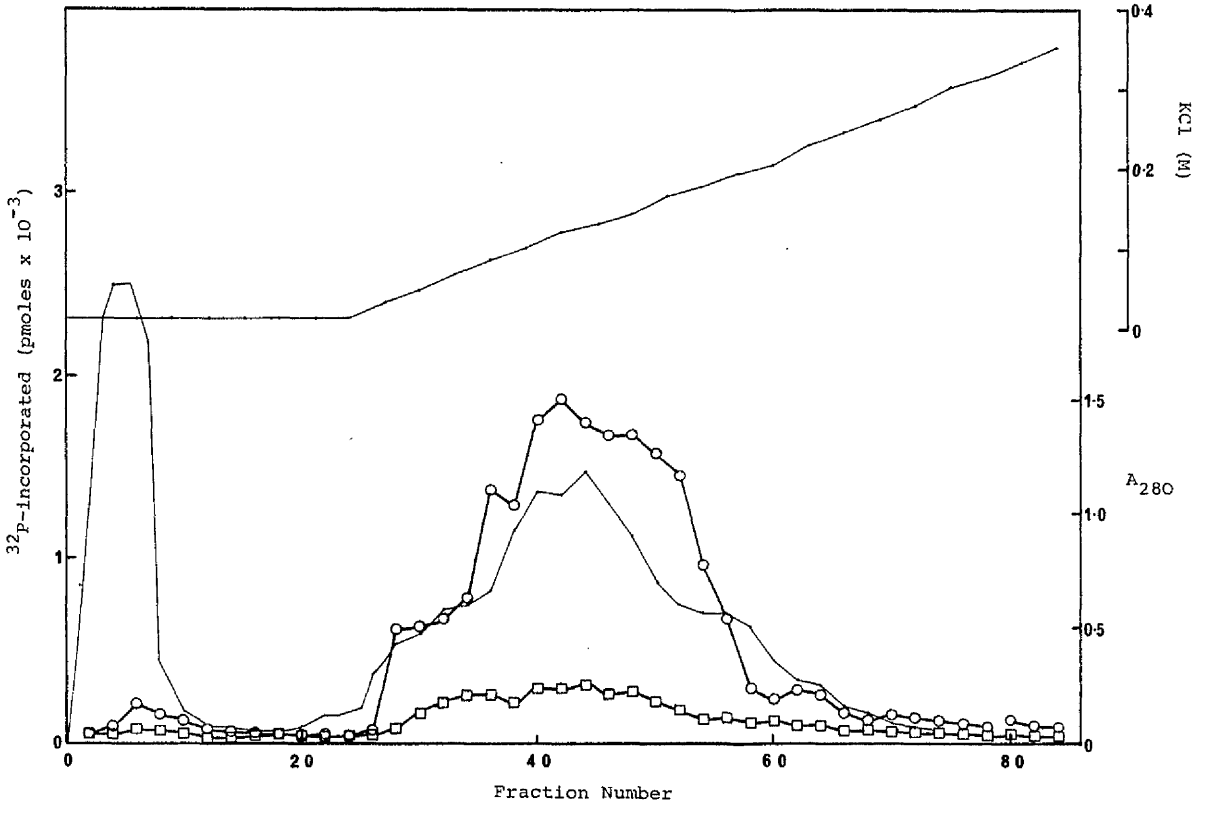

^{32}P-incorporated (pmoles x 10^{-3})
4
3
2
1
0
0
20
40
60
Fraction Number
1·0
1·5
0·5
0
NaCl (M)
1·0
A_{280}
0·5
0

Fig 3.12 : DEAE-Cellulose Chromatography of Ascites 'S-150' Protein Kinases.

Approximately 205mg of 'S-150' protein was subjected to chromatography on DEAE-cellulose (2.3.10) and assayed for protein kinase activity with,




^{32}P-incorporated (pmoles x 10^{-3})
3
2
1
0
0
20
40
60
80
Fraction Number
0·4
0·2
0
KCl (M)
1·5
1·0
0·5
0
A_{280}

equilibrated, and rechromatographed on phosphocellulose (Fig 3.13). As before (Figs 3.10 and 3.11) two distinguishable casein kinase activities were observed eluting at 0.4 - 0.57M NaCl and 0.6 - 0.78M NaCl, together with histone kinase eluting in the 'flow-through' fractions.

Table 3.2 shows that the combined protein kinase activity from phosphocellulose was purified approximately 17 fold compared with the original casein kinase in the 'S-150' fraction. If it is assumed that the relative amounts of CKI and CKII were unchanged throughout, then CKI was less purified than CKII, 6.3 compared to 27 times.

In view of the good resolution of the two kinase activities, the first priority was the characterisation of the ribosomal substrates for these kinases rather than their further purification.

Fig 3.13 : Phosphocellulose Chromatography of Protein Kinase Activity eluted from DEAE-Cellulose.

Fig 3.13

Pooled fractions from DEAE-cellulose chromatography (26 - 48, Fig 3.12) containing approximately 87.6mg of protein were concentrated (2.3.11), equilibrated and subjected to chromatography on phosphocellulose (2.3.12). Protein kinase assays were carried out (2.3.15.1) with,

casein and ATP ○——○

casein and GTP ●- - - -●

histone and ATP □——□

Protein concentration was monitored by absorbance at 280nm ——————

Concentration of NaCl ——————

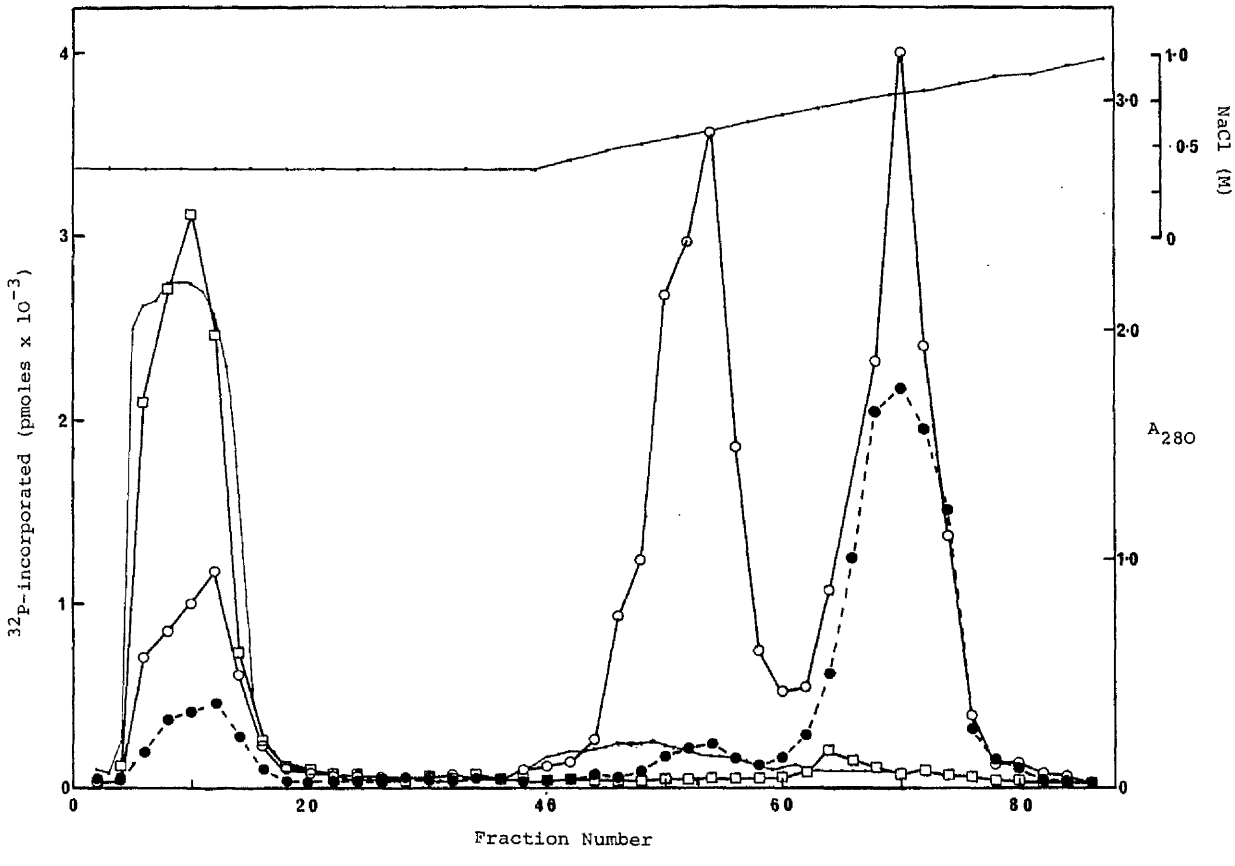

^{32}P-incorporated (pmoles x 10^{-3})
A$_{280}$
NaCl (M)
Fraction Number
0
20
40
60
80
1
2
3
4
1·0
2·0
3·0
0·5

Table 3.2 : Extent of Purification of Casein Kinases

Enzyme Fraction	Protein (mg)	Enzyme Units/mg ($\times 10^{-3}$)	Total Units ($\times 10^{-3}$)	Purification	Yield
'S-150'	205.2	0.32	65.7	1	100%
Pooled DEAE-cellulose kinase	87.6	0.8	70	2.5	106%
CKI	5.19	2.0	10.4	6.3	32%
CKII	1.19	8.9	10.6	27	32%

Protein kinase assays were performed as described in (2.3.15.1). Casein and ATP were used as substrates for all enzyme fractions. In order to obtain estimates for the purification and yield of CKI, and CKII, it was necessary to know their relative proportion in 'S-150'. As this could not be measured directly, it was assumed (from the final yields) that it did not change throughout the enzyme purification process. On the basis of this assumption, and the data obtained, purification was calculated.

3.5 Phosphorylation of Ribosomal Proteins with Resolved Protein Kinases from Ascites Cells

Before characterising the ribosomal substrates for the kinases resolved by phosphocellulose, the various DEAE-cellulose fractions were analysed using ribosomal subunits as substrate.

3.5.1 Phosphorylation by Kinases Resolved from the DEAE-Cellulose

Kinase fractions from the experiment shown in Fig 3.12 were used to phosphorylate 40S or 60S subunits. A portion of the reaction mixture was removed after termination of the reaction, and applied directly to a one-dimensional SDS slab gel and subjected to electrophoresis (2.3.17). When 40S ribosomal subunits were used as substrate, the main phosphoprotein had a molecular weight of approximately 32,000 which could correspond to S6, S2 or S3 (Fig 3.14). Proteins of higher molecular weight (approximately 42,000 and 90,000) were phosphorylated by fractions 31, 34, 37, 40 and 43 (tracks 4 - 8) and fractions 31, 34, 40 and 43 (tracks 4, 5, 7, 8). The 90,000 molecular weight protein is too large to be a ribosomal protein (Collatz *et al.*, 1977; Tsurugi *et al.*, 1977). The 42,000 molecular weight protein may correspond to Sa (molecular weight approximately 40,000) which was phosphorylated in intact ascites cells (Fig 3.4). A protein of approximate molecular weight 38,000 can be seen to be highly phosphorylated in track 7, but does not correspond to any stained ribosomal protein. A phosphoprotein band of similar

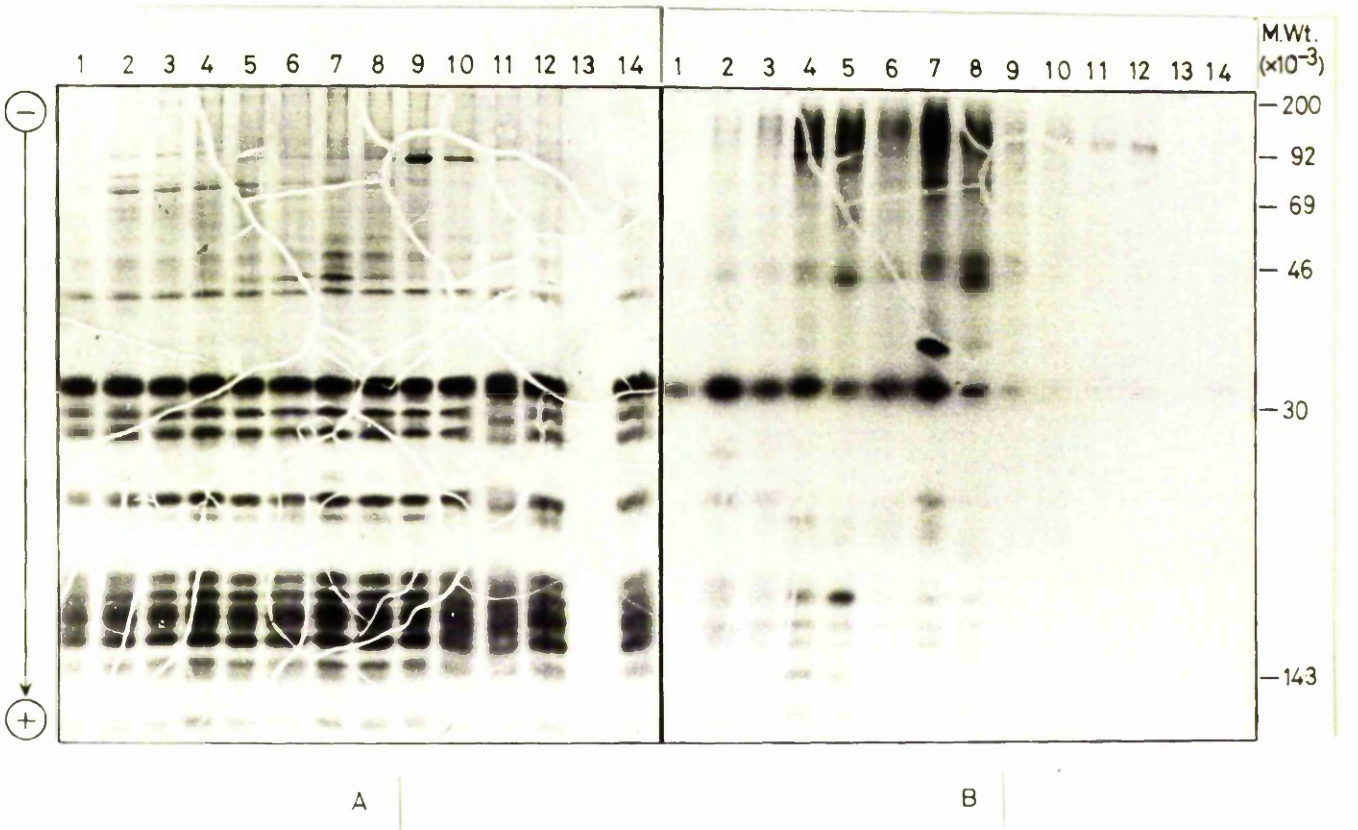
Fig 3.14 : Phosphorylation of 40S Ribosomal Subunits by Protein Kinase Fractions from DEAE-Cellulose.

Track	1	2	3	4	5	6	7	8	9	10	11	12	13	14
DEAE-Cellulose Fraction Number (Fig 3.12)	22 +S	25 +S	28 +S	31 +S	34 +S	37 +S	40 +S	43 +S	46 +S	49 +S	52 +S	55 +S	40 -S	- +S

S = substrate i.e. 40S subunits

Subunits were phosphorylated as described (2.3.15.2) in a reaction mixture which contained 5 A_{260} units of ascites 40S subunits, and DEAE-cellulose fractions (Fig 3.12) 22 - 55 as kinase and analysed by one-dimensional SDS gel electrophoresis (2.3.17).

A. Stain	B. Autoradiograph
Major Phosphoproteins (Approximate Molecular Weight)	Track Number
18,000	4, 5
25,000	7
32,000	1 - 8
38,000	7
42,000	4 - 8
92,000	4, 5, 7, 8


1 2 3 4 5 6 7 8 9 10 11 12 13 14
1 2 3 4 5 6 7 8 9 10 11 12 13 14
M.Wt. (×10⁻³)
200
92
69
46
30
143
A
B

molecular weight can also be seen when the same enzyme fraction is used to phosphorylate 60S subunits (Fig 3.15). Finally, two phosphoproteins of approximate molecular weights 18,000 and 25,000 were present.

Analysis of phosphorylated 60S subunits by one-dimensional SDS slab gel electrophoresis yielded two low molecular weight proteins 16,000 and 18,000 (Fig 3.15). The 16,000 molecular weight band is likely to be protein Lγ. Faint phosphoprotein bands at 27,500 and 31,000 were also observed, which could correspond to proteins L14 and S6. Fig. 3.14 shows clearly the heterogeneity of kinases (or their substrates or inhibitors) in the gradient fractions.

3.5.2 Phosphorylation by Protein Kinases Purified on Phosphocellulose

The protein kinases resolved by phosphocellulose chromatography (Fig 3.13) were used to phosphorylate 40S and 60S subunits (2.3.15.2). Reactions were terminated by addition of glacial acetic acid, and the protein then extracted (2.3.8). This protein was analysed by one-dimensional SDS gel electrophoresis. CKI was seen to phosphorylate mainly a 14,000 molecular weight protein of the 60S subunit, which probably corresponds to Lγ (Fig 3.16). Some fainter bands were also observed on 60S subunits. CKI also phosphorylated at least 4 protein bands on the 40S subunit. The most highly phosphorylated had a molecular weight of 31,000. The other phosphoproteins had molecular weights 22,000, 46,000 and 34,000. Further characterisation on two-dimensional 'Sweep' gel electrophoresis is shown in Fig 3.17. A faint spot corresponding to Lγ on the 60S protein autoradiograph and four

Fig 3.15 : Phosphorylation of 60S Ribosomal Subunits by Protein Kinase Fractions from DEAE-Cellulose.

Track	1	2	3	4	5	6	7	8	9	10	11	12	13	14
DEAE-Cellulose Fraction Number (Fig 3.12)	22	25	28	31	34	37	40	43	46	49	52	55	40	-
	+S	+S	+S	+S	+S	+S	+S	+S	+S	+S	+S	+S	-S	+S

S = Substrate i.e. 60S Ribosomal Subunits

Subunits were phosphorylated as described 2.3.15.2 in a reaction mixture which contained 5 A_{260} units of ascites 60S subunits, and DEAE-cellulose fractions (Fig 3.12) 22 - 55 as kinase and analysed by one-dimensional SDS gel electrophoresis. (2.3.17).

A. Stain	B. Autoradiograph
Major Phosphoproteins (Approximate Molecular Weight)	Track Number
16,000	2 - 8
18,000	2 - 8
27,500	2 - 6
31,000	2 - 6
38,000	7

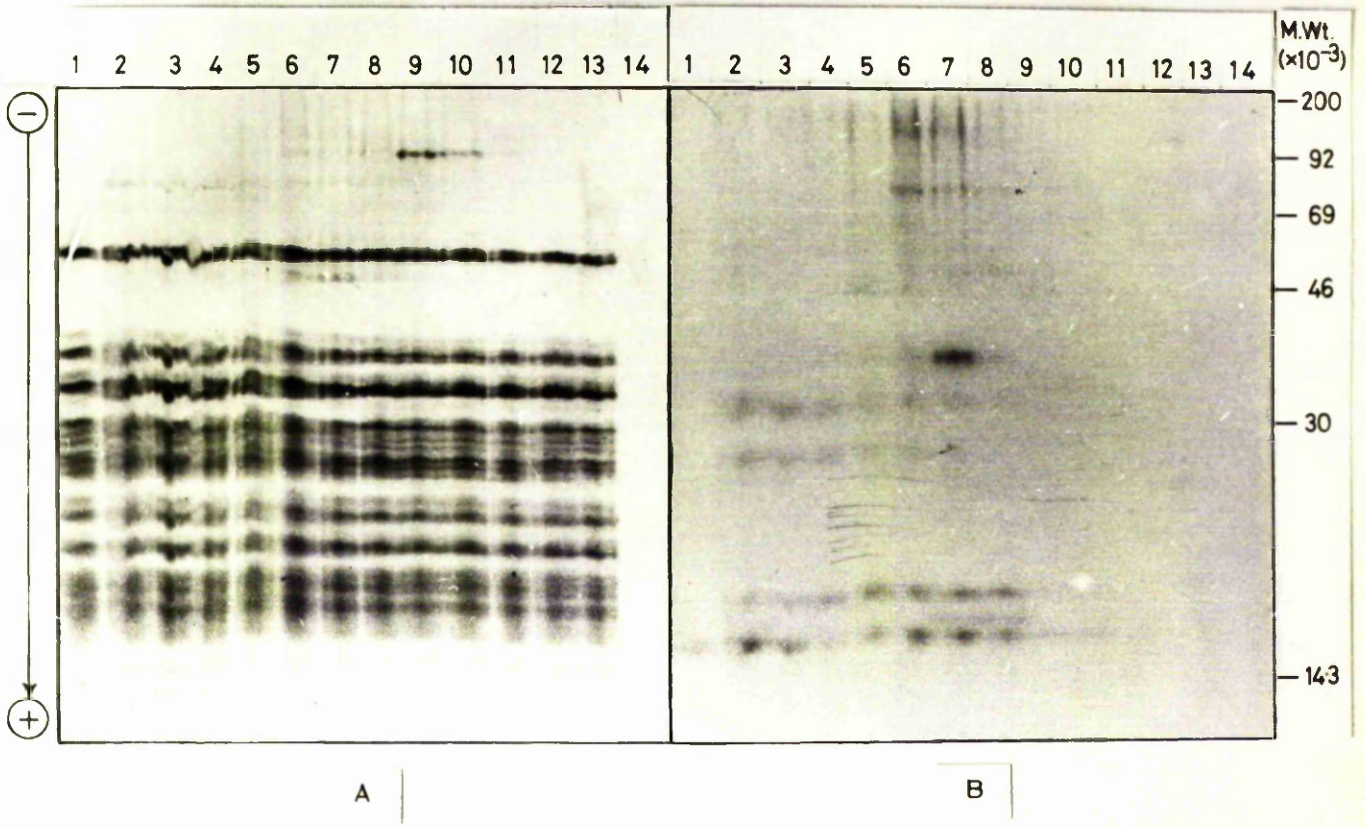

1 2 3 4 5 6 7 8 9 10 11 12 13 14
1 2 3 4 5 6 7 8 9 10 11 12 13 14
M.Wt. (×10⁻³)
200
92
69
46
30
143
A
B

Fig 3.16 : One-Dimensional Sodium Dodecyl Sulphate Gel Electrophoresis of Ribosomal Protein Phosphorylated by CKI.

Ribosomal subunits were phosphorylated by protein kinase activity CKI as described (2.3.15.2). Approximately 60μg of phosphorylated ribosomal protein was subjected to electrophoresis (2.3.17) and autoradiography. The specific activity of the protein was 6.5×10^4 cpm/mg for 40S protein, and 3.08×10^4 cpm/mg for 60S protein.

a. Stain of 60S protein
b. Autoradiograph of 60S protein
c. Stain of 40S protein
d. Autoradiograph of 40S protein

Approximate molecular weights of ^{32}P-labelled bands.

Band No.	Mol. wt.
1	14,000
2	31,000
3	13,500
4	22,000
5	34,000
6	46,000

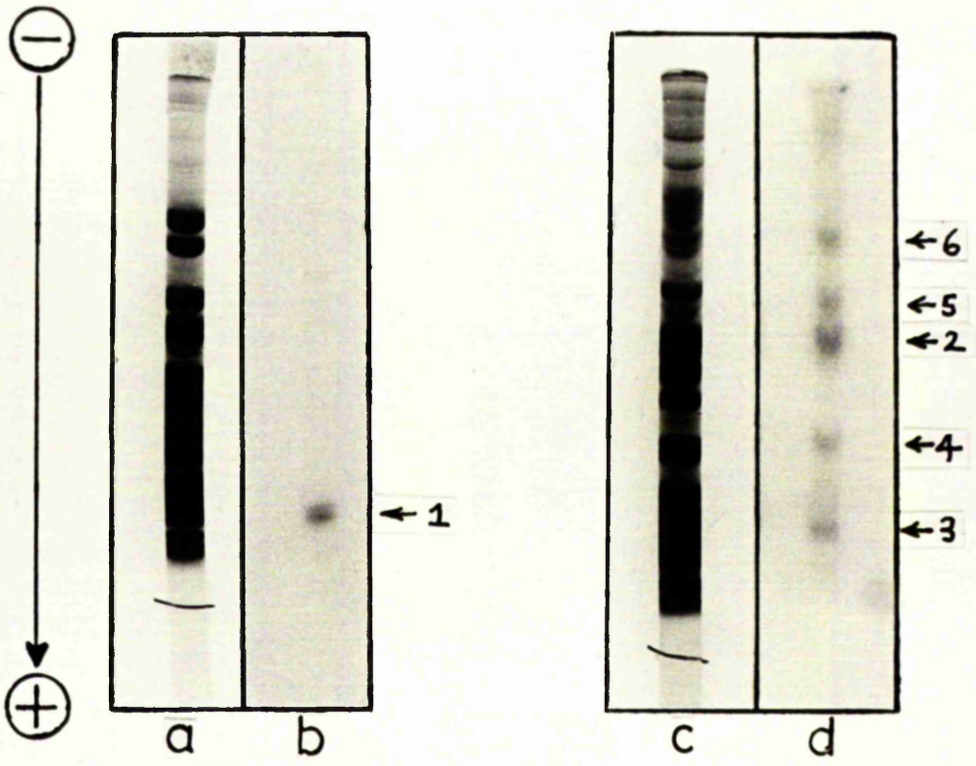

−
+
1
6
5
2
4
3
a
b
c
d

Fig 3.17 : Two-Dimensional 'Sweep' Gel Electrophoresis of Ribosomal Protein Phosphorylated by CKI

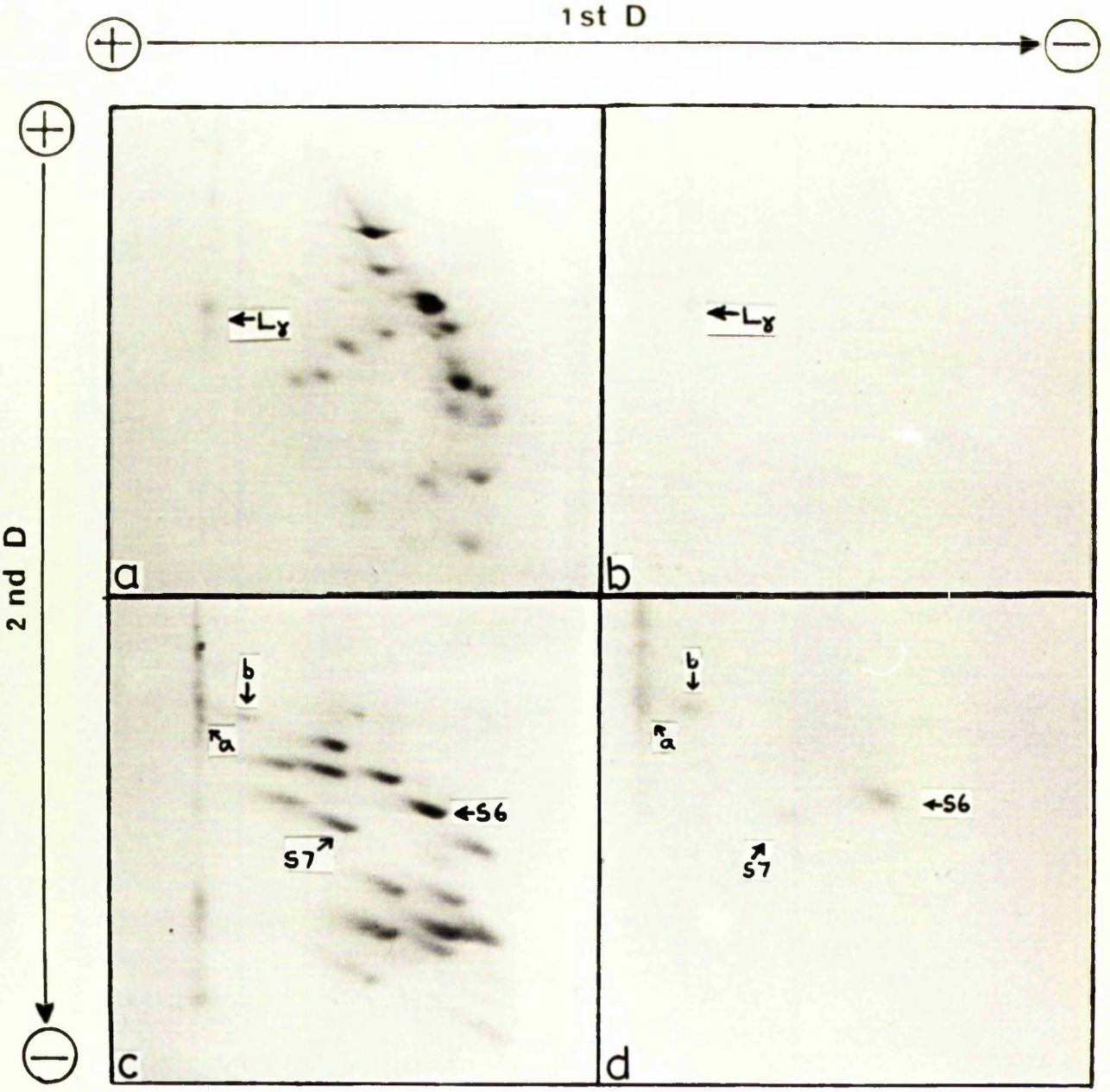
Approximately 100μg of 60S and 40S ribosomal protein (described in Fig 3.16) were subjected to electrophoresis (2.3.20) and autoradiography (2.3.20).

a. Stain of 60S protein

b. Autoradiograph of 60S protein

c. Stain of 40S protein

d. Autoradiograph of 40S protein


1st D
2 nd D
a
b
c
d
←Lγ
←Lγ
b
a
←S6
S7
b
a
←S6
S7

spots corresponding to proteins S6, S7, Sa and Sb on the 40S subunit autoradiograph were seen. The relative faintness is clearly due to the low amount of Lγ protein seen on the stained gel. When 'Acidic-Acidic' two-dimensional gel electrophoresis was performed on these phosphorylated subunits essentially the same pattern was obtained except that stained and phosphorylated Lγ was completely lacking (Fig 3.18). On the 'Basic-Acidic' two-dimensional gel system of Lastick and McConkey (2.3.19), only S6 and S7 were observed as phosphoproteins (Fig 3.19); this was expected since the acidic proteins Lγ, Sa, and Sb would not migrate into the gel in this system.

