

AN INVESTIGATION INTO THE BRAIN ISOENZYME OF CREATINE KINASE
FROM HUMAN BRAIN. WITH PARTICULAR EMPHASIS ON THE POSSIBLE
EXISTENCE OF AN INACTIVE FORM.

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ABSTRACT

This thesis is an investigation into the properties of the brain isoenzyme of creatine kinase (CKBB) from human brain. In particular, it is the attempt to clarify two suggestions in the literature (Burnam, 1981a, and Armstrong, 1977a) that there may be an inactive form of CKBB. Antibodies were raised in rabbits to purified human CKBB with the intention of using these antibodies as a tool to isolate and characterise inactive CKBB, which it was hoped would be immunologically similar to CKBB. Two attempts at antibody production were performed, one by classical inoculation (which yielded an antibody designated Ab-CKBB) and one by the inoculation of denatured CKBB. This antibody was designated Ab-DEN and was found to differ markedly in specificity compared to the Ab-CKBB. The bulk of the research presented in this thesis is the attempt to discover which protein(s) this Ab-DEN recognised, whether or not it was a creatine kinase molecule, and its subcellular location.

The Ab-DEN was found to recognise a protein with minimal, but measureable, creatine kinase activity, which migrated cathodally on isoenzyme electrophoresis. The properties of the target protein appeared to be affected by the presence of B-mercaptoethanol in the buffer. It had a high molecular mass of 250000 daltons as assessed by gel filtration, and a subunit molecular mass of 42000 daltons as assessed by SDS PAGE. The Ab-DEN recognised CKBB under denaturing conditions but not under non-denaturing conditions.

DECLARATION

I declare that this dissertation is my own, unaided work. It is being submitted for the degree of Master of Science in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other University.

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ABBREVIATIONS

BME	B-Mercaptoethanol
TBS	Tris-buffered saline
TTBS	Tris-buffered saline containing Tween 20
PBS	Phosphate-buffered saline
Tris/BME	50 mM Tris buffer pH 7,4, containing 5 mM B- mercaptoethanol

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1. INTRODUCTION

1.1. CREATINE KINASE ACTIVITY

Creatine kinase (CK;ATP Creatine Phosphotransferase EC 2.7.3.2.) catalyses the reversible reaction



Watts (1973) has extensively reviewed the structure and function of creatine kinase. The enzyme was first purified from rabbit skeletal muscle by Kuby in 1954 and since then has been studied in a wide range of animals and tissues.

Creatine kinase provides the ability to release energy stored in the form of creatine kinase for the production of ATP, and of course to store it by the reverse reaction, and is therefore vital in the metabolism.

In this introduction, I shall review the literature concerning the properties of creatine kinase. There is a considerable amount of research available on this enzyme, which reflects the central role which it plays in many systems. A large proportion of this research is on CKMM and CKMB (defined in the next section) but is included in this work since, although the isoenzymes have several distinct characteristics, they are essentially the same protein. The particular focus of this work is, however, CKBB and I have extensively reviewed the available literature on the isoenzyme(s) found in the brain. I have also paid attention to various membrane bound creatine kinase systems to establish whether the protein which

I characterise in this study has similar properties. To account for the differences between the CKBB and this protein, I survey the information available on the possible modifications to creatine kinase, both by inactivation and by post-translational modification.

1.2 ISOENZYMES

The subject of creatine kinase isoenzymes has recently been reviewed (Foreback and Chu, 1981; Nanji, 1983a).

1.2.1 CREATINE KINASE SUBUNITS

Cytosolic creatine kinase is a dimer composed of a combination of the M or B subunits; M so-called because it is principally found in Muscle and B because it is principally found in Brain. Thus the possible combinations are CKMM, CKMB and CKBB. The CKMB is found in cardiac tissue. The subunits can be dissociated with the use of agents like urea, and will hybridise with one another upon the removal of the dissociating agent (Watts, 1973). Renaturation, achieved by dilution or dialysis of the denaturant, leads to a 70% recovery of activity (Grossman et al, 1981). There is also a mitochondrial creatine kinase subunit, which has similar catalytic properties and molecular weight but which will not hybridise with the cytosolic forms.

The isoenzymes can be readily separated from one another electrophoretically due to the difference in charge of the different forms. This has diagnostic application in clinical medicine.

1.2.2. SOLUBLE AND MEMBRANE BOUND ISOENZYMES

Creatine kinase was originally thought to be a cytosolic enzyme. Although the majority of the enzyme is indeed soluble, it is also found in membrane-bound locations, where it seems to provide an essential energy link with other enzyme systems. Membrane bound creatine kinase has been found and investigated in mitochondria, synaptosomes, synaptic plasma membranes, crude microtubule preparations, myofibrils and mitotic spindles in dividing cells.

1.2.3. CK ISOENZYMES IN THE SERUM

Agarose or cellulose acetate electrophoresis of the three cytosolic isoenzymes yields three activity bands, roughly equidistant, with the CKMM being at the cathodic side, the CKMB intermediate, and the CKBB at the anodic side of the electrophoretogram. More powerful techniques such as isoelectric focusing and polyacrylamide gel electrophoresis can separate these activity bands further, based on slight charge differences caused by post-translational modifications. These will be discussed further in section 1.6.4. Mitochondrial CK migrates cathodically to CKMM.

A normal serum isoenzyme electrophoresis pattern shows mainly CKMM with a trace of CKMB. Any increase in these forms, or an appearance of the other forms, can provide information on the site of tissue damage. For example, the levels of CKMB are routinely used to monitor the severity and development of a myocardial infarct. There are, however, several factors which indicate that interpretation of creatine kinase electrophoretograms should be done with caution. This will become apparent in later sections.

In addition, there are two atypical forms of creatine kinase

which are found in the circulation (Nanji, 1983a). One (Macro CK type 1) is a complex of CKBB and immunoglobulin. CKBB complexed with IgA has an identical mobility to CKMB (Chen and Wong, 1987). The two bands can be distinguished with the use of specific antisera to IgA and to CKMM. CKBB complexed with IgG migrates between CKMM and CKMB on isoenzyme electrophoresis (Bohner et al, 1979). This complex is considerably more stable to heat than the uncomplexed CKBB, accounting for its persistence in the serum (Bohner et al, 1981). It does not seem to have any diagnostic value and is usually found in elderly ladies (Bohner, 1982). It is thought to represent an autoimmune response and Urdal and Kierulf (1981) have taken advantage of this autoantibody to develop a specific and sensitive radioimmunoassay for the measurement of CKBB. It is possible for serum to contain both IgA- and IgG-linked CKBB simultaneously as reported by Medeiros et al (1985) in an elderly woman.

The other atypical form (Macro CK type 2) is relatively heat stable, has a large molecular weight and a high energy of activation (Stein et al, 1985). It has an electrophoretic migration which is cathodic to CKMM and is assumed to be an oligomeric form of the mitochondrial enzyme (Bohner, 1982). The presence of dimeric or oligomeric mitochondrial bands in serum isoenzyme electrophoresis is a poor prognostic sign since release of mitochondrial enzyme signifies a breakdown of mitochondrial integrity (Grobbel et al, 1982; Nakagawa et al, 1982). A potential mitochondrial electrophoretic band must be distinguished from adenylate kinase, which migrates to a similar position, by the use of a control stain without substrate (Nanji and Blank, 1983b).

A variant CK isoenzyme which forms in the presence of glycine

and which migrates between CKMM and CKMB has been described by Olson et al (1982). The band was discovered after a transurethral resection of the prostate gland using an irrigation solution containing 15 g/L glycine. In vitro addition of glycine to serum containing CKMM and CKMB, and one containing CKMM, CKMB and CKBB resulted in a similar formation of this band.

The normal range of total serum creatine kinase activity is quoted as 80-200 IU/L. It has been observed that intensive care patients often have very low CK levels, and a suggested explanation is an impaired metabolism of the creatine substrate, as opposed to a decreased release from the cells (Delanghe et al, 1986). It has also been suggested that steroids can lead to low CK levels (Hinderks and Froblich, 1979). This observation has been confirmed for patients on chemotherapy, in particular prednisone treatment, where patients were shown to have particularly low CK levels (Fraser, 1980).

Duchenne's muscular dystrophy is characterised by an increase in serum CKMM. A screening test for the disease in neonates has been developed by measuring the creatine kinase of a blood sample spotted on to filter paper, using bioluminescence (Dellamonica et al, 1983). It has been shown that the levels of CKMM from normal skeletal muscle in vitro are significantly increased by increasing the calcium concentration in the medium (Anand and Emery, 1982) and that this can be inhibited by verapamil, which blocks calcium channels in the membrane. The fact that CKMM can be released from tissues by calcium suggests an active rather than a passive transport.

1.3 ISOENZYME FORMS FOUND IN BRAIN

There is an unresolved controversy about the tissue specific distribution of CK isoenzymes. The results obtained by different studies vary depending on whether electrophoretic or chromatographic separation is used, with detection of isoforms by enzymatic or immunological methods.

1.3.1. BRAIN ISOFORMS FOUND BY CHROMATOGRAPHY

During chromatographic separation of brain proteins, several workers have noted a small CK activity peak which elutes off an anion exchange column at a much lower salt concentration than the CKBB peak (Nealon and Henderson, 1975a; Yasmineh et al, 1976; Lindsey and Diamond, 1978; Kraft et al, 1978).

Lindsey and Diamond (1978) found that the CK activity in this peak could be inhibited by anti-M antibodies. On the basis of this, they concluded that 30% of CK activity in brain is due to CKMM. This finding has not been substantiated by other workers, and it is my opinion that the reaction with the commercial anti-CKMM antibody was due to recognition of an epitope which is present in both CKMM and this other CK form. The CK activity of this early peak was little affected by incubation at 37°C, whereas the CKBB was rapidly inactivated at 37°C.

Nealon and Henderson (1975a) found that the CK from this early peak migrated as a cathodic band on isoenzyme electrophoresis, compared to CKBB which migrated as an anodal band.

Using batch elution of proteins off DEAE -Sephadex A50 columns using fresh surgical specimens, Tsung (1976) found that 100% of the CK activity from normal brain was in the CKBB fraction. In a

subsequent study on CK isoenzymes found in neoplasms, he found 67% of activity from a glioblastoma multiforme tumour in the DEAE-Sephadex A50 fraction in which CKMM elutes (Tsung, 1983). Note, however, that the fact that CK activity is found in the same fraction as CKMM does not necessarily confirm that the CK in question is CKMM - it means that it has a similar overall charge.

Leroux et al (1977) found that chromatography of brain extract on DEAE-cellulose resulted in the resolution of three CK peaks. One of these was not retained on the column (the starting buffer contained 10mM NaCl) and was assumed to be Mt-CK. The peak which eluted at the start of the NaCl gradient was found in heart and brain tissue, and was called CK-Z. The third peak in brain was CKBB. CK-Z appeared to convert to a more basically charged form with time, and was not found in the absence of B-mercaptoethanol. Further characterization of CK-Z from the heart (Desjardins, 1982) showed that CK-Z migrated cathodically to CKMM on electrophoresis, but was not inhibited by anti-M or anti-B antibodies. It could assume two forms; one of MW 33-38000 daltons in a Tris/BME/EDTA buffer, and one of MW 62-68000 daltons in a phosphate/EDTA buffer. It was later shown to be associated with the mitochondrial fraction (Desjardins and Pesclovitch, 1983) and not with the 600 x g fraction as originally thought (Desjardins, 1982).

1.3.2. BRAIN ISOFORMS FOUND BY ELECTROPHORESIS

Using isoenzyme electrophoresis, Jockers-Wretou and Phleiderer (1975) found 100% activity in autopsy brain was due to CKBB, though as only one microlitre was applied, the sensitivity of the stain may not have picked up any other minor bands. Brain tissue was the only

tissue studied which had only one creatine kinase isoenzyme. They noted that small quantities of activity in the heart, brain, lung, intestine and thyroid could not be precipitated by antibodies to CKMM and CKBB, and this they attributed to a "particulate CK-enzyme known to migrate to the cathode during electrophoresis".

Urdal et al (1983), using fresh tissue obtained at surgery, also found only CKBB in the brain - this was 2,7% inhibited by anti-M antibodies. In contrast to Jockers-Wretou and Phleiderer, Urdal et al found that CKBB was the only isoenzyme present in 16 out of 19 tissues tested. A possible explanation for this may be that the Urdal group's w/v ratio of tissue to buffer was 7-33:1, in contrast to the Jockers-Wretou group's ratio of 2-3:1, thus resulting in a more dilute supernatant. In addition, the supernatants with high CK activity were further diluted to give 1000 U/l for electrophoresis, which they say enabled them to detect bands which constituted more than 2% of the total activity, since the limit of detection was 20 U/l. This dilution may have masked any other bands present. A comparison of results obtained using fresh and autopsy tissue showed that several autopsy tissues developed cathodic CK bands which were not seen in fresh material. In other words, the presence of only CKBB in brain may be a valid finding, not due to dilution of minor bands, and the additional bands found by other workers using autopsy tissue may represent breakdown products of CKBB, or membrane-bound forms released during autolysis.

Several groups have found one or two cathodically migrating bands in the brain, in addition to the anodal CKBB band. Murone and Ogata (1973) found that the 20,000 x g supernatant of crude brain extracts from foetal human and rabbit adult brain contained two CK

isozymes - as discerned by starch-gel electrophoresis - one anodal and one cathodic. The cathodic band was found to be very unstable and sometimes undetectable. Friedhoff and Lerner (1977) found a cathodically migrating CK band which was assumed to be synaptosomal in origin. Wevers et al (1982) found that electrophoresis of human autopsy brain tissue revealed two cathodic bands, also assumed to be mitochondrial, plus the CKBB band. Heinbokel et al (1982) used agarose gel isoelectric focusing to study the isoenzymes in brain frozen to -80°C within 30 minutes of death. They found a seven-band isoenzyme pattern which had the same immunological and electrophoretic properties as the CK isoenzymes from uterus - an anodic band which reacted with anti-CKBB antiserum, three intermediate bands which reacted with anti-CKMM antiserum, and three cathodic bands which did not react with either anti-CKMM or anti-CKBB antisera. These latter non-M, non-B bands are identified by the authors as identical to the two cathodic bands of Wevers. Chandler et al (1984) found a cathodic, non-M band in electrophoresis of autopsy tissue, which they assumed was mitochondrial.

1.3.3. DISTRIBUTION OF CREATINE KINASE ISOENZYMES IN THE BRAIN

Subcellular distribution:

The results of various groups investigating the subcellular distribution of CK isoenzymes in the brain are conflicting and this is probably due to the difficulty of obtaining pure, uncontaminated subcellular fractions.

As long ago as 1967, the presence of CK in the mitochondrial fraction of guinea pig cerebral cortex was suggested by Swanson et al (1967) who found that about 25% of the total CK activity in the brain

was found in mitochondria.

A study into the subcellular distribution of creatine kinase in rabbit brain and muscle was carried out by Murone and Ogata (1973). The CK isoenzymes associated with the membrane fractions were released by sonication. The microsomal fraction was found to have considerably more activity than the mitochondrial and nerve ending fractions, and exhibited the same migrational behaviour as CKBB. The striking aspect about this work is the suggestion that microsomal creatine kinase accounts for about 36% of the total creatine kinase in the brain, compared to 59% for cell sap, 2.8% for mitochondria, 2.0% for nerve endings and a negligible amount in nuclei.

The mitochondrial creatine kinase from rat brain has been studied (Booth and Clark, 1973) and found to account for approximately 5% of the total creatine kinase activity. It was shown to be tightly bound to the membrane. The neonatal level of Mt-CK was about a third of the adult level, until the 15th day, when it started to increase, taking about 10 days to reach the adult level, after which it remained constant.

Creatine kinase has been shown to be located in brain mitochondria by Desjardins (1982) and Wevers et al (1982). The mitochondrial origin of the two cathodically migrating bands in brain was established by Wevers using subcellular fractionation of fresh rat brain. In autopsy human brain, the molecular mass of the least cathodic (named Br-CKm1 i.e. mitochondrial band 1 from brain) of the two bands was found to be 65000 daltons by gel filtration. The other cathodic band (Br-CKm2) was given a tentative molecular mass of 184000 daltons - a more definite value could not be given, since the mobility of the form changed upon gel filtration in the presence and

absence of BME. No interconversion between the two forms could be discerned. Neither of the two cathodic bands reacted with anti-M and anti-B antiserum.

Although the research of Wevers et al and Desjardins was conducted using different techniques, i.e. electrophoresis and column chromatography respectively, their results have several features in common. They both found two basically charged CK isoforms in the brain. The most basic form which was not retained by the DEAE column was assumed by Desjardins to be mitochondrial in origin. This would correspond to the most cathodic band (Br-CKm2) obtained by Wevers et al. The form which was retained on the DEAE column and eluted by low salt concentration would correspond to the least cathodic (Br-CKm1) of the electrophoretic bands. Wevers et al obtained a molecular mass by gel filtration for this form of 65000 daltons, whilst Desjardins found a molecular mass of 68000 daltons in phosphate buffer, and 38000 daltons in Tris buffer.

The properties of mitochondrial CK derived from different tissues seem to vary quite widely. Wevers et al (1930) found two forms of CK from cardiac mitochondria, a faster cathodically migrating band which converted to a slower cathodically migrating band upon incubation with serum - a conversion which could not be mimicked with the two cathodic bands found in the brain. The slower band had a similar migration to CKMM. The two forms had an identical molecular mass - this also contrasts with the situation with the brain where the fastest cathodic band had a molecular mass about three times that of the slower band. The faster and slower cathodically migrating bands in the heart were named CKm(ox) or CKm2, and CKm(red) or CKm1 respectively. Murone and Kikuo (1973) found

that starch-gel electrophoresis of the mitochondrial fraction from rabbit cerebral cortex revealed two bands, one with more cathodic migration than the band from the crude extract, and one anodal band whilst the muscle mitochondrial fraction only revealed the cathodic band.

Friedhoff et al (1977) found that preparations of synaptosomal membranes and synaptic vesicles from rat brain, solubilised with Triton X-100 or sodium deoxycholate, contained an isoenzyme which migrated to a similar position to CKMM on isoenzyme electrophoresis. A later paper by the same authors (Lerner and Friedhoff, 1980) further characterised this isoenzyme. It was found to have a pI of 7.3, as compared with 6.8 for CKMM and 5.6 for CKBB. The molecular mass by gel filtration was 80000 daltons. It did not cross react with antiserum against CKMM or heart Mt-CK, but 75% of the enzyme was immunoprecipitated by antiserum against CKBB. Wevers et al (1982), on the other hand, found only minor amounts of CK in the same synaptosomal fraction, using a similar fractionation procedure.

Support for a synaptosomal CK fraction comes from the work of Lim et al (1983) who found CK in rat brain synaptic plasma membranes. The enzyme was tightly bound to the membrane and seemed to be associated with membrane-bound pyruvate kinase and neuron-specific enolase, which prompted the authors to suggest that the enzymes may constitute an "assembly for generating ATP"- enolase converting 2-phosphoglycerate to phosphoenolpyruvate, pyruvate kinase converting PEP and ADP to pyruvate and ATP, with the ATP being converted to creatine phosphate by creatine kinase. The measurement of enzyme activity associated with the synaptic plasma membranes required the presence of 0.25% Triton X-100 - in its absence the CK activity was

about 20-30% of this activity. Triton X-100 released substantially more CK activity from the membranes than salt treatment, plus the membrane-bound enzymes were unusually resistant to trypsin treatment. After release by Triton X-100, the enzymes were susceptible to trypsin digestion. These factors suggest an integral, protected position of the enzymes on the membrane, which may account for the failure of other workers to detect activity in this fraction.

Mahadevan et al (1984a) analysed crude microtubule preparations from rat brain and found three isoforms of CKBB on two-dimensional electrophoresis. Using the techniques of radioactive labelling and peptide mapping, they confirmed that the two more acidic forms of the enzyme were phosphorylated, i.e. CK is a phosphoprotein and possibly subject therefore to regulation by phosphorylation. A similar three-spot pattern, with identical molecular mass but differing pI, reacting to anti-CKBB in immunoblots, was found in the two-dimensional analysis of acetylcholine receptor rich membranes from Torpedo marmorata (Barrantes et al, 1983b and 1985).

Distribution in various regions of the brain:

An immunohistochemical technique using anti-CKBB was used to study the localisation of CKBB in human brain (Yoshimine et al, 1983). CKBB was found both in the neurons and the astrocytes, and was distributed throughout in the central nervous system.

Lindsey and Diamond (1978) studied the isoenzymes in cerebrum, cerebellum and basal ganglia. They found considerably more CKBB than the cathodally migrating form in cerebrum and cerebellum, but noted the reverse in basal ganglia.

Petronia et al (1980) investigated the CK electrophoretic bands from various regions of the brain. They studied autopsy tissue

homogenised in 55 mM Tris buffer pH 8.0. They found that the majority of regions from the brain (adult cortical grey matter, white matter, cerebellum and putamen) showed two cathodic bands in addition to CKBB. Foetal brain, obtained fresh from a therapeutic abortion, contained only CKBB, but newborn brain, derived at autopsy, had the additional cathodic bands. This difference may reflect the difference between fresh and autopsy tissue, rather than a valid difference between foetal and newborn isoenzyme distribution. The adult dura cauda equina, on the other hand, had a band which migrated to a similar position as CKMB, in addition to one cathodic band and the CKBB band.

Wevers et al (1982) found the two cathodically migrating bands in both cerebrum and cerebellum, though there was a consistently higher concentration of these bands in the cortex than in the medulla.

1.4. OTHER MEMBRANE BOUND CK SYSTEMS

1.4.1. MITOCHONDRIAL CREATINE KINASE

Location and Compartmentalisation:

Mitochondrial creatine kinase (abbreviated Mt-CK) was discovered in 1964 (Jacobs et al, 1964). It has been shown to be located on the outer surface of the inner mitochondrial membrane (Scholte et al, 1973). The mode of attachment of the Mt-CK to the mitochondrial membrane and its functional compartmentation with other enzyme systems is controversial. Recent research suggests that cardiolipin is the membrane receptor for Mt-CK, based on inhibition of binding of

Mt-CK to CK-depleted mitochondria by adriamycin, which specifically binds to cardiolipin (Muller et al, 1985). On the other hand, Gellerich and Saks (1982) propose that the ADP/ATP translocase serves as a membrane receptor, with a functional compartmentation of the ADP/ATP translocase and Mt-CK, whereby the ADP formed in the intermembranous space by oxidative phosphorylation is selectively transported back to the mitochondrial matrix by the translocase for rephosphorylation. Bessman et al (1981) propose that CK has a functional compartmentation with adenylate kinase, with the oxidative phosphorylation site, two adenylate kinase subunits and one creatine kinase subunit combined in what they call a 'comparticle'.

Mitochondrial genome:

Mt-CK has been shown by cell free translation of canine myocardial mRNA to be encoded by the nuclear genome and not by the mitochondrial genome (Perryman et al, 1983a). The primary translation product at 48000 daltons is 6000 daltons bigger than the final Mt-CK, and it is presumed that a hydrophobic leader sequence is cleaved when the protein is inserted into the mitochondrial membrane from the cytoplasm. The cytoplasmic CKMM, on the other hand, is translated to a protein with the same molecular mass as the mature subunit. Of interest is that the specific anti-MtCK antiserum immunoprecipitates actin (Perryman et al, 1983a).

Characteristics of Mt-CK:

Solubilisation of Mt-CK can be achieved by inducing mitochondrial swelling with 100 mM phosphate buffer or with mercurials such as parahydroxymercuribenzoic acid (Font et al, 1981). Swelling is a prerequisite for release, but the presence of ions or mercurials is necessary for efficient solubilisation.

Wevers et al have characterised the Mt-CK from human heart (1980) and brain (1982). The mitochondria from heart contained two cathodically migrating isoforms of CK with identical molecular masses. The most cathodic form was converted to the least cathodic form by incubation in human serum - this least cathodic form had an identical migration to CKMM, but neither form reacted with commercial anti-CKMM antiserum. The two forms were concluded to be redox conversions of the same molecule. As noted in section 1.3.2., the properties of the brain mitochondrial forms were slightly different since they differed widely in molecular mass (184000 and 65000 daltons), and were not shown to be interconvertible.

Kanemitsu et al (1982a) characterised the Mt-CK from normal human heart and liver tissues. They found that isolated mitochondria yielded two cathodically migrating bands with CK activity, the most cathodic being the major form. Both of these bands reacted with the antibody which they produced against their purified Mt-CK. The molecular mass of the most cathodic band was 350000 daltons whilst that of the least cathodic band was 80000 daltons. As did Wevers' group (1980), they found that incubation of heart Mt-CK in serum, and of liver Mt-CK in 2M urea (but not in serum) converted the most cathodic to the least cathodic, and finally to a third form which migrated to the same position as CKMM.

To summarise, these two authors report similar results with two cathodically migrating mitochondrial bands, with the most cathodic being a higher molecular mass form which can be converted to a less cathodic, lower molecular mass protein - except in the case of the brain where no interconversion was achieved.

Mitochondrial CK in the serum:

As noted earlier, the presence of Mt-CK in the serum is a bad prognostic sign since damage to the mitochondrial membrane must occur to release the enzyme. A method to separate cytoplasmic and mitochondrial isoenzymes from the serum using hydrophobic interaction chromatography has been published (Weselake and Jacobs, 1983) which allows differentiation between the CKMM and cathodic mitochondrial bands.

An experiment on rats exposed to hypoxic conditions showed the appearance of a cathodic Mt-CK in six out of eight rats during peak hypoxia, but only in two rats during recovery (Hayashi and Tanaka, 1985). In the animals which did not show the Mt-CK band, the CKMM was broad and atypical, possibly reflecting the different redox states suggested by Wevers et al (1980).

The crucial role that Mt-CK plays in mitochondria was recently highlighted by Bittl et al (1985) who showed that Mt-CK activity declined significantly within only 10 minutes of cardiac ischemia with a 70% loss within sixty minutes. The loss of enzyme activity correlated with loss of cardiac function, measured by left ventricular developed pressure.

Kanemitsu et al (1982b) studied the origin of a CK-staining band migrating cathodically to CKMM which was found in the serum of a patient with metastatic carcinoma of the liver and bone, and showed that it was identical to liver mitochondrial CK. Similar findings were reported for a patient with metastatic cancer of the liver (Heinz et al, 1980). The serum from this patient contained two bands cathodic to CKMM. The most cathodic had a high molecular mass. After treatment with 6M urea, the enzyme was retarded by a Sephadex

G-100 column, and the migration moved to the least cathodic position.