Similar experiments were carried out with subunits phosphorylated by CKII and [γ-^{32}P] ATP. On one-dimensional SDS gels (Fig 3.20) a single protein of molecular weight 14,000 was phosphorylated in the 60S subunit, and no 40S subunit protein appeared to be phosphorylated. Two-dimensional 'Sweep' gel electrophoresis (Fig 3.21) confirmed that Lγ was the only protein of either ribosomal subunit to be phosphorylated by CKII. 'Acidic-Acidic' two-dimensional gels (Fig 3.22) also indicated that Lγ was phosphorylated, but as before (3.1.1) it was present in much reduced amounts.

When HK was used to phosphorylate ribosomal subunits one 60S phosphoprotein of molecular weight 14,000 and four 40S subunit phosphoproteins of molecular weights 29,500, 19,500, 17,000 and 13,000 were obtained (Fig 3.23). From 'Acidic-Acidic' (Fig 3.24) and 'Basic-Acidic' (Fig 3.25) two-dimensional gels, the identities of these phosphoproteins

Fig 3.18 : Two-Dimensional 'Acidic-Acidic' Gel Electrophoresis of Ribosomal Protein Phosphorylated by CKI

Approximately 100μg of 60S and 40S ribosomal protein (described in Fig 3.16) were subjected to electrophoresis (2.3.21) and autoradiography (2.3.21).

a. Stain of 60S protein

b. Autoradiograph of 60S protein

c. Stain of 40S protein

d. Autoradiograph of 40S protein

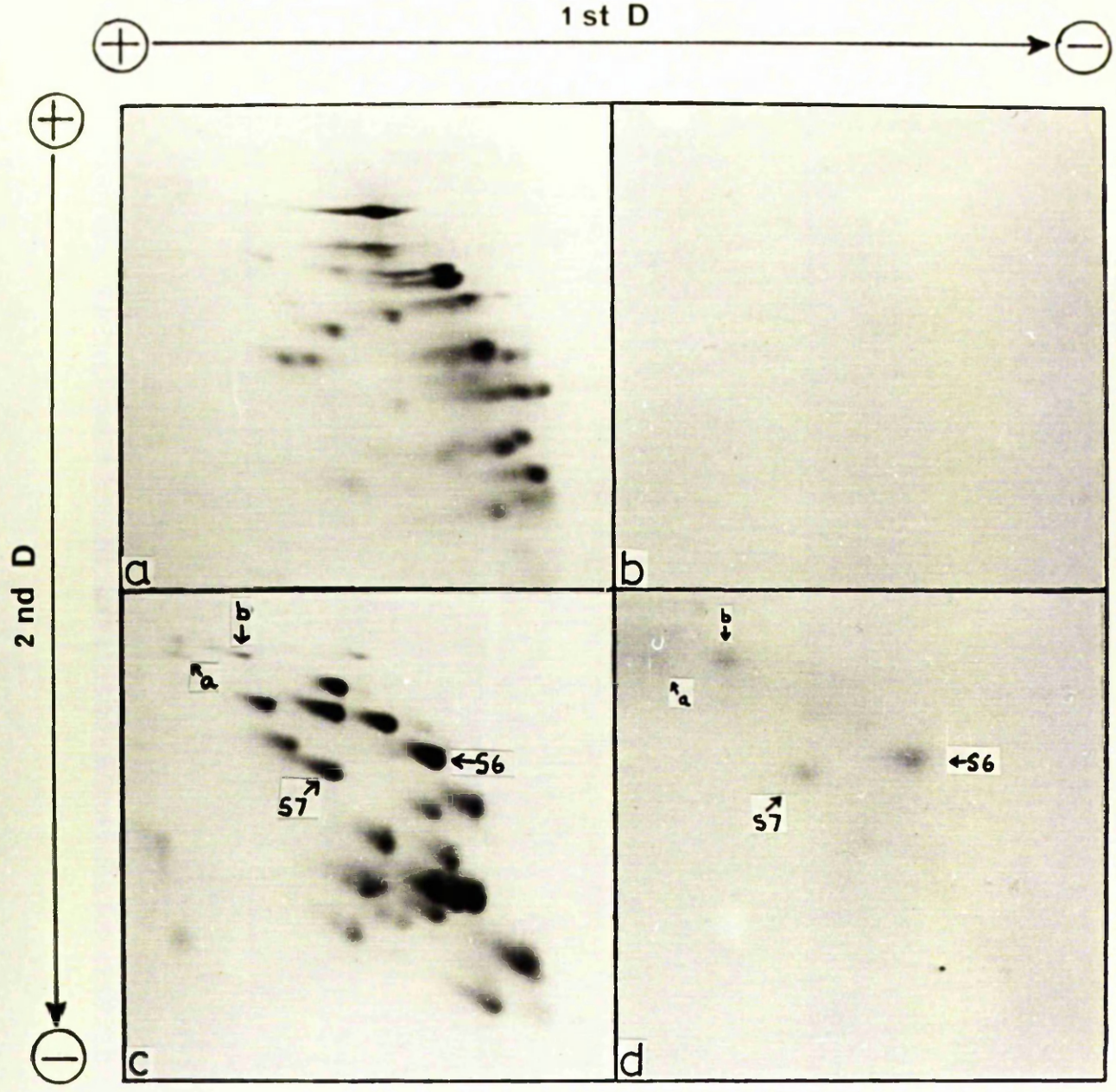

1 st D
2 nd D
a
b
c
d
b
a
S6
S7
b
a
S6
S7

Fig 3.19 : Two-Dimensional 'Basic-Acidic' Gel Electrophoresis of Ribosomal Protein Phosphorylated by CKI

Approximately 100μg of 60S and 40S ribosomal protein (described in Fig 3.16) were subjected to electrophoresis (2.3.19), and autoradiography (2.3.19).

a. Stain of 60S protein

b. Autoradiograph of 60S protein

c. Stain of 40S protein

d. Autoradiograph of 40S protein

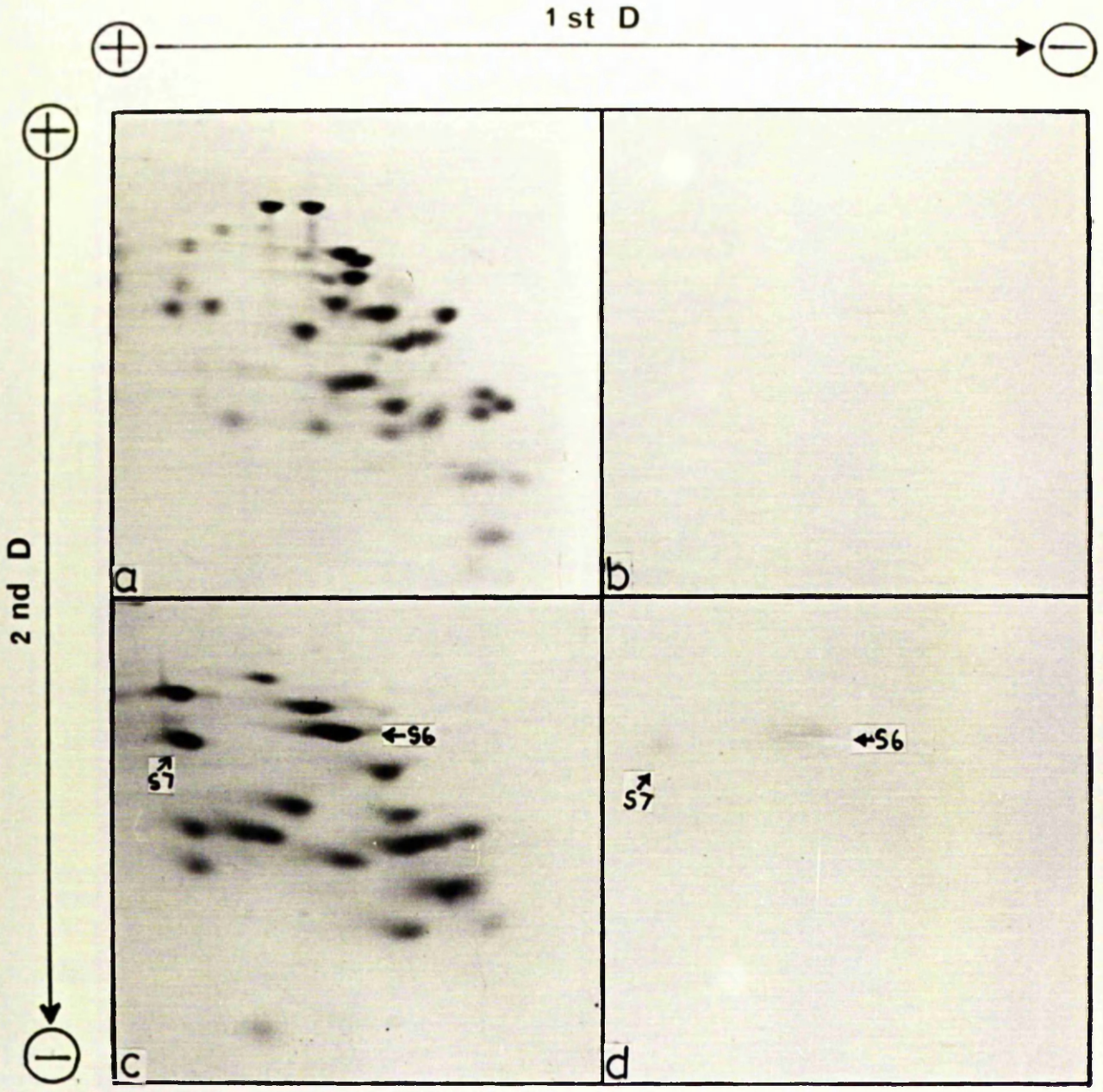

1 st D
2 nd D
a
b
c
d
←S6
S7
←S6
S7

Fig 3.20 : One-Dimensional SDS Gel Electrophoresis of Ribosomal Protein Phosphorylated by CKII

Ribosomal subunits were phosphorylated by protein kinase activity CKII as described, (2.3.15.1). Approximately 60μg of phosphorylated ribosomal protein was subjected to electrophoresis (2.3.17) and autoradiography. The specific activity of the protein was 6.8×10^4 cpm/mg for 60S protein, and 6×10^2 cpm/mg for 40S protein.

a. Stain of 60S protein

b. Autoradiograph of 60S protein

c. Stain of 40S protein

d. Autoradiograph of 40S protein

A single phosphoprotein of approximate molecular weight 14,000 is shown.

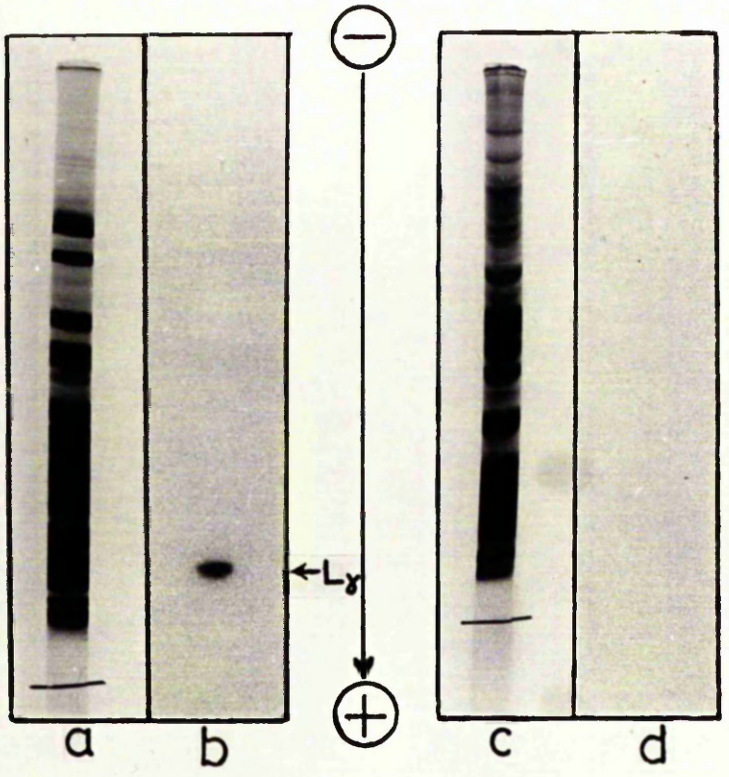

−
+
L_γ
a
b
c
d

Fig 3.21 : Two-Dimensional 'Sweep' Gel Electrophoresis of Ribosomal Protein Phosphorylated by CKII

Approximately 100μg of 60S and 40S ribosomal protein (described in Fig 3.20) were subjected to electrophoresis (2.3.20) and autoradiography.

a. Stain of 60S protein

b. Autoradiograph of 60S protein

c. Stain of 40S protein

d. Autoradiograph of 40S protein

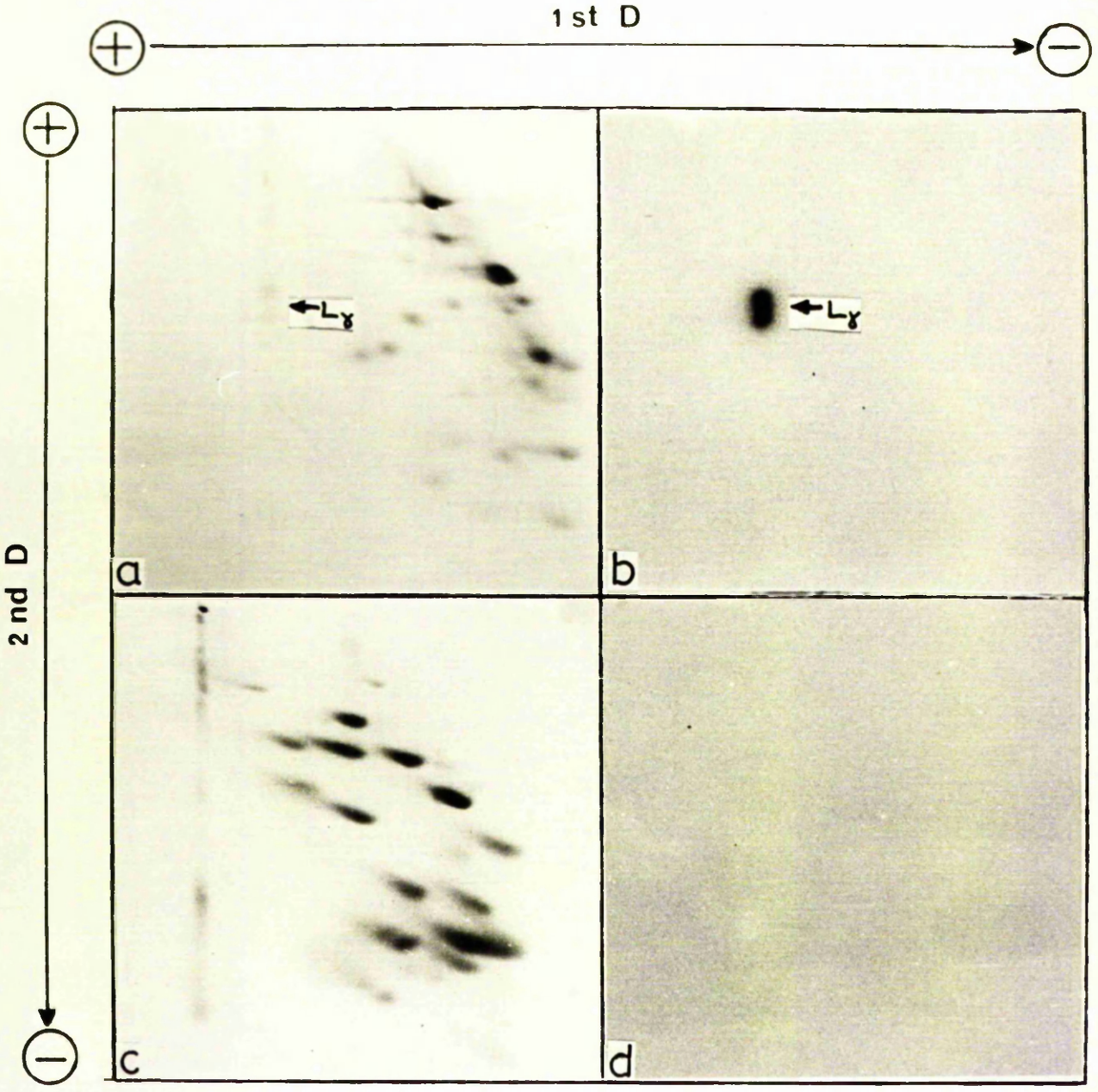

1 st D
2 nd D
+
−
+
−
L_γ
L_γ
a
b
c
d

Fig 3.22 : Two-Dimensional 'Acidic-Acidic' Gel Electrophoresis of Ribosomal Protein Phosphorylated by CKII

Approximately 100μg of 60S and 40S ribosomal protein (described in Fig 3.20) were subjected to electrophoresis (2.3.21) and autoradiography (2.3.21).

a. Stain of 60S protein

b. Autoradiograph of 60S protein

c. Stain of 40S protein

d. Autoradiograph of 40S protein

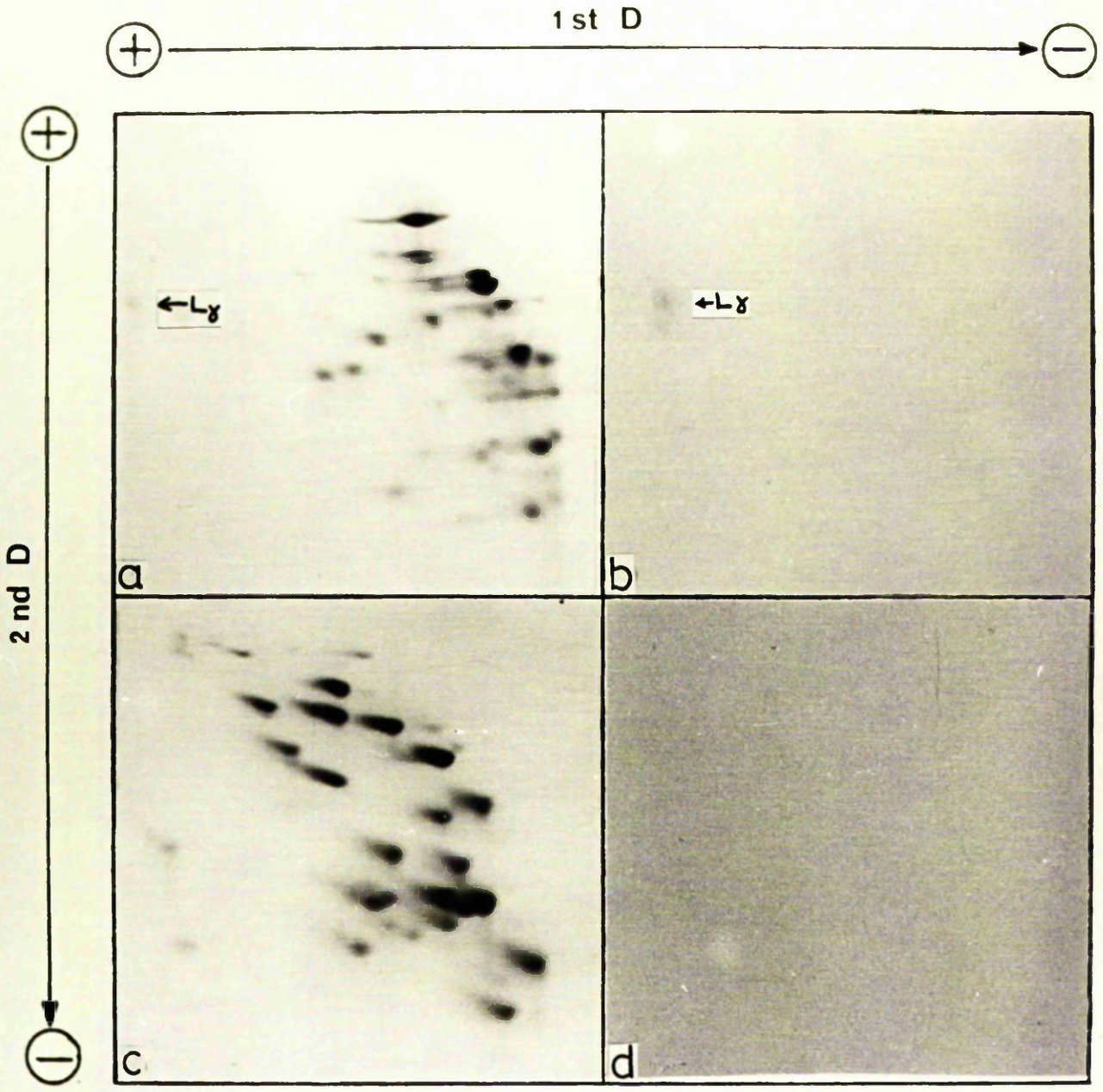

1st D
2nd D
←Lγ
←Lγ
a
b
c
d

Fig 3.23 : One-Dimensional SDS Gel Electrophoresis of Ribosomal Proteins Phosphorylated by HK

Ribosomal subunits were phosphorylated by protein kinase activity HK as described, (2.3.15.1). Approximately 60μg of phosphorylated ribosomal protein was subjected to electrophoresis (2.3.17) and autoradiography. The specific activity of the protein was 6.5×10^4 cpm/mg for 40S protein and 3.08×10^4 cpm/mg for 60S protein.

a. Stain of 60S protein

b. Autoradiograph of 60S protein

c. Stain of 40S protein

d. Autoradiograph of 40S protein

Approximate Molecular weights of ^{32}P-labelled protein bands.

Band No.	Mol. wt.
1	14,000
2	29,500
3	13,000
4	17,000
5	19,500

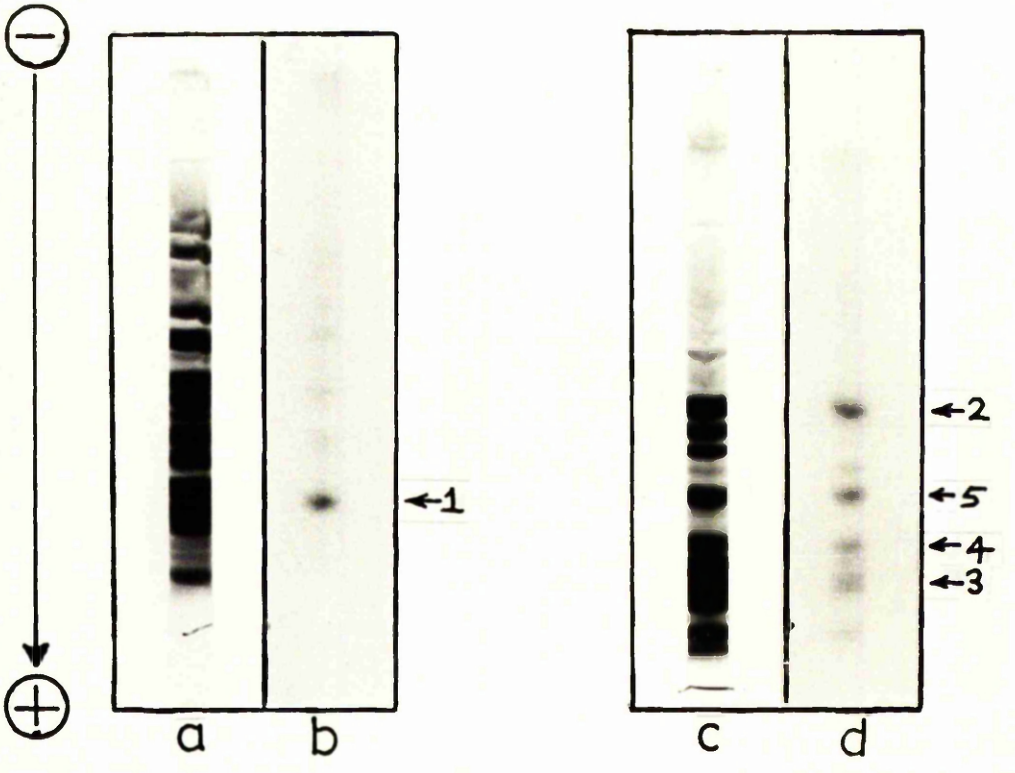

⊖
⊕
←1
←2
←5
←4
←3
a
b
c
d

Fig 3.24 : Two-Dimensional 'Sweep' Gel Electrophoresis of Ribosomal Protein Phosphorylated by HK

Approximately 100µg of 60S and 40S ribosomal protein (described in Fig 3.23) were subjected to electrophoresis (2.3.20) and autoradiography (2.3.20).

a. Stain of 60S protein

b. Autoradiograph of 60S protein

c. Stain of 40S protein

d. Autoradiograph of 40S protein

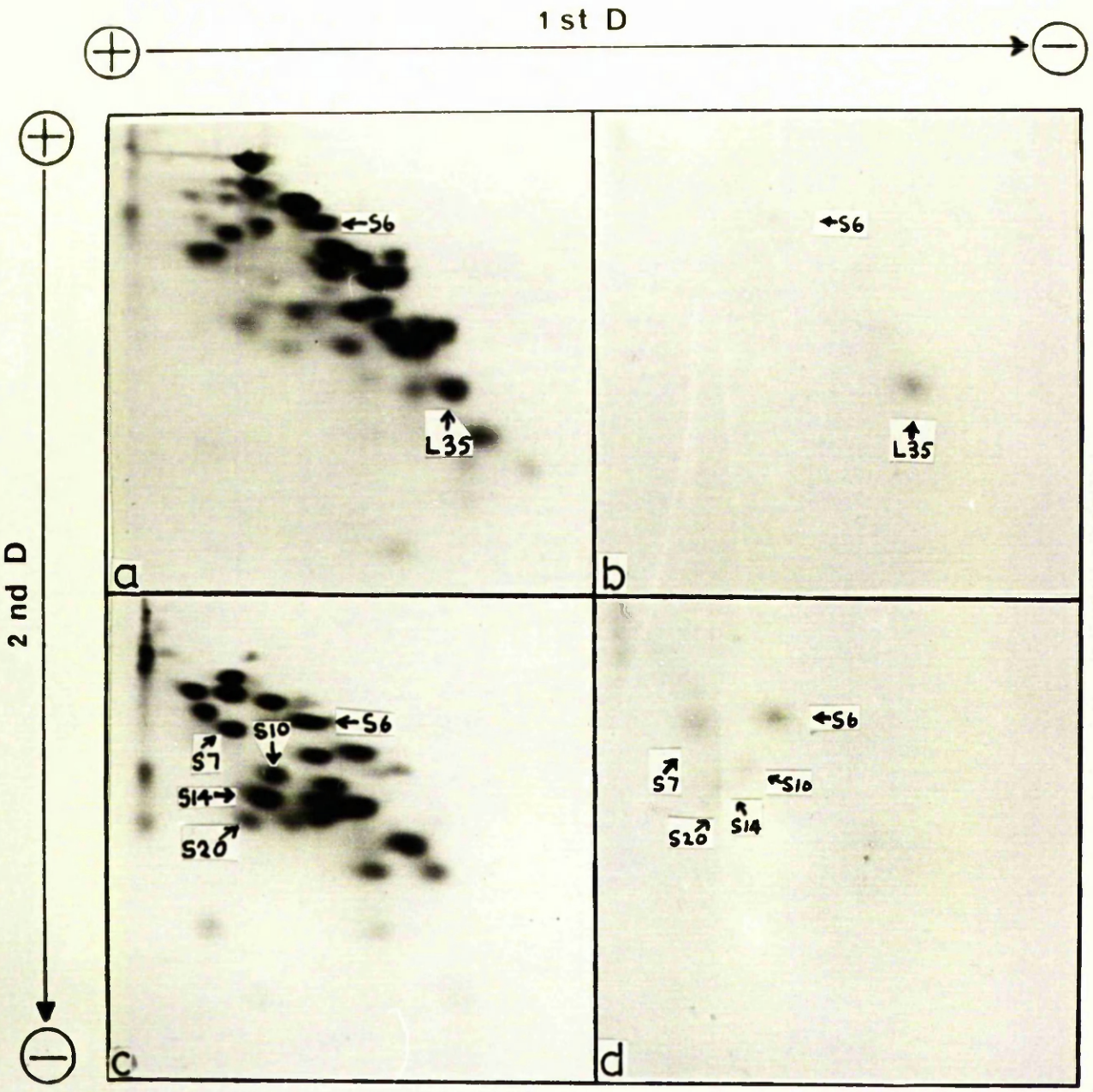

1 st D
2 nd D
S6
L35
a
S6
L35
b
S10
S6
S7
S14
S20
c
S6
S7
S10
S20
S14
d

Fig 3.25 : Two-Dimensional 'Basic-Acid' Gel Electrophoresis of Ribosomal Protein Phosphorylated by HK

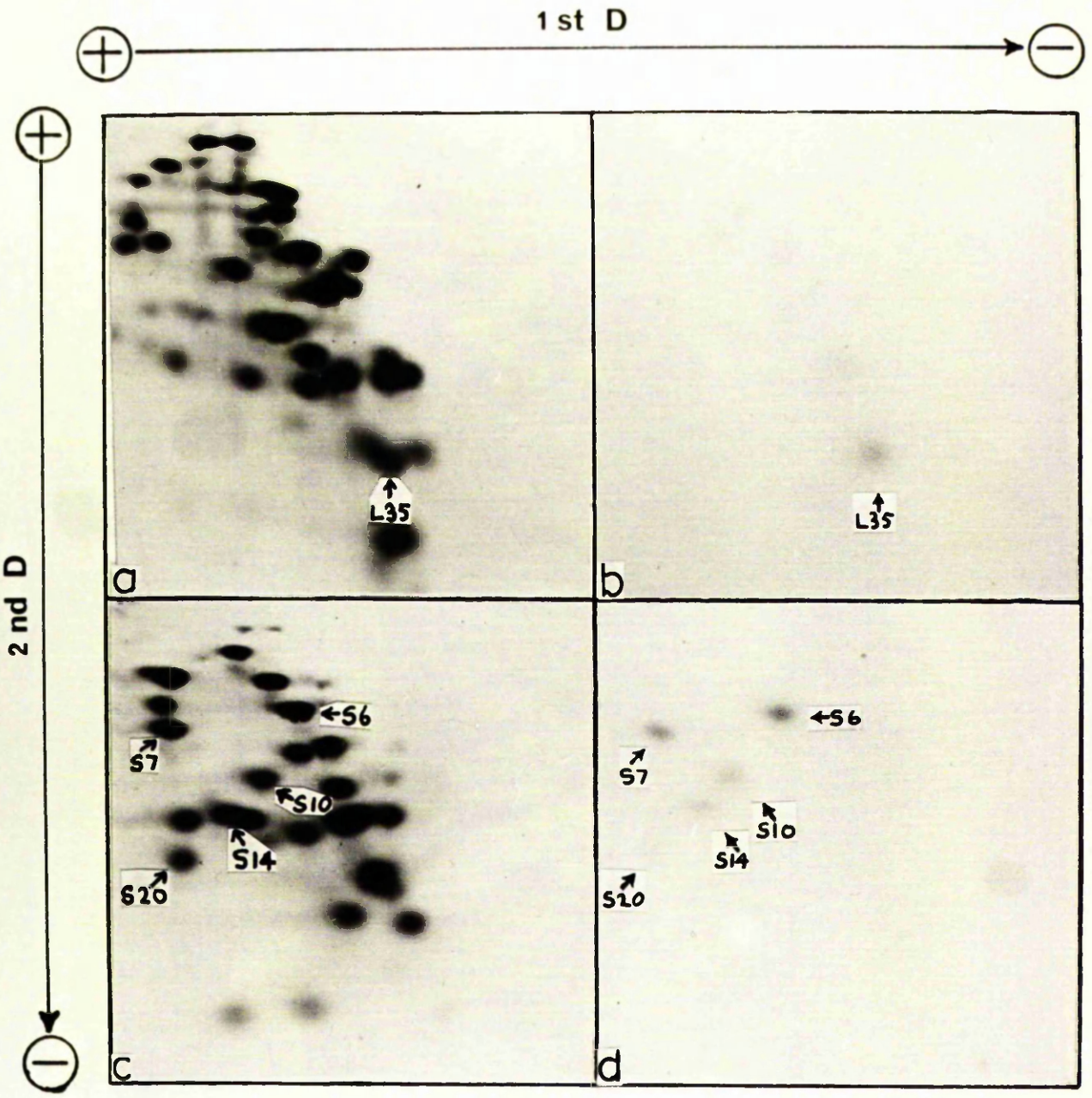
Approximately 100µg of 60S and 40S ribosomal protein (described in Fig 3.23) were subjected to electrophoresis (2.3.19) and autoradiography (2.3.19).

a. Stain of 60S protein

b. Autoradiograph of 60S protein

c. Stain of 40S protein

d. Autoradiograph of 40S protein


1 st D
2 nd D
L35
a
L35
b
S6
S7
S10
S14
S20
c
S6
S7
S10
S14
S20
d

appeared to be L35, S6, S7, S10, S14 and S20.

Phosphorylation of ribosomal 60S subunits by CKI or CKII was also carried out with [γ-^{32}P] GTP as phosphoryl donor. The results in Fig 3.26 show that Lγ is the only 60S subunit protein phosphorylated by CKII and GTP, and that no 60S proteins are phosphorylated by CKI and GTP. It had been suggested that GTP is not directly used by 'GTP utilizing protein kinases' but that the terminal phosphate of [γ-^{32}P] GTP was transferred by nucleoside phosphotransferases to form [γ-^{32}P] ATP which in turn was used by the protein kinases (Kuo, 1974). To test this possibility, samples taken from reaction mixtures in which CKII and [γ-^{32}P] GTP had been allowed to phosphorylate casein for 20 minutes were analysed by PEI-cellulose chromatography. As shown in Fig 3.27 no [γ-^{32}P] ATP could be detected, although the casein became labelled with ^{32}P in the reaction from which the sample was taken.

Phosphorylation of Extracted Ribosomal Protein with CKI and CKII.

It was decided to examine the proteins phosphorylated when isolated ribosomal proteins, rather than intact subunits, were presented to the resolved protein kinase. One particular reason why this was of interest was because the altered pattern of phosphorylation in ascites cells incubated in glucose might be due to altered ribosome conformation, presenting previously inaccessible sites for phosphorylation. Such sites would be expected to be accessible in the protein extracted from subunits.

Fig 3.26 : Two-Dimensional 'Sweep' Gel Electrophoresis of 60S Ribosomal Subunit Protein Phosphorylated by CKI and CKII using γ-^{32}P GTP.

60S ribosomal subunits were phosphorylated with γ-^{32}P GTP, as described (2.3.15.1). Approximately 100μg of protein was subjected to electrophoresis (2.3.20), and autoradiography. The specific activity of the protein was approximately 5.6 x 10^4 cpm/mg for 60S/CKII and 10^3 cpm/mg for 60S/CKI.

a. Stain of 60S protein/CKI

b. Autoradiograph of 60S protein/CKI

c. Stain of 60S protein/CKII

d. Autoradiograph of 60S protein/CKII

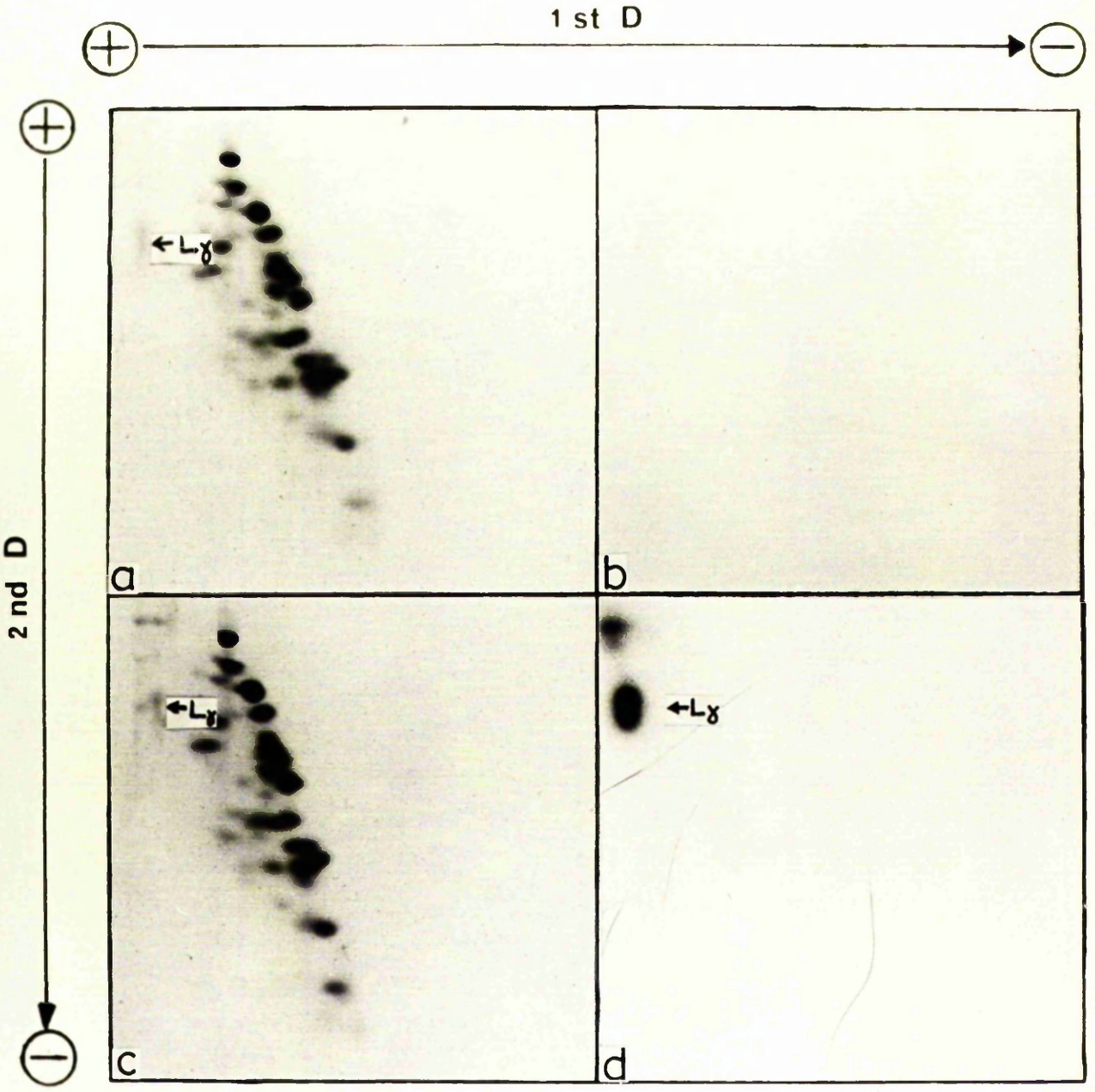
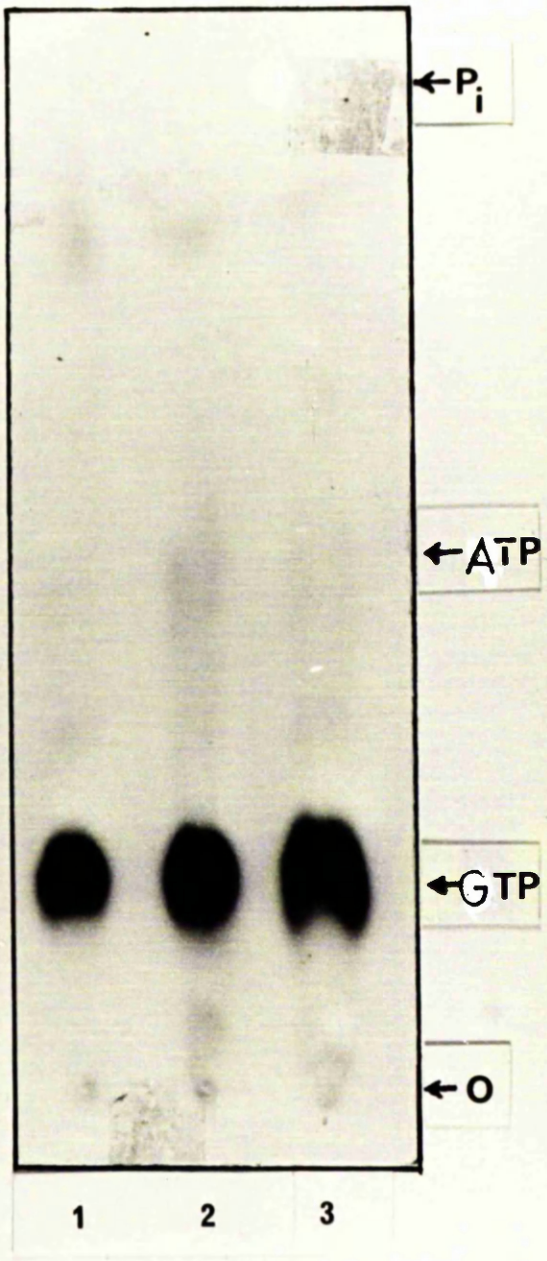

1 st D
2 nd D
a
b
c
d
←Lγ
←Lγ
←Lγ

Fig 3.27 : PEI-Cellulose Chromatography of GTP from CKII Phosphorylation Reactions of Casein

Samples of 10μl were removed from phosphorylation reactions containing CKII, casein and [γ-^{32}P] GTP (3.4.2) and chromatographed on PEI-cellulose as described (2.3.16). Samples contained approximately 25nmoles of [γ-^{32}P] GTP of specific activity 8 cpm/pmole. The positions of GTP and ATP were determined by using unlabelled ATP and GTP. Three phosphorylation reactions are shown.


←Pi
←ATP
←GTP
←O
1
2
3

Extracted 40S and 60S protein was phosphorylated by CKI and CKII with ATP as phosphoryl donor as described (2.3.15.2). After termination of the phosphorylation reaction samples were immediately resolved by one-dimensional SDS slab gel electrophoresis (Fig 3.28). CKI does not appear to phosphorylate any of the proteins, whereas CKII phosphorylates two protein bands of molecular weights 16,700 and 18,300 in the 60S subunit. These were also seen in the 40S subunits, though to a lesser extent, suggesting cross-contamination from the 60S subunit. It is likely that the 16,700 molecular weight band may correspond to Lγ.

3.5.3 Phosphorylation of Ascites Ribosomes by Exogenous Protein Kinases from Other Tissues

The protein kinases isolated from the cytosol of ascites cells were shown to be capable of phosphorylating both ribosomal and non-ribosomal substrates. It was of interest to compare the proteins phosphorylated by these kinases with those phosphorylated by exogenous protein kinases from other sources. Rabbit skeletal muscle peak I (PKI), and peak II (PKII) cyclic AMP dependent protein kinases (2.3.13), and bovine heart cyclic AMP-dependent protein kinase (2.1.3) were used to phosphorylate 80S ribosomes, and 40S and 60S subunits. The phosphorylation reactions were carried out as described (2.3.15.2). After termination a portion of the reaction mixture was resolved by one-dimensional SDS-slab gel electrophoresis, and subjected to autoradiography (Fig 3.29). As can be seen, a large number of proteins were phosphorylated, especially 80S

Fig 3.28 : One-Dimensional SDS Slab Gel Electrophoresis of Extracted Ribosomal Protein Phosphorylated by CKI and CKII.

Extracted ribosomal subunit protein (2.3.8) was phosphorylated as described (2.3.15.2), in a reaction mixture containing 200μg of ribosomal protein, [γ-^{32}P] ATP and either 11μg of CKI or 3.4μg of CKII in a volume of 250μl. Approximately 50μg of protein was resolved by one-dimensional SDS slab gel electrophoresis (2.3.17).

A. Stained Protein

B. Autoradiograph

Track	Sample
1	60S protein/CKI
2	60S protein/CKII
3	40S protein/CKI
4	40S protein/CKII

Main Phosphoproteins (Approximate Molecular Weight)	Track Number
16,700	2, 4
18,300	2, 4

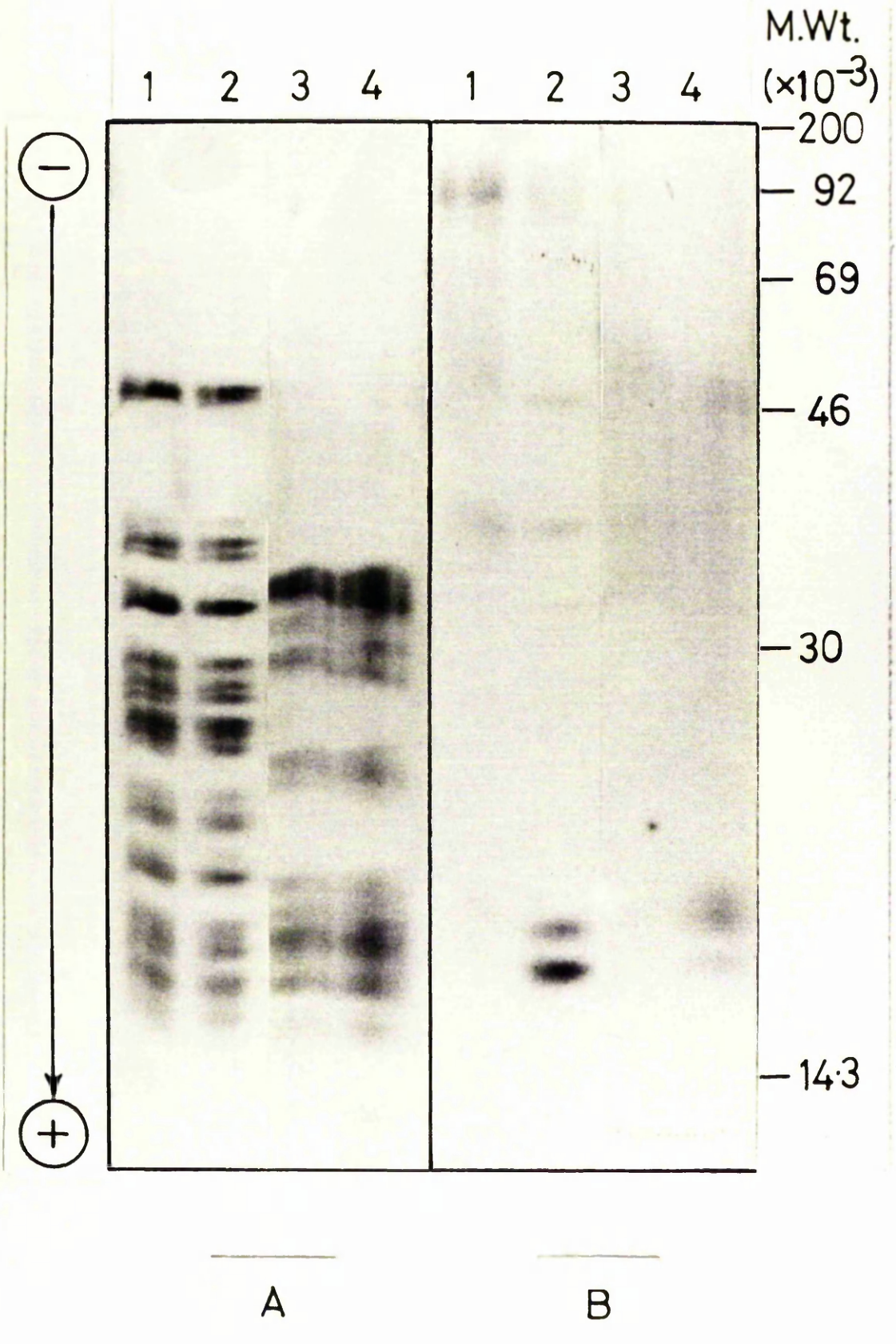

M.Wt.
(×10⁻³)
1 2 3 4 1 2 3 4
200
92
69
46
30
14·3
A
B

Fig 3.29 : The Phosphorylation of Ascites Ribosomes by Exogenous Protein Kinases from Other Tissues

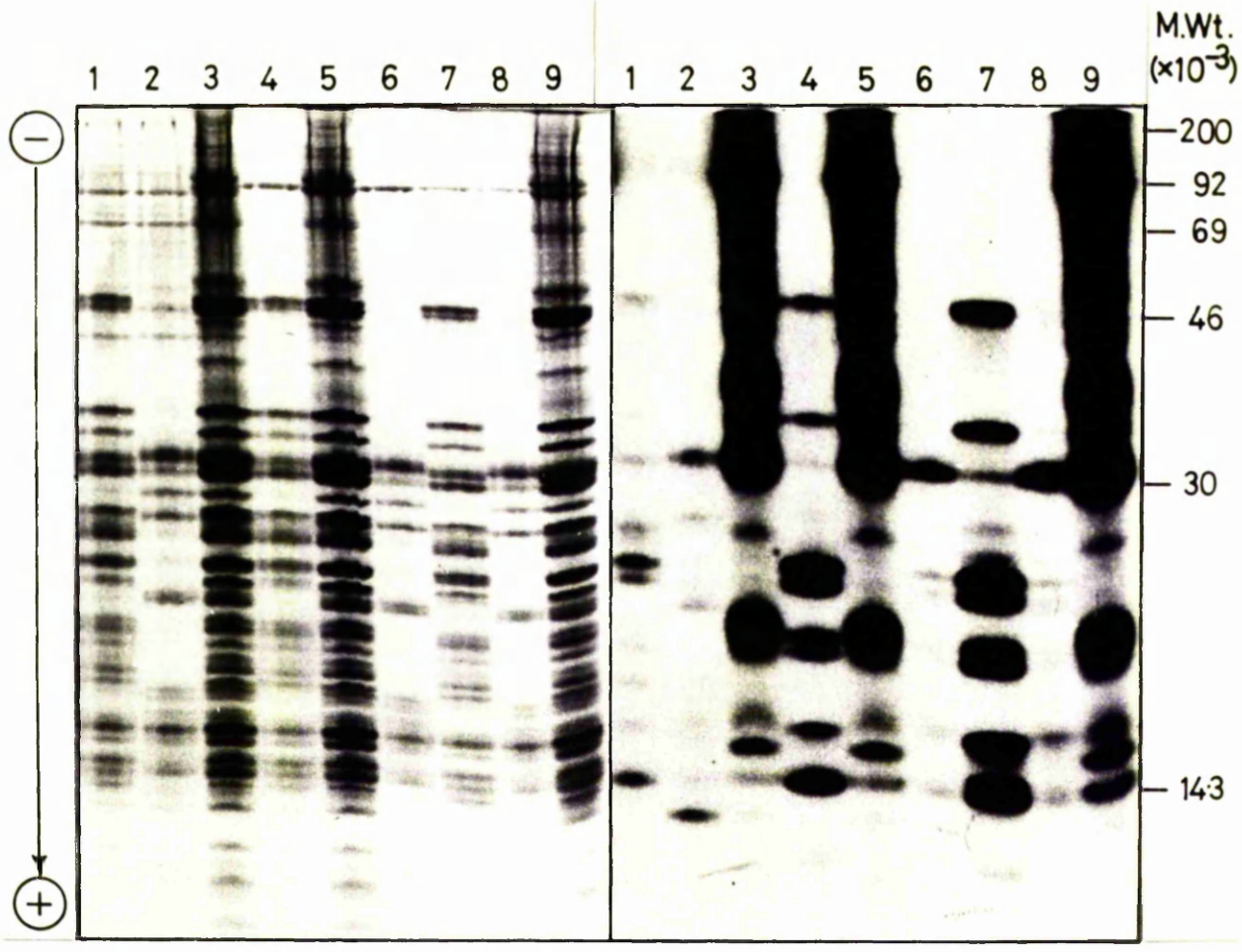
Track No.	1	2	3	4	5	6	7	8	9
Ribosomal Substrate	60S	40S	80S	60S	80S	40S	60S	40S	80S
Enzyme	bovine heart kinase			PKI			PKII		

80S ribosomes, and 40S and 60S ribosomal subunits were phosphorylated as described (2.3.15.2). The phosphorylation reaction mixture contained 20 A_{260} units of 80S ribosomes, or 5 A_{260} units of 40S or 60S subunits and either 40μg of PKI (8 units), or 55μg PKII (22.5 units) rabbit skeletal muscle protein kinases or 5μg of bovine heart protein kinase (10 units). Samples of approximately 50μg of phosphorylated substrate were resolved by one-dimensional SDS slab gel electrophoresis, and exposed to autoradiography.