1.4.2. MUSCLE

CKMM is bound to myofibrils, sarcoplasmic reticulum and plasma membranes in skeletal and cardiac muscle, and CKMB is bound to myofibrils in cardiac muscle. It has been proposed that the bound CK serves to regenerate the ATP hydrolysed during muscle contraction by the intramyofibrillar actin-activated Mg^{++} -ATPase (Walliman et al, 1984).

Bessman and Geiger (1981) recently reviewed a theory known as the "phosphorylcreatine shunt" in which phosphorylcreatine is proposed to be the carrier of the high energy phosphate from the Mt-CK bound to the mitochondria arranged mainly along the I band of the sarcomere to the CKMM bound to the M band. They showed that ATP produced by oxidative phosphorylation is directly supplied to creatine kinase, without mixing with the extramitochondrial pool of ATP. As phosphorylcreatine is the substrate for only creatine kinase, the energy is protected and stored for later release.

Three proteins have been shown to compose the M-line of the sarcomere - myomesin (Grove et al, 1984), M-protein and CKMM (Turner et al, 1973). Myofibrils of chicken heart do not contain the m-bridges of the M-line. It has been shown that microinjection of purified CKMM or CKMM mRNA into chicken heart cells resulted in the appearance of CKMM in the M-line, which proves that the mechanism for translation of mRNA and subsequent insertion of the translated protein into the M-line is available (Schafer et al, 1985).

Compartmentation of Membrane Bound CK in Muscle:

It is still controversial whether membrane bound CK has a

specific compartmentation with various enzyme systems so that the ADP/ATP flux is restricted to a specific pool. Grosse et al propose a functional coupling between CK and Na-K ATPase in cardiac sarcolemmal vesicles (Grosse et al, 1980). However, Philipson et al (1984) have re-examined this theory with a more sensitive technique for monitoring Na transport in cardiac sarcolemmal vesicles, and were unable to find evidence for exclusive pools of ATP. They found that the Na transport worked equally well with ATP produced endogenously in the sarcolemmal membrane or with ATP supplied in the reaction medium.

1.4.3. FISH ELECTRIC TISSUE

The distribution of CK isoenzymes in Torpedo tissues has been studied by Barrantes et al (1983b). Electric tissue contained mainly CKMM, with a hybrid presumed to be CKMB, and CKBB; brain contained CKMM and a band presumed to be mitochondrial CK; dorsal muscle contained CKMM and CKMB, whilst AcCh receptor rich membranes contained only CKBB. The CKMM and CKBB were identified with the aid of specific antisera.

SDS-PAGE of purified post-synaptic membranes from the electric tissue of Torpedo californica showed six major proteins (Neubig et al, 1979). Four of these were the subunits of the acetylcholine receptor. The other two were non-receptor proteins and had molecular masses of 43000 daltons and 90000 daltons. The 43000 dalton electrophoretic band could be further resolved into three proteins on the basis of charge differences and these were originally known as v1, v2 and v3 in the absence of further information about them. The v1 protein still remains unidentified, but the v2 was shown to

copurify with CK activity upon chromatofocusing, and the v3 was shown to be actin (Gysin et al, 1983).

Barrantes et al (1983a) positively identified the v2 protein as CKBB with the use of specific antiserum. The protein did not cross-react with antiserum against CKMM. Gysin et al (1983), on the other hand, found that the membrane-associated CK behaved like CKMM on isoenzyme electrophoresis. More recently, the location of CKBB in the Torpedo acetylcholine receptor rich membrane has been confirmed by Barrantes et al (1985) using immunofluorescence of electrocyte sections. The immunoblot of a two-dimensional separation of acetylcholine receptor rich membranes revealed two proteins which reacted with the antiserum to CKBB. This might be analogous to the discovery that microheterogeneity of CKBB in rat microtubules is due to phosphorylation (Mahadevan et al, 1984b).

Barrantes et al (1985) studied the differences between the soluble and membrane bound forms of creatine kinase. Tryptic fragment comparison showed small differences, which is additional proof that the membrane-bound form is not an artifact of purification. Kinetic analysis showed that the membrane bound form had a two-fold higher affinity for ADP binding to the binary enzyme-creatine-P complex.

Barrantes et al (1983b) suggest that there may be a correlation between the ultrastructural changes in the post-synaptic membrane found in muscular dystrophy with the effect on conformation of Torpedo ACh membranes caused by depletion of the v-proteins.

The results of Barrantes' group have been challenged by Gysin (1986) who failed to find CKBB associated with postsynaptic membranes in Torpedo californica. On the basis of immunological reactivity and

isoenzyme properties, he identified the membrane-bound form as CKMM with a molecular mass of 43000 daltons, as compared to 41000 daltons for the purified CKBB isoenzyme.

1.5 STABILITY AND INACTIVATION OF CREATINE KINASE

Creatine kinase appears to be subject to two kinds of inactivation - one involving the integrity of the essential cysteine residue which is reversible, and one which is temperature-dependent and irreversible (Dalal et al, 1972; Morin, 1977).

The stability of CK isoenzymes varies according to whether they are in a serum or buffer matrix. Since CK isoenzymes play a vital diagnostic role in the laboratory, the majority of the work concentrates on factors influencing stability of CK isoenzymes in serum and plasma.

1.5.1. INHIBITORS PRESENT IN THE SERUM

Dalal et al (1972) showed that an ultrafiltrate of human heat-inactivated serum could inactivate rabbit CKMM, an effect which could in part be prevented by the presence of DTT.

CKBB in serum is rapidly converted to a form which migrates on electrophoresis to a similar position to CKMB (Cho et al, 1976; Bayer et al, 1977; Heinbokel et al, 1981; Lott and Heinz, 1982). This new band was named CKBB' by Lott and Heinz. The transformation appears to involve;

a) a low molecular mass factor of less than 1000 daltons, since Cho found that an ultrafiltrate, and Lott found that a dialysate, of serum could produce the same effects.

b) cations, since Lott and Heinz found that the transformation did not occur when CKBB was incubated with plasma in which the anticoagulant was EDTA or citrate. Lott and Heinz also found the transformation was particularly marked in the presence of Cu^{++} and Zn^{++} ions, which may represent a competitive effect.

c) possibly sulphydryl groups, since Cho et al found that the presence of B-mercaptoethanol appeared to reduce the rate of formation of the CKBB' band.

d) no change in gross structure of the enzyme, since Cho et al found by gel filtration that the molecular mass was unaffected, and Bayer et al found by immunoprecipitation that the antigenic integrity of the molecule remained the same.

e) a dramatic loss of enzymatic activity. Heinbokel et al found that the rate of loss of activity increased with increasing temperature of incubation. Cho et al found that the loss of activity could only partially be restored by B-mercaptoethanol. These two factors suggest that the drop in activity represents the irreversible, heat-mediated inactivation mentioned at the beginning of the section, as a separate issue to the chemically-mediated transformation of CKBB to CKBB'. This suggestion is supported by the fact that Heinbokel et al found that incubation of CKBB in saline at 37°C led to a loss in activity of greater than 50% (where similar incubation in serum led to a greater than 90% loss), but with no change in the electrophoretic migration.

Two low molecular mass inhibitors of CK activity in serum have so far been identified:

Urate:

Urate in the serum has been shown to inhibit creatine kinase

activity (Warren, 1975). It was shown by Jacobs et al (1978) to have a specific inhibitor activity of only 45 anti-units per mg.

Cystine:

Jacobs et al identified another low molecular mass inhibitor of CKMM activity in serum as cystine (Jacobs, 1978). It was shown to be a very potent inhibitor with an activity of 600 anti-units per mg. There is a calculated 10000 fold excess of cystine to creatine kinase in the serum.

Creatine kinase conversion factor:

The presence of a carboxypeptidase in the serum which modifies CK subunits with concomitant loss of enzyme activity will be discussed in section 1.6.4. This conversion factor leads to the conversion of CKMM and CKMB to isoforms with changed electrophoretic mobility. The research on this effect has so far been limited to CKMM and CKMB. Note that this protease is inhibited by EDTA which may account for the stabilizing effect of EDTA on CK activity.

1.5.2. EFFECT OF OTHER FACTORS ON CK STABILITY

Effect of cations:

Ca⁺⁺ ions inhibit CK activity and this has been shown to be due to competition with Mg⁺⁺ ions which are essential to the activity of the enzyme (Nealon, 1980a). CKBB was the most susceptible to this inhibition with a K_i of 0,95 mM, followed by MB (1,85 mM) and MM (4,35 mM). K⁺, Na⁺, Fe⁺⁺, Mn⁺⁺, Zn⁺⁺ and Cu⁺⁺ ions had no significant effect of CK activity. Szasz et al found a K_i of 4,5 mM for CK in serum, competitively inhibited by endogenous polyvalent cations (1979). This could be reversed by adding chelators. However, storage with chelators was not recommended since protection

was not very successful and under some circumstances, chelators actually increased instability. They estimate that endogenous Ca^{++} contributes 50-60% to CK inactivation in serum, whilst Fe^{+++} and Cu^{++} each average only 20% and 25% respectively. The effect of EDTA is most marked in its protection of the thiol groups in the CK reagents. EDTA gave slight protection to purified CKBB added to a serum matrix, but EGTA caused marked inactivation.

Urdal et al (1979) report a procedure for measuring CK in CSF (cerebrospinal fluid). The CSF is concentrated tenfold, which naturally increases the inhibitory substances accordingly. They found that the addition of 15 mM Mg^{++} and 3 mM EDTA increased the activity of CKMM by 35% and CKBB by 60%, and that this was mainly due to the reversal of Ca^{++} inhibition.

An interesting study on the effect of lead on CK levels in rat plasma has been reported (Delahunty, 1984). The first thing of note was that normal rat plasma contained only CKBB (detected by electrophoresis and a specific RIA for CKBB), not CKMM. This contrasts markedly with the human situation. Secondly, the total activity was seven times that of human plasma. After three weeks of administration of lead in the drinking water, the specific activity of CKBB rose three-fold, with the appearance of an electrophoretic doublet. The lead dose was gradually increased over the next six weeks, which was accompanied by a decline in the specific activity of CKBB, to a level significantly below that of the controls. These results were interpreted to mean that the initial lead insult leads to an increased permeability of the cellular membranes, resulting in an increased leakage of creatine kinase. Thereafter, the declining specific activity reflects an inhibition of the enzyme activity by

the lead.

Effect of thiol and chelating agents:

The protection offered to CK activity by thiol agents is controversial. Nealon et al (1980b) showed that thiol agents or chelators gave little or no protection above pH 7,5 and at pH 8,5 actually increased inactivation. Rollo et al (1978) found that B-mercaptoethanol and N-acetyl cysteine increased the instability, but cation chelators markedly improved stability, of endogenous serum CK activity. Chelators caused a 16% increase in CK in fresh sera, independent of B-mercaptoethanol activation.

B-mercaptoethanol and/or EDTA are able to reactivate CKBB in serum. Dalal et al (1972) reported that addition of sulphhydryl groups to serum often resulted in an increased enzyme activity. Abbott and Lott (1984) found that the addition of BME and EDTA to serum increased the electrophoretic detection of CKBB in cancer patients from 34-78% of samples assayed. No CKBB was found in 15 controls, and in only 2 out of 50 non-cancer hospitalised patients but interestingly, 5 out of 8 patients with Alzheimer's senile dementia showed CKBB in the serum after reactivation.

Szasz et al (1978) also noted a marked increase in activity for CKMB and CKBB in serum on storage with thiols, but not for CKMM. They found that the thiol groups of added thiol agents were extremely unstable in serum and that this instability was temperature-dependent. This probably accounts for the inadequate protection of CK activity offered by these agents in serum. The thiol groups were reasonably stable in buffer, however. They recommend adding 50 mM thiol agent to serum to offset this problem. N-acetylcysteine seems to be the best since it resulted in less turbidity and gelling

problems. There was no significant loss of thiol groups when stored at -20°C . Purified isoenzymes were less stable than endogenous serum isoenzymes, which implies protection by bound moieties. Chelators were unable to protect thiol groups in serum (Szasz et al, 1979).

Effect of pH:

Storage of CK isoenzymes is profoundly affected by pH (Nealon et al, 1980b). The optimum pH for maximum stability of CK in serum appears to be 6,5. Increasing the pH above 7 decreases stability.

Effect of albumin:

Nealon and Henderson (1975) showed that albumin significantly increased the lability of CKBB in Tris buffer pH 7,0 at 37°C . In the presence of a less than physiological concentration of albumin, only 20% of the CKBB activity remained after 15 minutes at 37°C . By contrast, albumin seemed to have very little effect on the stability of CKMM and CKMB under similar conditions.

Dalal et al (1972) showed that the stability of rabbit CKMM in saline could be substantially improved by the addition of albumin, which was further enhanced by the inclusion of DTT.

Lemberg et al (1979) studied the effect of injecting bile acids, sodium oleate, albumin and saline into rats on the level of CKBB in the serum, as measured after isolation on an ion exchange column. As compared to the saline control (CKBB=13 U/l), both bile acids (CKBB=120 U/l) and sodium oleate (CKBB=70 U/l) led to a substantial increase of CKBB in the serum. This is apparently due to the detergent effects of the molecules on membrane integrity, resulting in leakage of intracellular proteins. The addition of 2,5 mM albumin to the bile acid plus sodium oleate injection resulted in no CKBB

being detected in the serum. The authors suggest that albumin prevents the leakage from intracellular compartments by binding the detergents, thus preventing their action. Another explanation could be, however, that the failure to detect CKBB in the presence of albumin resulted from an albumin-mediated inactivation, especially in view of the fact that the presence of albumin brought the serum CKBB level to below the control value. It could be that albumin binds to CKBB, masking its active site, or that it in some way increases the rate of inactivation of CKBB.

Effect of temperature:

Nealon and Henderson (1975b) showed that the activity of CKBB declined rapidly at 37°C, a fact which was confirmed by Morin (1977). Szasz found that above 30°C, irreversible inactivation of CK activity occurred, even with thiol protection (Szasz et al, 1978).

Effect of light:

Creatine kinase activity was found to be inactivated in the presence of light (Perry et al, 1979). This was not found in the absence of oxygen, so the mechanism is assumed to be a light-catalysed oxidative process.

1.6. POST TRANSLATIONAL MODIFICATIONS

1.6.1. GLYCOSYLATION

Creatine kinase has been shown to be a glycoprotein (Drummond and Silverman, 1982). McBride et al (1985) found two molecules of sialic acid plus twelve molecules of sulphate per molecule of creatine kinase. Treatment of the enzyme with sialidase resulted in a loss of activity, a 70% loss of immunoreactivity and the inability

to bind to specific glyco-affinity columns. Treatment with arylsulphatase, on the other hand, resulted in a loss of activity, with no loss of immunoreactivity and a partial loss of affinity for the lectin columns. The authors conclude that sialic acid is important in the antigenic site as well as for the active site of the enzyme. I think it would do well to remember that there are likely to be many possible epitopes in the creatine kinase molecule, and as there are only two sialic acid residues per subunit, the possibility exists that some antigenic sites would not be affected by sialidase treatment.

1.6.2. PHOSPHORYLATION

Gordon et al (1977) have provided evidence for an endogenous protein kinase activity in the acetylcholine receptor rich membranes of Torpedo californica. Several proteins were phosphorylated in the presence of labelled ATP, the major substrate being the delta subunit (65000 daltons) of the acetylcholine receptor. In a later publication, they showed that an alkali extract of T. californica postsynaptic membranes, in which the major protein component is the 43000 dalton band, is capable of phosphorylating casein in an exogenous assay system (Gordon et al, 1983). Labelled ATP rapidly bound to the 43000 dalton protein and on the basis of the fact that this protein was "virtually the only ATP binding protein in the alkaline extract" and that it could sustain exogenous phosphorylation, they proposed that the 43000 dalton protein (i.e. CKBB) is a protein kinase.

The work by Mahadevan et al on the phosphorylated CK isoforms associated with the microtubules in rat brain has already been

discussed in section 1.3.3.1. A further paper (Mahadevan, 1984a) describes a technique for separating membrane bound proteins and their associated protein kinases. This should prove to be a very powerful technique to probe membrane bound proteins which are controlled by phosphorylation.

Note that the work referred to on phosphorylation of creatine kinase involved membrane-bound CK, and the same mechanisms may not apply for the soluble forms.

1.6.3. PROTEOLYSIS OF CREATINE KINASE

Proteolytic digestion of creatine kinase with the fungal protease, proteinase K, leads to a rapid inactivation of the enzyme (Ebeling et al, 1974). The creatine kinase subunit appears to be susceptible to proteolysis at one internal site only. Williamson et al (1977) found that rabbit CK_{MM} consisted of two non-identical subunits of 42100 and 40300 daltons each. Treatment of these with proteinase K resulted in a cleavage of approximately 10% off each subunit resulting in two subunits of 37700 and 35500 daltons respectively. Similarly, Lebherz et al (1986) found that treatment of rabbit CK_{MM} with proteinase K led to a 98% loss of activity with a 10% loss of subunit molecular mass. The site of proteolytic cleavage was localised to a single site between alanine residues 328 and 329. A similar result was obtained with Pronase E digestion, which demonstrates the high degree of protection of creatine kinase's primary structure since one would expect that Pronase E, a mixture of non-specific proteases, would completely digest the protein. By labelling the essential cysteine with a fluorescent label, they were able to show that this thiol residue remains intact after proteolysis

- therefore loss of enzymatic activity is not caused by damage to the essential thiol group. The proteolytically modified creatine kinase subunit was still able to be denatured and rehybridise with both intact and modified subunits, showing that the modified protein refolds after denaturation in an appropriate conformation.

Price et al (1981) showed that proteolytically modified subunits of creatine kinase remain associated with one another with no loss of ability to bind the ADF substrate. Although they confirmed the decrease in subunit molecular mass as revealed by SDS-PAGE by the other workers, they showed that the native CK co-eluted with proteolytically modified CK during non-denaturing gel filtration. They conclude that proteolysis under non-denaturing conditions does not lead to a loss of molecular mass. I think a more appropriate explanation may be that the performance characteristics of the two techniques (SDS-PAGE and gel filtration) are too dissimilar to allow a direct comparison. In this case, the molecular mass must have changed since 51 amino acids have been removed, which represents a proportional molecular mass of approximately 5600 daltons.

1.6.4 SUBSPECIES OF CREATINE KINASE

Subspecies found in serum:

The three soluble CK isoenzymes appear to be divisible further into subspecies, due to the presence of dissimilar subunits probably formed by postsynthetic modification. Using isoelectric focusing, three CKMM and two CKMB subspecies were found in serum (Wevers et al, 1977), and hybridisation experiments showed that these were due to the presence of two different M subunits. Wevers et al (1978) found only one CKMM and one CKMB in tissue extracts, which when added to

the serum developed two more anodic CKMM bands and one more CKMB band. Another group isolated five CKMM sub-bands using thin-layer isoelectric focusing (Chapelle and Heusghem, 1980). Three bands (CKMM, pI 7.10; MM1, pI 6.88; MB1, pI 5.61) were dominant in the early stages of myocardial infarction, but rapidly disappeared with the concomitant appearance of bands with increased electrophoretic mobility (MM2, pI 6.70; MM3, pI 6.45; MM4, pI 6.25; MB2, pI 5.34). MM3 was always the dominant band at the end of the observation period. The changes in electrophoretic mobility were shown to be mediated by a thermolabile substance in normal serum. EDTA was found to stabilise the MM and MM1 forms, preventing the production of MM2-4 (Chapelle et al, 1981). Another group found four subspecies of myocardial CKMM in serum after an acute myocardial infarction, using isoelectric focusing, with a shift from the original form to the modified forms with time (Yasmineh et al, 1981). This shift could be mimicked by incubating heart tissue extracts with serum for increasing time intervals. They also noted two CKMB bands. The most recent paper on this issue (SiragEldin, 1986) found 3-4 CKMM bands in sera where the CK activity was below 80 U/l, using isoelectric focusing. In sera with elevated levels of CK, 3-11 bands of CKMM were found.

Creatine kinase conversion factor

Perryman et al (1984) found that incubation of human myocardial CKMM with carboxypeptidases N or B led to two other more anodally migrating forms. This modification of released CKMM and CKMB into further anodic forms has been shown to be mediated by a "creatin kinase conversion factor" which was partially purified from human plasma in 1983 (Edwards and Watts, 1983) and fully purified and

characterized in 1985 (van Landeghem et al, 1985). The molecular mass of the modifying protein was calculated to be 185000 daltons, with a pI of 4.35 (van Landeghem et al, 1985). This factor has been shown to be a carboxypeptidase (Edwards and Watts, 1984, Billadello et al, 1985) which removes the C-terminal lysine residue from each of the subunits in turn, accounting for the three different forms noted by Edwards and Watts in serum. Edwards and Watts make no mention of the above workers' findings of four and five MM sub-bands. Incubation of CKMM with the partially purified conversion factor resulted in the production of the more mobile MM forms, with concomitant loss of enzymatic activity. It can be shown that this is not due to the compromise of the essential thiol by protecting the thiol beforehand with potassium tetrathionate (Edwards and Watts, 1984). The other forms of CKMM still develop, but with no loss of activity.

This carboxypeptidase appears to differ from carboxypeptidases A,B and N on the basis of substrate specificity, pH-activity profile and the effects of metal ions (Edwards and Watts, 1984). Its action can be destroyed by heat inactivation or by adding EDTA to a final concentration of 10 mM. The same protein appears to modify human haemoglobin (Edwards and Watts, 1984) and alpha alpha enolase (Wevers et al, 1984) in a similar way. Wevers et al proposed the name 'modifying protein' rather than 'creatine kinase conversion factor', whilst Edwards and Watts named it carboxypeptidase K since kinase first discovered as a substrate.

Edwards and Watts (1987) have recently studied the developmental appearance of carboxypeptidase K in human plasma, and have found that carboxypeptidase activity is absent in third trimester fetuses but

present in equal amounts in third trimester fetuses, newborns and adults. This suggests that synthesis of this enzyme is activated between 24 and 30 weeks of gestation.

Subspecies in the tissues:

The subspecies discussed above are formed in the serum by the action of the carboxypeptidase which is also located in the serum. There is controversy as to how many subspecies there are in the tissues and so far there seems to be no suggestion as to how these forms are made.

Perryman et al (1984) found that CKMM in human myocardium exists in a single form which forms two additional forms upon release into the plasma. Yasmineh et al (1981) also found that heart contains primarily MM-1 (pI 6,90) with the formation of three additional bands in serum. Another group found one CKMM in canine myocardium, which was converted to two more acidic species after release into the circulation (Billadello et al, 1985).

Several groups, on the other hand, have found more than one CKMM type in the tissues, which may be a reflection on preparative procedures. Using isoelectric focusing, Heinbokel et al (1982) found five CK bands in human heart and skeletal muscle which reacted with antiserum to CKMM. In the uterus and the brain, they found similar patterns, with an anodic band which reacted with anti-CKBB antiserum, three intermediate bands which reacted with anti-CKMM antiserum, and three cathodic types did not cross-react with either antiserum. Vaidya et al (1984) found five CKMM variants using chromatofocusing, CKMM I-IV being found in human heart and skeletal muscle, and CKMM-V being found only in heart. SDS- PAGE revealed a doublet pattern for variants III-V which gave a single band on PAGE

without SDS. Guslits et al (1983) found twenty one subspecies of CKMM from human skeletal muscle using isoelectric focusing, which is consistent with the hybridisation of six different types of M subunit. Fresh serum contained four anodally migrating CK subspecies not present in tissue extract. Six bands on starch gel electrophoresis have also been found in rabbit skeletal muscle, and found by urea dissociation to represent six distinct CKMM subunits (Traugott and Massaro, 1973). Sulphydryl groups do not seem to be involved, since the same pattern was seen in the presence and absence of B-mercaptoethanol.

There is good correspondence between the MM subtypes found in serum and tissue by different groups as shown in Table 1. This table also demonstrates differences in nomenclature of the different MM subtypes used by different groups.

Table 1: MM ISOFORMS WITH ASSOCIATED pI VALUES

<u>Chapelle et al</u> (1980)	<u>Yasmineh et al</u> (1981)	<u>Mickelson et al</u> (1985)	<u>Wevers et al</u> (1977)
human serum	human serum	canine heart	human serum
MM (7,10)			
MM1 (6,88)	MM-I (6,90)	MM3 (6,90)	MM3 (6,86)
MM2 (6,70)	MM-II (6,62)	MM2 (6,70)	MM2 (6,49)
MM3 (6,45)	MM-III (6,36)	MM1. (6,45)	MM1 (6,24)
MM4 (6,25)	MM-IV (6,20)	MMx (6,25)	X (6,07)

The brain has been shown to have three isoforms of CKBB, whose difference in charge is due to phosphorylation (Mahadevan et al.

1981b) - see section 1.6.2.

1.7 CREATINE KINASE STRUCTURE

1.7.1 SUBUNIT MOLECULAR MASS

Molecular mass by SDS-PAGE:

Creatine kinase is a dimeric molecule with a subunit molecular mass in the region of 42000 daltons. Various publications have quoted molecular masses according to SDS-PAGE from 37000 to 500000 daltons, according to the type of subunit and the species of animal under investigation.

Perryman et al (1983b) showed that the M subunits from human, canine, rabbit, mouse and bovine tissue have identical molecular masses of 41000 daltons. The molecular masses of the B subunits on the other hand appeared to differ between species with values of 44500 daltons for human, 46000 daltons for canine, 44000 daltons for rabbit and 49000 daltons for mouse. A molecular mass for mouse CKBB of 50000 daltons was also obtained by Olson et al (1982) - the largest molecular mass quoted for creatine kinase. Under certain circumstances, the CK-B subunit appears to be in a form which gives a molecular mass on SDS-PAGE of 37000 daltons - Leykam et al (1983), who purified human CKBB for the production of monoclonal antibodies, found that they obtained a single band of molecular mass 37000 daltons. Their methodology differed from others in using HPLC as a final purification step.

All the amino acid sequences of creatine kinase so far published have shown the monomer to contain 380 or 381 amino acid residues (see next section). It seems probable therefore that the different

molecular masses obtained by SDS-PAGE are only apparent differences, due maybe to different degrees of post-translational modification affecting the mobility of the proteins during electrophoresis. It is possible that these differences in molecular mass determinations could also reflect the alleged inability of SDS to fully dissociate brain proteins (Katzman, 1972).