A. Stain B. Autoradiograph.

Major Phosphoproteins (Excluding 80S > 30,000 m.wt.)

(Approximate Mol. wt.)	Track No.
50,000	1,4,7
37,500	4,7
32,500	2,3,5,6,8,9
24,000	1,4,7
23,000	1,4,7
19,500	3,4,5,7,9
19,000	3,4,5,7,9
15,500	4,7
14,300	3,5,9
13,500	1,4,7,9
10,900	2


M.Wt.
(×10⁻³)
1 2 3 4 5 6 7 8 9
1 2 3 4 5 6 7 8 9
200
92
69
46
30
14·3
−
+
A
B

ribosomal proteins of molecular weight greater than 30,000. For this reason, identification of the molecular weights of 80S ribosome phosphoproteins greater than 30,000 was difficult. A protein of molecular weight 32,500 was phosphorylated in 40S subunits by all three enzymes, and also in 80S ribosomes. It is likely that this protein is S6 which has been shown by others to be phosphorylated readily by non-endogenous enzymes. A protein of molecular weight approximately 13,500 in 60S subunits is phosphorylated by all three enzymes, and this protein is probably Lγ. However the same protein was less phosphorylated in 80S ribosomes by either bovine heart kinase, PKI or PKII. The most striking difference between the phosphorylation of 60S subunits and 80S ribosomal proteins is the complete absence of a highly phosphorylated doublet of 60S protein of molecular weights 24,000 and 23,000 from 80S ribosomes. This suggests that these two proteins are much more accessible to protein kinases in 60S subunits than in 80S ribosomes.

3.5.4 Phosphorylation of Extracted Ascites Ribosomal Protein by Exogenous Protein Kinases from Other Tissues

Extracted ribosomal protein was phosphorylated with PKI, PKII and bovine heart protein kinase. Samples of the phosphorylation reactions were resolved on one-dimensional SDS slab gels and autoradiographed as described before (3.5.3). It can be clearly seen (Fig 3.30), that all three enzymes phosphorylate proteins of molecular weights 13,500 and 32,000 in the 60S and 40S subunits respectively. One other 60S

Fig 3.30 : The Phosphorylation of Extracted Ribosomal Proteins by Exogenous Protein Kinases from Other Tissues

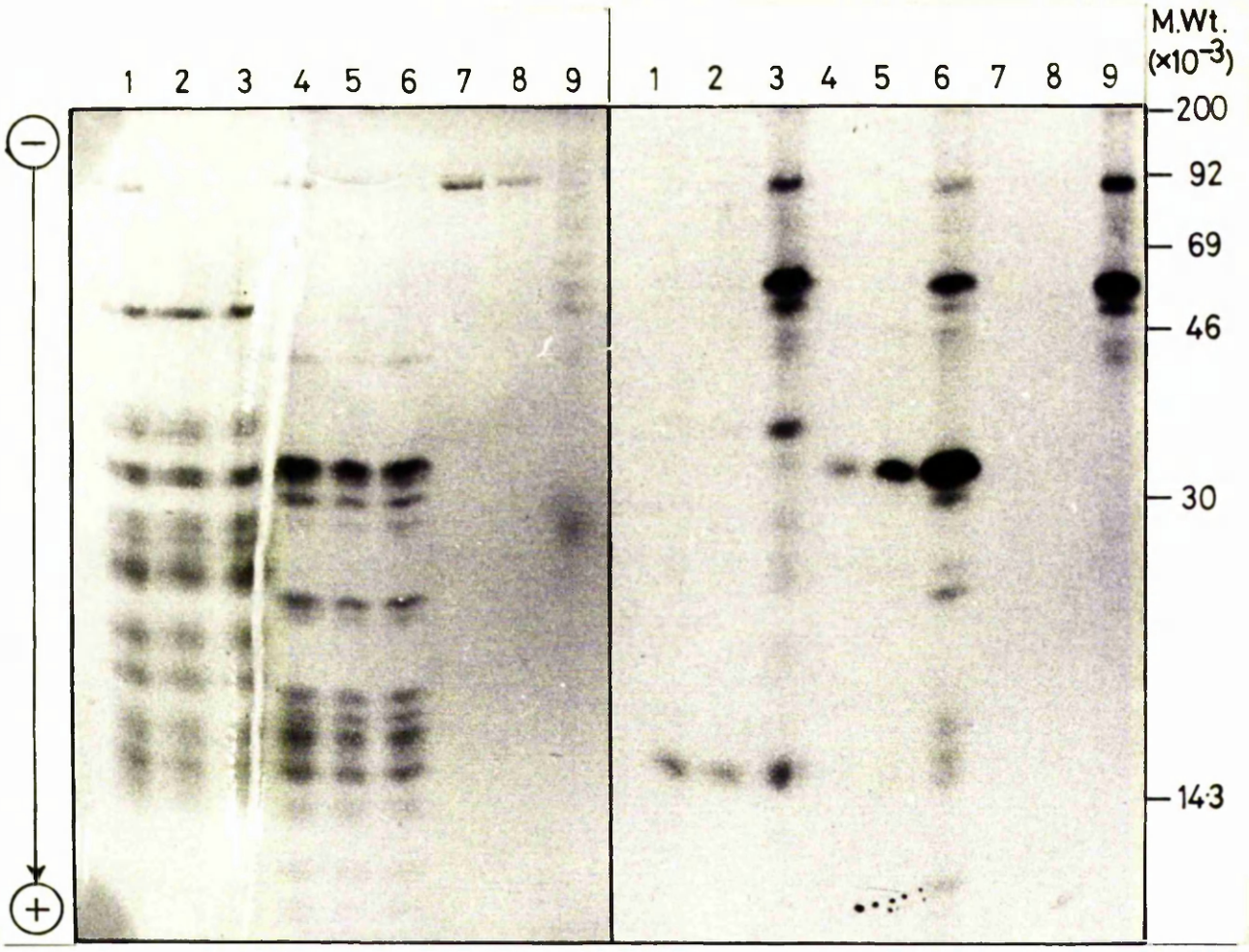
Track No.	1	2	3	4	5	6	7	8	9
Ribosomal Protein	60S	60S	60S	40S	40S	40S	-	-	-
Enzyme	PKI	PKII	bovine heart kinase	PKI	PKII	bovine heart kinase	PKI	PKII	bovine heart kinase

Extracted ribosomal protein was phosphorylated as described (2.3.15.2). The phosphorylation reaction mixture contained approximately 200μg of protein and either 5μg of PKI (1 unit) or 5μg of PKII (1 unit) rabbit skeletal muscle protein kinases, or 50μg of bovine heart protein kinase (100 units) in a volume of 250μl. Samples of approximately 50μg of phosphorylated protein were resolved by one-dimensional SDS slab gel electrophoresis, exposed to autoradiography.

A. Stain

B. Autoradiograph

Major Phosphoproteins (Approximate Molecular Weight)	Track No.
13,500	1 - 3
32,000	4 - 6
37,000	3
57,000	3,6,9
90,000	3,6,9


1 2 3 4 5 6 7 8 9
1 2 3 4 5 6 7 8 9
M.Wt.
(×10⁻³)
200
92
69
46
30
14·3
−
+
A
B

phosphoprotein of molecular weight 37,000 can be seen when bovine heart kinase was used; this enzyme also phosphorylates two proteins of 57,000 and 90,000 regardless of the substrate present.

3.6 Properties of Protein Kinases from Ascites Cytosol

General characteristics of the protein kinase activities present in ascites cytosol were examined.

3.6.1 Effect of Enzyme Concentration on Protein Kinase Activity

A variety of concentrations of S-150 fraction, pooled kinases purified by DEAE-cellulose chromatography, and CKI and CKII purified by phosphocellulose chromatography were assayed for incorporation of ^{32}P into casein (Fig 3.31). All experiments involving these kinase fractions (especially those used for calculation of the specific activities in Table 3.2) used enzyme concentrations in the ranges shown in these figures.

3.6.2 Effect of pH on Protein Kinase Activity

The optimal pH conditions for the various enzyme activities was determined in the range pH 5 to 9 (Fig 3.32). The optimal pH ranges were quite similar : 6.2 - 7.3 for CKI, 6.5 - 7.6 for CKII, and 6.5 - 7.7 for HK.

3.6.3 Effect of Magnesium and Potassium Concentrations on the Activities of CKI and CKII

The dependence of the enzyme activities of CKI and CKII on KCl and $MgCl_2$ concentration was determined (Fig 3.33). CKI had optima (i.e. 80 - 100% of maximum activity) for KCl in the range 80 - 200mM, and for $MgCl_2$ in the range 10 - 70mM. Optima for CKII were 100 - 250mM with KCl and 0 - 42mM for $MgCl_2$.

Fig 3.31 : Effect of Enzyme Concentration on the Activity of Protein Kinase Fractions

A number of concentrations of protein from either crude 'S-150', DEAE-cellulose purified CKI, or CKII were assayed for ^{32}P incorporation into casein, using γ-^{32}P ATP as phosphoryl donor (2.3.15.1).

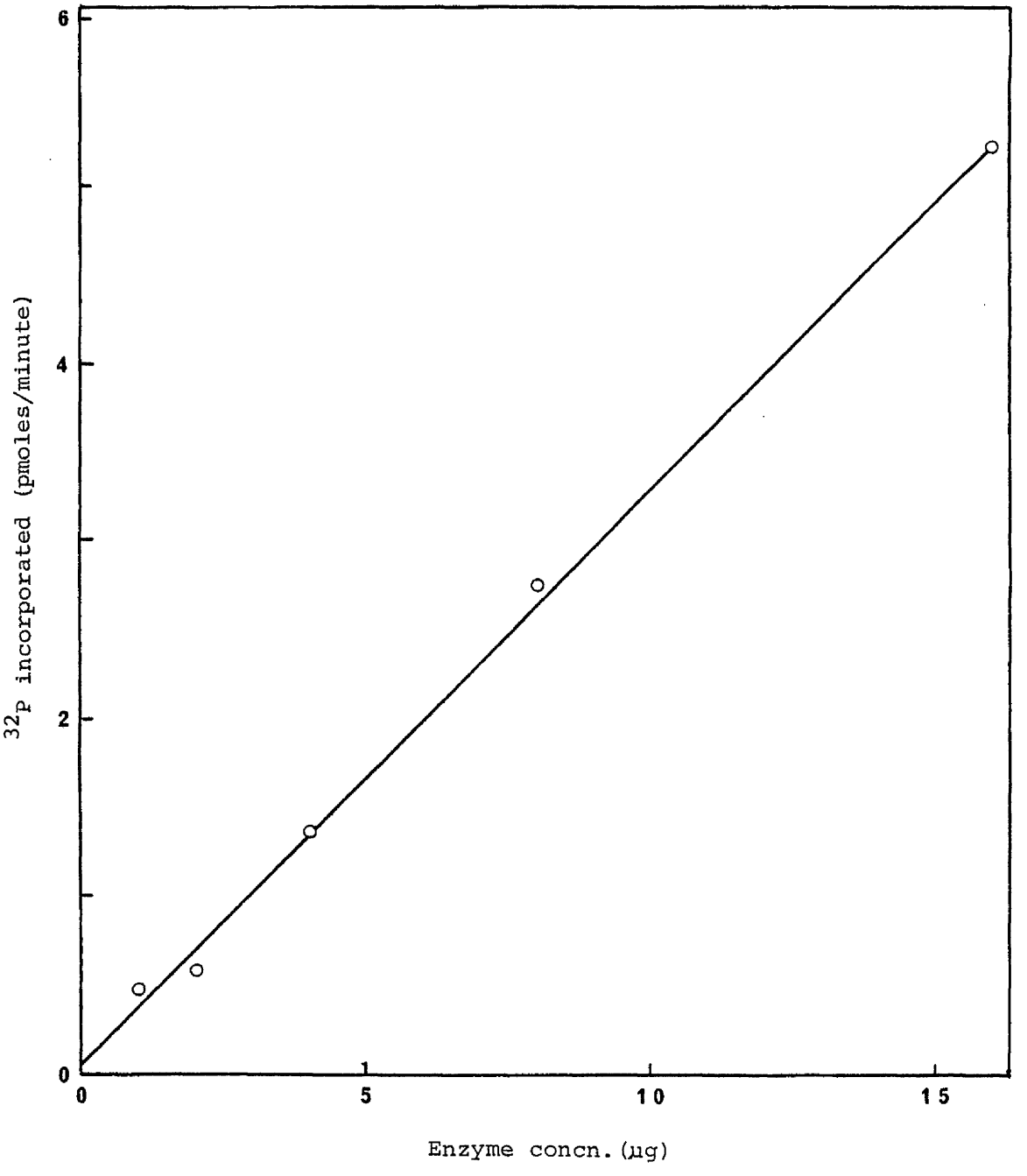
a. 'S-150' protein

b. DEAE-cell protein

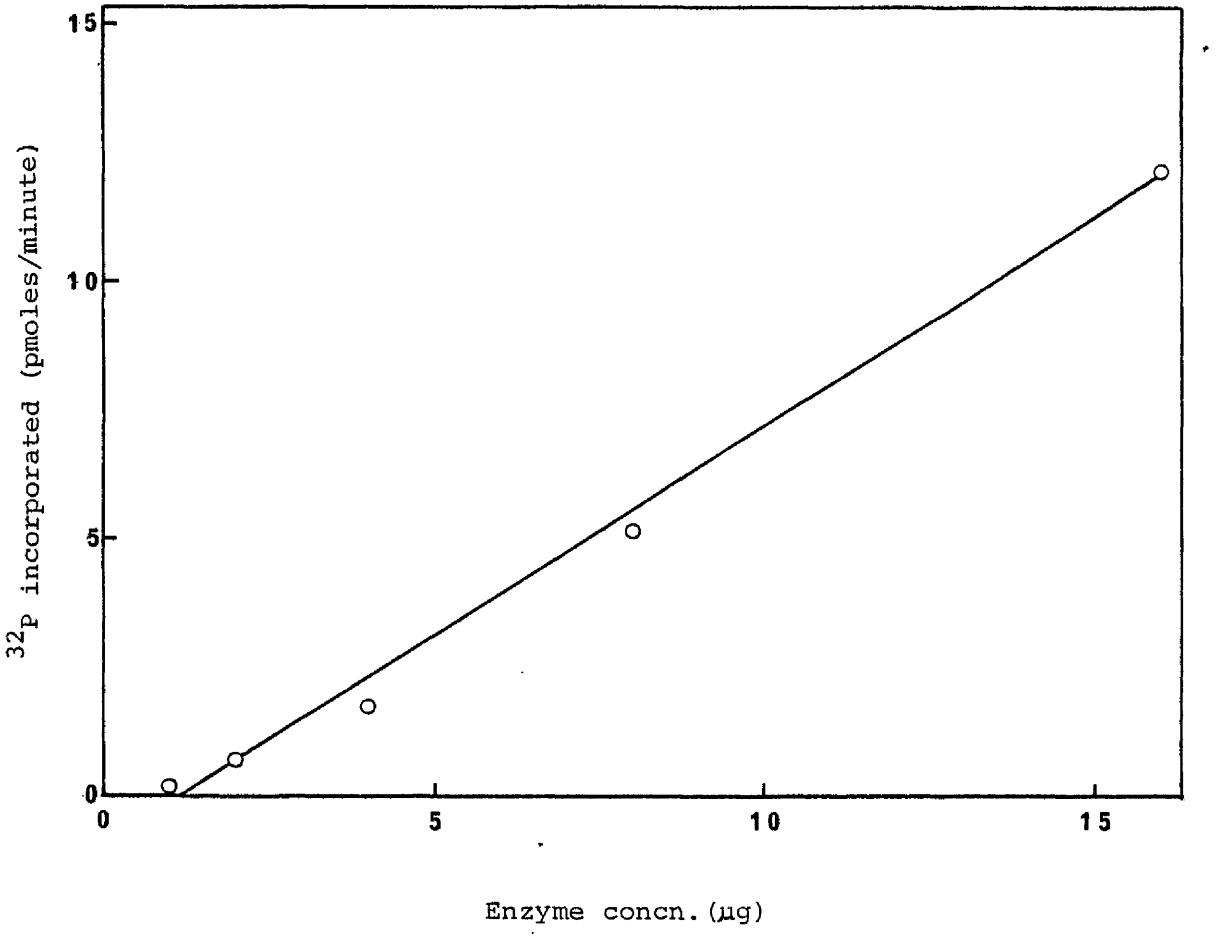
c. CKI protein

d. CKII protein

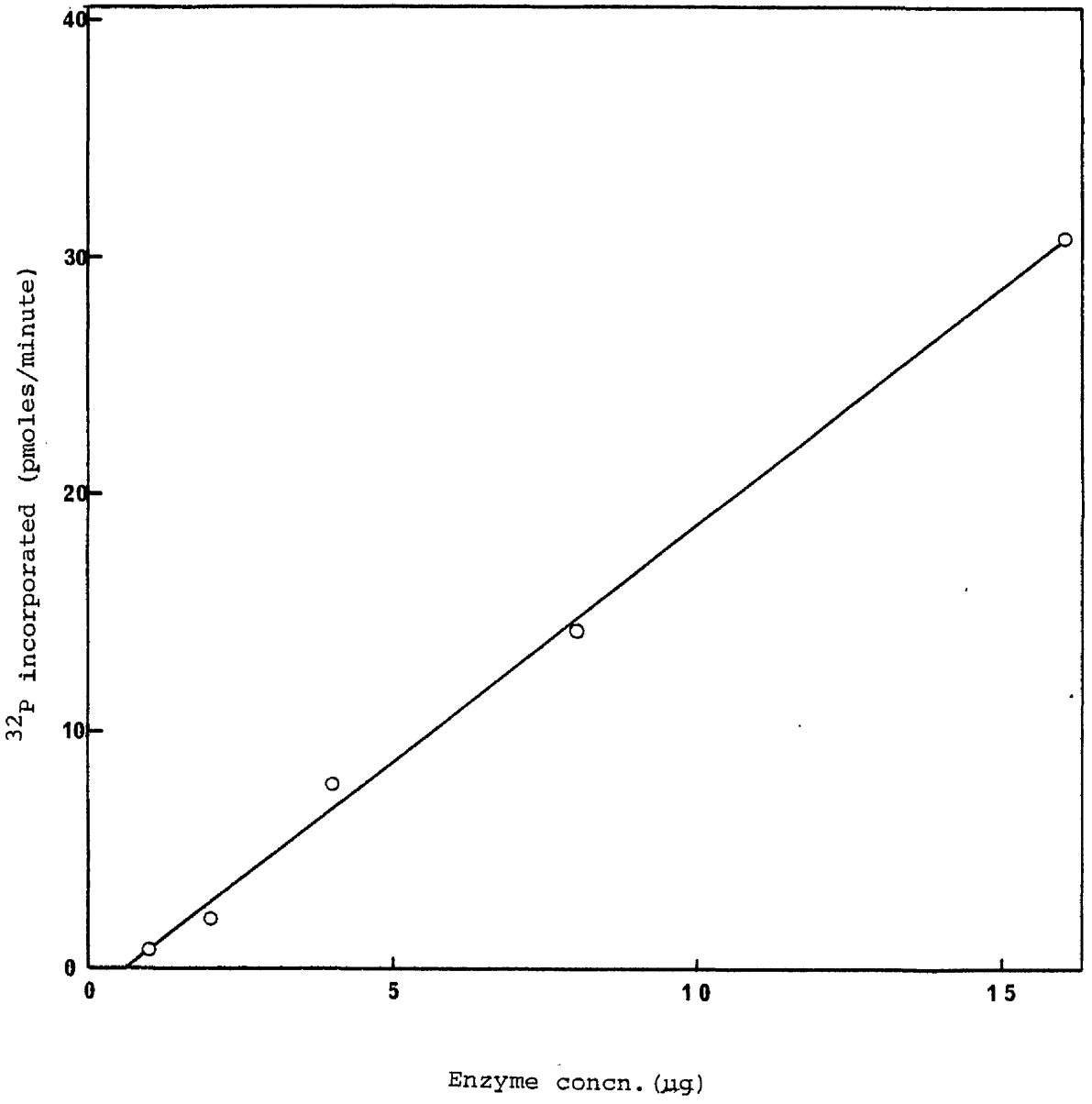
a.


6
4
2
0
0
5
10
15
^{32}P incorporated (pmoles/minute)
Enzyme concn. (µg)

b.


15
10
5
0
0
5
10
15
^{32}P incorporated (pmoles/minute)
Enzyme concn. (µg)

c.


40
30
20
10
0
0
5
10
15
^{32}P incorporated (pmoles/minute)
Enzyme concn. (µg)

d.

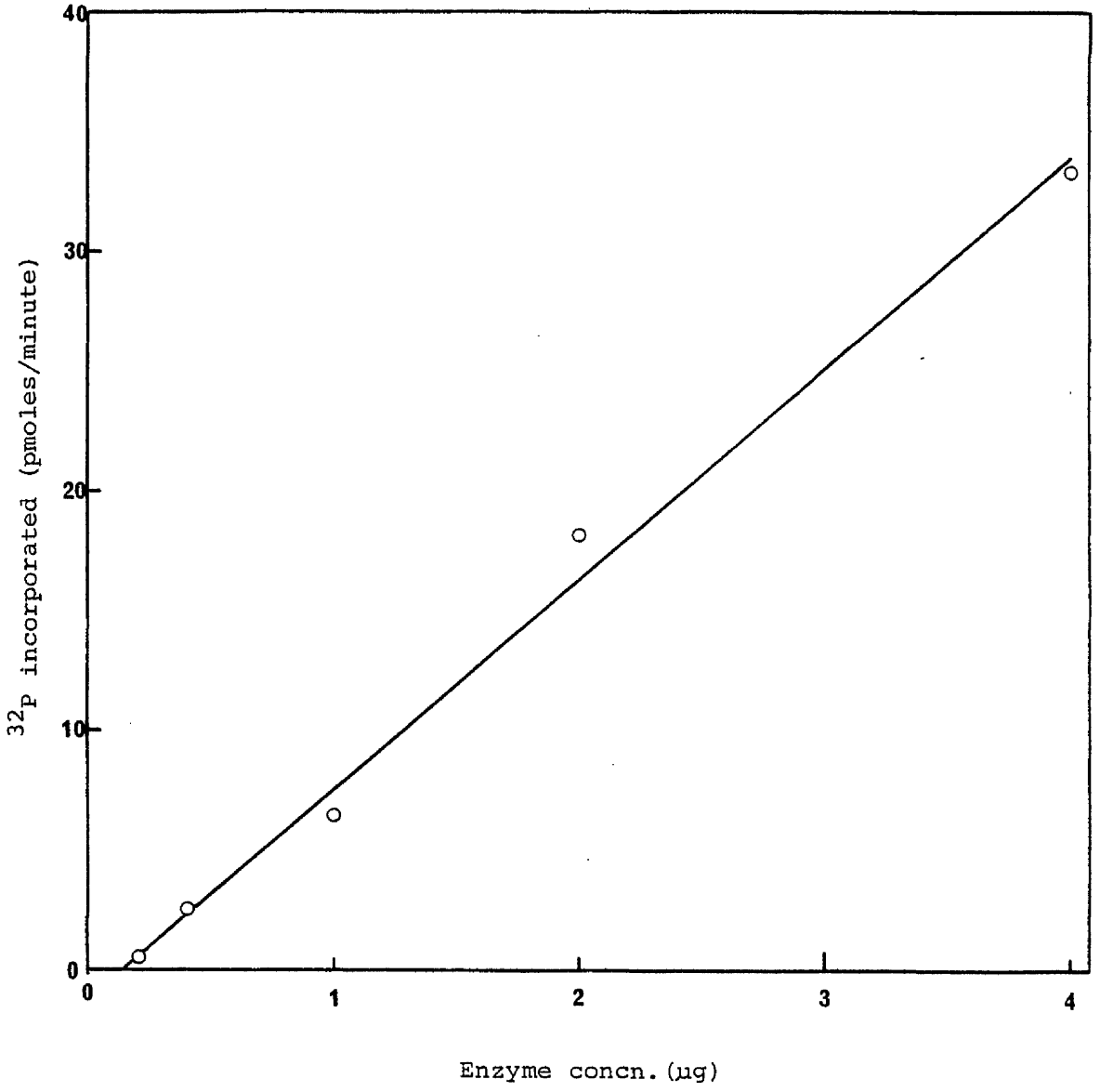

40
30
20
10
0
0
1
2
3
4
^{32}P incorporated (pmoles/minute)
Enzyme concn. (µg)

Fig 3.32 : Effect of pH on the Protein Kinase Activity of CKI, CKII or HK.

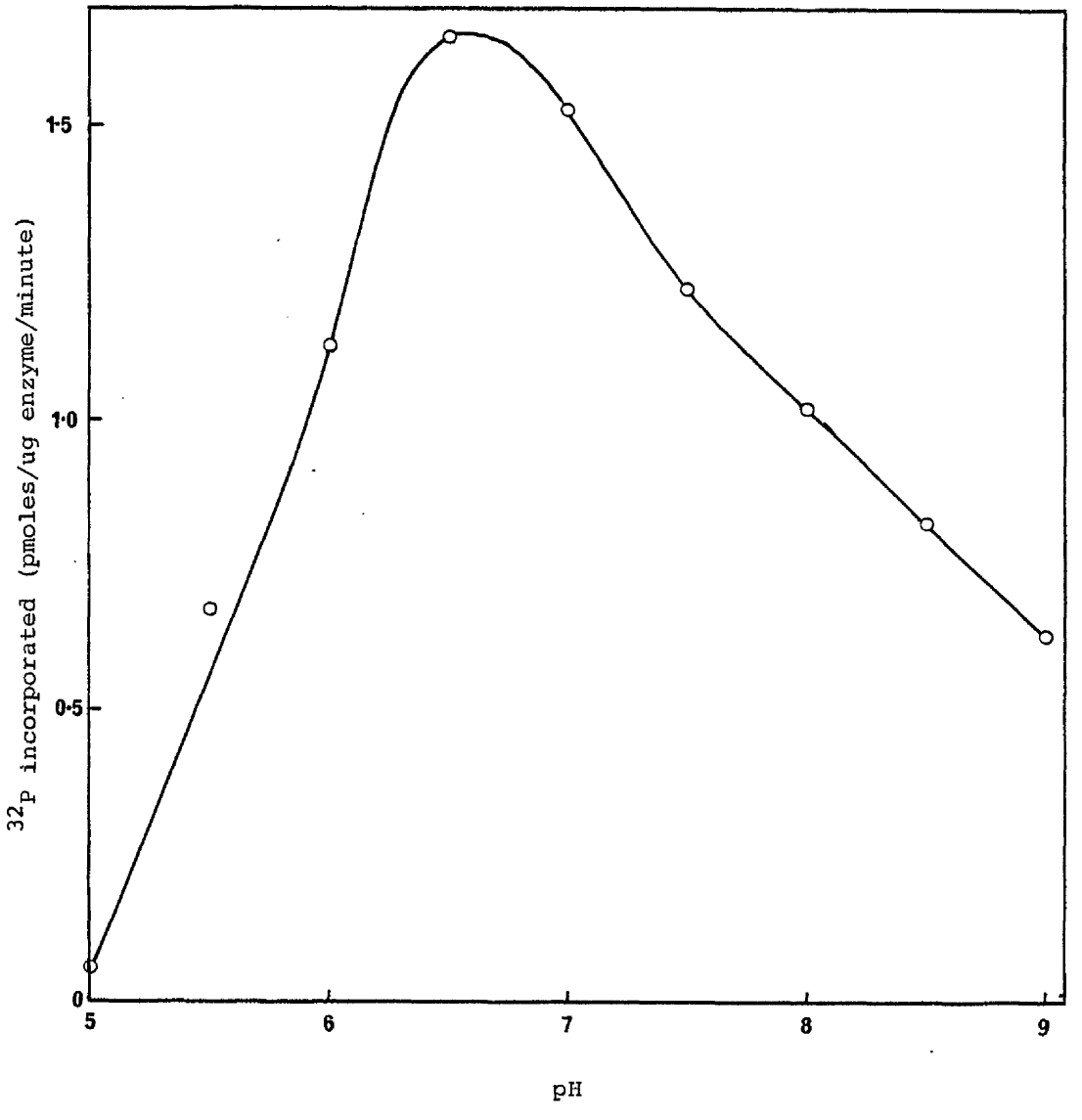
Protein kinase assays were carried out at between pH 5 - 9, using casein and ATP for CKI and CKII, and histone and ATP for HK.

a. CKI

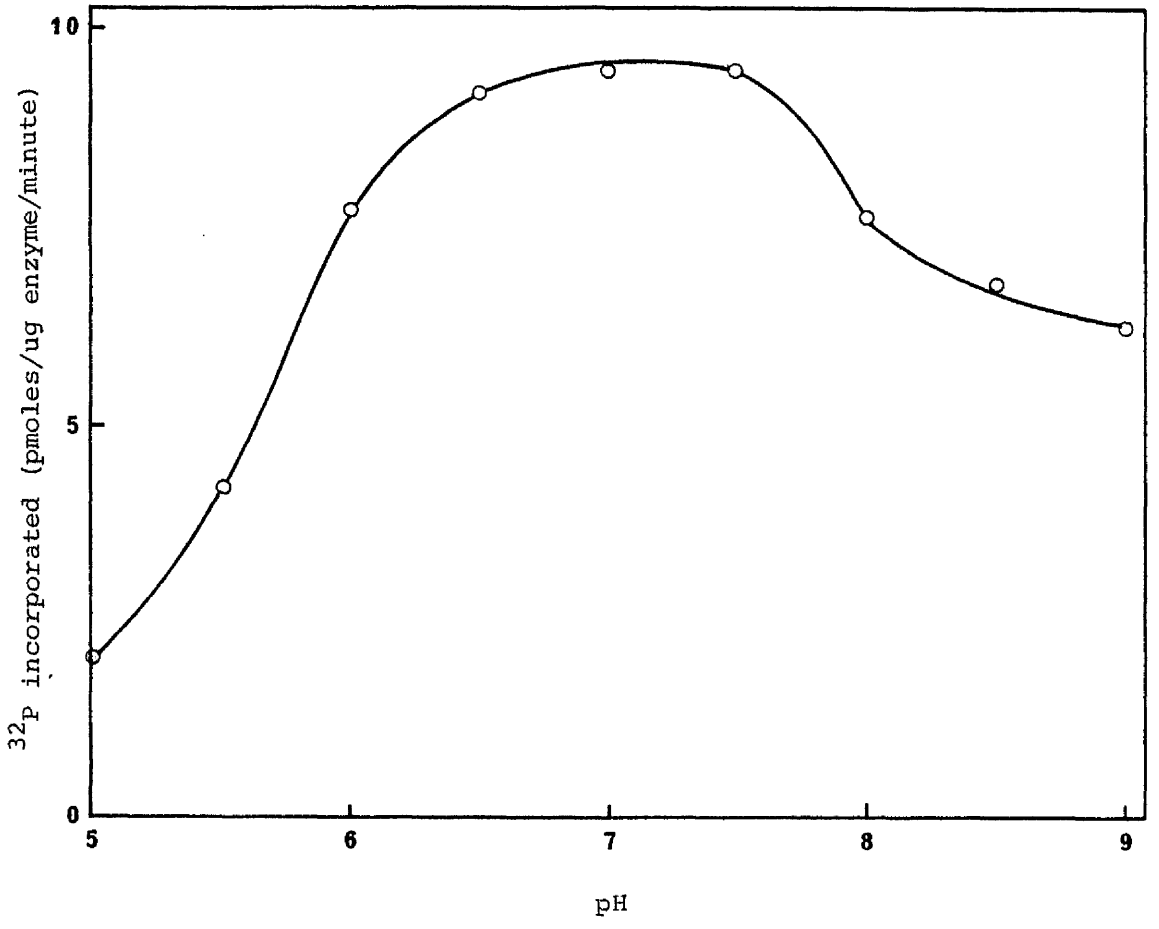
b. CKII

c. HK

a.



b.



C.

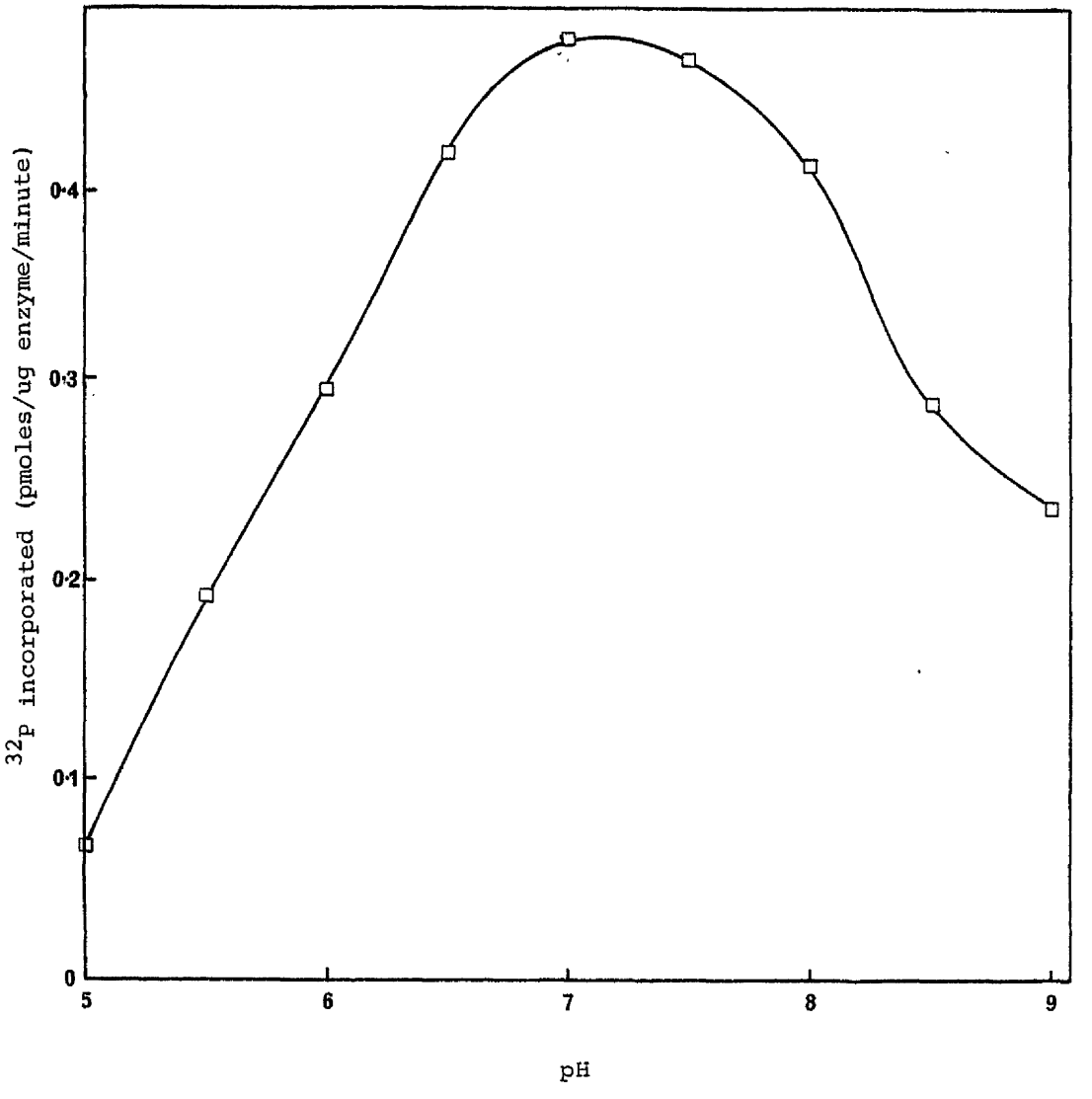
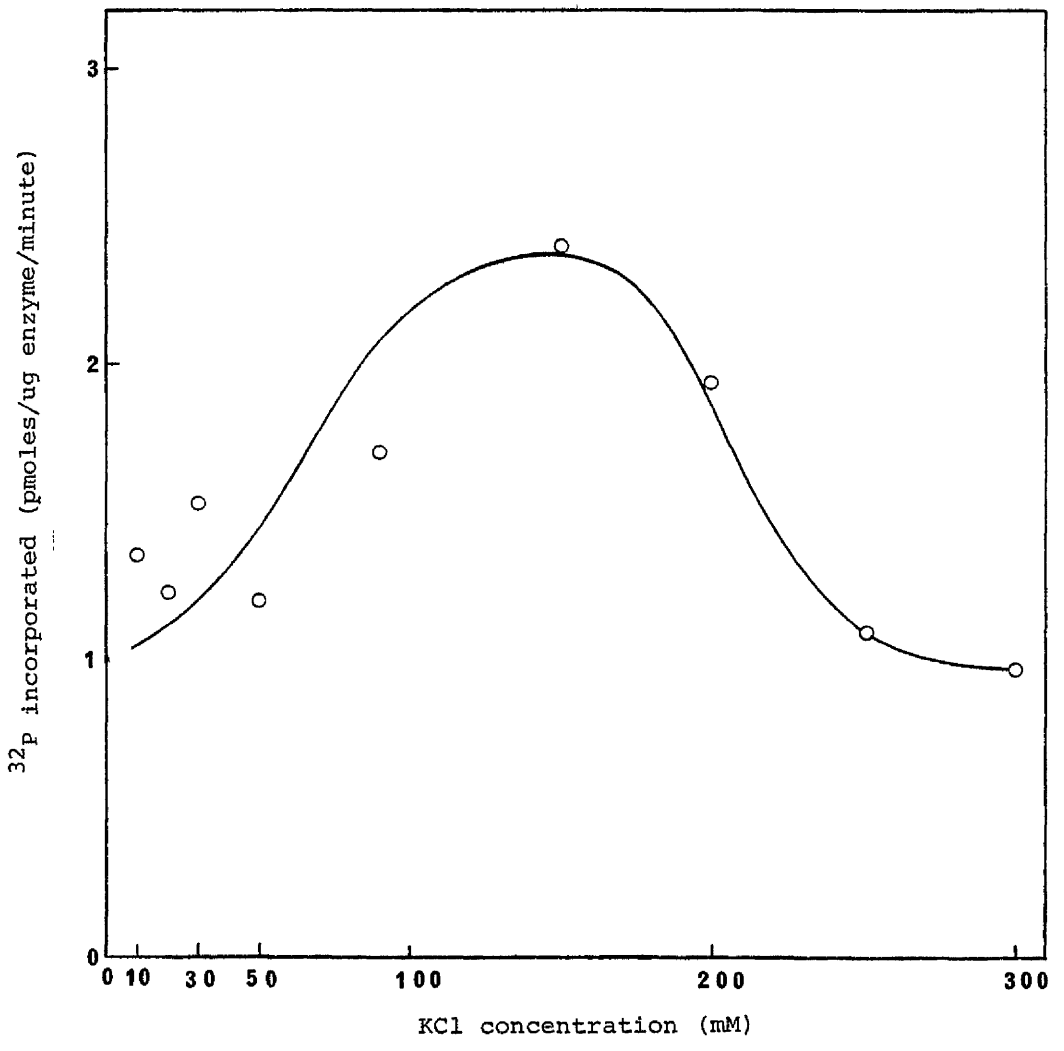

^{32}P incorporated (pmoles/ug enzyme/minute)
0·4
0·3
0·2
0·1
0
5
6
7
8
9
pH

Fig 3.33: Effect of Potassium and Magnesium Concentration on Activity of CKI and CKII

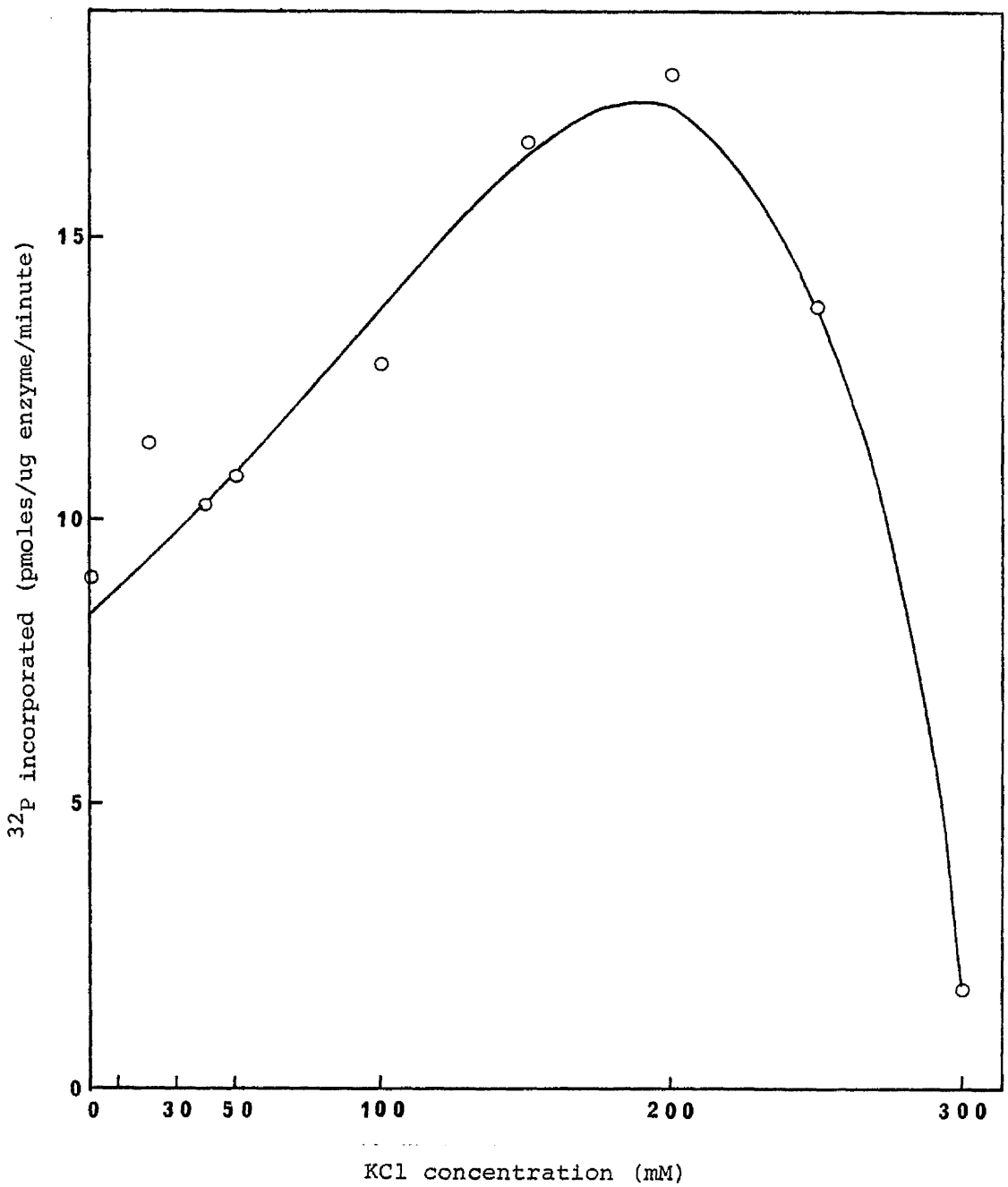
Protein kinase assays were performed as described in 2.3.15.1, except that either the potassium chloride, or magnesium chloride concentration was varied, as shown in the figures. Casein was used as substrate, and ATP as phosphoryl donor.

a. Activity of CKI vs KCl concentration
b. Activity of CKII vs KCl concentration
c. Activity of CKI vs $MgCl_2$ concentration
d. Activity of CKII vs $MgCl_2$ concentration

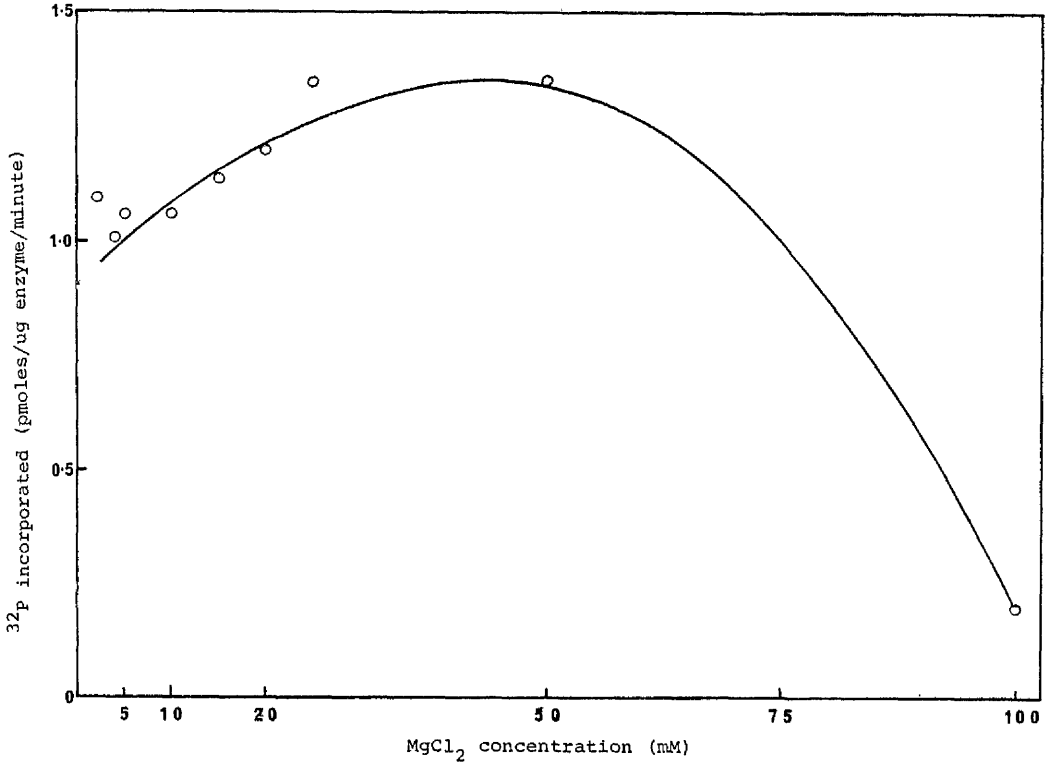
a.


3
2
1
0
0 10 30 50 100 200 300
KCl concentration (mM)
32P incorporated (pmoles/ug enzyme/minute)

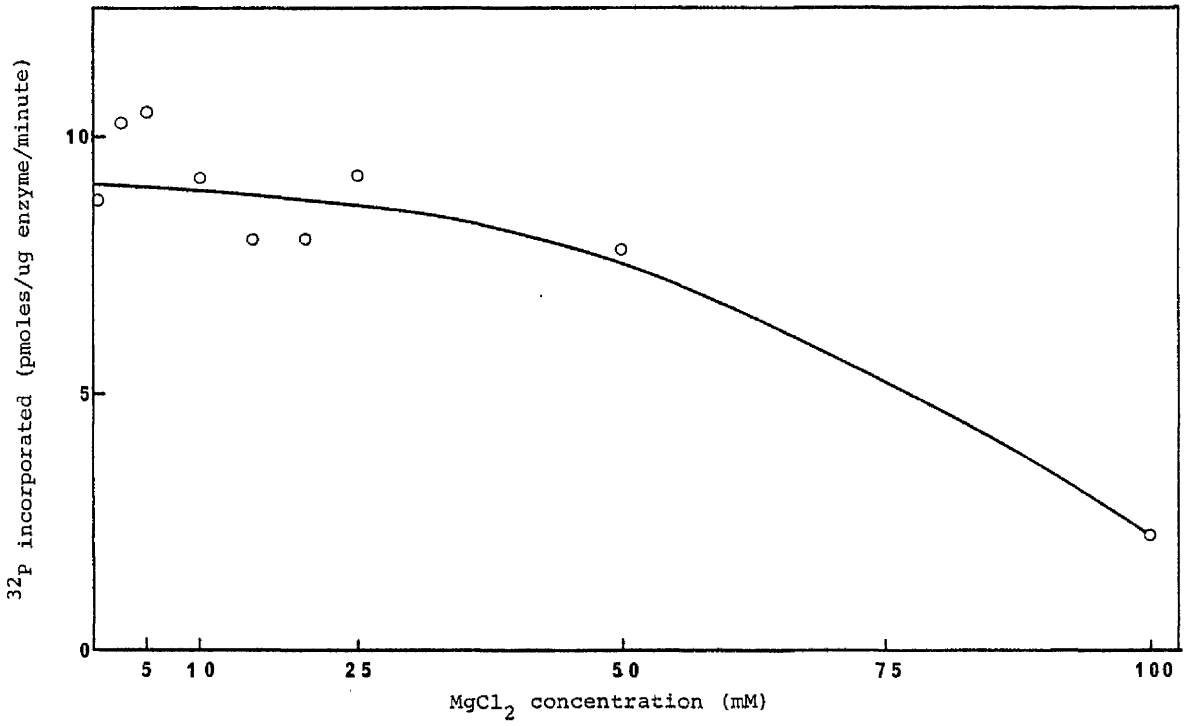
b.


15
10
5
0
^{32}P incorporated (pmoles/ug enzyme/minute)
0
30
50
100
200
300
KCl concentration (mM)

C.


1·5
1·0
0·5
0
5
10
20
50
75
100
^{32}P incorporated (pmoles/ug enzyme/minute)
$MgCl_2$ concentration (mM)

d.


^{32}P incorporated (pmoles/ug enzyme/minute)
10
5
0
5
10
25
50
75
100
$MgCl_2$ concentration (mM)

3.6.4 Effect of Incubation Time on Activities of CKI and CKII

The activities of CKI and CKII with time were determined at two ATP concentrations (Fig 3.34). Both CKI and CKII were found to incorporate ^{32}P linearly over the range 0 - 20 minutes at 100μM and 10μM,ATP, when 10μg of CKI or 3.4μg of CKII were used.

3.6.5 Determination of the Michaelis-Menten Constants for CKI and CKII, using Casein and ATP as Substrates

The apparent K_m values of CKI and CKII for casein and ATP were obtained from the initial velocities (at 2 minutes of reaction) of both enzymic reactions over a range of concentrations of ATP. Lineweaver-Burke plots of the data were made (Fig 3.35), and the apparent K_m values were found to be 14.0μM for CKI and 9.0μM for CKII.

3.6.6 Effect of Cyclic AMP on Protein Kinase Activities

As indicated in Fig 3.9 incorporation of ^{32}P into histone by HK was not stimulated by the presence of 10μM cAMP. Further analysis of the effect of cAMP on HK, CKI and CKII (Table 3.3) indicated that none of these enzyme activities was stimulated by cAMP.