Barrantes et al (1983b) have shown that the presence of reducing or alkylating reagents can change the real or apparent molecular mass on SDS-PAGE. In the absence of reducing or alkylating reagents, rabbit CKMM and Torpedo acetylcholine rich membrane preparations gave two CK bands on SDS-PAGE of roughly even intensity, differing in molecular mass by about 3000 daltons. Only one band was apparent after alkylation (higher molecular mass band remaining), or reduction followed or not followed by alkylation (lower molecular mass band remaining). These results demonstrate the difficulty in assigning an accurate molecular mass to creatine kinase by the use of SDS-PAGE.

This kind of explanation may account for the findings such as those by Williamson et al (19..) who found two subunits of CKMM of unequal molecular mass - 42100 and 40300 daltons respectively.

Molecular mass by gel filtration:

The molecular mass of creatine kinase has been determined to be between 78,500 and 85,100 daltons (Watts, 1973). This is consistent with a dimeric conformation of the enzyme.

1.7.2. cDNA AND AMINO ACID SEQUENCES

The CK-M, CK-B and Mt-CK subunits are coded for by different genes, and thus the isoenzymes are immunogenically distinct. It has long been established that there is no cross reactivity between the M

and B subunits by the respective antibodies (Eppenberger et al, 1967). The advances in cloning techniques have allowed the elucidation of the mRNA and amino acid sequences of proteins with considerable reliability. The cDNA sequence for the CK-M mRNA was first cloned in 1982 (Schweinfest et al, 1982). Since then, the nucleotide and amino acid sequences of both CK-B and CK-M have been established from several invertebrate, avian and mammalian sources, as indicated in Table 2.

The striking feature revealed by the creatine kinase sequences is how highly conserved the protein is evolutionarily. Buskin et al (1985) report a more than 80% homology between the sequences reported so far. The M sequences show more homology between species than do the M and B sequences from the same species (Perryman et al, 1983a) suggesting that the M sequence is more highly conserved than the B. Roman et al (1985) note that if conservative substitutions are allowed for the M sequences, then there is approximately 98% homology between species.

Buskin et al (1985) estimate that the gene duplication which led to separate M and B genes took place some 600 million years ago, the approximate time of vertebrate/invertebrate divergence. However, there is remarkable conservation of the integrity of the molecule. The unit evolutionary period (the time in 10 to the power 6 years required for 1% amino acid substitution) has been calculated to be 30, which is one of the highest values obtained for proteins so far characterised. Examples of other proteins which are more highly conserved are alpha-1 collagen (36), glutamate dehydrogenase (55) and histone H4 (400), whilst less conserved proteins include cytochrome c (15), histone H1 (8) and alpha-haemoglobin (36) (Wilson et al, 1977).

Table 2: PUBLISHED SEQUENCES

<u>SPECIES</u>	<u>SUBUNIT</u>	<u>AUTHOR</u>	<u>YEAR</u>
T. marmorata	M	Giraudat et al	1984
T. californica	M	West et al	1984
Chicken	M	rdahl et al	1984
Chicken	M	Kwiatkowski et al	1984
Mouse	M	Buskin et al	1985
Rat	B	Benfield et al	1985
Rabbit	M	Putney et al	1984
Rabbit	B & M	Pickering et al	1985
Human	M	Perryman et al	1986
Human	B	Villarreal-Levy et al	1987

Another interesting point is that the interspecies nucleotide sequences vary more than the amino acid sequences suggesting that the protein sequence is actively selected for. For instance, a comparison between mouse and rat DNA sequences reveals 39 "silent" base changes with only two amino acid differences (Buskin et al, 1985). This critical conservation of the primary sequence implies that it is vital for the function of the enzyme. Roman et al (1985) suggest that this high degree of conservation implies a structural as well as functional importance. Creatine kinase has been found in many membrane bound locations - this will be discussed in more detail later.

The N terminal region of the protein seems less critical than the C terminal since for the known mammalian sequences, half of the

amino acid differences occur within the first 45 residues. The other differences occur in the same places in the sequence and seem to be highly conserved.

There is marked conservation in the 5' non-coding region of the mRNA which suggests that this area may have a regulatory function (Buskin et al, 1985). There is less homology in the 3' non-coding area.

Perryman et al (1983b) showed that the tryptic fragment pattern of CKMM was identical between species, whilst that of CKBB appeared to be specific for each species. Differing proteolytic digest patterns for different isoenzymes have been noted by other authors (Eppenbarger et al, 1967; Barrantes et al, 1985).

1.8 STRUCTURE WITH RESPECT TO FUNCTION

There has been considerable fundamental research done to determine structure/function relationships, reviewed in 1978 by Bickerstaff et al and in 1983 by Kenyon and Reed. Now that the amino acid sequence is available, the opportunity arises for the first time to speculate about the function of the various regions of the molecule.

1.8.1. ESSENTIAL CYSTEINE RESIDUE

Creatine kinase has been known for some time to contain a cysteine which is essential for catalytic activity. Alkylating reagents, for example iodoacetate, bind to this cysteine with a concomitant loss of enzyme activity (Watts, 1973). In 1964, Thomson did a partial sequence of the area surrounding this 'essential'

cysteine (Thomson et al, 1964). By comparison with the full amino sequence of the creatine kinase subunit, this cysteine has now been confirmed to be located at position 283 (Ordahl et al, 1984).

1.8.2. NUCLEOTIDE BINDING SITE

It has been shown by comparison of several nucleotide binding proteins that an "invariant" glycine sequence GXGXXG is required for nucleotide binding (Wierenga et al, 1983). In the case of creatine kinase, this sequence is found at positions 288-294 (Buskin et al, 1985). It has also been shown that a tryptophan residue is required for the binding of adenine (Vasak et al, 1979; Messmer and Kagi, 1985). There are only four try residues in the sequence of CK, localised at 211, 218, 228 and 273. One of these must be in association with the 288-294 'invariant' region, either by a continuous or discontinuous arrangements of amino acids. Nucleotide binding has also been shown to require an arginine (Vasak et al, 1979), which is found at position 291, within the invariant region. The transfer of the phosphoryl group is dependent on lysine residues (James and Cohn, 1974) and at least three histidine groups to act as acid-base catalysts (Rosevear et al, 1981).

1.8.3. CREATINE BINDING SITE

It is possible to pinpoint the creatine binding site by a similar comparative approach. There is homology between creatine kinase and the invertebrate guanidino kinases, in the particular a 33 amino acid sequence which in the case of creatine kinase is localised at positions 162-194 (Buskin et al, 1985). The homology implies a functional similarity, which in turn predicts this sequence to be a

guanidino binding sequence - arginine in the case of the invertebrate kinases and creatine in the case of creatine kinase. It is known that a carboxyl group is required for binding of creatine (Kenyon, 1983).

1.9 COMPARISON OF CREATINE KINASE WITH ALPHA-1 ACID GLYCOPROTEIN AND NEURON-SPECIFIC ENOLASE.

There are two proteins with similar physical properties which could easily be confused with creatine kinase - these are alpha-1 acid glycoprotein (or orosomucoid) and enolase (particularly the neuron-specific isoenzyme). These three proteins are acidically charged and so copurify in systems which exploit the charge of the molecule. This has been shown to be the case by Ganz et al (1984) who found that alpha-1 acid glycoprotein co-purified with CKBB on DEAE-chromatography, and by Gerbitz et al (1983) who found that the only purification system which could distinguish CKBB from neuron-specific enolase was hydroxylapatite chromatography. These facts have obvious implications for this research, which is why this comparison is presented.

Alpha-1 acid glycoprotein (AGP) is synthesized mainly in the liver. It is an acute phase protein, which means that its levels rise in the serum of patients with inflammation, neoplasm or surgical trauma. The function of this protein is still controversial. The fact that it is synthesized in the liver and released into the circulation means that confusion between AGP and CKBB need only be considered when dealing with serum, or where contamination by blood is suspected. The two proteins migrate to a similar position anodal

to albumin on electrophoresis (Hollinshead et al, 1977; personal experience). Both are raised in certain cancers, so a positive identification should be made immunologically, which was not the case with Hollinshead et al, who assumed that that band was AGP where it could very easily have been CKBB.

Enolase is a glycolytic enzyme which converts 2-phosphoglycerate to phosphoenolpyruvate. There are three possible subunit types; alpha, beta and gamma. Alpha-alpha enolase is found in various tissues and in glial cells in adult nervous tissue; it is commonly called non-neuronal enolase (NNE). The beta-beta enzyme is found in muscle. The gamma-gamma is supposedly only found in nervous tissue and is called neuron-specific enolase (NSE). Immature cells contain alpha-alpha enolase, which is switched to beta-beta in the case of muscle and gamma-gamma in the case of nervous tissue cells. This is a comparable situation to creatine kinase, where the embryonic form is CKBB which is switched to CKMM in maturing muscle. The hybrid alpha-gamma enolase isoenzyme is found in brain. The fact that NSE is found in the brain singles this isoenzyme out as a possible contaminant in the purification of brain CKBB, especially in light of the finding of Gerbitz's group (1983) concerning the co-purification of NSE and CKBB.

1.9.1 MOLECULAR MASS BY SDS-PAGE

These three proteins give very similar molecular masses when analysed by SDS-PAGE, which is obviously important to bear in mind when trying to identify them in this way. As noted in section 1.7.1., creatine kinase has a molecular mass on SDS-PAGE anywhere between 42000 and 50000 daltons, depending on the isoenzyme and the

species. AGP has a band equivalent to 41000 daltons (Schmidt, 1975). A membrane-bound form of AGP of 52000 daltons has been reported on normal human T and B lymphocytes. As in the case of CK, enolase gives different molecular weights depending on the isoenzyme and species: In rat brain, Marangos et al (1978) found that the NNE had a molecular mass of 44000 daltons whilst the NSE had a molecular mass of 39000 daltons. Heydorn et al (1985), on the other hand, found that rat NNE had a molecular mass of 49000 daltons and rat NSE had a molecular mass of 44000 daltons (a 5000 dalton difference in each case). Heydorn et al also showed that human NSE had a molecular mass of 47000 daltons whilst human NNE had a molecular mass of 49000 daltons. Shimizu et al (1983) found that purified alpha and gamma subunits both from human brain and skeletal muscle had molecular masses of 46000 daltons, whilst the beta subunit has a molecular mass of 44000 daltons. On two-dimensional gels, the rat and human NNE could be resolved into subspecies with differing pI's, which suggests that they may be subject to phosphorylation as in microtubule CKBB (Mahadevan et al, 1984b). Shimizu et al (1983) found that purified alpha and gamma subunits both gave molecular masses of 50000 daltons.

1.9.2. PROTEIN STRUCTURE

A comparison of the structure of the proteins shows that CK and NSE have much more in common than AGP. CK is a dimer, with each subunit having 381 amino acids (Benfield et al, 1985). Enolase is also a dimer, with the subunit size being 433 amino acids; the amino acid sequence of chicken skeletal muscle enolase (Russell et al, 1986) and the nucleotide sequence of rat non-neuronal enolase (Sakimura et al, 1985) have been determined. AGP, on the other hand,

is a monomer of 180 amino acids (Dente et al, 1985).

Enolase has a tyrosine at position 43 which is subject to phosphorylation by tyrosine kinase (Coope et al, 1984). A comparison of the surrounding sequence of the tyrosines which are phosphorylated in enolase, lactate dehydrogenase and virus transformed proteins with CK shows that the tyrosine at position 38 in the CK sequence has reasonable homology, which suggests that this may be a site for phosphorylation.

Another obvious similarity between enolase and creatine kinase is that they are both subject to modification by the "modifying protein" in serum (Wevers et al, 1984)

A marked difference between AGP and CK is the degree of glycosylation. AGP has a particularly high carbohydrate content of about 45% (Schmidt, 1975).

1.10. INDUCTION OF CK SYNTHESIS

The induction of CKMM in differentiating muscle cells to replace the foetal CKBB can be used as a marker of cell differentiation. One study compared the differentiation of normal human muscle cultures with cultures from patients with a variety of neuromuscular diseases and with Duchenne's muscular dystrophy (Franklin et al, 1981). In the normal cultures, CK total and specific activities increased at least ten-fold during myoblast fusion, with a change in isoenzyme distribution of only CKBB to a profile containing all three soluble isoenzymes. The muscular dystrophy cultures contained significantly less total CK, but with a significantly higher proportion of CKBB than the cultures from neurogenic diseases. This reflects a poor

morphological differentiation. The CKBB proportions were similar to the cultures from myopathic diseases, confirming that muscular dystrophy is a myopathic disease.

It has been shown that CKMM is synthesized after the fusion of myoblasts in culture (Schutzle et al, 1984) and that this is coincident with a rise in cellular cAMP. Inhibition of the increased cAMP levels by indomethacin suggests that this rise is mediated by prostaglandins. This is probably a consequence of phosphatidyl inositol turnover in the membrane, since Ca^{++} is essential for the fusion step.

The regulation of creatine kinase induction has been studied in differentiating MM14 mouse myoblasts (Chamberlain et al, 1985) which begin terminal differentiation when changed to a mitogen free medium. The myoblasts contained traces of CKBB, but within 48 hours of differentiation, CKMM levels had been induced 400-fold, CKMB levels 150-fold, and CKBB 10-fold. Antibodies were used to show that the amounts as well as the activities of these isoenzymes increased. No CK-M mRNA was present in the myoblasts, but by 24 hours, the myotubes contained 1,100 copies of mRNA per nucleus.

Bone cells in culture have been shown to have increased CK activity after treatment with parathyroid hormone or prostaglandin E_2 - combined treated was additive, suggesting different pathways of action (Scmjén et al, 1984). Calcitonin did not stimulate CK activity. Bone cells in low Ca^{++} medium responded to PTH stimulation, but not to PGE_2 , confirming the proposal above that Ca^{++} is required for the action of prostaglandins on CK activity. The increase in CK was both dose and time dependent, and was inhibited by protein synthesis inhibitors, which shows that the increased CK

activity is the result of de novo synthesis, rather than activation of an extant CK pool. The isoenzyme which was induced was CKBB.

1.11. INACTIVE CREATINE KINASE

Burnam's group published three papers on the subject of a protein which they identified as an inactive form of CKBB and which they called CK-Bi. The first paper was the isolation of this protein and the development of a radioimmunoassay (Burnam and Shell, 1981a). The method was essentially a classical isolation of CKBB with an alcohol fractionation step followed by ion-exchange chromatography. The so-called CK-Bi was then formed by repeated alcohol inactivation. They found an inverse relationship between CK activity and the level of CK-Bi as measured with the RIA. The two following papers described elevated levels of CK-Bi in patients with malignancy (Burnam et al, 1981b) and with cardiac disease (Burnam et al, 1982).

The original aim of my research was to substantiate this claim of the existence of an inactive CKBB and its levels in various clinical conditions. This promised to have great diagnostic potential in the laboratory. After extensive efforts to produce the CK-Bi by the published method (Burnam and Shell, 1981a), which failed, I decided to change my approach somewhat. I decided to make an assumption that the protein was there, and to try and purify and characterize it using different methodological approaches. To do this, I decided to raise antibodies to CKBB from human brain. I then hoped that the inactive CKBB would be immunologically similar to CKBB, and that I would be able to isolate it using an affinity antibody column followed by the separation of the active from the

inactive form.

As I gained experience in the isolation of the CKBB from human brain, I realised the inadequacies of Burnam and Shell's methodology. Firstly, the initial step of their isolation was a 60-70% ethanol fractionation after the method of Carlson et al (1976). I discovered that at 60% ethanol, 95% of the CK is precipitated out already - i.e. a 60-70% fractionation loses 95% of the enzyme in the first step. Secondly, they used a batch elution of CKBB off their ion exchange column. Even the use of a gradient elution does not result in a pure CKBB, so there is no doubt that their CKBB preparation must have been contaminated by a number of other proteins. Though they do not say so, I must assume this is why they decided to attempt a further purification by ethanol washing. This resulted in the loss of CK activity, which is hardly surprising.

That they became aware of this contamination is apparent in a subsequent paper (Ganz et al, 1983) where it is stated that a glycoprotein with a similar molecular weight often co-purifies with CKBB. They refer to a promising study of an initial RIA for a mixture of these components - the original paper refers only to CKBB and not to a mixture of components. The contaminating glycoprotein was identified as alpha-1-glycoprotein. (orosmucoid).

It was not until 1984 that the identity of the protein detected by the RIA for CK-Bi was revealed and this in a paper which I was extremely lucky to come by, as the keyword 'CK-Bi' was in no way featured in the abstract and which would never therefore have been revealed by a computerised library search. Ganz et al (1984) disclosed on the second page of paper entitled 'Monitoring the therapy of lung cancer with alpha-1-glycoprotein' that "in 1980, a

newly developed RIA, thought to measure an immunologically distinct inactive form of the BB isoenzyme of creatine kinase was made available to us for evaluation of its utility in monitoring the clinical activity of neoplastic disorders. Our subsequent investigations, which included amino acid composition, peptide mapping, double immunodiffusion, and electrophoresis, have established that the material measured by this RIA is AGP." These investigations are not included in their publication.

By this stage, I had already done about two years of research in search of the inactive CKBB. There is one other group of workers who found an inactive CKBB which gave me some assurance. Armstrong et al (1977a) raised antibodies to rabbit brain CKBB. The antigen (CKBB) was prepared by running purified CKBB on disc polyacrylamide electrophoresis and injecting the excised, homogenised band of CKBB. This ensured the purity of the injected antigen, since they observed eight minor contaminants in the CKBB preparation, as revealed by SDS-PAGE. The resultant antibody to CKBB showed no cross-reaction with CKMM or CKMB.

They then showed that the antibody to rabbit CKBB cross-reacted only partially with the CKBB from rat, guinea pig, sheep, monkey and man, confirming that the B subunit appears to be more species specific than the M subunit (Armstrong et al, 1977b). In addition, they found large quantities of immunoreactive CKBB in mature rabbit cardiac and skeletal muscle which had no enzyme activity. This inactive CKBB was also shown in human skeletal muscle. Furthermore, the active and inactive forms could be separated on ion-exchange chromatography - the inactive form eluting in a fraction between CKMB and CKBB. The active and inactive forms appear to have similar

diffusional and antigenic properties, based on their identical behaviour in agar gel double immunodiffusion studies. Note that their antigen was prepared by the excision of an electrophoretically purified CKBB band. This may have direct comparability with my results.

Their final publication (Armstrong et al, 1977c) noted a three-fold increase in immunoreactive BB in dystrophic gastrocnemii. Enzymatic CKBB was dramatically increased in the muscle, but not in the heart or brain, when compared to controls. There was no statistical difference between the control and dystrophic muscles when the enzyme activity was expressed as a function of immunoreactivity - therefore they concluded that both forms were increased, and that the increased CKBB activity in dystrophy was unlikely to be due to activation of an inactive pool of CKBB. I think this may have been an overhasty conclusion, and needs to be substantiated.

1.12. AIM OF THIS STUDY

As noted above, this study was originally intended to develop an assay for the inactive CK-Bi by the method of Burnam and Shell, and use this assay in the laboratory to study its incidence in cancer and coronary patients.

When the difficulties mentioned in section 1.10., and the complexity of the CK isoenzymes, particularly in serum where their enzymatic activity and electrophoretic mobility are very rapidly modified became apparent, I decided to confine my efforts to more fundamental research, and to study CKBB principally in brain.

It was with the aim of investigating the existence of an inactive CKBB that antibody production was attempted. The first attempt resulted in a multivalent antibody. The second attempt by a different method resulted in an antibody with unusual properties and it was the investigation of these properties which then constituted the bulk of the study.

2. METHODS

2.1 PURIFICATION OF CKBB FROM HUMAN BRAIN

Human brain was obtained post mortem and stored at -20 degrees C. if immediate purification was not possible. The purification was a combination of the procedures published by Grace and Roberts (1982) and Wang and Cushman (1980), and consisted of an initial alcohol fractionation followed by three column chromatography steps.

Alcohol fractionation

The first step was an alcohol fractionation step. The brain was homogenised in a Waring blender in three bursts of 15 secs, separated by 1 minute intervals, in 50mM Tris/HCl buffer pH 7.4 containing 5mM BME (B-mercaptoethanol) in the ratio of 2 volumes of buffer per weight of brain. The homogenate was centrifuged at 10000xg for 30 minutes. The supernatant was transferred to a metal container which was housed in an ice container on top of a magnetic stirrer in the cold room. The supernatant was brought to 50% ethanol by the slow addition, stirred, of absolute ethanol which had been cooled to -20°C. The precipitation of proteins was allowed to proceed for 30 mins, after which time the solution was centrifuged at 5000xg for 15 minutes. The supernatant from this step was brought to 70% ethanol as above and recentrifuged. The precipitate from this step was suspended in Tris/BME buffer with the aid of a hand homogeniser and spun at 10000xg for 30 minutes.

Ion exchange chromatography

The clarified 50-70% ethanol fraction was chromatographed on a 5

by 1 cm column of DEAE Sephrose CL6B as described in section 2.4. The fractions containing the peak of CK activity were pooled.

Hydroxylapatite chromatography

The fractions containing the CK activity from the ion exchange column were chromatographed on a 5 by 1 cm hydroxylapatite column as described in section 2.4. It was established that the presence of NaCl in the pooled peak of activity from the DEAE column did not interfere with the binding of the CK to the hydroxylapatite column. The CK activity of the eluted fractions was monitored and the peak of activity was pooled. This pool was immediately dialysed or buffered exchanged on a Pharmacia PD10 column equilibrated with Tris/BME buffer to remove the phosphate which appears to inactivate the CK activity. The volume of the hydroxylapatite CK peak was reduced to about 1 ml using an Amicon B15 chamber.

Gel filtration

The concentrated peak of CK from the hydroxylapatite column was applied to a Sephacryl S200 gel filtration column and developed as described in section 2.4. The CK activity peak was pooled, concentrated and stored at -20°C .

2.2 PREPARATION OF ANTISERUM

In this study, two antibodies were made using two different approaches to antibody production. This was because the initial attempt yielded an antibody with contaminants. A further attempt was made in order to overcome these problems and it will be shown that the antibody formed in this attempt had interesting properties which became the main focus of this work.

2.2.1 NON DENATURED ANTIGEN ANTIBODY

Preparation

This approach was used in the early days of the project. The CK peak from the DEAE column was used as an antigen, after Burnam and Shell (1981a). The 'purified' CKBB was emulsified with Freund's complete adjuvant and injected into 6 sites intradermally in the backs of 4 rabbits. Two boosters at one month intervals were given in Freund's incomplete adjuvant. The animals were bled out at the end of this schedule. This antibody was designated Ab-CKBB.

Purification

It was only subsequent to the production of this antibody that the extent of the contaminating proteins in the injected fraction was appreciated, and thus its unsuitability without further purification. Further purification was achieved by affinity absorption. A partially purified preparation of CKBB was electrophoresed on a 10% SDS preparative gel and electroblotted to nitrocellulose paper at 60V for three hours. The CKBB band was visualised with a Coomassie blue stain and cut out. The remainder of the binding sites on the nitrocellulose strip were blocked with 3% gelatin in TBS and the strip was then incubated with the Ab-CKBB to absorb the specific antibody. The strip was washed three times with TTBS to remove contaminating proteins and the bound antibody was eluted with 2 mL 0,2 M glycine HCl pH 2,8 for 2 minutes. At the end of this time the solution was buffer exchanged immediately to Tris/BME on a Pharmacia PD10 column to minimise the deleterious effects of the low pH. The antibody solution was then stabilised with 10% BSA and 0,01% merthiolate.

2.2.2. DENATURED ANTIGEN ANTIBODY

When the first antibody was characterised, it was realised that it contained a number of contaminating antibodies, owing to the injection of non-pure antigen. I decided to make another antibody using a technique recommended in a Bioradiation technical bulletin (Knudsen, 1984) which ensures that the injected antigen is pure. The CK peak from the DEAE column was subjected to SDS-PAGE on a 10% preparative plate. The electrophoresed gel was electroblotted onto nitrocellulose paper at 60V for 3 hours in the cold room using the Biorad electroblotting apparatus. The CK protein band was visualised by staining a side strip of the electroblotted nitrocellulose paper with a 0,2% solution of Coomassie blue R-250 in 40% methanol, 10% acetic acid for 5 minutes, followed by rapid destaining in 90% methanol, 2% acetic acid, and its position was confirmed by comparison to an independent gel run concurrently with commercial low molecular mass markers. The horizontal band containing the CKBB was excised from the nitrocellulose using a scalpel blade, thus separating it from the contaminating proteins. The nitrocellulose paper strip was then dissolved in approximately 0,5 ml dimethyl sulphoxide, and combined with Freund's complete adjuvant. The antigen cocktail was injected in 6 sites intradermally on the backs of two rabbits (approximately 100 mg creatine kinase per rabbit). The remainder of the antigen mixture was frozen and used for two booster inoculations at 1 month intervals. The rabbits were bled out after this time. The antibody formed by this procedure was designated Ab-DEN (i.e. antibody to denatured protein) to differentiate it from the Ab-CKBB.

2.3. ASSESSMENT OF ANTIBODY TITRE

2.3.1. PASSIVE HAEMAGGLUTINATION

The first antibody (non-denatured preparation, or Ab-CKBB) was assessed using passive haemagglutination using tanned sheep's red blood cells by the method of Adler and Adler (1980). Sheep's blood collected into citrate tubes was obtained from the serology department of the South African Institute for Medical Research. The cells were washed three times in phosphate buffered saline pH 7.2 (PBS) and suspended to give a 5% (v/v) suspension. The red cells were tanned by the addition of an equal volume of 1:20000 (w/v) tannic acid in PBS and incubated at 37°C for 15 minutes. The tanned cells were centrifuged at 750xg for 10 minutes, drained by inversion on tissue paper and resuspended to 5% in PBS. They were then coated with CKBB by mixing 1 volume of tanned cells with 4 volumes of CKBB (approximately 0.5 mg) in PBS for 15 minutes at 37°C. The cells were washed three times in PBS and suspended to 5% in PBS. Round bottomed microtitre plates were used for this assay. Doubling dilutions of antibody in PBS containing 1% normal rabbit serum were aliquoted out into the wells of the plate, and 1 drop of tanned, coated cells was added to each well. The plates were then shaken to mix the contents, covered and set aside for several hours to allow haemagglutination to occur. The plates were assessed visually. In the wells where no agglutination occurred, the cells settled by gravity into a large, smooth 'button'. With increasing agglutination, the cells stick together and form tighter, smaller deposits. The reciprocal of the dilution in the last well to be positive for agglutination (with the

appearance of 'half a button') was taken as the titre of the antibody.

2.3.2. ELISA (enzyme linked immunoassay)

The second antibody (denatured preparation or Ab-DEN) was assessed using an ELISA technique developed by myself. Doubling dilutions of antigen were coated onto flat-bottomed microtitre plates (Nunc-Immuno Plate 11) and left to coat at 4 degrees overnight, or at 37 degrees for an hour. The plates were washed three times with Tris buffered saline (TBS) containing 0,05% Tween 20 (then known as TTBS). Additional binding sites on the plates were blocked with 10% bovine serum albumin (Boehringer 735086) for 30 minutes at room temperature. 100 ul of the first antibody solution (Ab-DEN) - 1 in 100 dilution in 1% BSA in TBS - was added to each well and incubated at 37°C for 30 minutes (or 1 hour at room temperature, or overnight in the fridge). The wells were then washed three times with TTBS and 100 ul of the second antibody (goat anti-rabbit IgG horseradish peroxidase conjugate Bio-rad 170-6516) solution - 1 in 2000, 3000 or 4000 dilution in 1% BSA in TBS depending on the amount of antigen present - and incubated as for the first antibody solution. The plates were washed three times with TTBS and 100 ul of colour reagent (100 mg % ortho-phenylene diamine.2HCl [Sigma P1526] in pH 5,0 buffer) added. The plates were protected from light whilst the colour was developing, and then the reaction stopped with the addition of 100 ul 1M H₂SO₄. The absorbance of each well was then measured using a Titertek Multiscan reader (Flow Laboratories). Each plate had a column of blank wells containing all reagents except the antigen, and the reader was blanked against this column; this compensates for slight differences in the plate itself as well as giving an appropriate background absorbance.

2.4. CHROMATOGRAPHIC TECHNIQUES

2.4.1. ION EXCHANGE CHROMATOGRAPHY

The fraction to be chromatographed was pumped onto a 5 by 1 cm column of DEAE Sepharose CL6B (Pharmacia) [which gives an improved separation of isoenzymes compared to Sephadex (Bondar et al, 1980)] equilibrated with Tris/BME at about 2 ml/min. The column was washed with Tris/BME buffer until the A_{280} of the discarded fraction was negligible. A linear sodium chloride gradient formed with 50 ml each of Tris/BME buffer containing 0 and 300 mM NaCl respectively was then pumped onto the column at about 1 ml/min and 1 minute fractions were collected. The collection of fractions was begun in all the columns at the same time that the eluting buffer was applied. The gradient was assessed by measuring the NaCl in the eluted fractions with a flame photometer. The fractions were monitored for CK activity using a Boehringer NAC-activated kit on the Encore centrifugal analyser.

2.4.2. HYDROXYLAPATITE CHROMATOGRAPHY

The fraction to be chromatographed was pumped onto a 5 by 1 cm hydroxylapatite (Bio-rad) column equilibrated with Tris/BME. The column was washed with Tris/BME until the A_{280} of the discarded buffer was negligible, and then eluted in a batch fashion with 10mL of 100mM phosphate buffer pH 7.4 containing 5mM BME pumped on to the column at 1 ml/min with the collection of 1 min fractions.

2.4.3. BLUE SEPHAROSE CHROMATOGRAPHY

The fraction to be chromatographed was pumped onto a 5 by 1 cm

column of Blue Sepharose CL6B (Pharmacia) equilibrated with Tris/BME and washed until the A_{280} of the discarded buffer was negligible. Several elution procedures were tried - one using an increase in salt concentration after Thompson et al (1975) and Miller and Wei (1984), one using batch elution with 10 mM ADP after Walliman (1985) and one using an increase in pH from 6,0 to 8,0 after Fischer and Whitt (1979) and Walliman (Walliman, 1985).

2.4.4. GEL FILTRATION

The fraction to be chromatographed was concentrated to a volume of not more than 1 ml and pumped onto a 40 by 2,5 cm column of Sephacryl S200 (Pharmacia). It was developed overnight with Tris/BME at a pump speed of about 0,2 ml/min, with the collection of 10 minute fractions. The CK activity peak was pooled, concentrated and stored at -20°C .

The gel filtration column was calibrated for determining molecular masses with reference proteins from the Combithek kit (Boehringer 104540). The void volume was marked with dextran blue.

2.5. ELECTROPHORESIS

Non-denaturing and SDS polyacrylamide electrophoresis were performed on standard vertical slab gel electrophoresis equipment using the method from the Hoeffer electrophoresis catalogue. Routinely non-denaturing gels were 7,5% T 2,7% C, SDS gels were 10% T 2,7% C and both had a 4% T 2,7% C stacking gel. Calibration of SDS gels for molecular mass determinations was with a low molecular weight marker kit (Pharmacia or Bio-rad).

Visualisation of proteins bands was achieved by fixing the gels in 50% methanol, 10% acetic acid and then staining with a 0,1% solution of Coomassie blue R250 in the same solution for at least half an hour. The unbound stain was washed off overnight in 5% methanol 7% acetic acid. Staining for carbohydrate moieties was with the periodic acid Schiff (PAS) stain.

2.6. ELECTROBLOTTING

Electroblotting of PAGE gels to nitrocellulose paper was achieved using the Bio-rad electroblotting equipment and visualisation of immunoreactive bands was done with the reagents and method supplied with the Bio-rad immuno-blot (GAR-HRP) kit.

2.7. PROTEIN ESTIMATIONS

Proteins concentrations were estimated using the Bio-rad protein assay kit (Bio-rad 500-0001) using bovine serum albumin as a reference protein.

2.8. ISOENZYME ELECTROPHORESIS

The isoforms of creatine kinase were separated at 140 V for 25 minutes on Paragon CK agarose gels (Beckman) using the Helena marker for identification and characterisation of the bands. The bands were visualised into bands visible under ultraviolet light using the stain supplied with the Paragon kit. The gels were photographed immediately under the ultraviolet light since the bands tend to fade

with time.

2.9. LIMITED PROTEOLYSIS

Limited proteolysis of bands excised from stained SDS PAGE gels was performed after Cleveland et al (1977). The excised band was soaked for 30 minutes in 10 ml 125 mM Tris/HCl pH 6,8 containing 0,1% SDS and 1 mM EDTA. The soaked band was pushed into the bottom of a sample well in the stacking gel of a 15% T SDS gel with the aid of a microspatula. This was overlaid first with 20% glycerol and then with a solution containing 20% glycerol and 2,5 or 50 ug/ml protease (endoproteinase Glu-C from Staph aureus V8 - Boehringer 791156). The proteolysis takes place whilst the gel is stacking and then once the proteins start to separate out according to size, the protease is removed from contact from the peptides formed.

2.10. DIFFERENTIAL CENTRIFUGATION

Brain was fractionated into subcellular components by the method of Hemminki and Suovaniemi (1973), without the sucrose centrifugation steps, to purify further the particulate fractions. Brain was homogenised in Tris/BME buffer and centrifuged at 3500xg for 10 minutes. The precipitate was washed, and the combined supernatants were centrifuged at 20000xg for 20 minutes. The precipitate was washed, and the combined supernatants were centrifuged at 100000xg for 30 minutes.

2.11. MEASUREMENT OF CREATINE KINASE ACTIVITY

CK activity was measured using a Boehringer NAC-activated kit on the Encore centrifugal analyser, and the activity was expressed in International Units per milligramme (U/mg).

3. RESULTS

3.1. PURIFICATION OF CKBB

The brain isoenzyme is extremely difficult to purify to homogeneity in good yield whilst still maintaining high specific activity - firstly because the enzyme activity is labile, and secondly because there appear to be many forms of the enzyme with differing charges. A variety of approaches have been used by different researchers in the purification of the enzyme, utilising differences in charge, thiol content, dinucleotide folding, phosphate content, and size.

In my experience the best purification scheme was an initial 50-70% ethanol fractionation, followed by gradient elution off a DEAE Sepharose CL-6B (Fig 1a), batch elution off a hydroxylapatite column (Fig 1b) and gel filtration on a Sephacryl S-200 column (Fig 1c). The CK peak fraction off the DEAE column could be loaded, without dialysis, onto the hydroxylapatite column which saved a lot of time. Time is obviously of the essence, since any delay results in loss of activity. The ethanol fractionation and first two chromatographic steps (i.e. DEAE and HTP) could be achieved in one working day, and the gel filtration step run overnight, resulting in a virtually homogenous enzyme preparation within 24 hours. Obviously specific activities varied quite widely with each preparation, particularly since the brain is post mortem, but characteristically the specific activity of the purified CKBB was about 700 U/mg.

The results of two different purifications of CKBB are outlined in Tables 3 and 4. The specific activities obtained compared very

favourably with others quoted in the literature (see Table 5).

TABLE 3: PURIFICATION OF CKBB FROM 110,5 g HUMAN BRAIN

<u>FRACTION</u>	<u>CK</u>	<u>VOL</u>	<u>PROTEIN</u>	<u>SPECIFIC</u>	<u>YIELD</u>
	(U/l)	(ml)	(mg/ml)	<u>ACTIVITY</u> (U/mg)	(mg)
initial homogenate	920000	176	9,1	101	1605
50-70% alcohol step	820000	41	6,2	132	254
peak from DEAE	970000	17	1,4	697	23,7
peak from HTP	540000	4,7	0,5	1080	2,4

TABLE 4: PURIFICATION OF CKBB FROM HUMAN BRAIN

<u>FRACTION</u>	<u>CK</u>	<u>PROTEIN</u>	<u>SPECIFIC</u>
	(U/l)	(mg/ml)	<u>ACTIVITY</u> (U/mg)
initial homogenate	152	14,0	11
50% alcohol supernatant	71	1,1	65
70% alcohol precipitate	900	9,4	95
peak from DEAE column	1180	3,0	388
peak from HTP column	700	1,2	583
peak from S-200 column	174	0,3	615

SDS PAGE of the purified CKBB consistently gave a molecular mass of 45000 Daltons, in contrast to the published molecular mass of

43000 Daltons. It proved extremely difficult to remove a contaminating band at 48000 Daltons, which is assumed to be neuron

TABLE 5. SPECIFIC ACTIVITIES OF PURIFIED CKBB FROM DIFFERENT LABORATORIES

<u>AUTHOR</u>	<u>SPECIES</u>	<u>SPECIFIC ACTIVITY OF PURIFIED CKBB (U/mg)</u>
Olson et al (1982)	rat	190
Armstrong et al (1977a)	rabbit	1412
Keutal et al (1972)	rabbit	122
"	human	102
Carlson et al (1976)	human	90
Thompson et al (1980)	human	123
Wang et al (1980)	human	182
Urdal et al (1981)	human	470
Roberts et al (1982)	human	487
Grace et al (1982)	human	506
Gerbitz et al (1983)	human	470
Stephens et al (1983)	human	119
Miller et al (1984)	human	170
McBride et al (1984)	human	1122

specific enolase (NSE). Gerbitz et al (1983) found that in a purification procedure using ammonium sulphate fractionation, DEAE, gel filtration and hydroxylapatite chromatography, CKBB and NSE

cochromatographed in all steps except for the hydroxylapatite column where a slowly increasing phosphate gradient could differentiate a CKBB peak at about 23 mM phosphate, followed immediately by a NSE peak at about 33 mM phosphate. Batch elution off a hydroxylapatite column with a 10 mM phosphate buffer is a convenient way to remove the majority of the NSE, though if an overloaded SDS PAGE gel of 'purified CKBB' is run, there is invariably a trace of the 48000 dalton NSE.

**Fig 1a. DEAE SEPHAROSE CL6B CHROMATOGRAPHY
OF THE 70% ETHANOL PRECIPITATE OF BRAIN
HOMOGENATE**

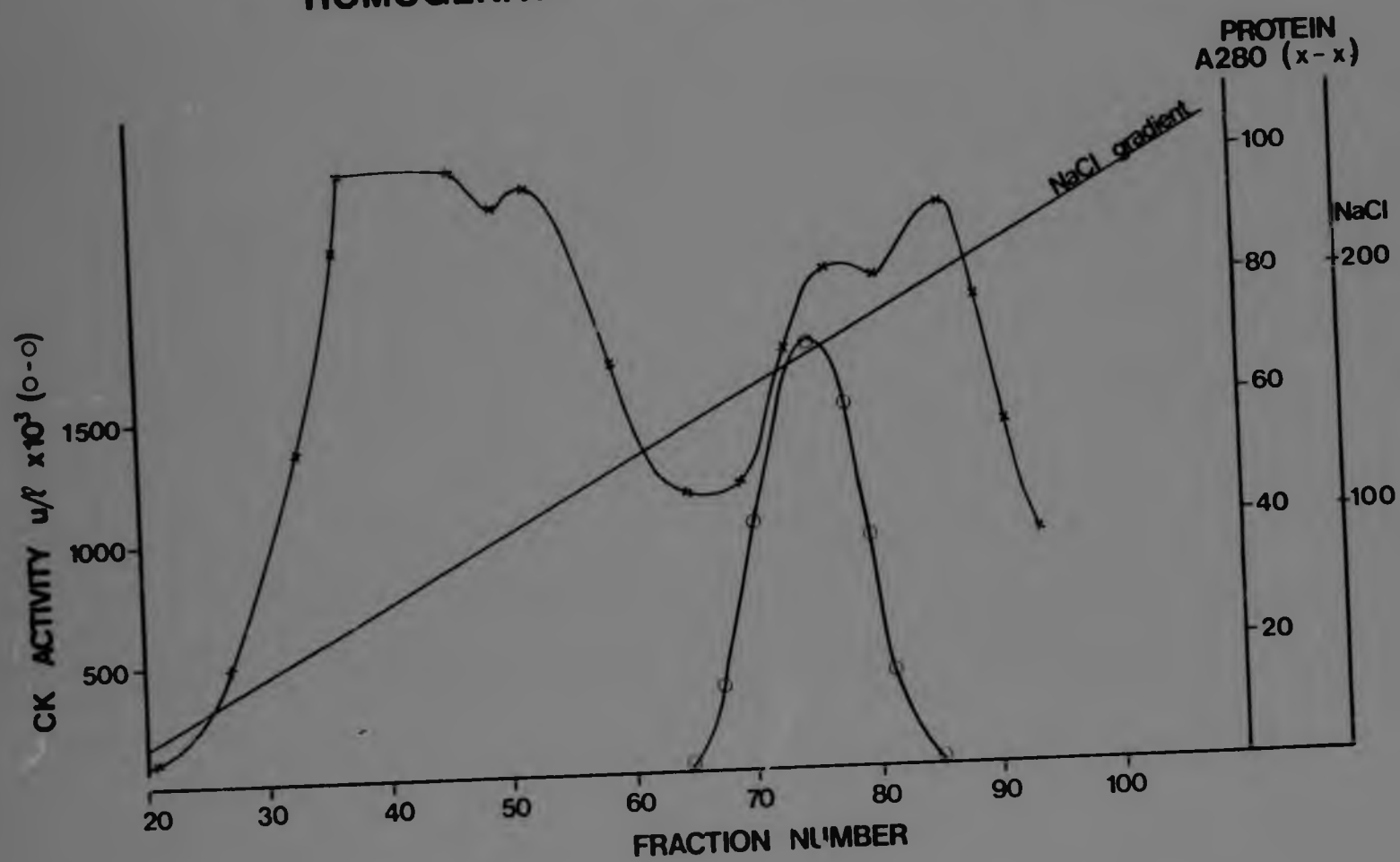


Fig. 1b
HYDROXYLAPATITE CHROMATOGRAPHY OF
BRAIN PROTEINS, previously purified by 50 - 70%
ethanol precipitation and DEAE Sepharose CL6B
chromatography

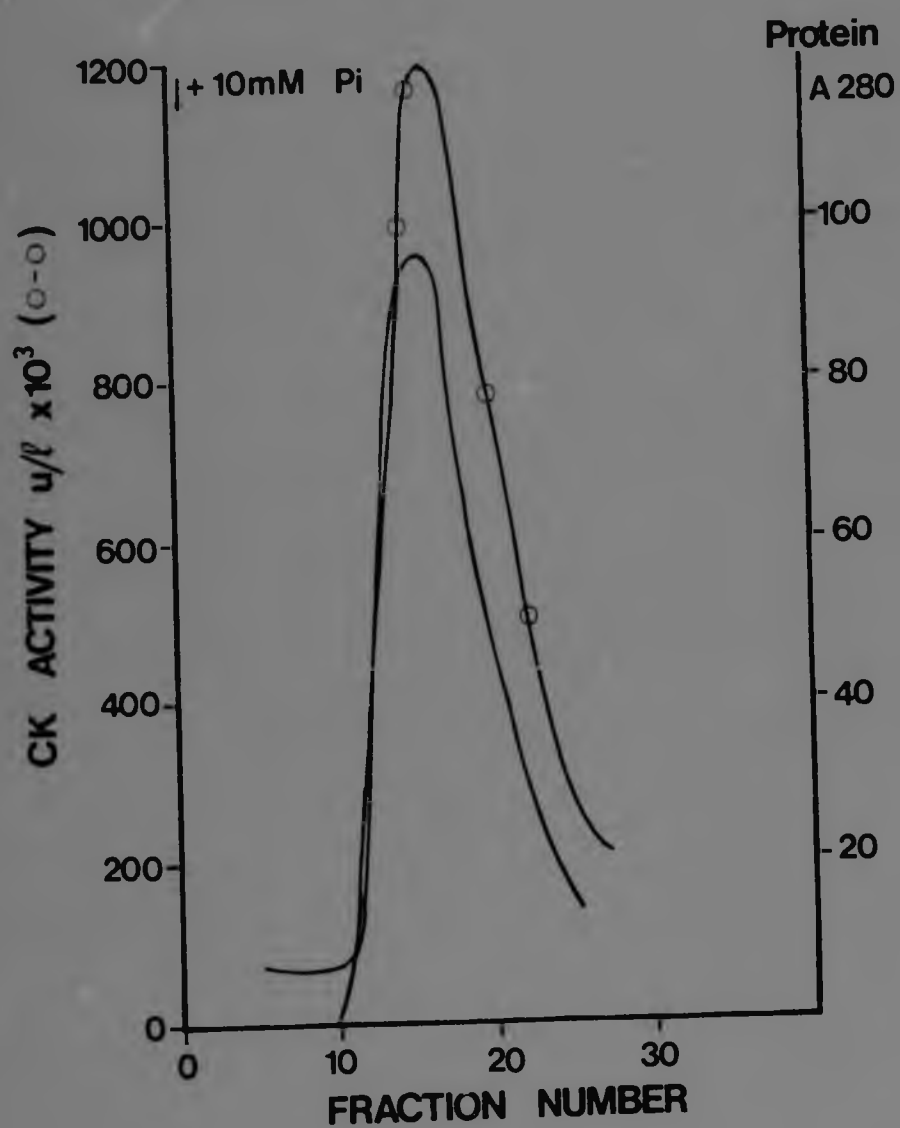
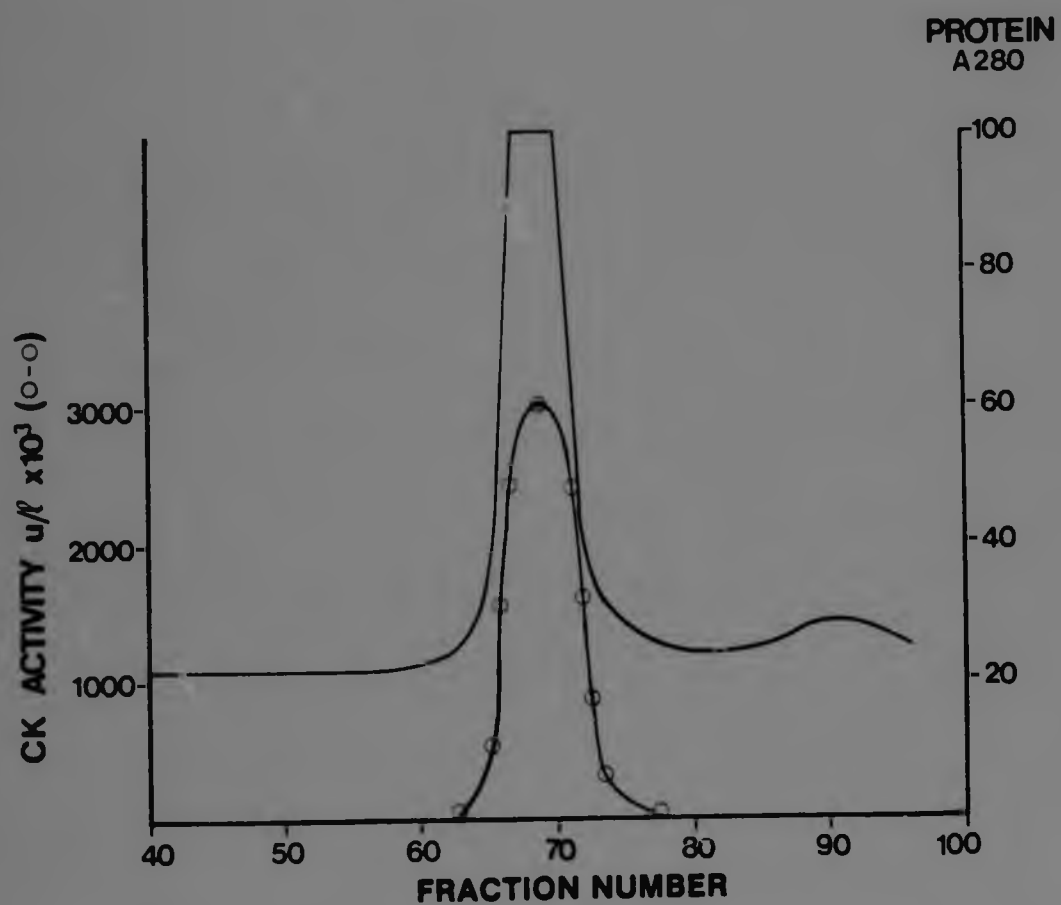


Fig. 1c
SEPHACRYL S200 CHROMATOGRAPHY OF CKBB,
previously purified by 50 - 70% ethanol fractionation,
DEAE Sepharose CL6B chromatography and
hydroxylapatite chromatography.



3.2. PRODUCTION OF ANTIBODY

Two antibodies were produced in this study. The first was made by inoculating rabbits with CKBB in the conventional manner. The CKBB used was the peak of activity off the DEAE Sepharose CL6B column which therefore contained several contaminating proteins. The production of antibodies, as monitored by the passive haemagglutination technique, is shown in Fig 2. Rabbits 2 and 4 responded well to the inoculation, whereas rabbits 1 and 3 made a negligible quantity of antibody.

The second antibody was made by injecting purified CKBB electroblotted to nitrocellulose and excised, a technique which guaranteed that no contaminating proteins were injected (or only those proteins with virtually identical physical properties which cannot be completely ruled out.) The production of antibodies, as monitored with an ELISA technique, is shown in Fig 2b. Rabbit 2 produced much more antibody than rabbit 1 and this antiserum was used in all the following experiments. It has been designated Ab-DEN (antibody to denatured protein) to distinguish it from the AB-CKBB (antibody to native CKBB as above).