3.6.7 Effect of Cyclic AMP Dependent Protein Kinase Inhibitor Protein on Purified 'S-150' Protein Kinases.

Cyclic AMP dependent protein kinase inhibitor protein (cAMP-PrKI) was prepared according to Walsh et al., (1971)

Fig 3.34 : Effect of Incubation Time on Activities of CKI and CKII.

Protein kinase assays were performed with CKI and CKII at two concentrations of ATP with casein as substrate (2.3.15.1).

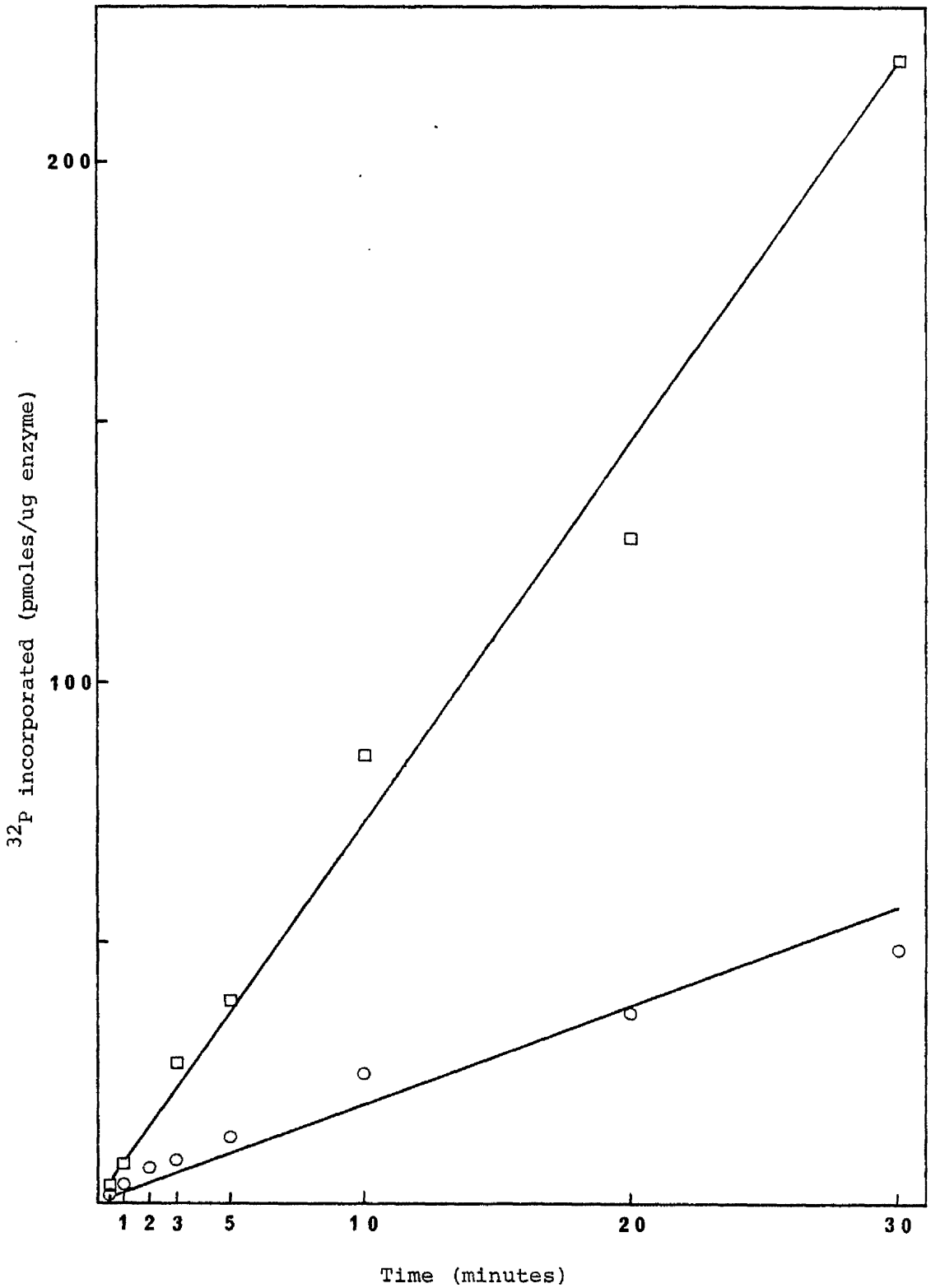
100μM ATP

10μM ATP

a. CKI

b. CKII

a.


200
100
^{32}P incorporated (pmoles/ug enzyme)
1 2 3 5 10 20 30
Time (minutes)

b.

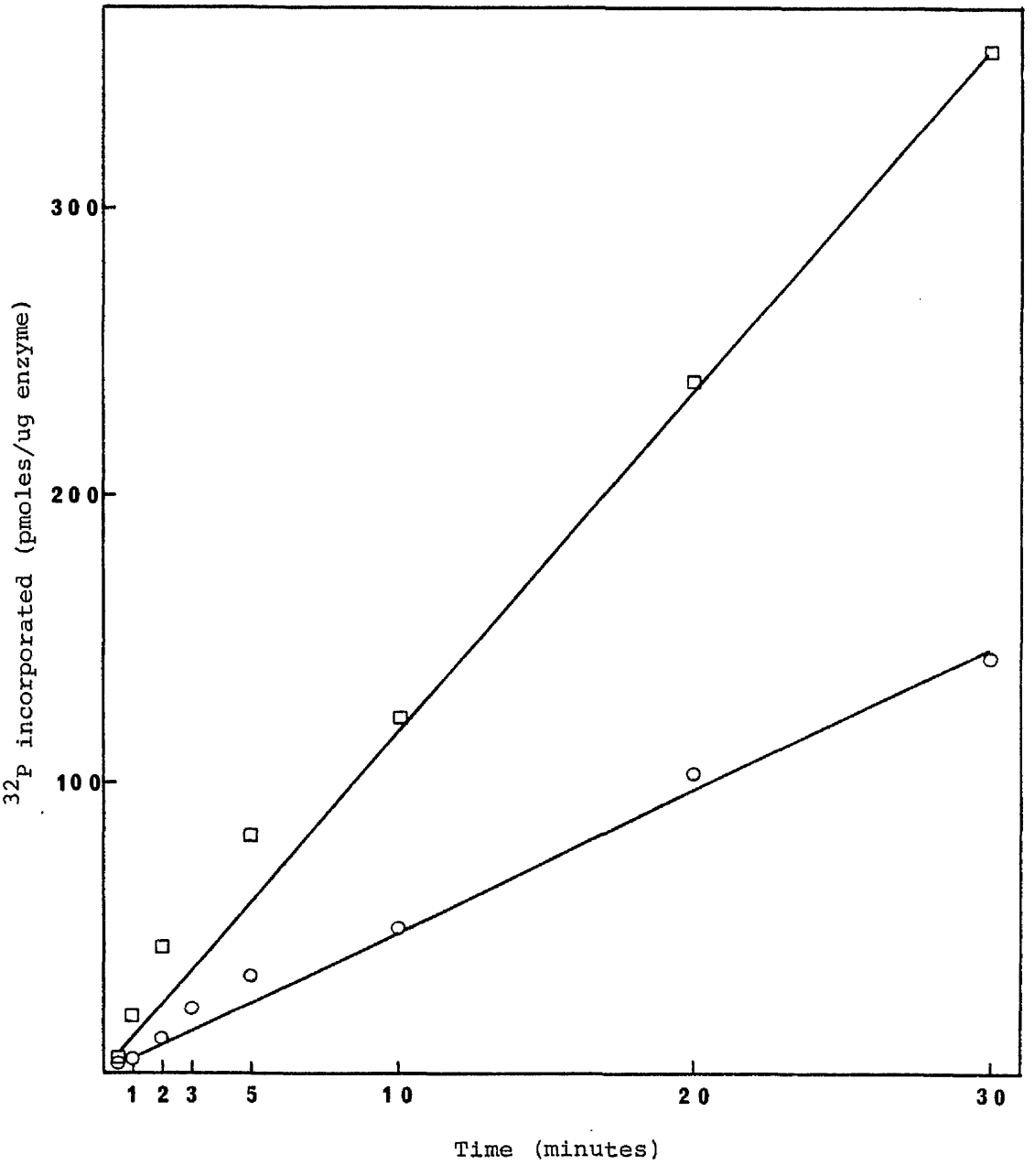

300
200
100
^{32}P incorporated (pmoles/ug enzyme)
1 2 3 5 10 20 30
Time (minutes)

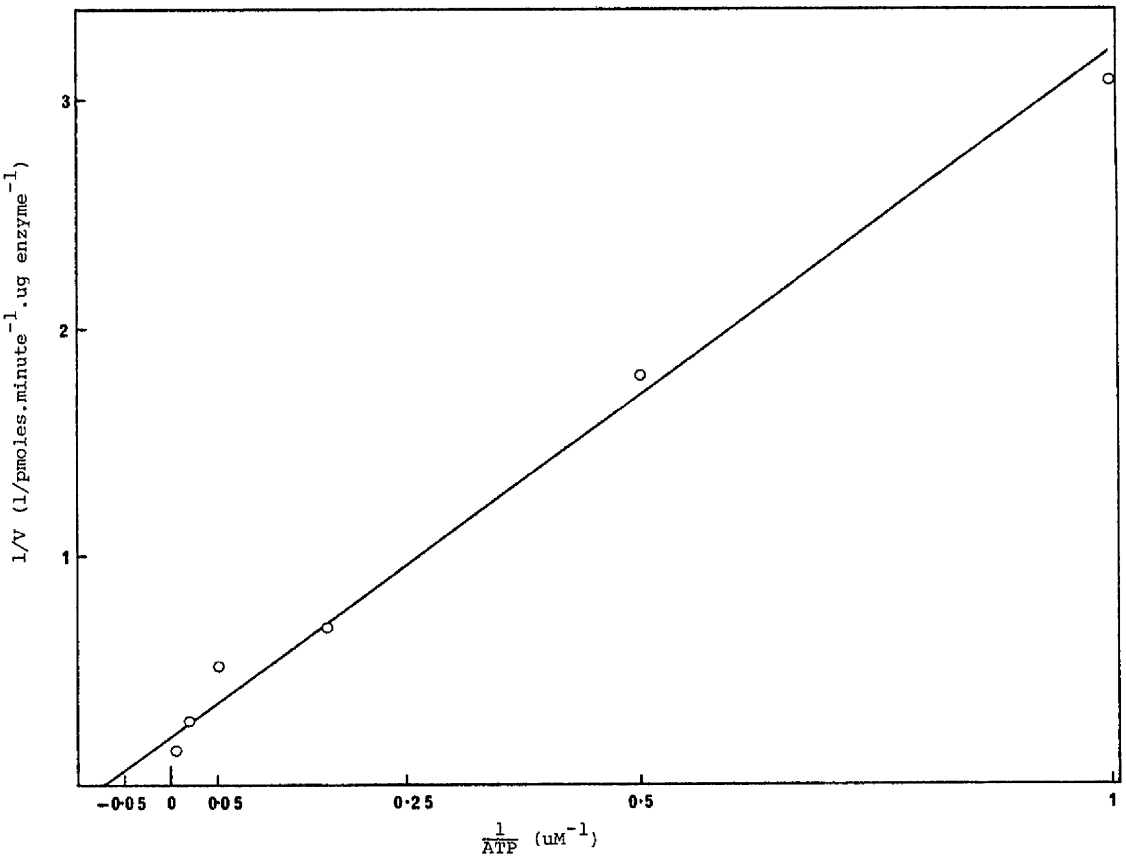
Fig 3.35 : Lineweaver-Burke Plots of CKI and CKII using Casein and ATP

The initial velocities of reaction were obtained at a variety of ATP concentrations. The reciprocals of the velocities (pmoles/minute/μg enzyme) were plotted against the reciprocals of ATP concentrations.

a. CKI Apparent K_m =14.0μM

b. CKII Apparent K_m = 9.0μM

a.


3
2
1
1/V (1/pmoles.minute^{-1}.ug enzyme^{-1})
-0·05
0
0·05
0·25
0·5
1
$\frac{1}{ATP}$ (uM^{-1})

b.

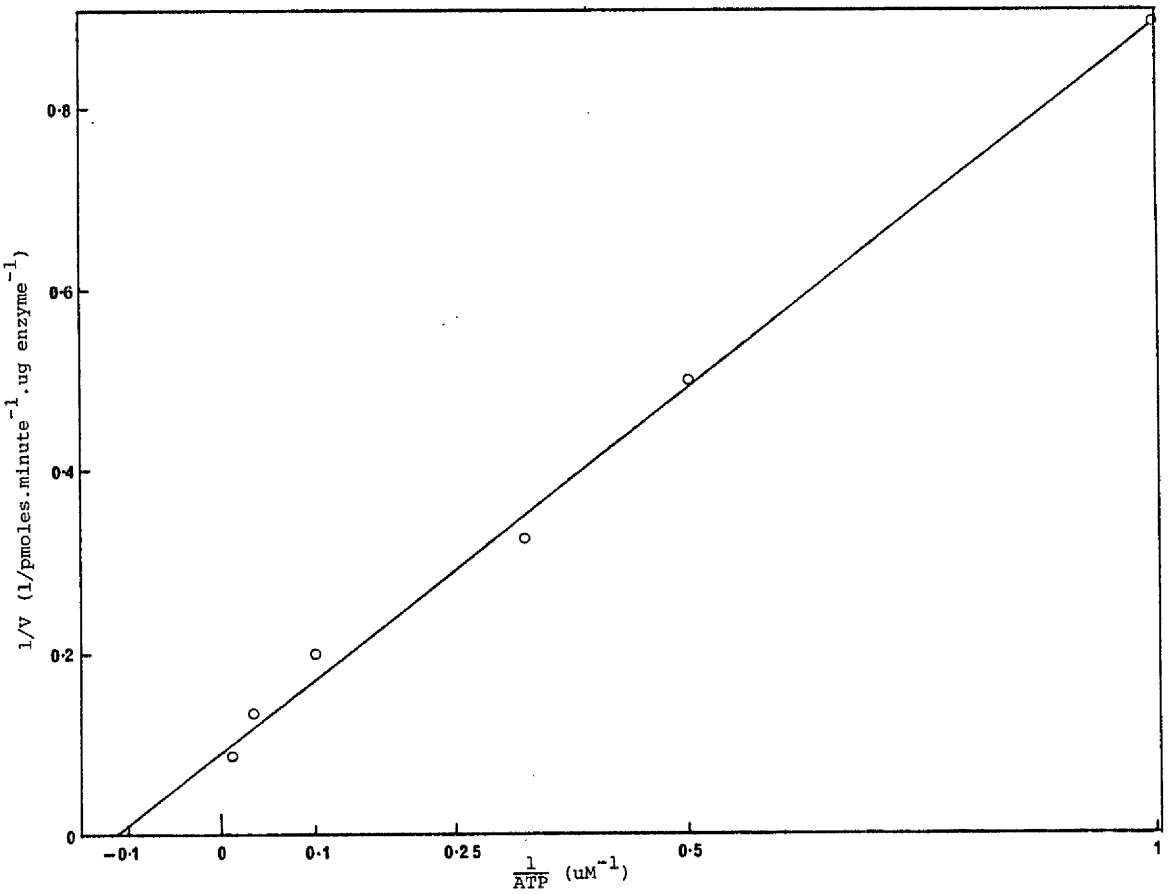

0·8
0·6
0·4
0·2
0
1/V (1/pmoles.minute^{-1}.ug enzyme^{-1})
−0·1
0
0·1
0·25
0·5
1
$\frac{1}{ATP}$ (uM^{-1})

Table 3.3 : Effect of Cyclic AMP on Resolved Protein Kinase Activities

Enzyme	^{32}P Incorporated (pmoles/min/μg enzyme)	
	+cAMP	-cAMP
CKI	2.4	2.4
CKII	12.6	12.6
HK	0.2	0.2

Protein kinase assays were performed for CKI, CKII and HK in the presence or absence of 10μm cAMP (2.3.15.1). Casein and ATP were substrates for CKI and CKII. Histone and ATP were substrates for HK.

as described in 2.3.14 and its effect on CKI, CKII and HK was examined. Two known cyclic AMP-dependent protein kinases (PKI and PKII rabbit skeletal muscle protein kinases of Reimann et al., (1971), see 2.3.13) were included as controls in the same experiment. As seen from Table 3.4 neither CKI, CKII or HK were inhibited by this inhibitor protein whereas the activities of PKI and PKII were reduced by 84% and 83% respectively.

3.6.8 Effect of Calmodulin on Protein Kinase Activity

The effect of calmodulin (Cheung, 1970) on CKI, CKII and HK was tested. Neither CKI nor CKII appeared to be significantly effected by the presence of calmodulin at either 5 or 50μg/ml (Table 3.5). HK activity however showed stimulation of approximately four-fold in the presence of 50μg/ml calmodulin.

3.6.9 Effect of Casein Kinase Inhibitor Protein 'CKGI' on Protein Kinase Activity

Inhibitor protein, 'CKGI', (Job et al., 1979) was examined for inhibitory effect on CKI, CKII, and HK. This inhibitor protein, described by Job et al., (1979), selectively inhibited casein kinases similar to CKII, i.e. kinases that use either ATP or GTP. The sample of 'CKGI' obtained (2.1.1) was stated to have an activity such that a 1:300 dilution of this material would give 50% inhibition of susceptible casein kinase from bovine adrenal cortex. A range of dilutions of this material was tested with CKI, CKII, and HK. Inhibition of approximately 29% was observed with CKII but CKI and HK were uneffected (Table 3.6).

Table 3.4 : Effect of cAMP-Dependent Protein Kinase Inhibitor Protein on Protein Kinases.

Enzyme	^{32}P-Incorporated (pmoles/min/μg enzyme)		
	Inhibitor Protein (μg/assay)		
	0	16	160
CKI	2.4 (100)	2.4 (100)	2.6 (100)
CKII	12.6 (100)	12.6 (100)	12.7 (101)
HK	0.2 (100)	0.2 (100)	0.2 (100)
PKI	1.2 (100)	N.D.	0.19(16)
PKII	1.74(100)	N.D.	0.29(17)

Protein kinase assays were carried out as described (2.3.15.1). Casein and ATP were substrates for CKI and CKII. Histone and ATP were substrates for HK, PKI and PKII. Figures in parenthesis are the percentages of activity in the absence of inhibitor.

Table 3.5 : Effect of Calmodulin on Resolved Protein Kinases

Enzyme	^{32}P Incorporated (pmoles/min/μg enzyme)			
	EGTA (1mM)	$CaCl_2$ (0.1mM)	Calmodulin/$CaCl_2$ (5μg/ml)/(0.1mM)	Calmodulin/$CaCl_2$ (50μg/ml)/(0.1mM)
CKI	2.89(100)	2.81(98)	2.92 (101)	2.92 (101)
CKII	15.6 (100)	13.9 (90)	14.7 (94)	12.2 (78)
HK	0.17(100)	0.20(116)	0.30 (178)	0.66 (395)

Protein kinase assays were performed as described in (2.3.15.1) with the additions shown in the table. Casein and ATP were substrates for CKI and CKII. Histone and ATP were substrates for HK. Figures in parenthesis represent the percentages of activity found in the presence of 1mM EGTA.

Table 3.6 : Effect of Inhibitor Protein 'CKGI' on Resolved Protein Kinases

Enzyme	^{32}P Incorporated (pmoles/min/μg enzyme)			
	'CKGI' (Dilution of Standard Material c.f. 3.6.9)			No 'CKGI'
	1:300	1:100	1:50	
CKI	2.5 (105)	2.45 (102)	2.8 (116)	2.4 (100)
CKII	12.2 (96)	10.2 (81)	8.9 (70)	12.6 (100)
HK	0.18 (90)	0.22 (108)	0.22 (108)	0.20(100)

Protein kinase assays were performed as described in (2.3.15.1) with addition of 'CKGI' as indicated in the table. Casein and ATP were substrates for CKI and CKII. Histone and ATP were substrates for HK. Figures in parenthesis represent percentages of control experiments performed in the absence of 'CKGI'.

3.6.10 Effect of Heparin on Protein Kinase Activity

Heparin has been reported to selectively inhibit protein kinases similar to CKII (Hathaway, Lubben and Traugh, 1980). Protein kinase assays of CKI and CKII with casein and ATP, and HK with histone and ATP, were carried out in the presence of heparin (Table 3.7). CKI showed a 60% inhibition in the presence of heparin at 40 units/ml, and 80% inhibition with 400 units/ml of heparin. CKII showed 93% inhibition at 40 units/ml and 94.5% at 400 units/ml. Conversely HK appeared to be stimulated by the presence of heparin showing a maximum of 5-fold stimulation with 40 units/ml.

3.6.11 Self-Phosphorylation of Protein Kinases

It has been reported (e.g. Hathaway and Traugh, 1979) that various protein kinases, including cAMP-independent casein kinases, could phosphorylate themselves. To investigate this possibility, samples of the three ascites protein kinase activities were incubated in the standard phosphorylation mixture (2.3.15.1) in the presence of $[\gamma\text{-}^{32}P]$ ATP, but in the absence of exogenous protein substrates. After the reaction was terminated, the samples were prepared for one-dimensional SDS gel electrophoresis, (2.3.17) and electrophoresis performed (Fig 3.36). On the stained gel bands can only be seen in tracks 3 and 6 corresponding to HK at 60μg and 12μg. Neither CKI, nor CKII had any stained material. The autoradiograph of this gel shows only a faint blackening at the top of track 3, but it is not clear which bands are phosphorylated. This experiment does not show self-phosphorylation of

Table 3.7 : Effect of Heparin on Purified Protein Kinase

Enzyme	^{32}P Incorporated (pmoles/min/μg enzyme)			
	Heparin (units/assay)			
	0	10	40	100
CKI	2.4 (100)	0.96 (40)	0.51 (21)	0.32 (13)
CKII	12.4 (100)	0.9 (7)	0.78 (6)	0.68 (6)
HK	0.2 (100)	1.0 (500)	0.64 (320)	0.66 (330)

Protein kinase assays were performed as described in (2.3.15.1) with addition of heparin as indicated in the table. Casein and ATP were substrates for CKI and CKII. Histone and ATP were substrates for HK. Figures in parenthesis represent percentages of the activity found in the absence of heparin.

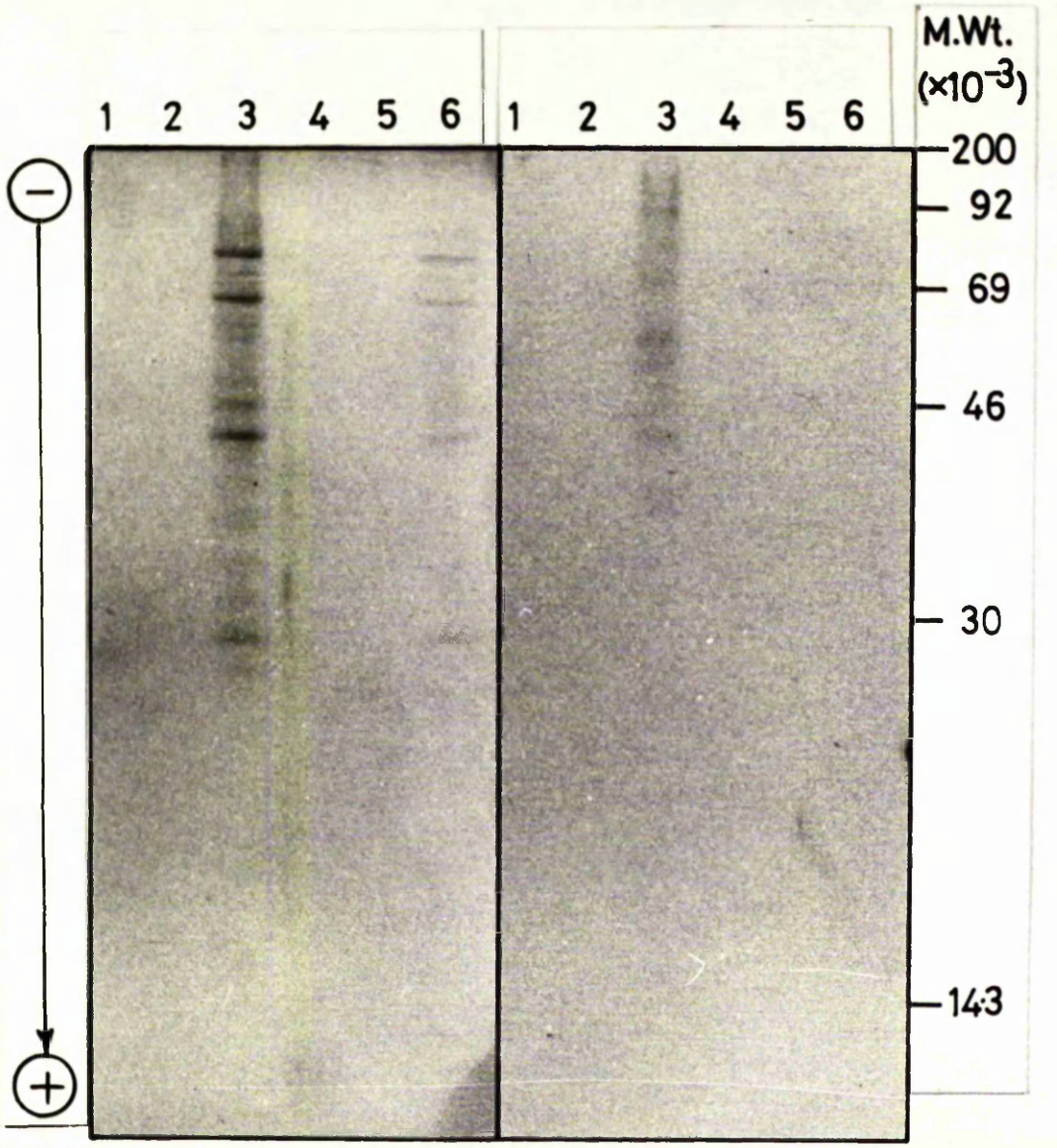
Fig 3.36 : Self-Incorporation of Phosphate by Purified Ascites Protein Kinases

Fractions of Protein kinases CKI, CKII and HK were incubated in the phosphorylation reaction described, (2.3.15), in the absence of other protein substrates. The enzyme fractions were immediately resolved by one-dimensional SDS slab gel electrophoresis (2.3.17) and subjected to autoradiography on termination of the reactions.

A. Stain

B. Autoradiograph

Track	Sample	
1	CKI	(10μg)
2	CKII	(3.5μg)
3	HK	(60μg)
4	CKI	(2μg)
5	CKII	(0.7μg)
6	HK	(12μg)


M.Wt.
(×10⁻³)
1 2 3 4 5 6
1 2 3 4 5 6
200
92
69
46
30
143
A
B

of the ascites kinases, but it is possible that insufficient quantities of the enzyme protein were present in the preparations used.