Fig. 2a
ASSESSMENT OF ANTIBODY (Ab - CKBB) TITRE by
passive haemagglutination — antiserum from 4 rabbits

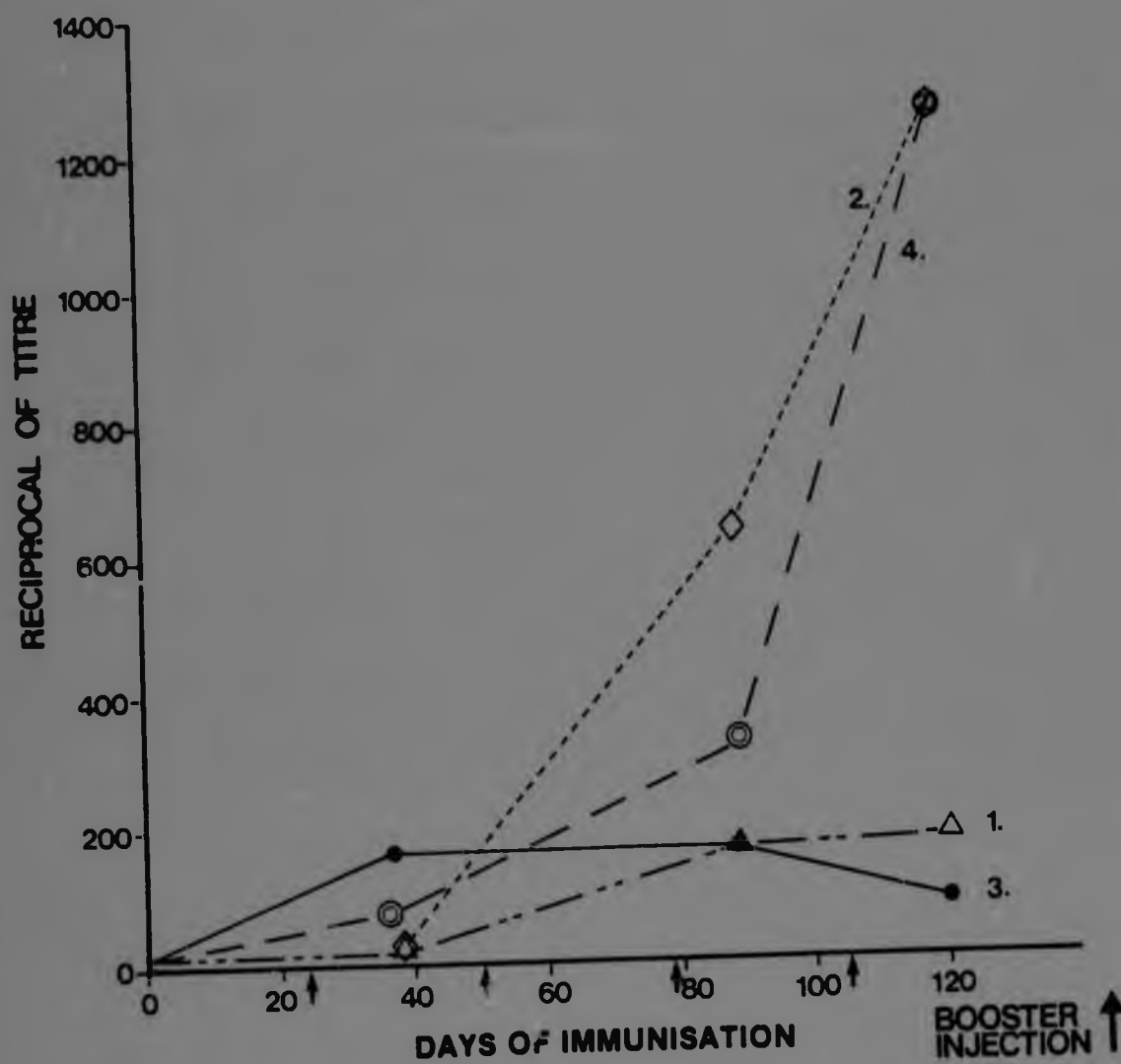
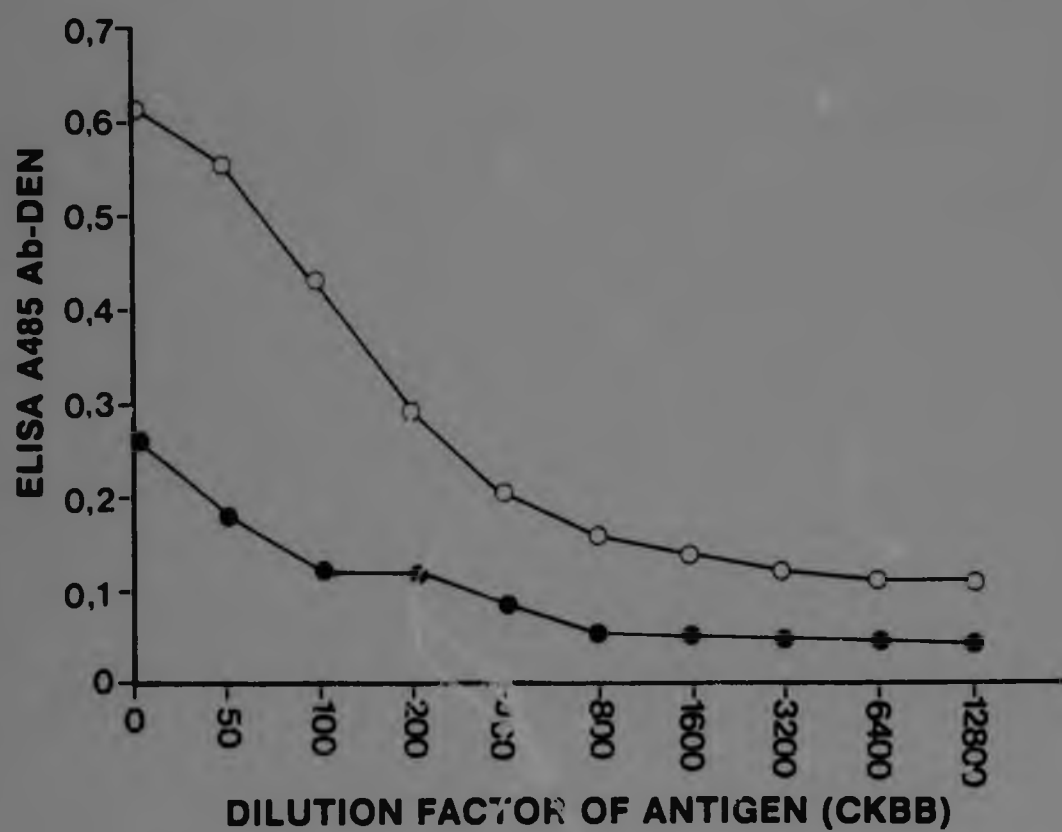


Fig. 2b
ASSESSMENT OF ANTIBODY (Ab - DEN) TITRE by
ELISA antiserum from rabbit 1 (● - ●) and rabbit 2 (○ - ○)



3.3. SPECIFICITY OF Ab-DEN

The specificities of the Ab-DEN and Ab-CKBB were tested by immunoblotting a 10% SDS PAGE gel of the 70% ethanol precipitated brain proteins. The Ab-CKBB reacted strongly with one band and weakly with apparently three higher molecular mass and one lower molecular mass bands. The Ab-DEN reacted weakly only with the same band which reacted strongly with Ab-CKBB. It was thought at this stage, therefore, that Ab-DEN was monospecific for CKBB, if not very sensitive. Later experiments showed, however, that it did indeed recognise other molecular mass bands and the problem was then to try and show whether these other bands were CKBB, CK-like or contaminating proteins. An example of such an experiment was the incubation of Ab-DEN coupled to a sheet of nitrocellulose paper with brain supernatant (1:2 w/v which had been stored at -20°C for 25 days) followed by elution of bound proteins with glycine-HCl. This process yielded 144 ug protein from 10 ml brain supernatant - i.e. roughly 5 g brain - and when these proteins were electrophoresed on a 10% SDS PAGE gel, three proteins of molecular mass 66000, 56000 and 46000 daltons were seen.

In another instance, brain proteins were separated into two groups by collecting the discarded proteins from a DEAE Sepharose CL6B column and batch eluting the bound proteins with NaCl. These two groups of proteins were then run on a 10% SDS PAGE gel, stained for protein with Coomassie Blue and immunoblotted with Ab-DEN from rabbit 1 (the only instance that this Ab was used because of low titre), The discarded fraction had an faint immunoreactive band at 34000 daltons and about 10 extremely faint bands (only visible by holding the nitrocellulose paper up to a strong light). The eluted

fraction had a main immunoreactive band at 44000 daltons with a minor band at 67000 and bands at 50000 and 74000 daltons which could be seen if the nitocellulose sheet was held up against the light. It is most unlikely that these higher molecular mass species could reflect contaminating antibodies since a narrow band of molecular weight section was cut from the gel in the preparation of the antibody.

The first indication that Ab-DEN recognised proteins other than CKBB, the protein which had been used as antigen, came when first brain supernatant (fig 3a), then breast tumour cytosol [a gift from the Nuclear Medicine Department] (fig 3b), were chromatographed through DEAE Sepharose CL6B and all the eluted fractions were monitored for CK activity and reaction to Ab-CKBB and Ab-DEN using the ELISA technique.

These experiments were performed in pursuit of the CK-Bi of Burnam and Shell, with the rationale that the antibody formed to CKBB may well recognise any inactive form of the protein. It must be pointed out that subsequent to these experiments I discovered a publication in which it was revealed that Burnam and Shell's "CK-Bi" was in fact AGP (see section 1.11).

The discovery of an antigen peak which responded to the Ab-DEN at fraction 30 of the DEAE Sepharose column and which had a measureable but negligible CK activity, in contrast to the CKBB peak at fraction 74, which also did not respond to the Ab-CKBB, raised hopes that an inactive CK had, in fact, been found.

It was discovered that this protein, hereafter referred to as the 'antigen', was precipitated by 50% ethanol, a factor which allowed for a convenient separation of antigen from CKBB (Fig 3c).

**Fig. 3a DEAE SEPHAROSE CL6B CHROMATOGRAPHY
OF BRAIN SUPERNATANT, assayed for CK activity and
ELISA using Ab - DEN**

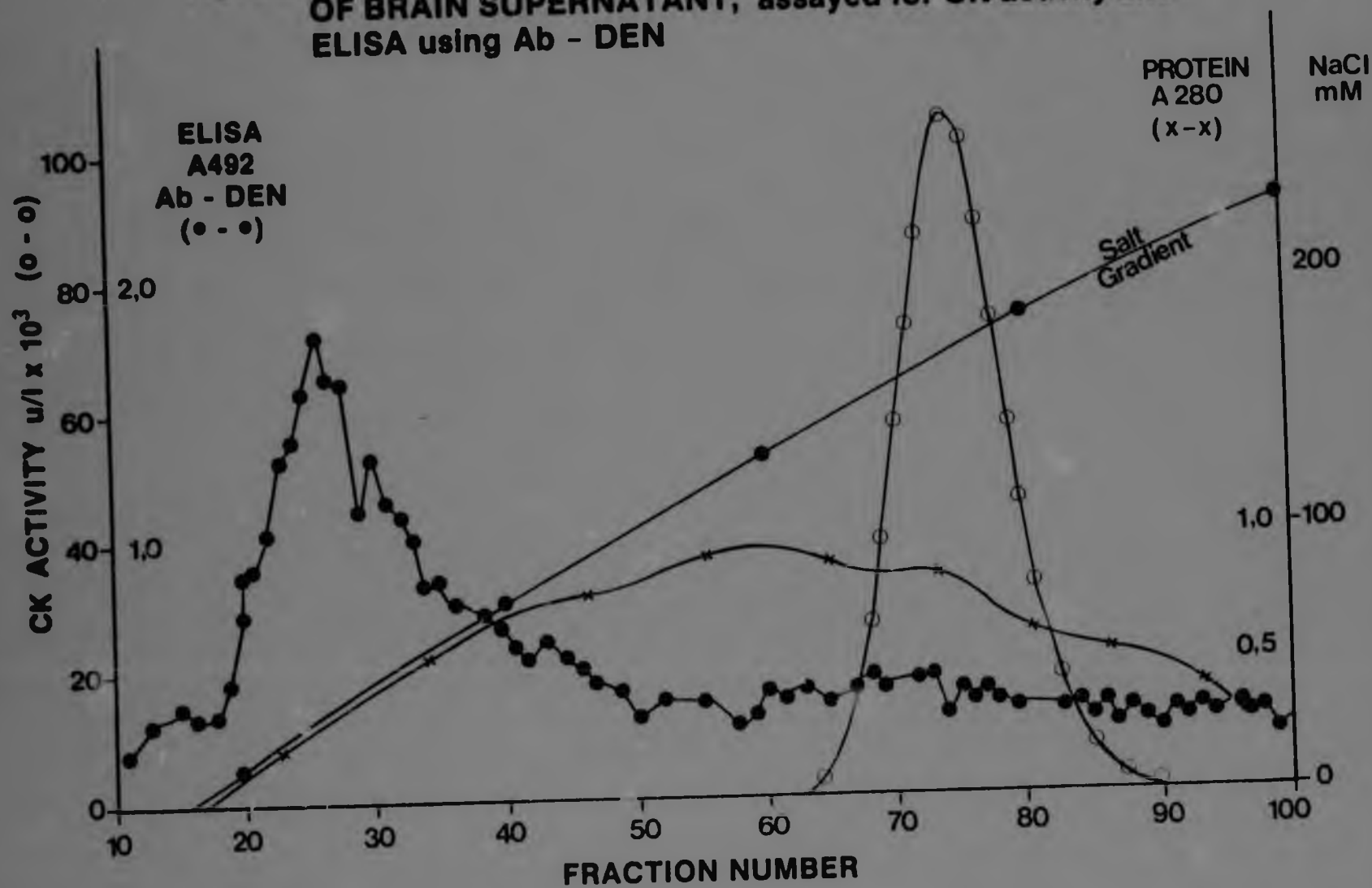


Fig. 3b DEAE SEPHAROSE CLEP CHROMATOGRAPHY OF BREAST TUMOUR CYTOSOL

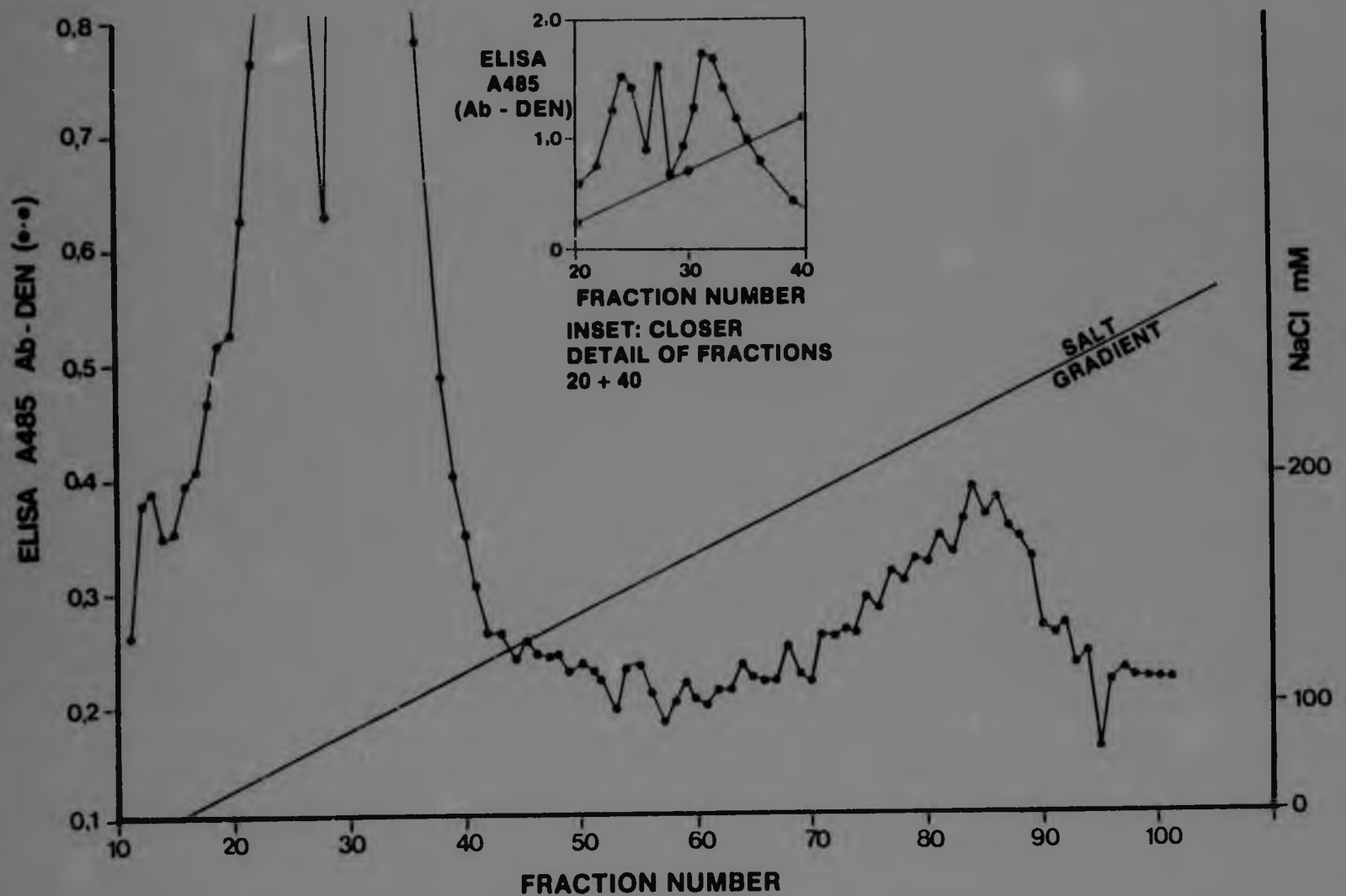
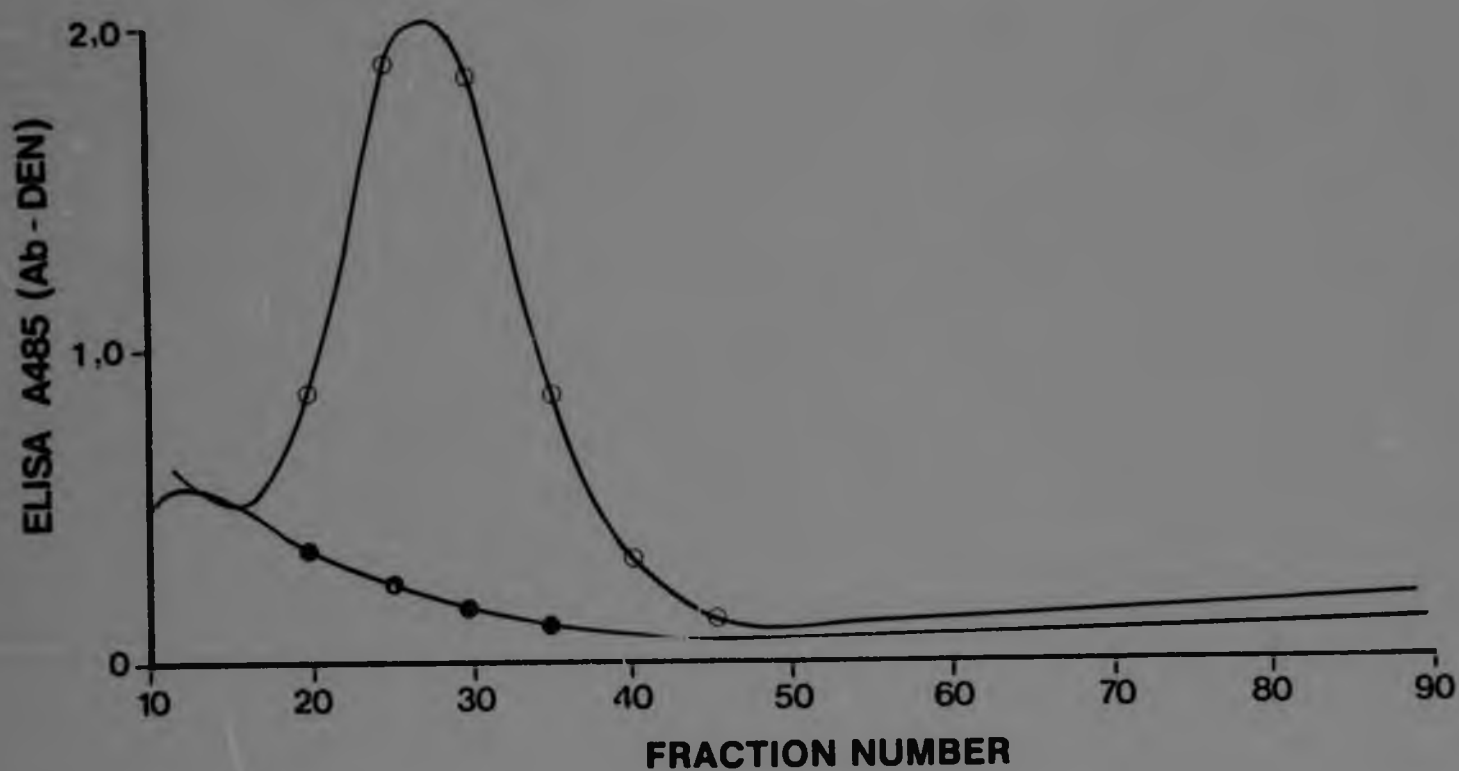


Fig. 3c
DEAE SEPHAROSE CL6B CHROMATOGRAPHY, of
50% ethanol precipitate (o - o) and 50-70% ethanol
fraction (● - ●) of brain, assayed by ELISA
using Ab-DEN



3.4. PURIFICATION OF 'ANTIGEN'

The experiments shown in this section were performed on brain which had been stored in the ice chest of the refrigerator, i.e. at 0°C, for two weeks. This point is relevant since the results differ from those obtained using fresh brain with respect to the presence and height of peaks on DEAE Sepharose.

The DEAE Sepharose CL6B chromatography of the 50% ethanol precipitate of brain is shown in Fig 4a. Only one peak was obtained with the ELISA using Ab-DEN, and this was concurrent with a CK activity peak at fraction 30 (55 mM NaCl). In addition, there were two other roughly equal CK activity peaks, one at fraction 65 (165 mM NaCl) and one at fraction 74 (190 mM NaCl). The ELISA with Ab-CKBB (unpurified) gave a broad region of reaction at the beginning of the gradient, and two later peaks, one concurrent with the fraction 74 CK activity and one at fraction 56 which I surmise to be reaction with contaminating albumin antibodies. This is the only occasion when a CK activity peak was obtained at fraction 65, and must be formed by degradative breakdown of the CKBB since the brain had been stored at 0°C instead of -20°C.

The 50-70% ethanol fraction of the same brain, chromatographed through DEAE Sepharose CL6B, is shown in Fig 4b. Two peaks of CK activity were obtained at fractions 73 (185 mM NaCl) and 77 (195 mM NaCl). Similar treatment of fresh brain routinely yielded only one CK activity peak.

The SDS PAGE electrophoresis of the peak fractions from DEAE Sepharose CL6B chromatography of the respective 50% ethanol precipitate and the 50-70% ethanol fraction is shown in Fig 4c. The lanes of particular interest are 5 (the antigen peak - fraction 30 -

from the DEAE Sepharose CL6B chromatography of 50% ethanol precipitate) where a dominant protein of molecular mass 42,000 daltons is seen, and lane 8 where the peak CKBB activity fraction (fraction 72) is seen with a molecular mass of 45,000 daltons. Also noteworthy is lane 9 (fraction 76) which has a marked band of molecular weight 48,000 daltons. This band is assumed to be neuron specific enolase which, as noted in section 1.9, is extremely difficult to separate completely from the CKBB.

The same fractions as were run on the SDS PAGE were electrophoresed under non-denaturing conditions using a Paragon CK gel and stained for activity. The isoenzyme bands obtained are shown in Fig 4d. All fractions gave a band equivalent in migration to the CKBB marker, and these were especially strong in the fractions 72 and 76 from the 50-70% ethanol column. In addition, fraction 30 from the 50% ethanol column gave a cathodic band with an equivalent migration to the CKMM marker.

Fractions 20-43 from the 50% ethanol precipitate column, representing the antigen peak, were pooled, concentrated and chromatographed on a Sephacryl S200 gel filtration column (Fig 4e). A peak of antigen was obtained directly after the dextran blue void volume marker, at $V_e/V_o = 1.06$. This is equivalent to a non-denaturing molecular mass of 250,000 daltons (see calibration curve Fig 9d). The ELISA using Ab-CKBB reacted weakly with this peak.

This purified antigen was electrophoresed on a 10% SDS PAGE gel in duplicate - one lane was stained for protein with Coomassie Blue, and one lane was immunoblotted with Ab-DEN. The results are shown in Fig 4f. The antigen had a subunit molecular mass of 42,000 daltons by reference to the molecular mass markers, and was essentially pure,

with the presence of a number of minor contaminating bands. The antigen band reacted very strongly with the Ab-DEN in the immunoblot, with possibly three much higher molecular mass proteins reacting slightly, and these could easily be aggregated forms of the molecule.

Fig. 4a DEAE SEPHAROSE CL6B CHROMATOGRAPHY of 50% ethanol precipitate of brain, dissolved in 50 mM TRIS buffer pH 7,4 containing 5 mM β - mercaptoethanol

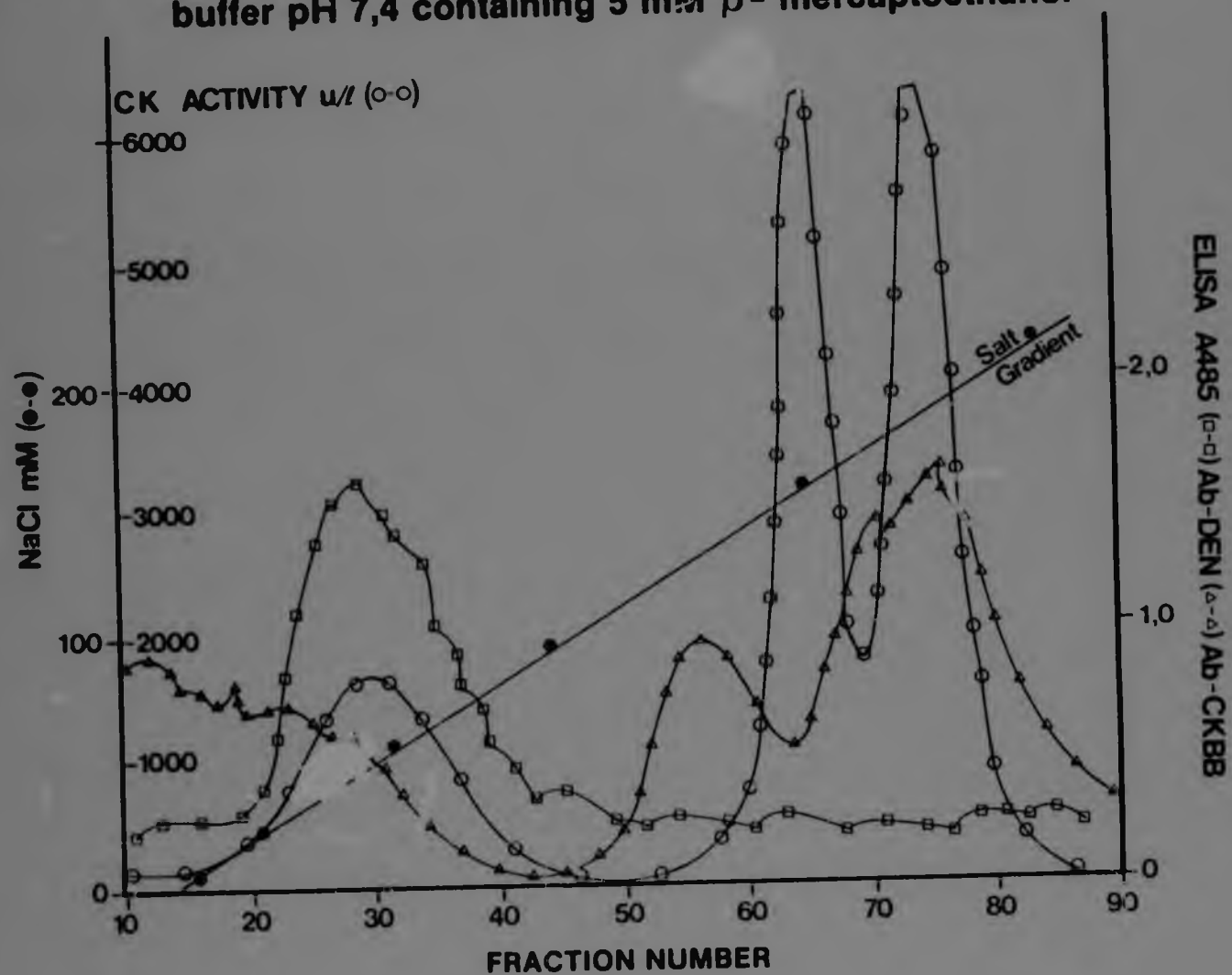


Fig. 4b
DEAE SEPHAROSE CL6B CHROMATOGRAPHY OF
50 - 70% ETHANOL FRACTION OF BRAIN

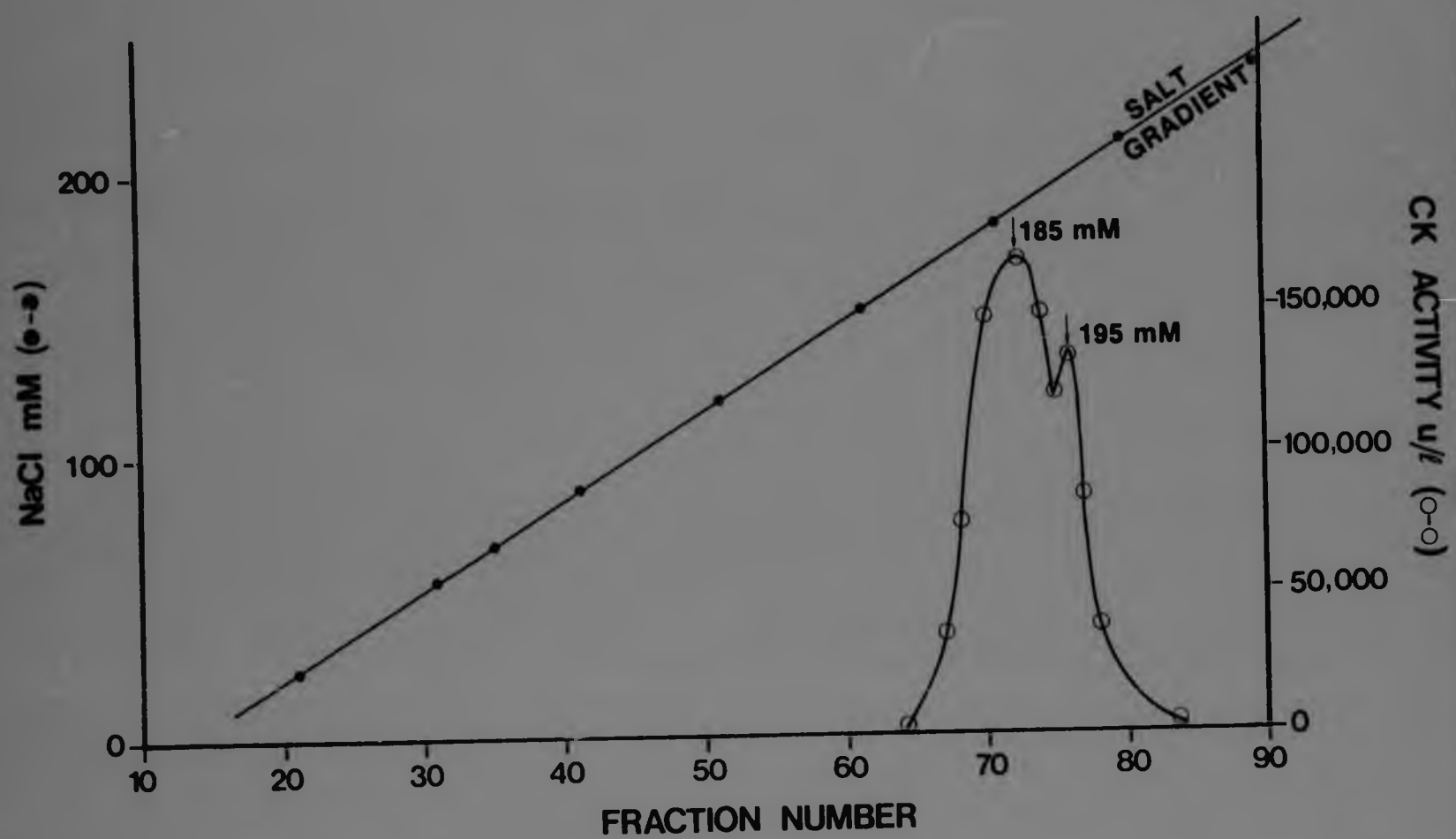


FIG 4c: SDS-PAGE OF THE FRACTIONS OBTAINED IN THE PURIFICATION OF CKBB FROM HUMAN BRAIN.

(Lane 1 = molecular mass markers; lane 2 = fraction 76 from the DEAE Sepharose CL6B chromatography of 50-70% ethanol fraction; lane 3 = fraction 72 from same column; lane 4 = fraction 74 from the DEAE Sepharose CL6B chromatography of 50% ethanol precipitate fraction; lane 5 = fraction 65 from same column; lane 6 = fraction 30 from same column; lane 7 = the 50-70% ethanol fraction; lane 8 = 50% ethanol precipitate fraction; lane 9 = 50% ethanol supernatant fraction; lane 10 = supernatant of brain homogenate.)

fractions as per Fig 4a

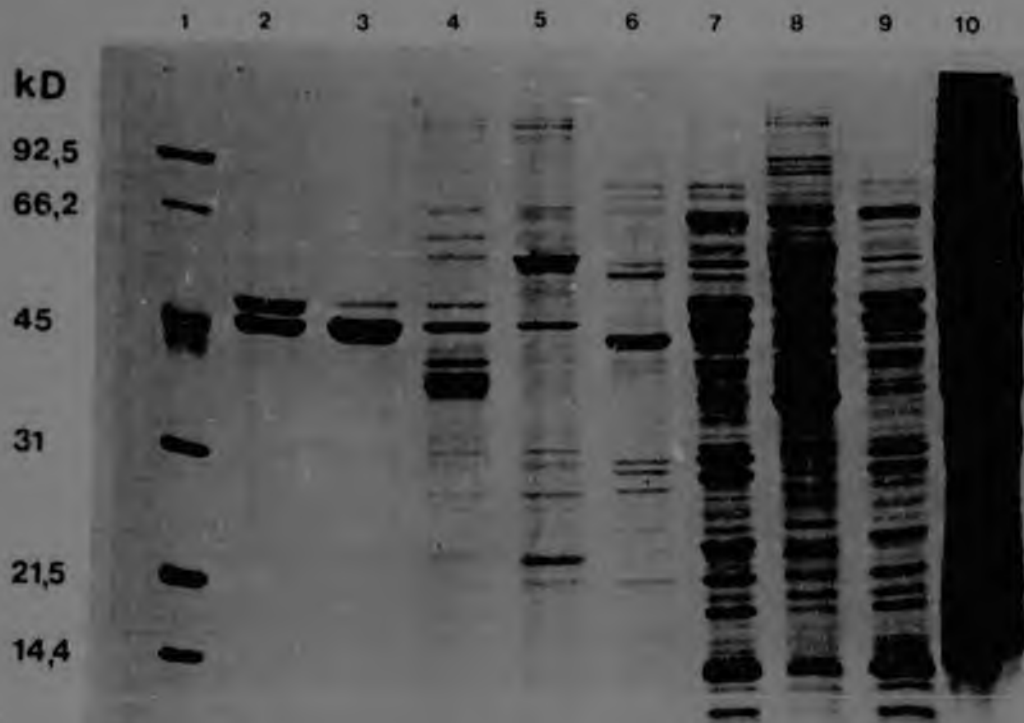


FIG 4d: ISOENZYME ELECTROPHORESIS OF THE PEAK FRACTIONS FROM THE DEAE SEPHAROSE CL6B CHROMATOGRAPHY OF THE 50% ETHANOL PRECIPITATE AND 50-70% ETHANOL FRACTIONS OF HUMAN BRAIN.

(Lane 1 = isoenzyme markers; lane 2 = fraction 30 from the DEAE Sepharose CL6B chromatography of the 50% ethanol precipitate fraction; lane 3 = fraction 64 from same column; lane 4 = fraction 72 from the same column; lane 5 = fraction 72 from the DEAE Sepharose CL6B chromatography of the 50-70% ethanol fraction; lane 6 = fraction 76 of the same column)

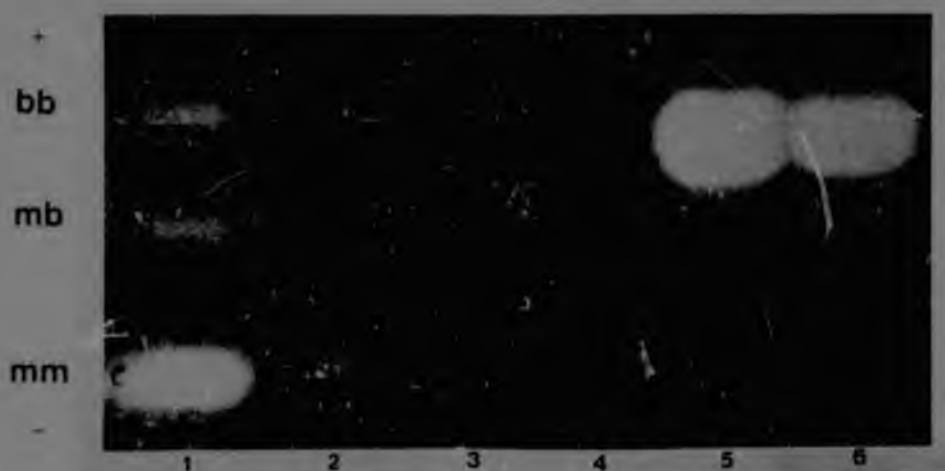


Fig. 4e
SEPHACRYL S200 CHROMATOGRAPHY OF
'ANTIGEN' PEAK, previously purified by 50% ethanol
precipitation and DEAE sepharose CL6B
chromatography. Peak 'antigen' fraction 38 has V_{Vo} of
1,06, molecular mass of 250,000 daltons and creatine
kinase specific activity of 0,8 u/mg - estimated
separately

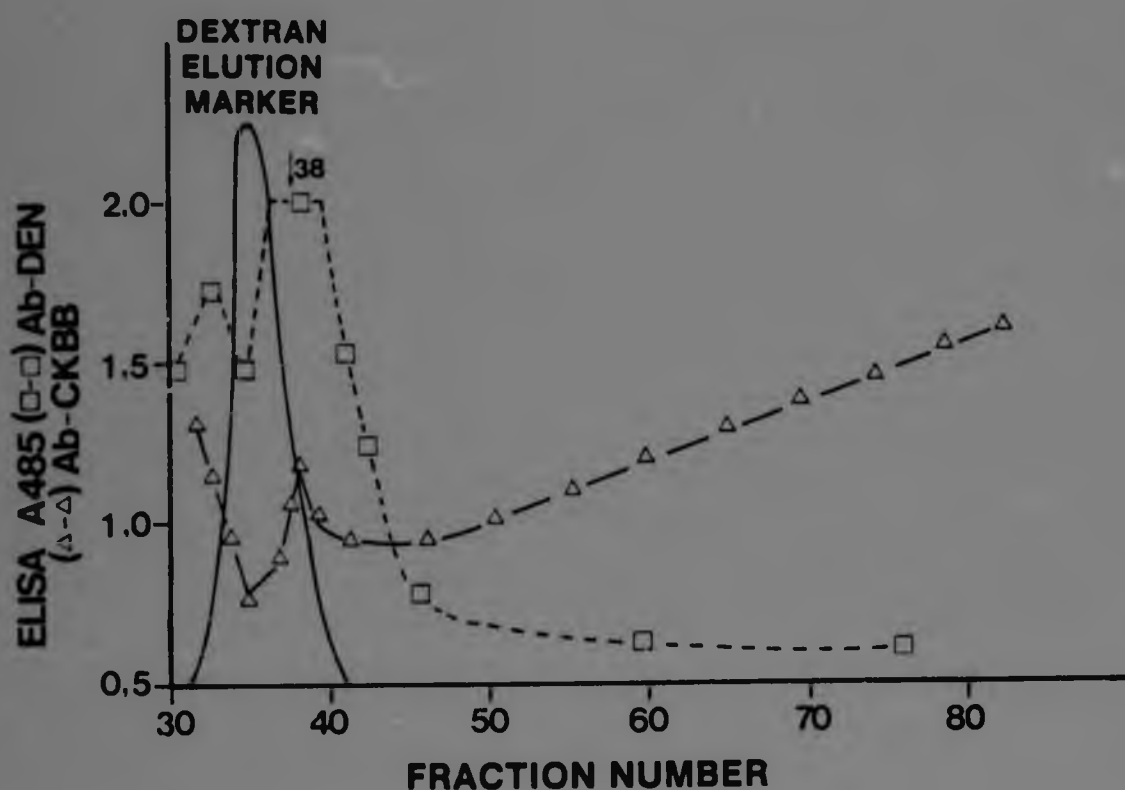
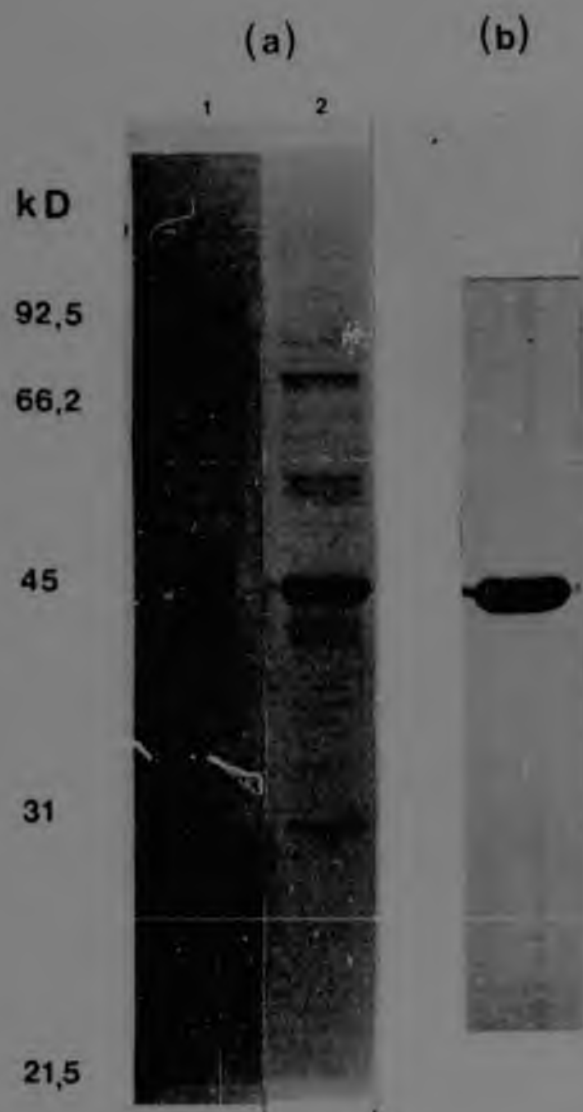


FIG 4f: SDS-PAGE (10%T) OF PURIFIED 'ANTIGEN'

(a) Coomassie Blue stain (lane 1 = molecular mass markers; lane 2 = 'antigen')
(b) immunoblot using Ab_{GEN}



3.5. HYDROXYLAPATITE CHROMATOGRAPHY

The fact that the ELISA (Ab-DEN) and CK activity peaks were coincident in fraction 30 of the DEAE Sepharose CL6B column of the 50% ethanol precipitate did not necessarily mean that these methods were recognising the same protein. Therefore, I attempted to resolve this issue on another column system, in much the same way that one would confirm thin layer chromatography results using two different solvent systems. I decided to use a hydroxylapatite column for this since hydroxylapatite has been used by several groups to purify CK isoenzymes.

Fig 5a shows the hydroxylapatite (HTP) chromatography of the purified antigen shown in lane 2, Fig 4f(i). I anticipated that I would either get two peaks, one ELISA and one CK activity, which would show that there were two proteins involved, or that I would get one peak containing both ELISA and CK activity, which would show that there was only one protein involved.

A large protein peak was eluted with a phosphate gradient, as indicated by the A_{280} trace, peaking at about 36 mM phosphate, with two small peaks at the beginning of the gradient. The fractions containing the large protein peak were pooled and contained approximately 2.5 mg protein. This protein was electrophoresed on a 10% SDS PAGE gel in duplicate - one lane was stained for protein using a silver stain (Fig 5b), and the other was immunoblotted using Ab-DEN. Two surprising facts were revealed - firstly, the protein stain showed the presence of about 5 major bands plus about a dozen fainter bands (Lane 3, Fig 5b). This should be compared with lane 2 from Fig 4f(i), which was the fraction submitted to HTP chromatography, where there was only one major protein band.

Secondly, there was no reaction with the Ab-DEN in the immunoblot. This should also be contrasted with Fig 4f(ii) where a very strong band was obtained in a similar experiment. Exposure of the antigen to hydroxlyapatite, therefore, had in some way altered the molecule, and judging by the number of protein bands formed, this alteration appears to have been a breakdown of structure rather than a conformational change.

In an attempt to clarify the position, a 50% ethanol precipitate of brain (which is known to contain the antigen) was submitted to HTP chromatography and eluted with a phosphate gradient. The results of this column are shown in Fig 5c. Two CK activity peaks were obtained - one at fraction 29 (8.5 mM phosphate) and one at fraction 68 (43 mM phosphate). The fractions were assessed by ELISA using Ab-DEN and there was the suggestion of an increased immunoreactivity at the very start of the gradient. The elution of a large protein peak after the chromatography of the purified antigen (Fig 5a) proves that this protein does in fact bind to the column, and the column was eluted with a greater phosphate concentration than that which achieved elution in Fig 5a. - therefore one can be reasonably confident that the protein was not discarded by the column or irreversibly bound.

The two CK activity fractions 29 and 68 were electrophoresed on a 10% SDS PAGE and stained for protein with a silver stain (Fig 5b - lanes 1 and 2). One interesting thing which is evident from this gel is the presence of only one band of subunit molecular mass 38,000 daltons in lane 1. As this fraction had CK activity, one can conclude that this band is CKBB, but in a form which is either of smaller molecular mass or which migrates differently on SDS PAGE. This is additional evidence that HTP induces a change in the CK

molecule.

Fig 5d shows the results of chromatographing brain supernatant through HTP. In this column there was only one CK activity peak at fraction 39, and there was a more pronounced immunoreactivity at the beginning of the gradient. This separation of ELISA and CK activity suggests that the antigen peak fraction from the DEAE column (fraction 30) may indeed contain two proteins. Notice, however, that the SDS PAGE of every tenth fraction from this HTP column (Fig 5e) reveals heavy bands of low molecular mass proteins (about 20,000 daltons) in lanes 3 to 9, suggesting marked degradation of protein structure. Notice, also, that fraction 21 which has one of the highest ELISA readings of the column contains undetectable protein. The silver stain is extremely sensitive. This fraction also gave a negligible reaction with the Biorad protein reagent. This suggests that the Ab-DEN reaction in this case is not against a protein. Evidence will be presented later that the Ab-DEN recognises ATP which proves that this antibody recognises other organic molecules as well as proteins.

To test whether the phosphate used to elute the HTP column in some way modifies the antigen to conceal its antigenic sites, an experiment was performed to determine the effect of phosphate concentration on the ELISA reaction with Ab-DEN. The 'antigen' peak from the DEAE Sepharose CL6B chromatography of the 50% ethanol precipitate of brain was pooled, and 10 uL was aliquoted into a microtitre dish containing 100 uL of doubling dilutions of phosphate buffer from 100 - 0,2 mM. The results of this study are presented in Fig 5f. There was a linear relationship between the phosphate concentration and the intensity of the antibody-antigen reaction with

higher concentrations of phosphate yielding higher ELISA values, to a critical concentration of 0,8 mM phosphate, after which point decreasing phosphate gave increasing ELISA values. These are results are difficult to explain since it appeared in the hydroxylapatite chromatography that the presence of phosphate was actually masking the presence of antigen in some way. This leads to the conclusion that passage through the hydroxylapatite column in some way leads to loss of antigenicity of the 'antigen', rather than a direct effect of the phosphate in the eluting buffer.

Fig 5g shows the results of HTP chromatography of the 50-70% ethanol fraction of brain which is known to contain the peak of CKBB activity. Three peaks of CK were obtained - one at fraction 21 (the very beginning of the gradient), one major peak at fraction 29 (12 mM phosphate), and a very small peak at fraction 68 (78 mM phosphate). When the profile of this column is compared to the equivalent column for the 50% ethanol precipitate (Fig 5c), it can be seen that both columns yield CK activity peaks at fractions 29 and 68. Differing estimates were achieved of the phosphate concentrations in these fractions which may be experimental error caused by the dilution required to measure the phosphate on the Gemini centrifugal analyser with a reagent designed to measure the low levels of phosphate in serum. The fraction 68 CK activity peak is relatively more enriched in Fig 5c with respect to the main CK peak at fraction 29. The CK activity peak at fraction 21 is found only in Fig 5g.

**Fig. 5a HYDROXYLAPATITE CHROMATOGRAPHY OF
'ANTIGEN', which was previously purified by 50%
ethanol precipitation, DEAE sepharose CL6B
chromatography and Sephacryl S200 gel filtration**

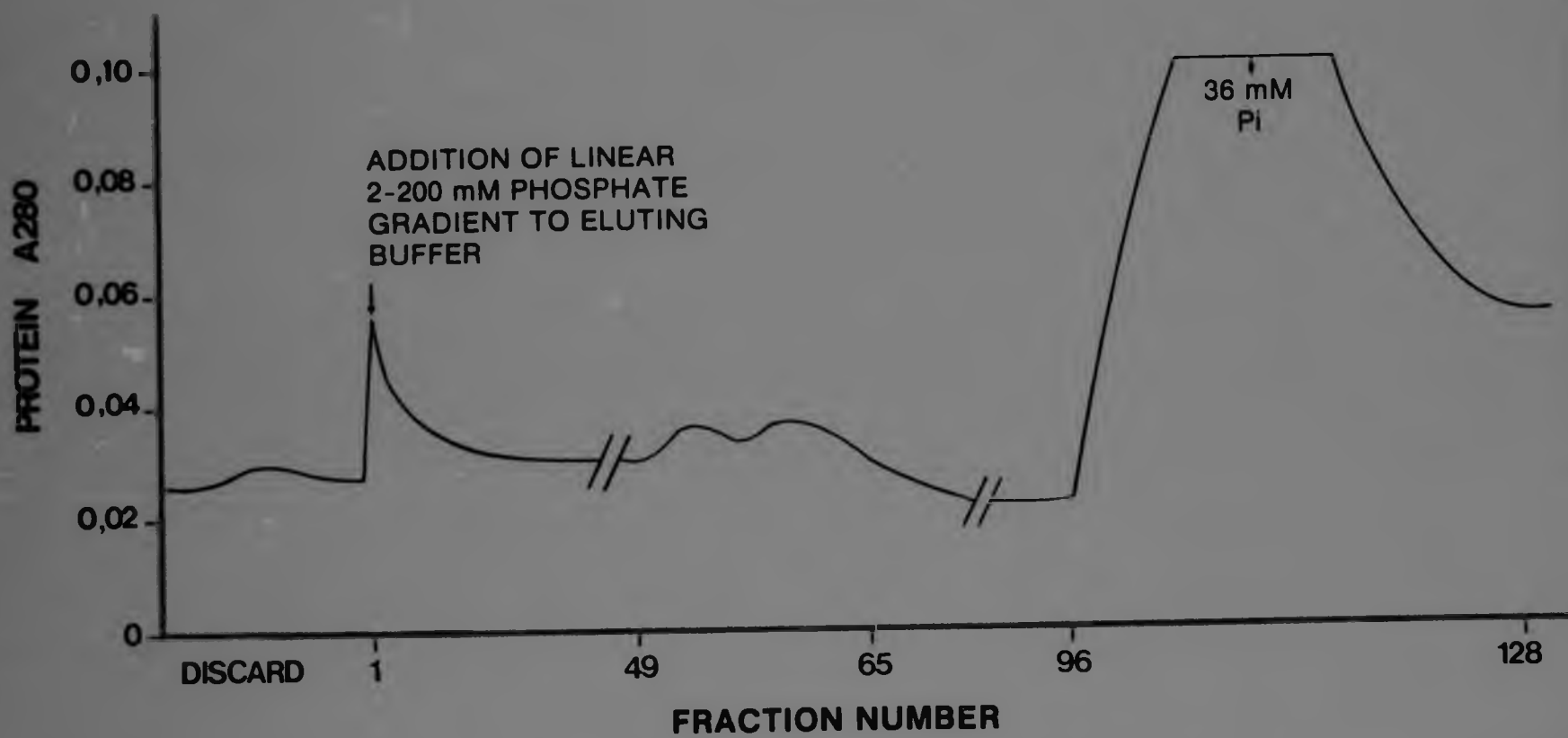
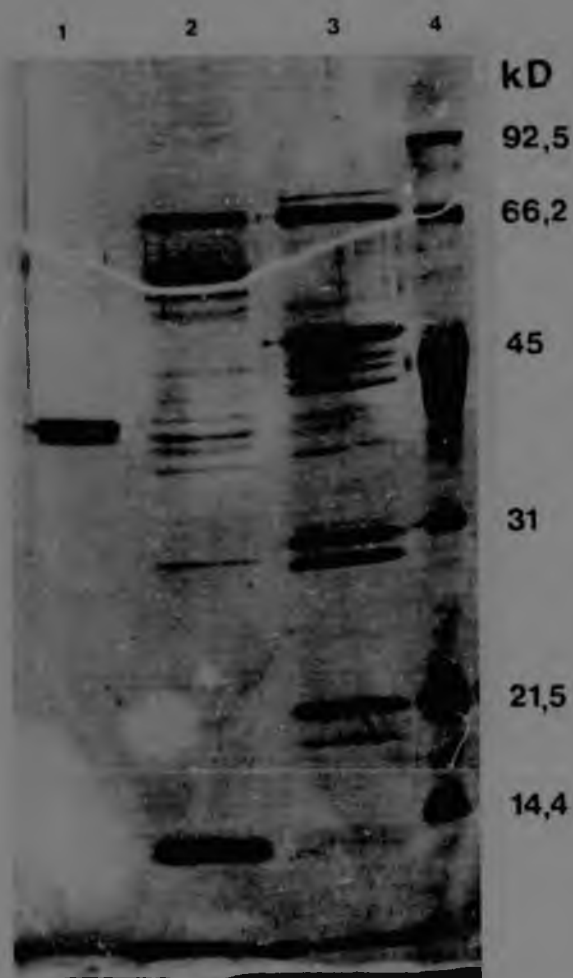


FIG 5b: SDS-PAGE (10%T) OF FRACTIONS FROM A HYDROXYLAPATITE COLUMN, stained for protein with a silver stain.