3.7 Protein Kinase Activity in Ascites Cells Incubated in the Presence of Glucose

As described earlier (3.1.2), under conditions in which intact ascites cells are incubated with glucose *in vitro*, the phosphorylation pattern of the ribosomal proteins becomes altered. It was of interest to examine the protein kinases in cytosol from cells incubated under these conditions to see if any alterations could be detected.

3.7.1 DEAE-Cellulose Chromatography of 'S-150' Fraction

The 'S-150' fraction from ascites cells incubated for three hours in medium containing glucose was resolved on DEAE-cellulose as described (2.3.10). The elution profile of the casein and histone kinase activities appeared similar to that obtained with normal ascites cells (Fig 3.37).

3.7.2 Phosphocellulose Chromatography

The fractions containing active protein kinase from DEAE-cellulose chromatography (34 to 57) were pooled, concentrated (2.3.11), equilibrated, and re-chromatographed on phosphocellulose (2.3.12). Protein kinase assays using casein and ATP, histone and ATP, and casein and GTP were performed (Fig 3.38). As before with normal ascites fractions, casein kinase resolved into two peaks of activity; the first (eluting at 0.42 - 0.55M NaCl) used only ATP and the second (eluting at 0.61 - 0.73M NaCl) used either ATP or GTP. However, the chromatographic behaviour of histone kinase activity had become altered. Although there was histone

Fig 3.37 : DEAE-Cellulose Chromatography of 'S-150' Fraction from Ascites Cells Incubated in Medium Containing Glucose

Approximately 320mg of 'S-150' fraction (2.3.6) from ascites cells incubated for three hours in medium containing glucose (2.3.3) was chromatographed on DEAE-cellulose (2.3.10) and assayed for protein kinase activity (2.3.15.1) with,

casein and ATP ○——○

histone and ATP □——□

Protein concentration was monitored by absorbance at 280 nm ————

Concentration of KCl ————

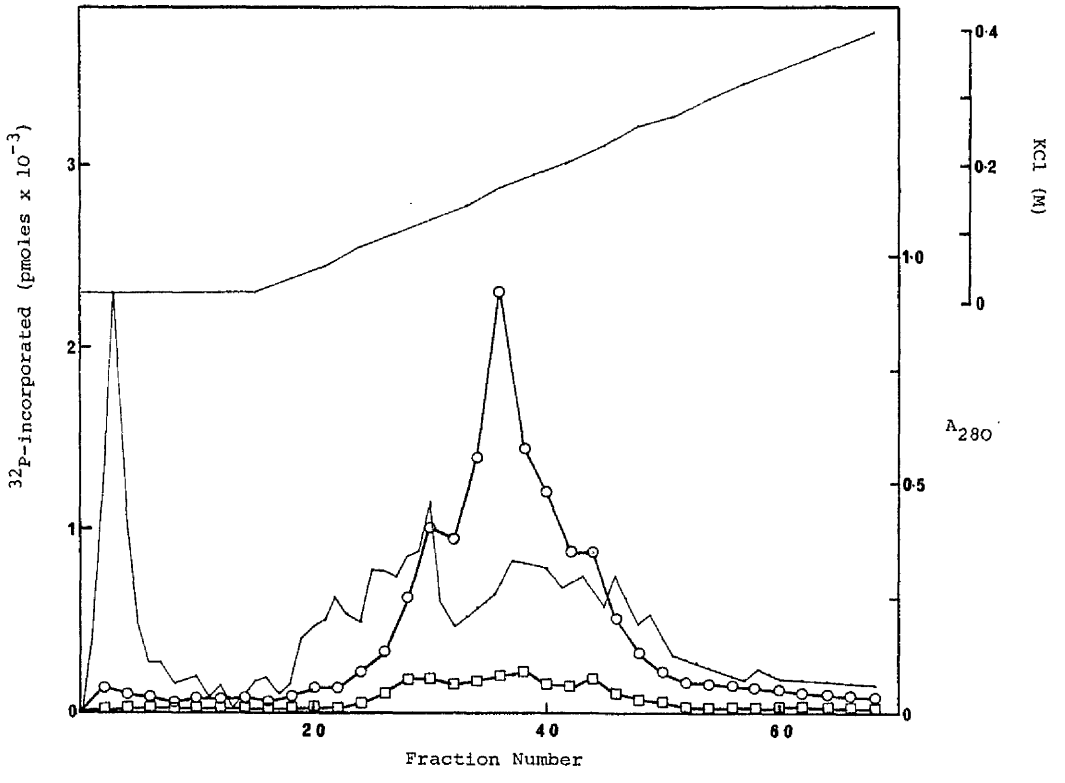

^{32}P-incorporated (pmoles x 10^{-3})
3
2
1
0
20
40
60
Fraction Number
1·0
0·5
0
A_{280}
0·4
0·2
0
KCl (M)

Fig 3.38 : Phosphocellulose Chromatography of Protein Kinase Activity from Ascites Cells Incubated in Medium Containing Glucose

Pooled fractions (30 - 56, Fig 3.37) containing approximately 51mg of protein were concentrated (2.3.11), equilibrated, and chromatographed on phosphocellulose (2.3.12). Protein kinase assays were carried out (2.3.15.1) with,

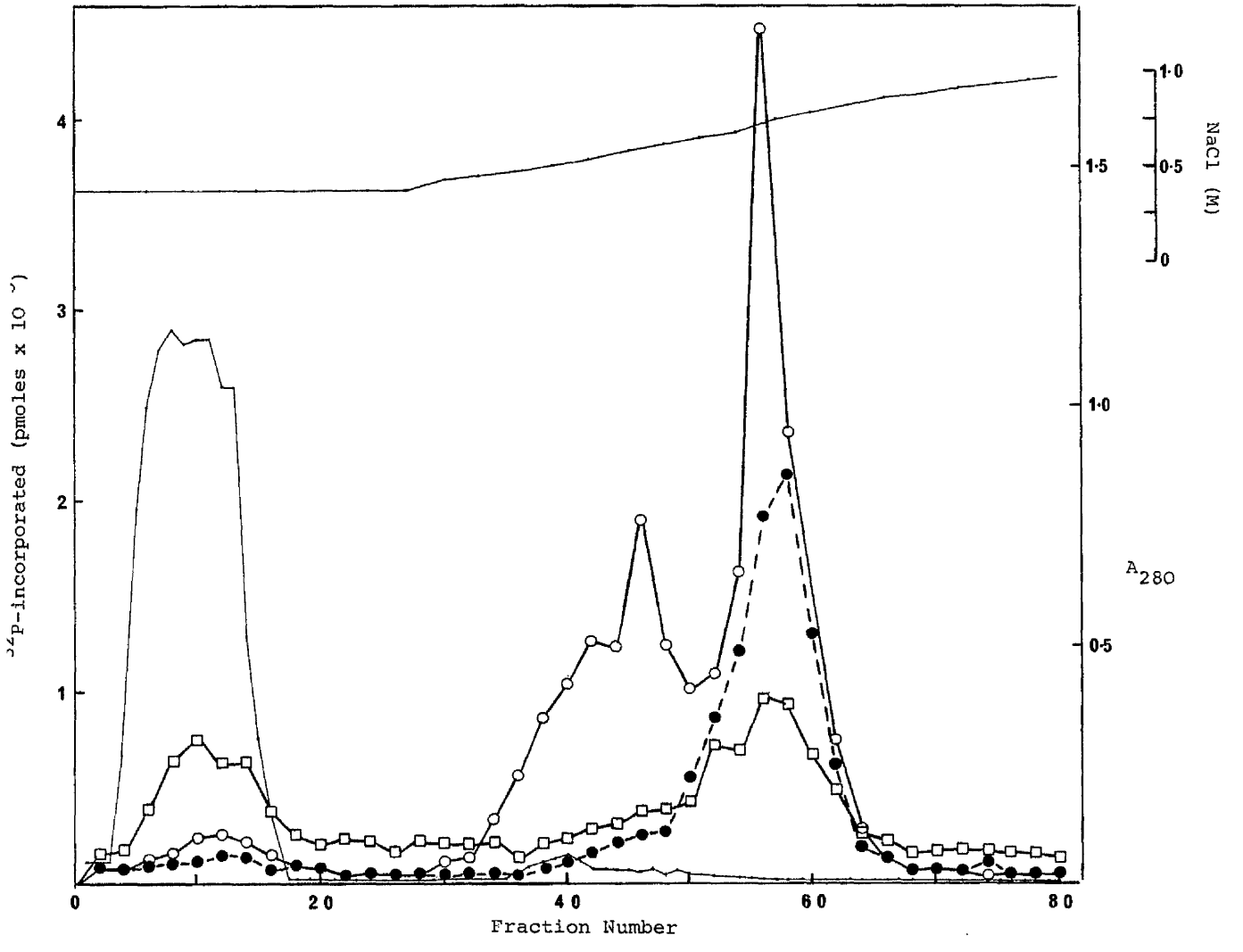
Casein and ATP ○——○

Casein and GTP ●------●

histone and ATP □——□

Protein concentration was monitored by absorbance at 280nm ————

Concentration of NaCl ————


^{32}P-incorporated (pmoles x 10^{-1})
4
3
2
1
0
20
40
60
80
Fraction Number
1·5
1·0
0·5
A_{280}
1·0
0·5
0
NaCl (M)

kinase activity in the 'flow-through', as found before, there was also a distinct peak of histone kinase activity which eluted with CKII. However, unlike HK, CKI and CKII, this new histone kinase (which was stored at -20°C) was extremely labile to freezing and thawing. After 6 days at -20°C the activity of the second histone kinase had been completely abolished. It remains to be seen whether the new histone kinase activity can phosphorylate ribosomal proteins, or whether there have been any changes in the ability of the other protein kinases to phosphorylate ribosomal proteins.

3.8 The Effect of Pseudorabies Virus Infection on the Ribosomal Protein Kinase Activities of BHK Cells

It has been observed by Kennedy, Stevely and Leader (1981) that infection of BHK cells with pseudorabies virus can alter the phosphorylation of ribosomal proteins *in vivo*. The phosphorylation state of protein S6 was greatly increased after virus infection and a protein (S16 or S18) not normally phosphorylated in BHK cells became phosphorylated. This suggested that pseudorabies virus infection might influence the protein kinase activities present in BHK cells. To examine this possibility, protein kinase activities in BHK cells infected by pseudorabies virus and in uninfected BHK cells were compared.

3.8.1 DEAE-Cellulose Chromatography of BHK Cell 'S-150' Fraction

Uninfected BHK cells were fractionated to give an 'S-150' fraction (2.3.6), and this was resolved on DEAE-cellulose. Protein kinase assays with casein and histone were performed using ATP as phosphoryl donor. Casein kinase eluted between 0.065 - 0.225M KCl as a broad peak whereas histone kinase eluted initially as a sharp peak at 0.006 - 0.026M KCl followed by a broad peak of lower activity eluting between 0.105 - 0.225M KCl, (Fig 3.39). A similar experiment was performed with 'S-150' fraction from BHK cells infected for 6 hours with pseudorabies virus (2.3.4). Again the casein kinase activity eluted between 0.075 - 0.240M KCl (Fig 3.40).

Fig 3.39 : DEAE-Cellulose Chromatography of S-150 Fraction from Uninfected BHK Cells

Approximately 8mg of 'S-150' fraction was chromatographed on DEAE-cellulose (2.3.10) and assayed for protein kinase activity (2.3.15.1), with

casein and ATP ○——○

histone and ATP □——□

Protein concentration was monitored by absorbance at 280nm ————

Concentration of KCl ————

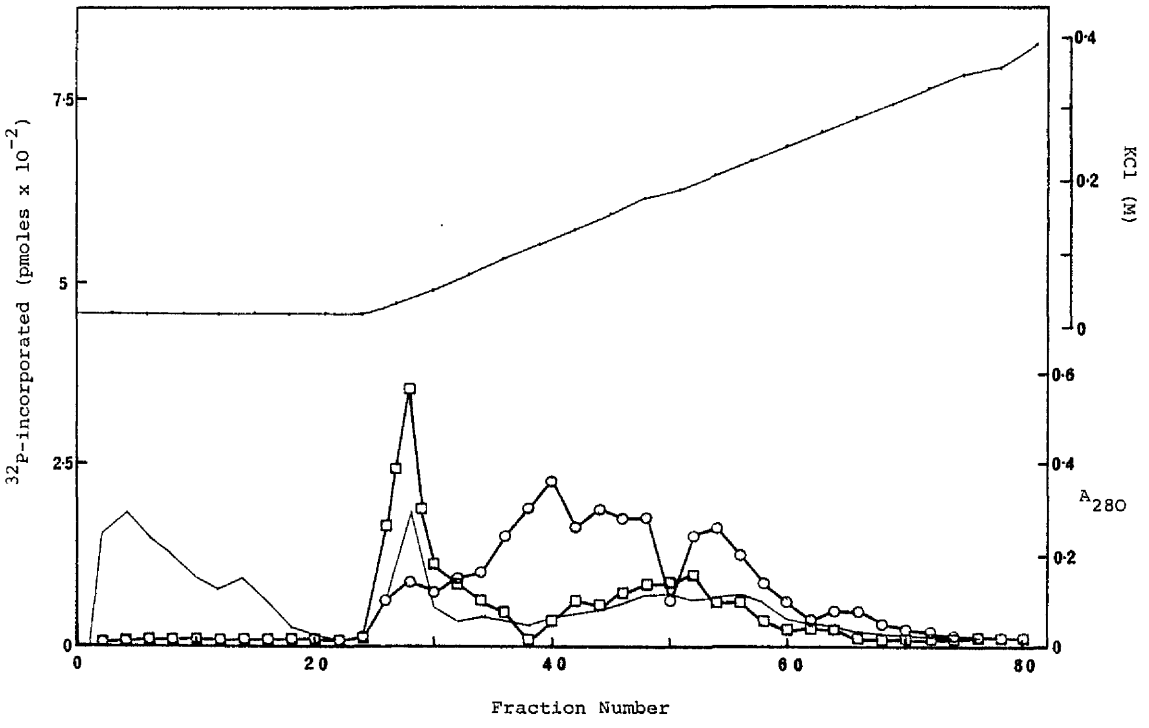

^{32}P-incorporated (pmoles x 10^{-2})
7·5
5
2·5
0
0
20
40
60
80
Fraction Number
0·4
0·2
0
KCl (M)
0·6
0·4
0·2
0
A_{280}

Fig 3.40 : DEAE-Cellulose Chromatography of S-150 Fraction from BHK Cells Infected with Pseudorabies Virus

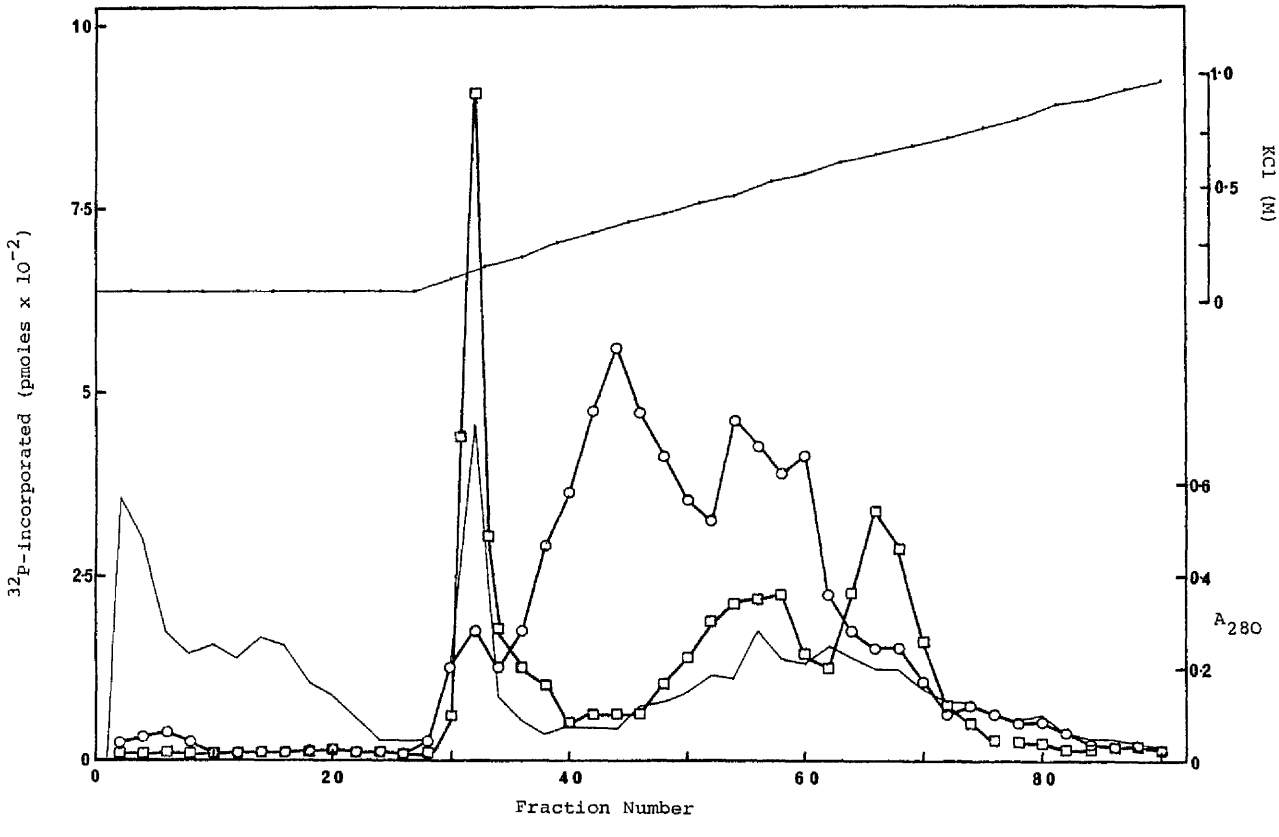
Approximately 20mg of 'S-150' fraction was chromatographed on DEAE-cellulose (2.3.10) and assayed for protein kinase activity (2.3.15.1), with,

casein and ATP ○——○

histone and ATP □——□

Protein concentration was monitored by absorbance at 280nm ————

Concentration of KCl ————


10
7·5
5
2·5
0
0
20
40
60
80
Fraction Number
^{32}P-incorporated (pmoles x 10^{-2})
1·0
0·5
0
KCl (M)
0·6
0·4
0·2
0
A_{280}

The histone kinase eluted as a sharp peak at 0.015 - 0.050M KCl followed by a broad peak of activity as before. However, there was a new peak of histone kinase activity eluting at 0.240 - 0.275M KCl, from infected BHK cells. The actual amount of ^{32}P incorporated in the protein kinase assays was greater for the chromatographic fractions from the infected cells than for the fraction from uninfected cells. This however is due to more 'S-150' protein fraction from infected cells being applied to the DEAE-cellulose column (The assays showed linear incorporation with respect to protein concentration).

3.8.2 Phosphorylation of Ribosomal Proteins by Exogenous Protein Kinases from BHK Cells

The ability of these DEAE-cellulose fractions to phosphorylate the proteins of intact 40S ascites ribosomal subunits was determined. Using conditions described (2.3.15.2) the subunit protein was phosphorylated, and then analysed by immediate electrophoresis on one-dimensional SDS gels. A protein of approximate molecular weight 31,000 was phosphorylated by all the fractions tested from uninfected BHK cells (Fig 3.41). Other 40S ribosomal subunit proteins were not phosphorylated.

Fractions from cells infected with pseudorabies virus also phosphorylated a protein of molecular weight 31,000 (Fig 3.42). Furthermore, a new phosphorylated protein was observed when the fractions which had shown the new histone kinase activity were used. This protein had a molecular

Fig 3.41 : Phosphorylation of 40S Ribosomal Subunits by DEAE-Cellulose Fractions from Uninfected BHK Cells.

Track	1	2	3	4	5	6	7	8	9	10	11	12
DEAE-Cellulose Fraction Number (Fig 3.39)	28	32	36	40	44	46	48	50	54	58	60	62

Subunits were phosphorylated as described (2.3.15.1), and analysed by one-dimensional SDS slab gel electrophoresis (2.3.17). Phosphorylation reaction mixtures contained approximately 5 A_{260} units of 40S subunits and various DEAE-cellulose fractions.

Major Phosphoproteins (Approximate Molecular Weight)	Track Number
31,000	1 - 12

A. Stained Protein

B. Autoradiograph

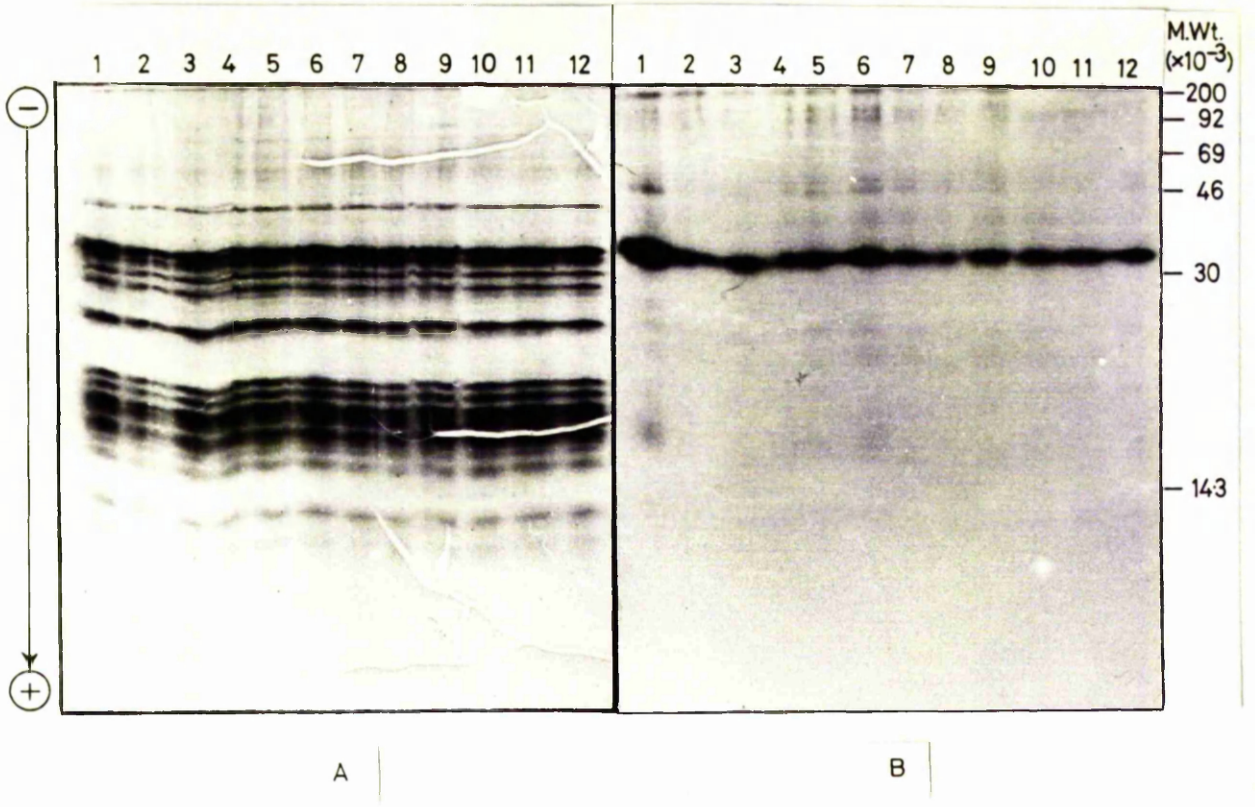

1 2 3 4 5 6 7 8 9 10 11 12
1 2 3 4 5 6 7 8 9 10 11 12
M.Wt.
(×10⁻³)
200
92
69
46
30
143
A
B

Fig 3.42 : Phosphorylation of 40S Ribosomal Subunits by DEAE-Cellulose Fractions from BHK Cells Infected by Pseudorabies Virus.

Track	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
DEAE-Cellulose Fraction Number (Fig3.40)	32	36	40	44	48	52	56	58	62	66	70	32	44	56	66
	+S	+S	+S	+S	+S	+S	+S	+S	+S	+S	+S	-S	-S	-S	-S

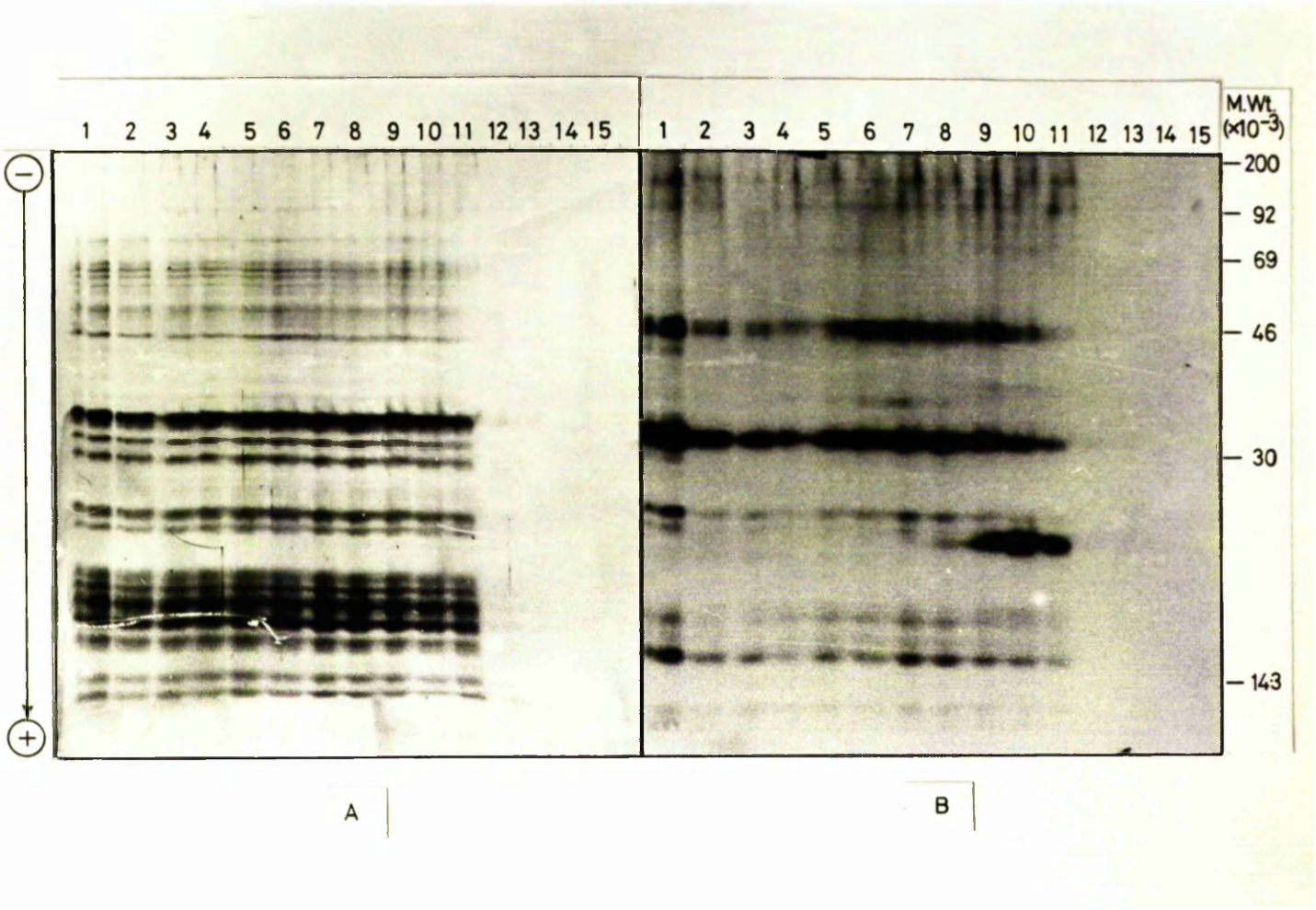
S = Substrate i.e. 40S subunits

Subunits were phosphorylated as described (2.3.15.2), and analysed by one-dimensional SDS slab gel electrophoresis (2.3.17).

Major Phosphoproteins (Approximate Molecular Weight)	Track Number
14,000	1 - 11
16,000	1 - 11
22,000	9 - 11
31,000	1 - 11
48,000	1 - 11

A. Stained Protein

B. Autoradiograph


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
M.Wt. (×10⁻³)
200
92
69
46
30
143
A
B

weight of approximately 22,000. Two phosphorylated proteins of molecular weights 16,000 and 14,000 were also observed with most of the kinase fractions tested. A relatively strong band was also seen at 48,000 molecular weight but is unlikely to be a ribosomal protein. Some other minor phosphoproteins were also observed.

The most striking difference between the infected and uninfected conditions was the appearance of a phosphorylated protein of molecular weight 22,000 when particular fractions of protein kinase from infected cells were used to phosphorylate 40S subunits.

4. DISCUSSION

4.1 Comparison of Ribosomal Protein Kinases from Krebs II Ascites Cells with those from Other Tissues

The protein kinases resolved by phosphocellulose chromatography in this study are compared to those described by other workers in Table 4.1. All of the authors included in Table 4.1 described the presence of one 'casein AG' kinase which can use either ATP or GTP (CKII : Hathaway and Traugh, 1979; III : Schmitt, Kempf and Quirin-Stricker, 1977; K3 : Kudlicki, Grankowski, and Gasior, 1978; III : Becker-Ursic and Davies, 1976; CKII : present study). Similarly each described at least one other casein kinase which can only use ATP as phosphoryl donor (casein A kinase) and also histone kinase activities.

The most interesting feature of ascites protein kinase CKII described here is that it is highly specific for the 60S subunit protein L_γ, phosphorylating no other ribosomal proteins (Fig 3.21) The phosphorylation of other ribosomal proteins in 40S and 60S subunits by both ascites (Figs 3.16 and 3.23) and non-ascites (Fig 3.29) protein kinases indicates that the specificity of CKII for L_γ cannot be due to a lack of potential phosphorylation sites on other ribosomal proteins. Other workers have also shown that a casein AG protein kinase can phosphorylate an acidic 60S subunit protein in other cell types (yeast; Kudlicki, Grankowski and Gasior, 1976; HeLa cells : Horak and Schiffman, 1977 : rabbit reticulocytes : Issinger, 1977). It seems likely then that CKII will phosphorylate L_γ in intact ascites cells. However whether CKII is the sole enzyme responsible for the phosphorylation of L_γ *in vivo* is less clear, since CKI can also phosphorylate L_γ. The phosphorylation of L_γ by CKI is less extensive than with CKII and CKI also phosphorylates other ribosomal proteins (S6, S7, Sa and Sb).

Ascites CKII (Fig 3.27) and similar protein kinases from

Table 4.1 A Comparison of Ribosomal Protein Kinases

	Designation*	Stimulated by cAMP	Protein Substrates			Phosphoryl Donor	
			Casein	Histone	Ribosomal	ATP	GTP
1	HK	+	-	+	+	+	-
	CKI	-	+	-	+	+	-
	CKII	-	+	-	+	+	+
2	I	+	-	+	-	+	-
	II	-	+	+	-	+	-
	III	-	+	-	+	+	low
3	K1	-	+	-	+	+	-
	K2	ND	+	ND	ND	+	ND
	K3	-	+	-	+	+	+
4	I	+	+	+	+	+	-
	II	+	+	+	+	+	ND
	III	-	+	+	+	+	+
5	HK	-	-	+	+	+	-
	CKI	-	+	-	+	+	-
	CKII	-	+	-	+	+	+

1 = Rabbit reticulocytes ; Hathaway and Traugh (1979), 2 = Plasmacytoma cells; Schmitt, Kempf and Quirin-Stricker (1977), 3 = Yeast ; Kudlicki, Grankowski and Gasior (1978), 4 = Yeast ; Becker-Ursic and Davies (1976), 5 = Krebs II ascites cells; Present Study.

N.D. = not determined.

* Protein kinases are presented in order of elution from DEAE-cellulose or phosphocellulose.

other tissues use GTP as phosphoryl donor. If the terminal phosphate of [γ - ^{32}P] GTP were to undergo a nucleoside exchange reaction with ATP thereby producing [γ - ^{32}P] ATP to be used as phosphoryl donor as suggested by Kuo (1974) then such nucleoside phosphotransferases would have to be tightly coupled to the casein kinase. In the present study CKII was resolved from CKI (which would also use any [γ - ^{32}P] ATP produced by nucleoside phosphotransferases) and purified approximately 27 fold. Casein AG kinases similar to ascites CKII have been more highly purified (e.g. yeast K3 purified 1860-fold: Kudlicki,Grankowski and Gasior, 1978; rabbit reticulocyte CKII purified 380-fold : Hathaway and Traugh, 1979) without loss of 'GTP-utilizing' activity which would occur if it were resolved from the putative phosphotransferase. Given that kinases that use GTP preferentially have been described (Ventamiglia and Wool, 1974), it seems most likely that CKII uses GTP directly as a phosphoryl donor.

The lack of phosphorylation of L_γ with ascites CKI when GTP is used as phosphoryl donor (Fig 3.26) strongly suggests that the ability of CKI to phosphorylate L_γ with ATP is not due to cross-contamination with CKII.

Yeast casein A kinase (K1 : Kudlicki,Grankowski and Gasior, 1978), like ascites CKI, could phosphorylate acidic proteins from the 60S subunit (analogous to L_γ). They also reported that yeast casein AG kinase K3 (equivalent to ascites CKII) could phosphorylate these acidic proteins. In contrast to CKII from ascites cells however, K3 will phosphorylate an acidic protein of the 40S subunit (possibly corresponding to Sa or Sb) and

an uncharacterized basic protein (Kudlicki, Grankowski and Gasior, 1976) which they believe to be S6. The results of the present study and those of Kudlicki, Grankowski and Gasior (1978) are unambiguous in their identification of which of the two types of casein kinase is highly specific for L_γ or its equivalent. This apparent reversal in the substrate specificities of the two types of casein kinase is rather surprising despite the considerable evolutionary distance between these two cell types.

There are differences between the results reported by other workers : e.g. Becker-Ursic and Davies (1976) described a cAMP-independent GTP utilizing kinase from yeast which could phosphorylate casein or histone, whereas Kudlicki, Grankowski and Gasior (1978) described a kinase (also from yeast), which might be assumed to be analogous, that is cAMP-independent, utilizes GTP but only phosphorylates casein. However it is possible that further resolution of the kinase described by Becker-Ursic and Davies (1976) would resolve the casein kinase from histone kinase. Kudlicki *et al.*, (1980) have recently reported that the casein A kinase (K1) from yeast can be further resolved to give a casein phosphorylating kinase and a highly specific ribosomal protein kinase which phosphorylates the yeast acidic proteins analogous to ascites L_γ.

Although ascites protein kinase activities CKI and HK both phosphorylate protein S6 (Figs 3.19 and 3.24) it is possible that it is cross-contamination of HK with casein kinase (or vice-versa) which phosphorylates S6 (Figs 3.10, 3.11 and 3.13).

However the fact that both these protein kinase activities each phosphorylate a number of other proteins (Table 4.3), does suggest that their abilities to phosphorylate S6 are indeed separate. As mentioned in 1.3.2. there is evidence that S6 can be phosphorylated by more than one type of protein kinase (e.g. Wettenhall and Howlett, 1979). The phosphorylation of S6 by ascites CKI and HK suggests that more than one mechanism for controlling S6 phosphorylation could exist in ascites cells.

From the evidence presented here (Fig 3.9, Tables 3.3 and 3.4) both of the casein kinases (CKI and CKII) and the histone kinase (HK) from ascites cells seem to function independently of cAMP. The lack of stimulation of HK with cAMP is in contrast to the stimulation of histone kinase activity found by other workers mentioned in Table 4.1 (HK : Hathaway and Traugh 1979 ; I : Schmitt, Kempf and Quirin-Stricker, 1977; I and II : Becker-Ursic and Davies, 1976).

Ascites histone kinase (HK) was stimulated by calmodulin (Table 3.5) suggesting that it is regulated by calcium. It is interesting in this respect that a rabbit reticulocyte protease activated histone kinase (which phosphorylates S6) reported by Del Grande and Traugh (1979) is neither stimulated by cAMP nor inhibited by the heat stable inhibitor protein. Also the protease was activated by calcium.

Hathaway, Lubben and Traugh (1980) have recently reported that casein kinase AG (CKII) from rabbit reticulocytes is specifically inhibited by heparin. Ascites CKII was also strongly inhibited by heparin with up to 94% inhibition with 50-100 units of heparin per assay (3.6.10). In contrast to

the results of Hathaway, Lubben and Traugh (1980), who found no effect of heparin on any other reticulocyte protein kinase, ascites CKI showed an 87% inhibition and HK was stimulated 3-5 fold. The concentrations of heparin were higher in the present study than those of Hathaway, Lubben and Traugh (1980) and this could explain these differences.

Casein kinases from heparin containing cells such as liver have been shown to be inactive until an initial chromatography step has been performed (Sommarin and Jergil, 1978; Meggio, Donnella-Deana and Pinna, 1979). It may be for this reason in part that the 60S subunit acidic proteins analagous to L_Y were not seen to be phosphorylated in earlier studies in vivo with rat liver (Gressner and Wool, 1974).

The apparent K_m values of ascites CKI(14.0μM)and CKII (9.0μM) with casein as substrate and ATP as phosphoryl donor (Fig 3.35) are of the same magnitude as the values obtained by others (yeast : Kudlicki, Grankowski and Gasior, 1978 obtained K_m values of 33μM and 7.6μM for protein kinases K1 and K3 ; rabbit reticulocytes : Hathaway and Traugh, 1979 obtained K_m values of 13μM and 10μM for CKI and CKII). Although K_m values with GTP as phosphoryl donor have not been obtained in the present study others have found that these match the ability or inability of the enzymes to use GTP. Kudlicki, Grankowski and Gasior (1978) obtained a value of 55μM for K3 but were unable to obtain a value for K1.Hathaway and Traugh (1979)found that when GTP was used as phosphoryl donor the K_m values were 40μM for CKII and 900μM for CKI. Whether ascites CKII or corresponding enzymes in other tissues use ATP or GTP or both in vivo is not yet known.

From evidence presented here, protein kinases capable of phosphorylating ribosomal proteins appear to be present only in the cell fraction of ascites cells derived from the soluble components of the cytoplasm. No protein kinase activity associated with ribosomes was detected, despite using a variety of different conditions both to prepare the ribosomes and to carry out the phosphorylation reaction (3.2).

However many workers have reported ribosome associated kinase from other tissues (c.f. 1.3.2) when ribosomes were prepared under similar conditions to those in the present study. One possible explanation is that there are genuine differences in the strength of association of protein kinases with ribosomes from ascites cells and those from other cell types.

4.2 Comparison of Ribosomal Proteins Phosphorylated in Intact Cells and in Cell-Free Phosphorylation Systems

The ribosomal proteins phosphorylated in intact ascites cells and in isolated ribosomal subunits phosphorylated by ascites cytosol protein kinases are listed in Tables 4.2 and 4.3. In both cases only proteins characterized by two-dimensional gel electrophoresis are considered. This is necessary since it is not possible to unambiguously identify a protein merely on the basis of its molecular weight. When 60S subunits were phosphorylated by ascites HK one-dimensional SDS gel electrophoresis revealed that a protein of similar molecular weight to L_γ (14,000) was phosphorylated. However, further analysis on two-dimensional gel electrophoresis showed that only protein L35 (molecular weight 13,700) was phosphorylated in 60S subunits.

In intact ascites cells protein S6 was weakly-labelled with ^{32}P (c.f. 4.3) in agreement with the observations of Leader and Coia (1977). Phosphorylation of ascites 40S subunits by CKI or HK also resulted in a relatively weak-labelled autoradiographic spot on two-dimensional gel electrophoresis (Figs 3.19, 3.25). The low extent of S6 phosphorylation can be further seen by the absence of any 'anodic tail' (Gressner and Wool, 1974) in the stained patterns of two-dimensional gels (Figs 3.5, 3.19, 3.25). The results of the present study indicate that there is relatively little protein kinase in ascites cells that will phosphorylate S6.

Protein L_γ was much more highly labelled, in both intact cells (Fig 3.2) and in isolated 60S subunits phosphorylated by

4.2 Ascites Ribosomal Proteins Phosphorylated in Intact Cells.

Protein Phosphorylated	Cellular Conditions	Figures
L_γ	Normal	3.2, 3.3
S3	Normal/Acidic	3.2,3.3/3.4,3.5
L14	Acidic	3.4, 3.5
L27/28	Acidic	3.4, 3.5
S6	Acidic	3.4, 3.5
S2	Acidic	3.4, 3.5
Sa	Acidic	3.5
Sb	Acidic	3.5

This Table lists the proteins phosphorylated in intact ascites cells incubated in medium which did not contain glucose or amino acids (Normal : c.f. 3.1.1) or which did contain glucose and amino acids (Acidic : c.f. 3.1.2). The figures which show these phosphorylated proteins are also listed.

Table 4.3 Ascites Ribosomal Proteins Phosphorylated by Cytosol Protein Kinases

Protein Phosphorylated	Protein Kinase	Phosphoryl Donor	Figures
L_γ	CKI	ATP	3.17,3.18
	CKII	ATP/GTP	3.21,3.22/3.26
S6	HK	ATP	3.24,3.25
	CKI	ATP	3.17,3.18,3.19
S7	HK	ATP	3.24,3.25
	CKI	ATP	3.17,3.18,3.19
Sa	CKI	ATP	3.17,3.18
Sb	CKI	ATP	3.17,3.18
L35	HK	ATP	3.24,3.25
S10	HK	ATP	3.24,3.25
S14	HK	ATP	3.24,3.25
S20	HK	ATP	3.24,3.25

This Table lists the ribosomal proteins in isolated subunits phosphorylated by ascites cytosol protein kinases (shown in Table) and characterized by two-dimensional gel electrophoresis. The figures which show these phosphorylated proteins are also listed.

CKII using [γ - ^{32}P] ATP (Fig 3.21) or [γ - ^{32}P] GTP (Fig 3.26) as phosphoryl donor, than any other ascites ribosomal protein. Alkaline phosphatase treatment of 60S subunits and 'Sweep' gel analysis (2.3.20) of ^{32}P-labelled L_γ have been used to show that L_γ has two phosphorylation sites (Leader and Coia, 1978c). In the present study the autoradiographic spot corresponding to L_γ in 'Sweep' gels of 60S protein ^{32}P-labelled in intact cells (Fig 3.2) or with CKII (Figs 3.21 and 3.26) is resolved into two components (as indicated by the vertically elongated shape of the radioactive spot). This suggests that CKII can phosphorylate both of the available phosphorylation sites in L_γ.

In a number of experiments (e.g. Figs 3.3, 3.8, 3.17) L_γ is only weakly-labelled. This could be because the amount of ^{32}P actually incorporated is low. Madjar *et al.*, (1979a) have however recently suggested that acidic 60S subunit proteins (equivalent to L_γ) can be solubilized in the staining and destaining solutions used to process two-dimensional gels, thereby reducing the apparent labelling of these proteins.

Some workers have reported that protein S3a was phosphorylated *in vivo* (Roberts and Morelos, 1979) whereas others stated that protein S3 rather than S3a (Rankine, Leader and Coia, 1977) was phosphorylated. The two-dimensional gel electrophoresis systems used by these workers (those of Howard *et al.*, 1976; and Lastick and McConkey, 1976) do not resolve S3 from S3a. Hence, it was not possible to unambiguously identify which of these proteins was phosphorylated. The 'Acidic-Acidic' gel electrophoresis system ('system IV' : Madjar *et al.*, 1979b, described in 2.3.21) does allow the resolution of these two

proteins and as can be seen from Fig 3.3 S3 is phosphorylated whereas S3a is unphosphorylated.

Proteins Sa and Sb were phosphorylated in intact ascites cells (Fig 3.4) and in isolated 40S subunits by protein kinase CKI (Fig 3.18). These phosphoproteins have not been observed before in ascites cells (Leader and Coia, 1977) since they do not migrate on the two-dimensional gel electrophoresis system (Lastick and McConkey, 1976 described in 2.3.19) used to characterize ascites ribosomal phosphoproteins. Both Sa and Sb have been purified from rat liver ribosomes by Collatz et al., (1977). These proteins were however obtained at a lower yield than other ribosomal proteins, and they have higher than average molecular weights (41,500 and 33,000) for ribosomal proteins c.f. Table 1.1). This suggests they may not in fact be ribosomal proteins. The phosphorylation of S2 and L14 is discussed in 4.3.

A number of proteins were phosphorylated in isolated 60S and 40S subunits, by ascites cytosol protein kinases, which were not phosphorylated in intact cells (S7, S10, S14, S20 and L35). It is quite likely that some or all of these phosphorylations are artifactual due to the increased accessibility of serine and threonine residues, which are normally unphosphorylated,to protein kinases (Bylund and Krebs, 1975). Protein S7 has been reported as phosphorylated in intact rabbit reticulocytes (Issinger and Beier, 1978) as well as by CKI and HK in isolated ascites 40S subunits. It is interesting that a protein of similar molecular weight (22,000) to S7 (though as yet uncharacterized) is phosphorylated by a novel protein kinase

which appears after infection of BHK cells with pseudorabies virus (c.f. 4.3).

Proteins of similar molecular weight to L_γ and S6 (13,500 and 32,000) are phosphorylated in 40S and 60S subunits by cAMP-dependent protein kinases from rabbit skeletal muscle and bovine heart (Fig 3.29). Proteins of similar molecular weight are also phosphorylated in isolated 40S and 60S subunit protein by these enzymes (Fig 3.30). In 80S ribosomes, although a protein of 32,000 becomes labelled when they are phosphorylated by these cAMP-dependent protein kinases there is much less radioactive label in a 13,500 molecular weight protein (which might correspond to the 13,500 molecular weight protein labelled in 60S subunits). Although the evidence presented here is limited it could be speculated that the 13,500 molecular weight protein (possibly L_γ) is inaccessible to protein kinase in 80S ribosomes. This might be because it is located at the subunit interface though equally it could be masked by non-ribosomal proteins in the 80S ribosome. Horak and Schiffman (1977) have reported that the acidic 60S subunit protein L40/L41, analogous to L_γ, is only phosphorylated in isolated 60S subunits but not isolated 80S ribosomes when phosphorylated with protein kinases. They believe that this is because L40/L41 is located at the subunit interface.

4.3 Alterations in Ribosomal Protein Phosphorylation

In the present study, L_γ became dephosphorylated when ascites cells were incubated in medium which contained glucose and amino acids (Fig 3.4). Conversely L14 and S2 became phosphorylated under these conditions (Figs 3.4 and 3.5), as had been shown previously by Leader and Coia (1978).

One difference between the present study and that of Rankine, Leader and Coia (1977) was the absence of S6 phosphorylation and also the phosphorylation of S3 when cells were incubated in medium which contained neither glucose or amino acids. The rather low level of phosphorylation of S6 in ascites cells (approximately 0.1 moles of phosphate per mole of 40S subunits) and the presence of a second 40S subunit phosphoprotein (Rankine, Leader and Coia, 1977) might make this difference in the ribosomal protein phosphorylation pattern less anomalous. The reason why Sa and Sb (Fig 3.4) have not been previously reported to be phosphorylated has been mentioned earlier (4.2).

Proteins S2, S3 and L14 were not phosphorylated *in vitro* on 40S subunits by any of the three protein kinase activities isolated from ascites cells taken from mouse peritonea (2.3.1) whereas proteins L_γ, S6, Sa and Sb (together with several proteins not observed as phosphorylated *in vivo* (Table 4.3) could be phosphorylated by these protein kinase activities. The lack of phosphorylation of S2 and L14 is not too surprising since neither of these proteins are phosphorylated in ascites cells *in peritonea*. It might be expected that the protein kinases responsible for phosphorylating these proteins would be present in cells incubated under conditions that cause the phosphorylation of these proteins in intact cells. Protein

kinases resolved from ascites cells incubated in medium containing glucose and amino acids (2.3.3) do indeed include a peak of histone kinase not previously seen, but this was found to be very labile to freezing and thawing (3.7.2). It is possible that this new histone kinase activity can phosphorylate L14 or S2.

It is necessary to account for the presence, in extracts of these incubated cells, of protein kinase corresponding to CKII in similar amounts to that found in normal ascites cells (Fig 3.38). As shown previously this protein kinase is highly specific for L_γ when presented with ribosomal subunits. It might be expected that there would be less CKII from cells in which L_γ was dephosphorylated. One possibility is that the protein kinase which phosphorylates L_γ is in fact separate from the casein phosphorylating activity of CKII and that the acidic conditions diminish the 'L_γ kinase' but not the 'casein kinase' component of CKII. As mentioned (4.1) Kudlicki *et al.*, (1980) have shown that the 'casein phosphorylating' and the 'ribosomal protein phosphorylating' activities of yeast casein A kinase (K1 : Kudlicki, Grankowski and Gasior, 1978) can be resolved. An alternative explanation for the presence of CKII in cells incubated with glucose and amino acids is that the extent of phosphorylation of L_γ *in vivo* is controlled by changes in the pattern or activity of phosphoprotein phosphatases. Yet another possibility is that protein kinase or phosphatase levels could be modulated by transient effector molecules, and purification might resolve the enzymes from their effectors.

A rather different explanation is that alterations in cell conditions such as extremes of pH could alter the conformation of the ribosome thereby previously inaccessible sites become capable of being phosphorylated. For this reason the ability of CKI and CKII to phosphorylate isolated ribosomal protein was examined since it was felt that the isolation of proteins could expose phosphorylation sites which were normally only exposed under abnormal conditions. Phosphorylation of extracted 60S subunit protein with CKII showed that two proteins (molecular weights 16,700 and 18,000) were labelled (Fig 3.28). CKII normally phosphorylates L_γ (molecular weight 14,000) and it would seem likely that one or both of these proteins are L_γ (though having a somewhat higher molecular weight than that normally obtained from L_γ). As mentioned in 1.3.1.2 there is considerable evidence that there are in fact two acidic 60S subunit phosphoproteins and this could explain the appearance of two phosphorylated proteins. No protein of similar molecular weight to L14 (approximately 25,800) was phosphorylated in this experiment which suggests that its phosphorylation is not simply due to increased availability of potential phosphorylation sites.

The inability of CKI to phosphorylate extracted ribosomal protein is surprising in view of the fact that the enzyme phosphorylated a number of proteins in the intact 40S and 60S subunits. It is unlikely that some conformational effect due to isolation of the protein from subunits prevents their phosphorylation by CKI since CKII was able to phosphorylate these two proteins under similar conditions.

In the present study, a comparison of the protein kinase activity of BHK cells before and after infection with pseudo-

rabies virus was made (Figs 3.39 and 3.40). As mentioned in 1.3.1, Kennedy, Stevely and Leader (1981) have shown that in BHK cells infected with pseudorabies virus the phosphorylation state of S6 is greatly enhanced together with the appearance of a newly phosphorylated ribosomal protein (S16 or S18). One difference in the pattern of protein kinase activity was the appearance of a new protein kinase in the cytosol of BHK cells infected with virus (Fig 3.39).

One-dimensional gel electrophoresis of 40S ribosomal subunits phosphorylated with DEAE-cellulose resolved protein kinases from infected and uninfected cells revealed a ^{32}P-labelled band of molecular weight 31,000 in both cases (Figs 3.41 and 3.42). The molecular weight and extent of labelling of this protein makes it likely that it is protein S6. Little evidence of other ^{32}P-labelled protein was obtained with cytosol kinases from uninfected cells (Fig 3.41). However as seen in Fig 3.42 cytosol kinases from BHK cells infected with pseudorabies virus were also able to phosphorylate a number of other proteins. The most striking change in the phosphorylation activity of BHK cytosol kinases following virus infection was the appearance of a 22,000 molecular weight protein (Fig 3.41) when the new histone kinase was used (Fig 3.40). The identity of this protein cannot be ascertained merely from one-dimensional SDS gel electrophoresis, but its molecular weight is somewhat larger than most estimates of S16 or S18 (17,100 and 18,500; Collatz et al., 1977). From the position of the stained band corresponding to this labelled protein it would appear to be either protein S5 or S7 (molecular weight 21,500 Collatz et al., 1977). Neither S5 nor S7 have been observed as phosphorylated in BHK

cells infected by pseudorabies virus (Kennedy, Stevely and Leader, 1981) and it will require a more rigorous identification of this protein by two-dimensional gel electrophoresis before its identity can be established unequivocally.

Proteins of molecular weight 14,000 and 16,000 were also phosphorylated(Fig 3.42) with protein kinase from virus infected BHK cells. Whether either of these labelled protein bands corresponds to S16 or S18 will also have to await their further characterization.

Another interesting feature of the 40S subunit protein phosphorylated by cytosol kinases from BHK cells infected with virus is the appearance of a ^{32}P-labelled protein of molecular weight 48,000. A ^{32}P-labelled protein of this molecular weight was reported to be present in one-dimensional SDS gels of 40S ribosomal subunit protein from HeLa cells infected with herpes simplex virus (Fenwick and Walker, 1979). This protein is certainly too large to be a ribosomal protein (Wool, 1980) however it could be a protein synthesis factor (e.g. the β-subunit of initiation factor eIF-2 : molecular weight 53,000; or a 50,000 molecular weight component of elongation factor EF-1).

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