(lane 1 = fraction 29 from the hydroxylapatite chromatography of the 50% ethanol precipitate fraction of brain; lane 2 = fraction 68 from the same column; lane 3 = pooled fractions 96-120 of the hydroxylapatite chromatography of brain proteins purified by precipitation by 50% ethanol, followed by DEAE Sepharose CL6B chromatography and Sephacryl S200 chromatography; lane 4 = molecular mass markers)



**Fig. 5c HYDROXYLAPATITE CHROMATOGRAPHY OF
THE 50% ETHANOL SUPERNATANT OF BRAIN,
assayed for CK activity and ELISA using Ab-DEN**

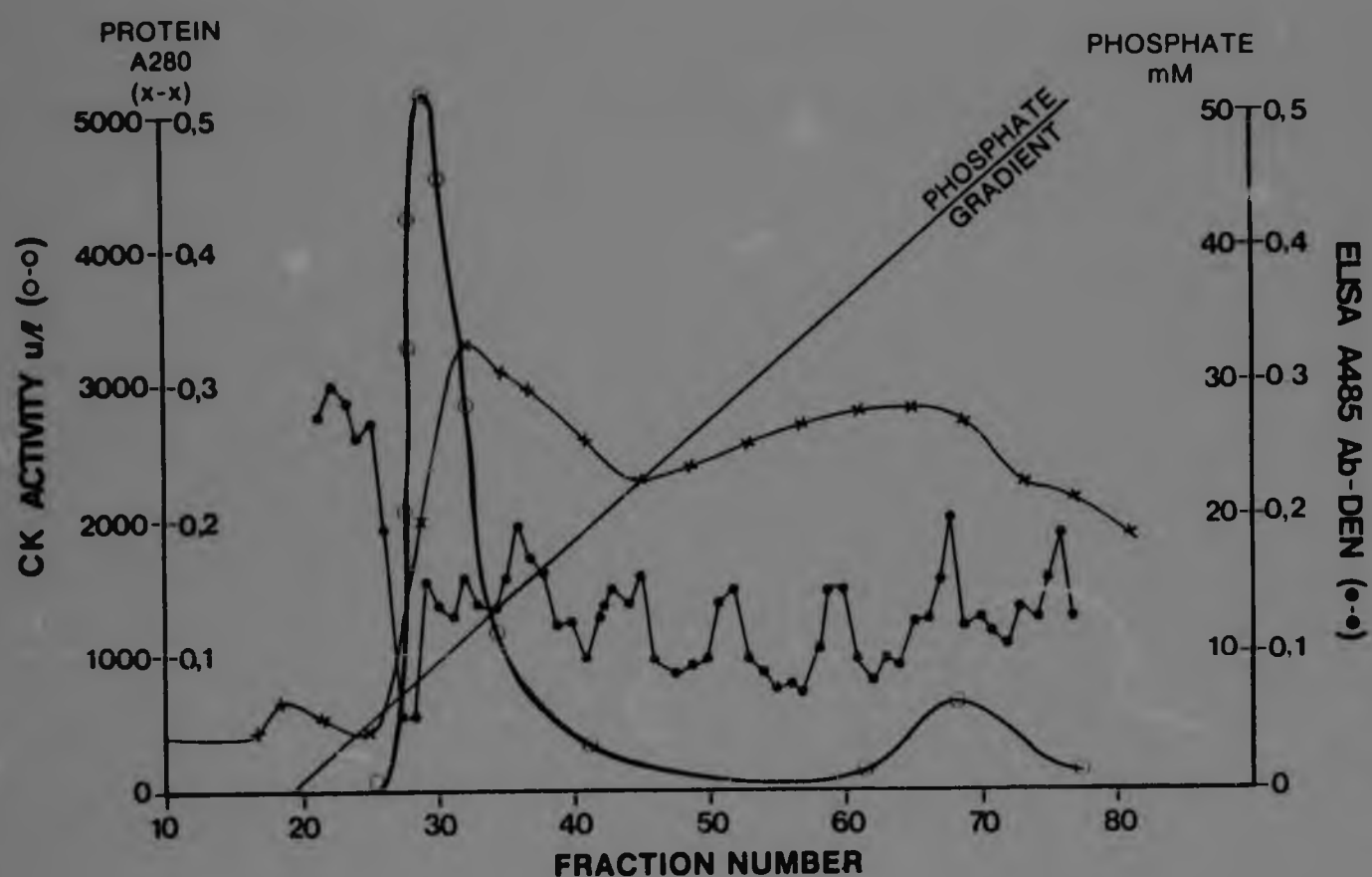


Fig. 5d
HYDROXYLAPATITE CHROMATOGRAPHY OF
2,5 ml BRAIN SUPERNATANT, assayed for CK activity
and ELISA using Ab-DEN

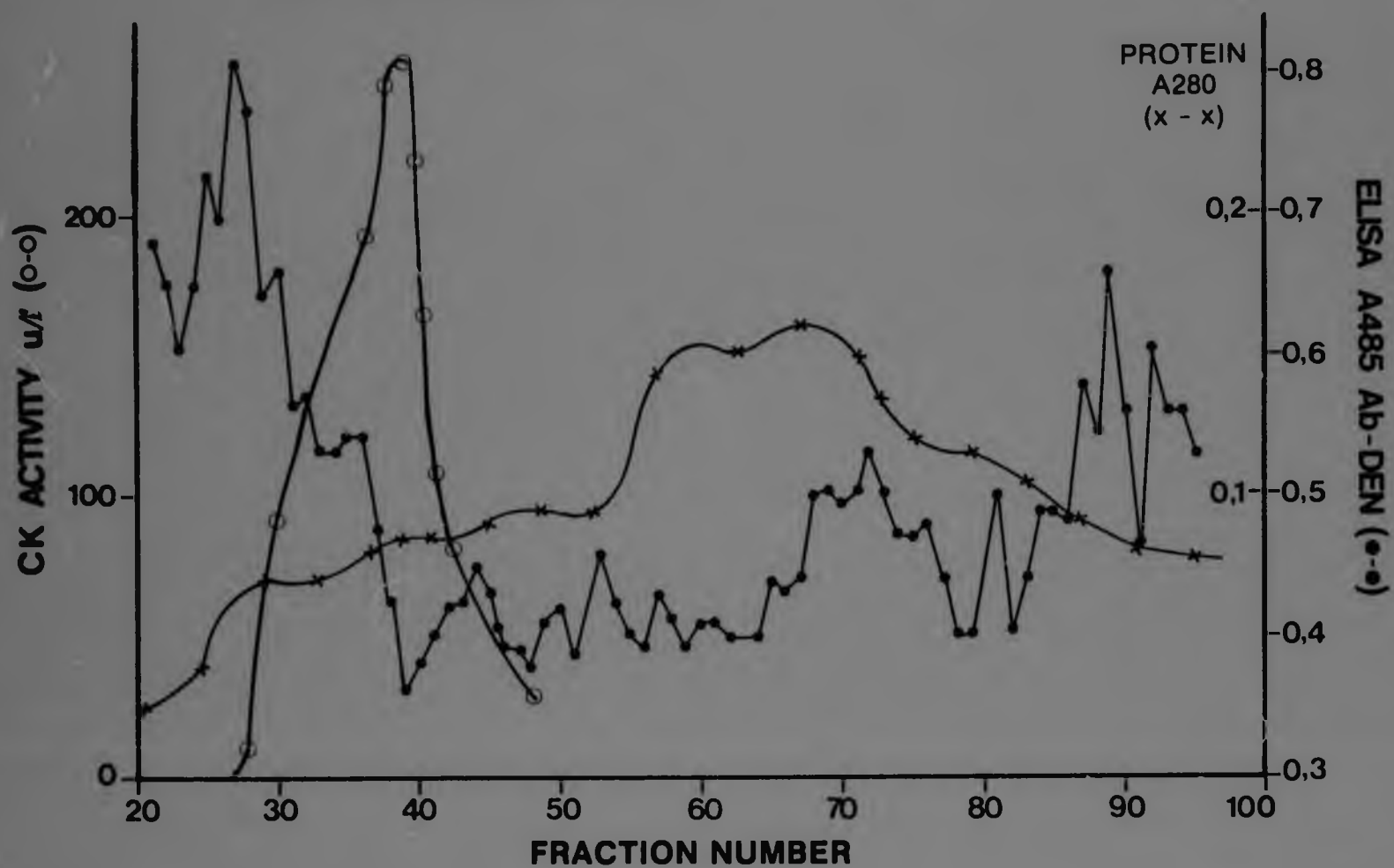
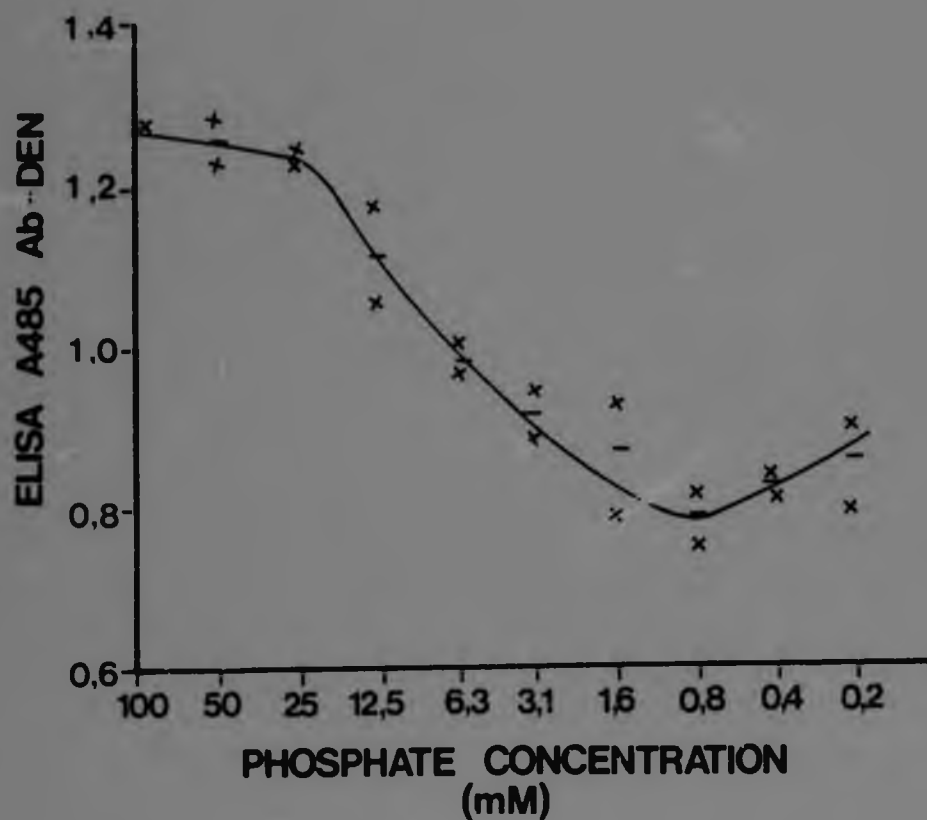


FIG 5e: SDS-PAGE (10%T) OF FRACTIONS FROM THE HYDROXYLAPATITE CHROMATOGRAPHY OF BRAIN SUPERNATANT, stained for protein with a silver stain.

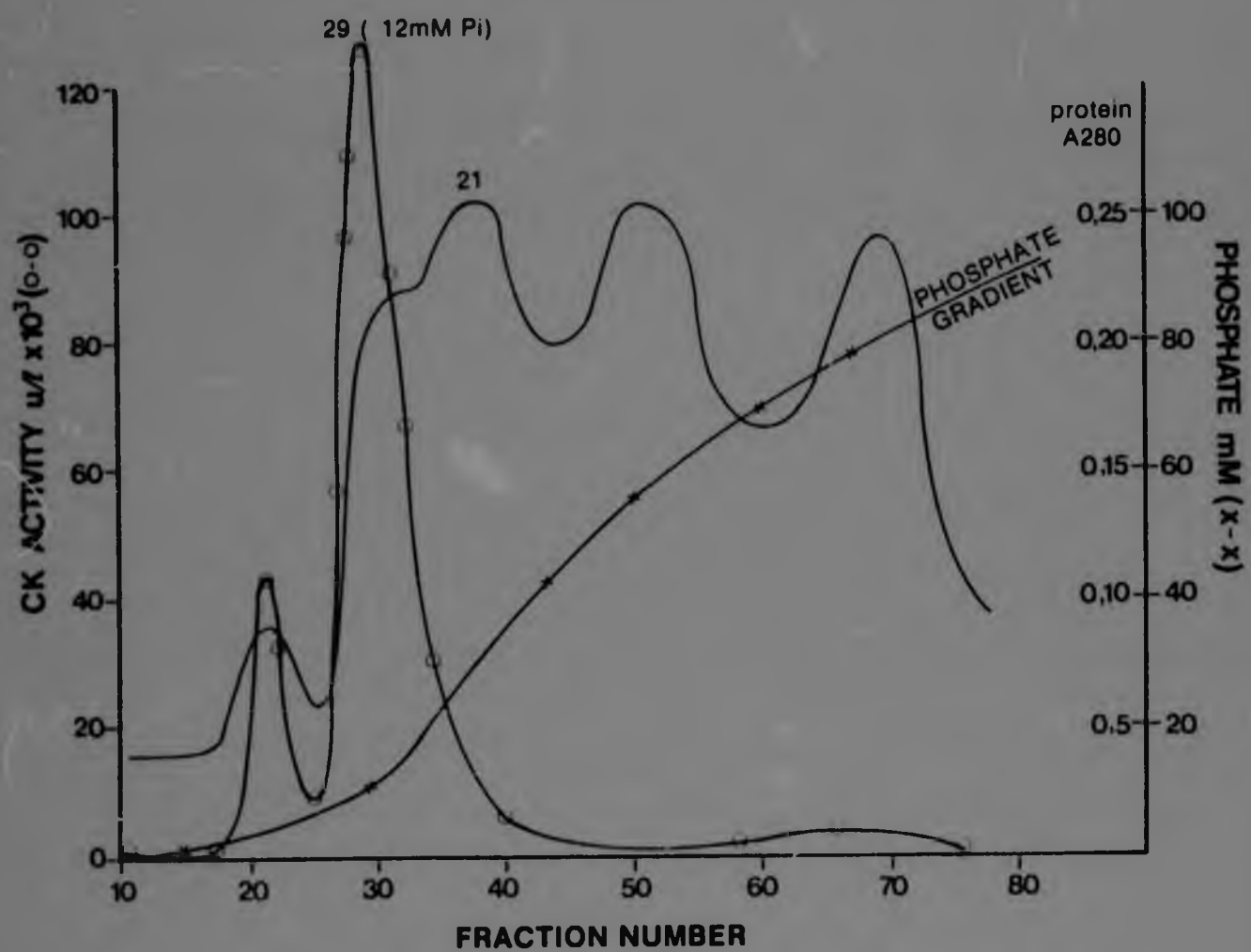
(lane 1 = fraction 21, lane 2 = fraction 31, lane 3 = fraction 41; lane 4 = fraction 51; lane 5 = fraction 61; lane 6 = fraction 71, lane 7 = fraction 81, lane 8 = fraction 91; lane 9 = molecular mass markers)



Fig. 5f
EFFECT OF PHOSPHATE CONCENTRATION ON
IMMUNOREACTION OF 'ANTIGEN' WITH Ab-DEN,
as assessed by the ELISA technique



**Fig. 5g HYDROXYLAPATITE CHROMATOGRAPHY OF
50-70% ETHANOL FRACTION OF BRAIN**



3.6. BLUE SEPHAROSE CHROMATOGRAPHY AND ANTIGENICITY OF ATP

Since the results obtained using the hydroxylapatite column were equivocal in the attempt to see whether the coincident ELISA and CK activity peaks obtained with DEAE Sepharose CL6B chromatography could be separated, I decided to try a Blue Sepharose column. This attempt eventually had to be abandoned when it was found to be unsuitable for three reasons. Firstly, the elution buffer contains ATP which interferes with the measurement of the CK activity since it is one of the components in the reagent buffer, and therefore no accurate assessment of the CK activity in each fraction could be obtained. Secondly, the ATP absorbs at A_{280} so the protein trace off the column is difficult to interpret. And thirdly, and unexpectedly, the ATP was found to be immunogenic to the AB-DEN and therefore gave a positive ELISA.

This finding was confirmed using a DEAE Sepharose CL6B column with the dual intention of establishing the fraction in which ATP elutes from the column. Fig 6a demonstrates the results obtained by chromatographing ATP through the DEAE Sepharose CL6B. There was no A_{280} in the discarded fraction, and elution with a salt gradient produced two peaks - a main peak at fraction 48 corresponding to ATP and a shoulder at about fraction 30 corresponding to ADP formed by hydrolysis from ATP. The ELISA, however, shows only reaction with ATP and not with the ADP.

To establish beyond doubt that the antigen in study was not merely a small inorganic or organic molecule, a peak of antigen activity from a DEAE Sepharose CL6B column of breast tumour cytosol was pooled, dialysed extensively against several changes of buffer, and rechromatographed (Fig 6b). The second column contained an ELISA

peak in the same position as the first, proving that the antigen is non dialysable, with the addition of two smaller peaks at fractions 41 and 49.

It should at this stage be pointed out that many of the ELISA results, when plotted, give an eight fraction periodicity, which suggests that this may be due to an artifact resulting from the microtitre plate. The microtitre plate contains eight rows and there is an acknowledged effect known as the 'edge effect' in which the outer rows may react slightly differently to the inner ones owing to the proximity to the environment. One would expect that the use of an entire column as a blank, which was my routine practice, would nullify this effect. However, one must always be cautious when interpreting ELISA curves at eight fraction intervals.

Fig. 6a
DEAE SEPHAROSE CL6B CHROMATOGRAPHY OF
5 mM ATP (ADENOSINE TRIPHOSPHATE)

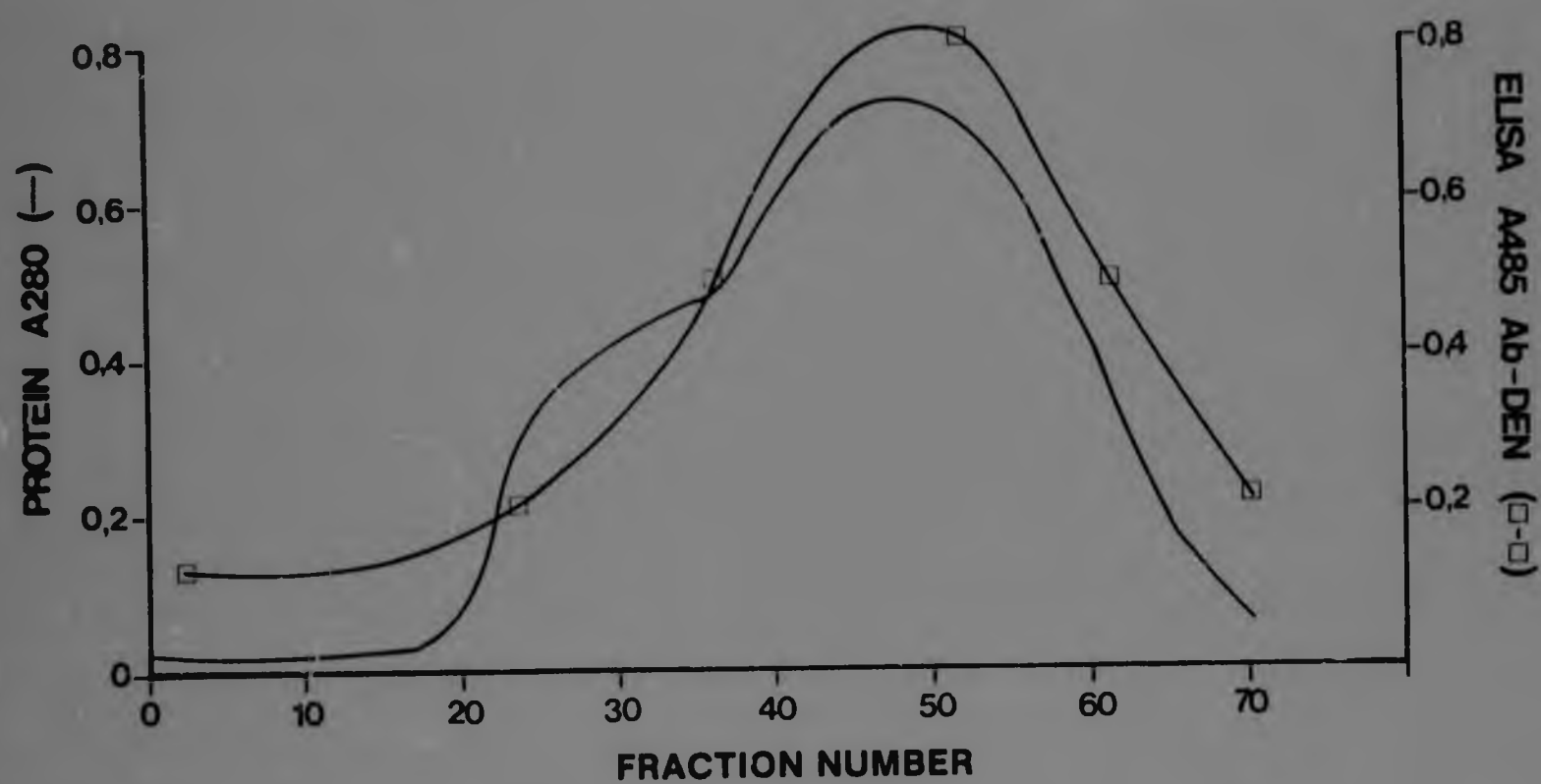
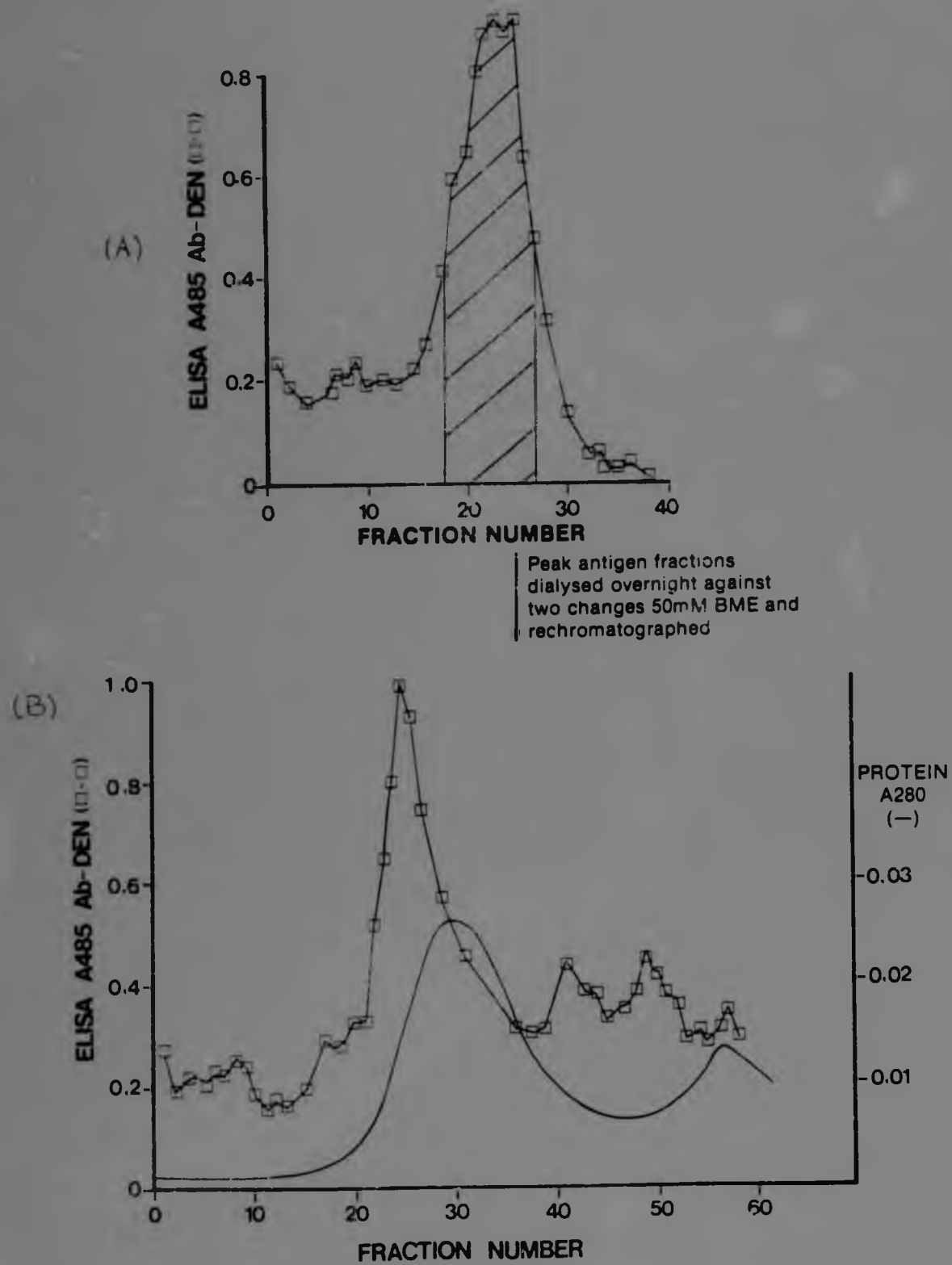


Fig 6b

DEAE SEPHAROSE CL6B CHROMATOGRAPHY OF
(A) BREAST TUMOUR CYTOSOL and (B) dialysed
antigen peak from (A). NO CK activity was detected in (B).



3.7. IS THE 'ANTIGEN' CK?

When the use of column chromatography seemed to be incapable of resolving whether fraction 30 from the DEAE Sepharose CL6B column represented one or two proteins of interest, other approaches were tried.

It was noted that new attempts of DEAE Sepharose CL6B chromatography of the 50% ethanol precipitate of brain yielded different results to the earlier runs, in that the ELISA and CK peaks now did not seem to be coincident, and indeed that there seemed to be two ELISA peaks - one at fraction 18 and one at fraction 24. Fig 7a illustrates these differences. When the two columns were compared carefully, it was noted that in the case where the two peaks were coincident, the brain had been stored at 0°C for two weeks, during which time degradation would have occurred. Notice that Fig 7a(i) was of 70g original weight of brain and yielded a CK peak at fraction 30 of 1700 U/l, whereas 7a(ii) was of 135g original weight of very fresh brain and yielded a CK activity peak at fraction 29 of 28 U/l.

This gave rise to speculation that the CK activity peak at fraction 30 is derived from CKBB, either as a breakdown product or by aggregation. This was demonstrated to be the case by pooling the fractions containing the CKBB activity off a DEAE Sepharose CL6B column (i.e. fractions 71-90) after they had been stored in the refrigerator for ten days, and rechromatographing these proteins. The results are shown in Fig 7b. Storage of the CKBB did indeed give rise to a CK activity peak at fraction 32, with the suggestion of three other small peaks at fractions 42, 53 and 59.

I could now reasonably conclude that the CK activity peak obtained at fraction 30 is in fact a breakdown product of CKBB. This

did not, however, clear up the issue of whether the 'antigen' was a CK molecule at all. It was beginning to look likely that the 'antigen' and the denatured CKBB were separate entities, particularly with results showing a separation of the peaks on DEAE Sepharose CL6B as shown in Fig 7a(ii). Notice, also, in Fig 4d, that isoenzyme electrophoresis of fraction 30 reveals two CK activity bands, one equivalent in migration to the CKBB marker, and one to the CKMM marker. In another identical experiment, the cathodic band was cathodic to the CKMM marker, not similar in migration to it as here (results not shown).

To investigate further whether the antigen is formed from the ageing of CKBB, 310 ml of brain supernatant (1:2 w/v) was stored at 0°C for 5 days to encourage transformation of CKBB to the more basically charged forms, and then precipitated with 50% ethanol and chromatographed on DEAE Sepharose CL6B. The discarded proteins and every tenth fraction off the column were electrophoresed on a 10% SDS-PAGE gel and then electroblotted to nitrocellulose paper. The electroblot was immunoblotted using the Ab-DEN and then stained for protein by Coomassie Blue. The results are shown in fig 7c.

Unfortunately it was not possible in this case to calculate molecular mass because the 15th lane of the gel containing the molecular mass markers had to be sacrificed in the electroblotting procedure since the nitrocellulose paper was not wide enough to accommodate all the lanes. The immunoblot gave one faint band in all the eluted fractions which from past experience can be assumed to be 42,000 daltons. This band was strongest in the fraction 70 lane which corresponds to the CK activity peak. The discarded fraction had one lower molecular mass protein on the immunoblot, estimated to

be 38,000 daltons, and this band was also faintly represented in the first section of the column in fractions 20 and 25. Fractions 20 through 35 had, in addition to the 42000 and 38000 dalton bands, a third higher molecular mass immunoreactive protein (?48000 daltons). The important findings from this experiment are firstly that there are three immunoreactive bands in the section of the DEAE column under study - i.e. fractions 20 to 40 - and secondly that the 38,000 dalton form of the antigen is not retained on the DEAE column.

The Ab-DEN was raised against a protein (CKBB) which had been denatured by SDS PAGE, and the question arose whether the antigenic determinants recognised by Ab-DEN were in a section of the molecule which under non-denaturing conditions was normally concealed. This concept was investigated by incubating purified CKBB with increasing amounts of SDS (Fig 7d) and urea (Fig 7e) and then measuring the immunogenicity with the ELISA reaction. The results show an almost linear relationship between the degree of denaturation of the CKBB molecule and the response to Ab-DEN, proving that the antigenic determinants which the Ab-DEN recognises are indeed normally concealed in the enzymatically active molecule.

In a separate experiment, brain was homogenised in Tris/BME containing 0,6% sodium deoxycholate. A 5 mL aliquot of this homogenate (equivalent to about 1,5 g brain) was buffer exchanged to Tris/BME to remove the detergent and chromatographed on DEAE Sepharose CL6B. A comparatively large peak of CK activity was found peaking at fraction 28, with a coincident peak of ELISA activity peaking at fraction 29 (Fig 7f). The peak CK value of 140 u/l from a 1,5 g sample of brain reveals a significantly higher activity than that in Fig 7a where 135 g brain gave rise to a peak CK activity of

28 U/l. The fact that the CK and ELISA peaks are coincident with a high level of CK activity is further evidence that relaxation of the conformation of the CKBB molecule reveals the antigenic site.

Further evidence that the antigen is a CK molecule came from an experiment in which brain supernatant which had been homogenised in six different buffers was incubated with nothing, purified Ab-CKBB and Ab-DEN, and then submitted to isoenzyme electrophoresis. Fig 7g(i) shows that incubation of brain in Tris buffer yielded a major CKBB band and a cathodic band (to be called here Br-CM1 or cathodally migrating band 1 from brain), which appears slightly anodic to the CKMM marker here. The phosphate buffer series contained an additional band (to be known as Mt-CK or mitochondrial CK since phosphate is known to release this isoenzyme), cathodic to CKMM. Incubation of the supernatants with Ab-CKBB [Fig 7g(ii)] considerably lessened the intensity of the CKBB band without affecting the other bands. On the other hand, incubation of the supernatants with Ab-DEN [Fig 7g(iii)] led to a change in migration of the Br-CM1 to a position intermediate between its original position and that of MtCK to a position to be known as Br-CM2. The broadening of the and that of MtCK band in the phosphate series shows that the Br-CM1 has not been converted to MtCK, which is evidence that the Br-CM1 band is not mitochondrial as proposed by Wevers (Wevers, 1982).

The fact that an antibody raised against a denatured form of CKBB reacts with cathodic band Br-CM1 shows that this band is CKBB-like and not mitochondrial-like.

A similar experiment showed that very similar results to those achieved with incubation with Ab-DEN could be obtained with time (results not shown). After a period of several days of storage of

the supernatants in the refrigerator, the isoenzyme electrophoresis pattern was the same as Fig 7f(iii). This accounts for the observation that in two separate experiments, the cathodic band was seen in one instance in the Br-CM1 position and in the other instance in the Br-CM2 position upon isoenzyme electrophoresis of fraction 30 (DEAE Sepharose CL6B of 50% ethanol precipitate), probably reflecting different degrees of freshness of the autopsy brain. It should also be noted that storage of these same supernatants in the freezer for 10 days, followed by defrosting and isoenzyme electrophoresis led to the development of an additional band equivalent in migration to the CKMB marker (results not shown).

Fig. 7a
DEAE SEPHAROSE CL6B CHROMATOGRAPHY OF
BRAIN SUPERNATANT, homogenised in 50 mM TRIS
buffer pH 7,4 containing 5 mM BME (A) 20g brain which
had been stored at 0°C for two weeks and (B) 135g
brain, homogenised immediately without storage.

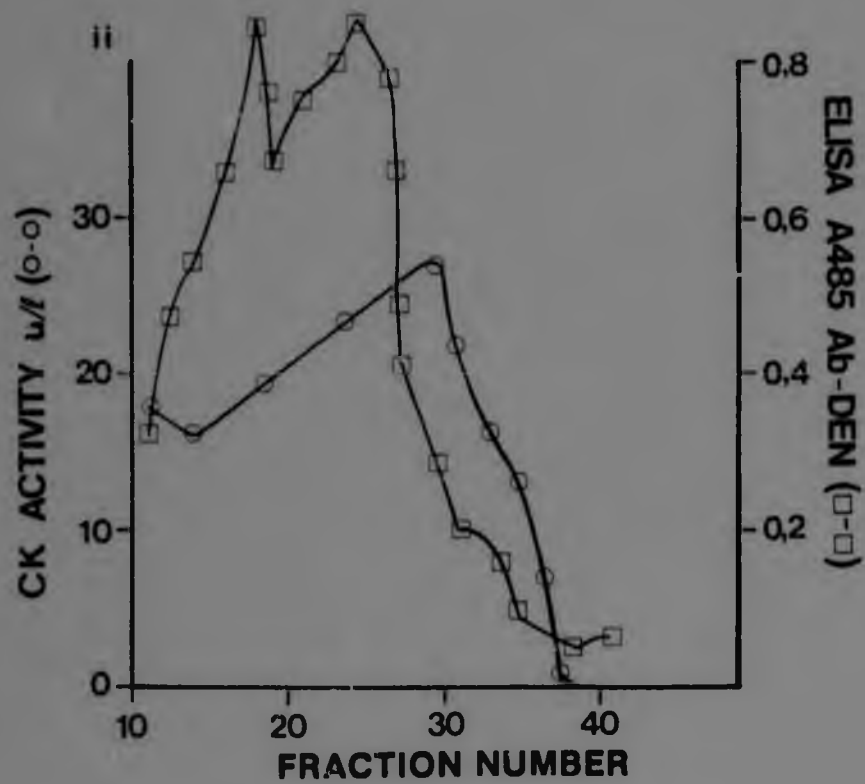
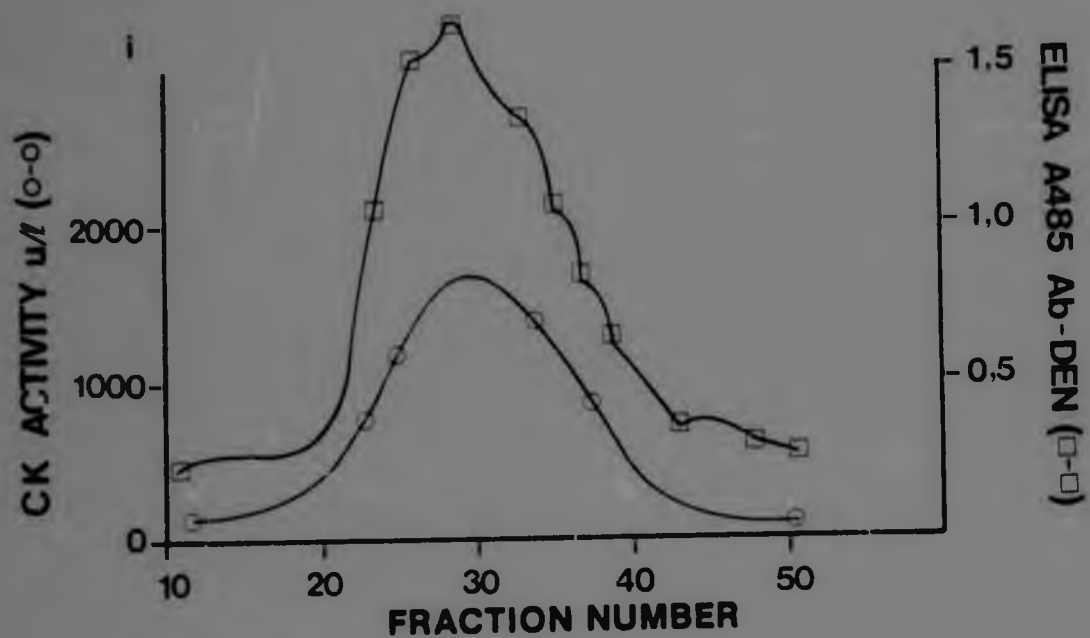


Fig. 7b

DEAE SEPHAROSE CL6B CHROMATOGRAPHY, on (A) purified CKBB and (B) fractions 71-90 from (A), pooled after 10 days at 4°C, concentrated and buffer exchanged to 50 mM TRIS buffer containing 5 mM β -mercaptoethanol.

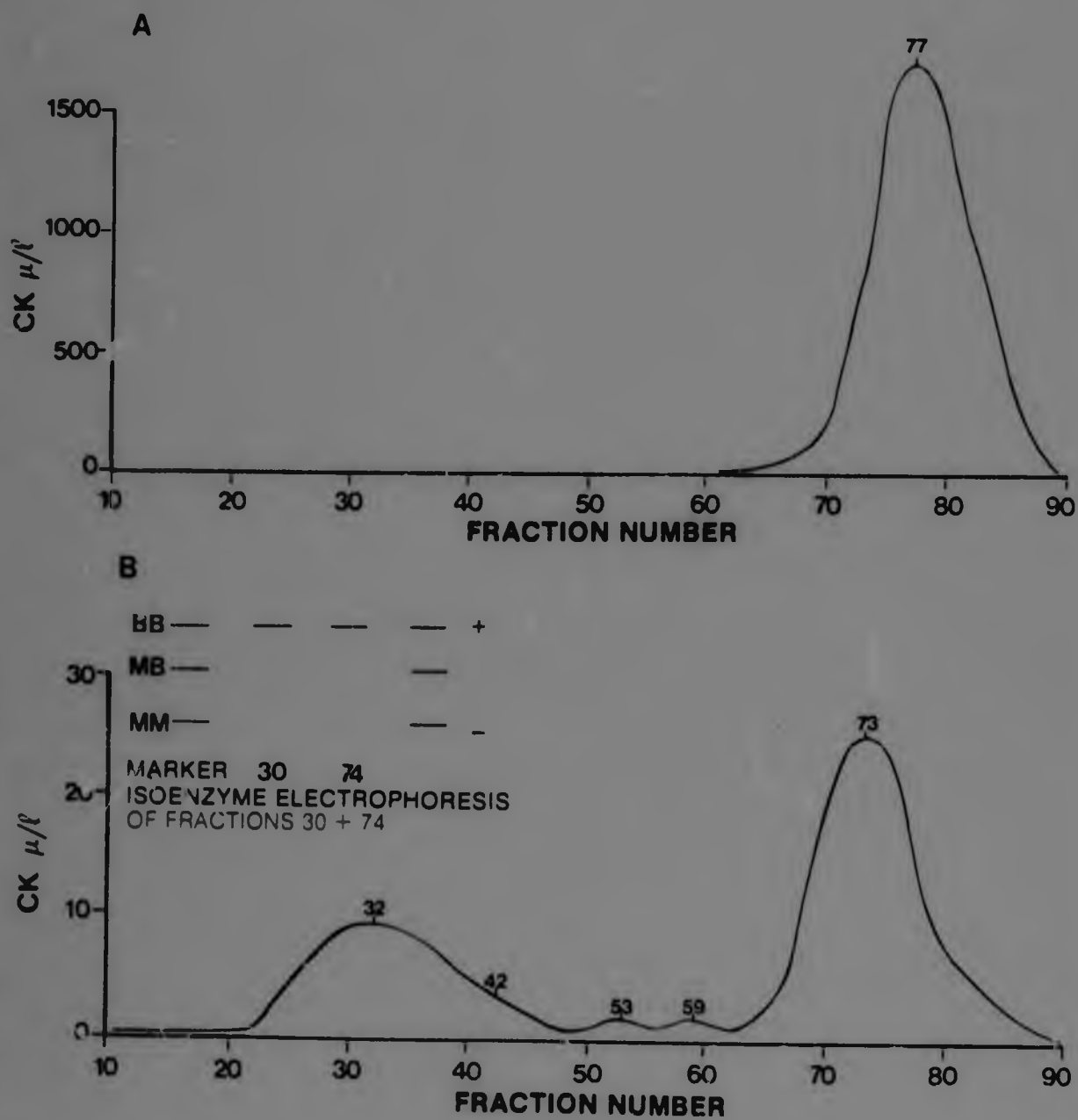


FIG 7c: SDS-PAGE OF FRACTIONS FROM DEAE SEP-HAROSE CL6B CHROMATOGRAPHY OF PROTEINS PRECIPITATED FROM HUMAN BRAIN BY 50% ETHANOL

(a) stained by Coomassie Blue. (b) immunoblotted using Ab-DEN

(Lane 1 = fraction 80; lane 2 = fraction 75; lane 3 = fraction 70; lane 4 = fraction 65; lane 5 = fraction 60; lane 6 = fraction 55; lane 7 = fraction 50; lane 8 = fraction 45; lane 9 = fraction 40, lane 10 = fraction 35, lane 11 = fraction 30; lane 12 = fraction 25, lane 13 = fraction 20; lane 14 = fraction discarded by the column)

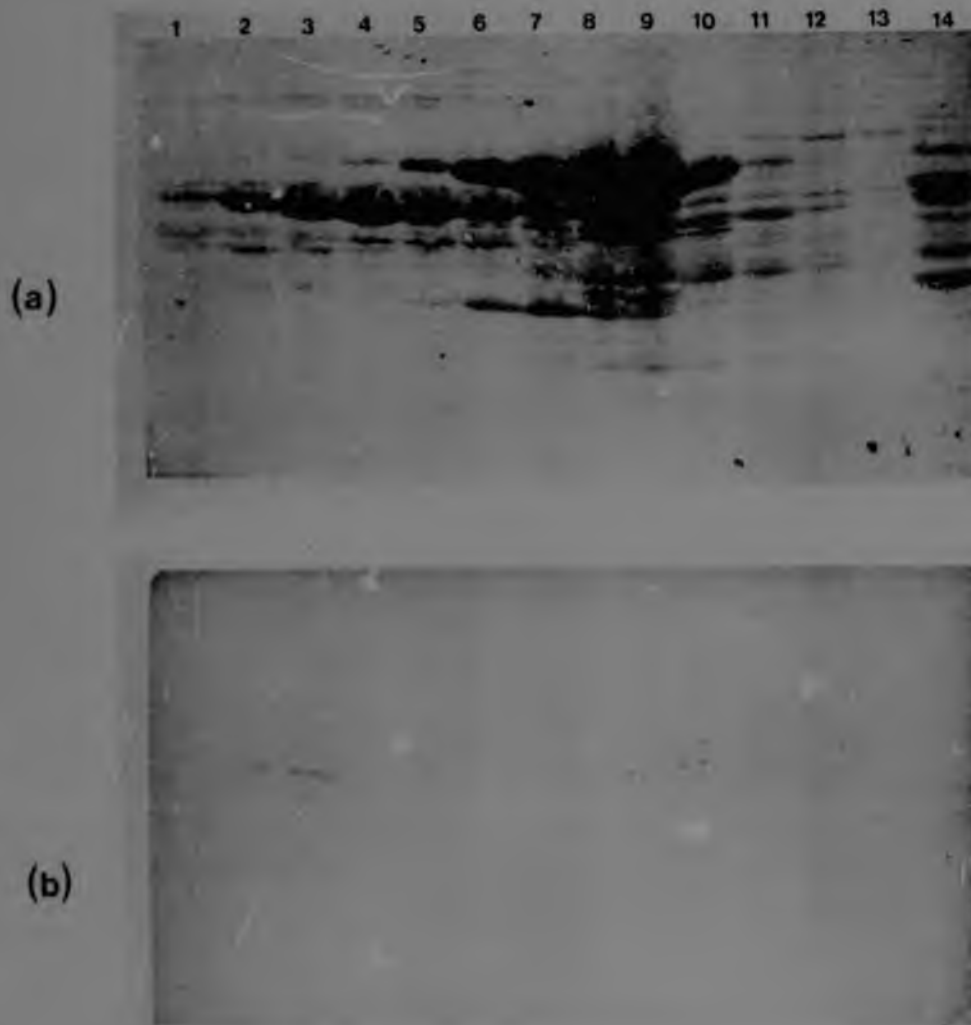


Fig. 7d
EFFECT OF SDS (SODIUM DODECYL SULPHATE)
CONCENTRATION ON THE IMMUNOREACTION
OF PARTIALLY PURIFIED CKBB WITH Ab-DEN, as
assessed by the ELISA technique.

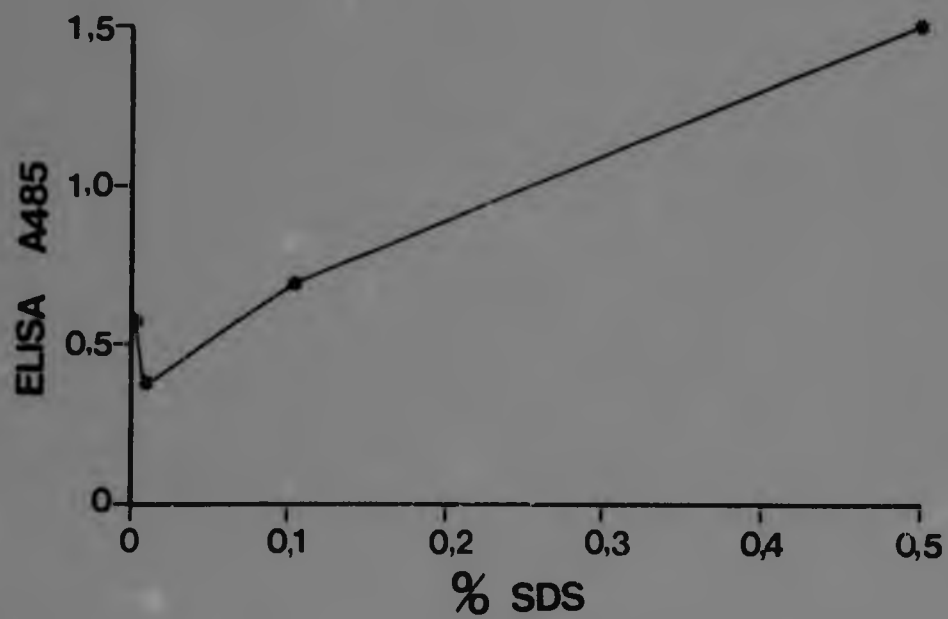


Fig. 7e
EFFECT OF UREA CONCENTRATION ON THE
IMMUNOREACTION OF PARTIALLY PURIFIED
CKBB WITH Ab-DEN as assessed by the
ELISA technique

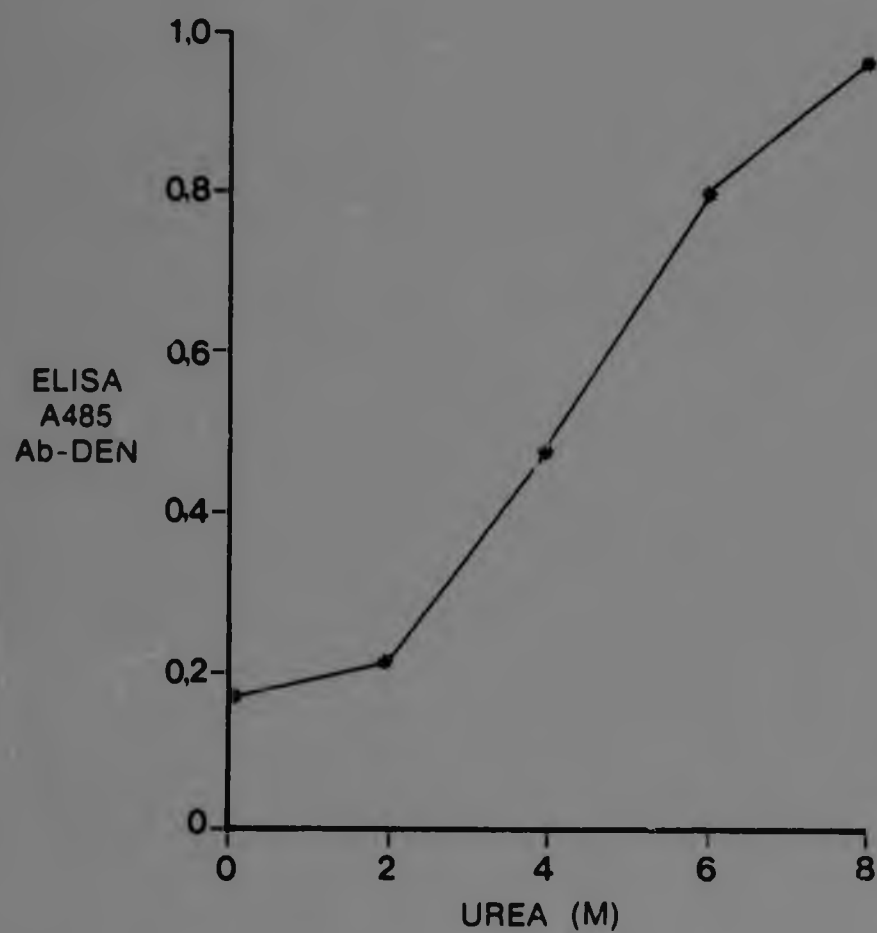


Fig. 7f
DEAE SEPHAROSE CL6B CHROMATOGRAPHY OF
BRAIN homogenised in 50 mM TRIS buffer pH 7,4
 containing 5 mM β - mercaptoethanol and 0,6% sodium
 deoxycholate. Fractions assayed for CK activity and
 ELISA using Ab-DEN.

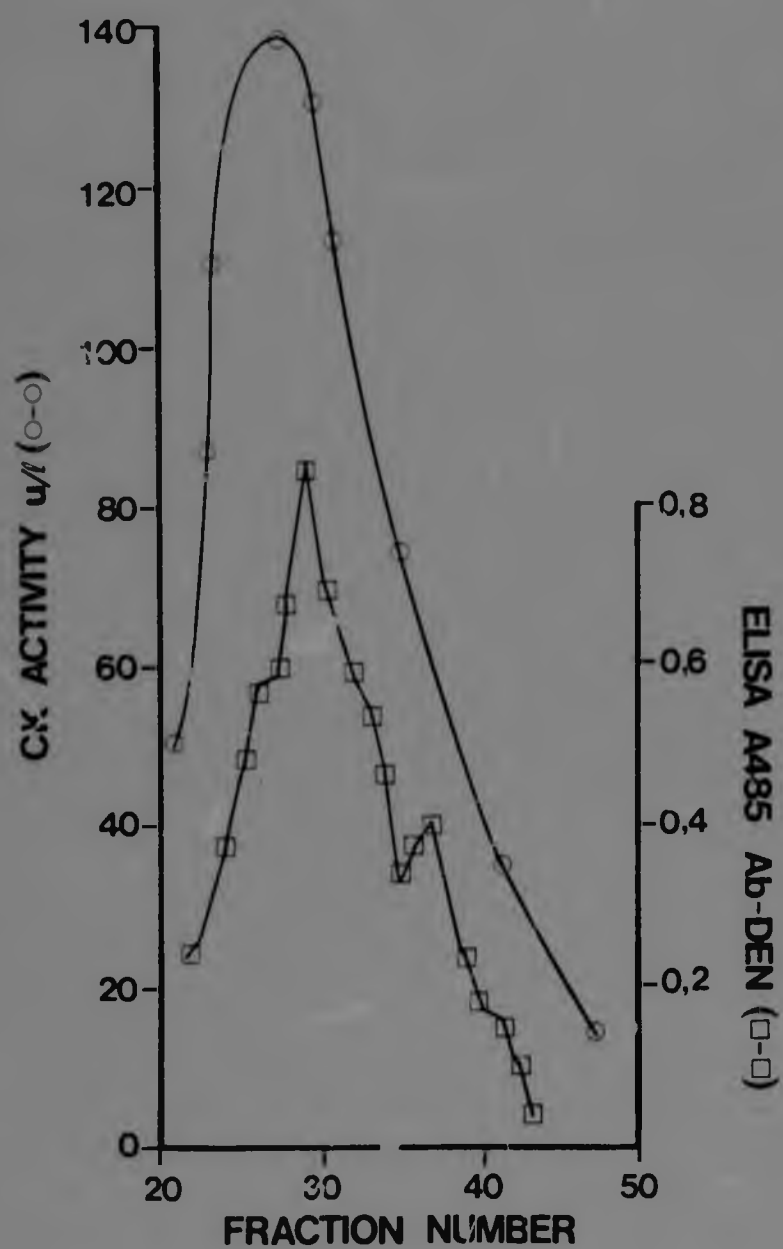
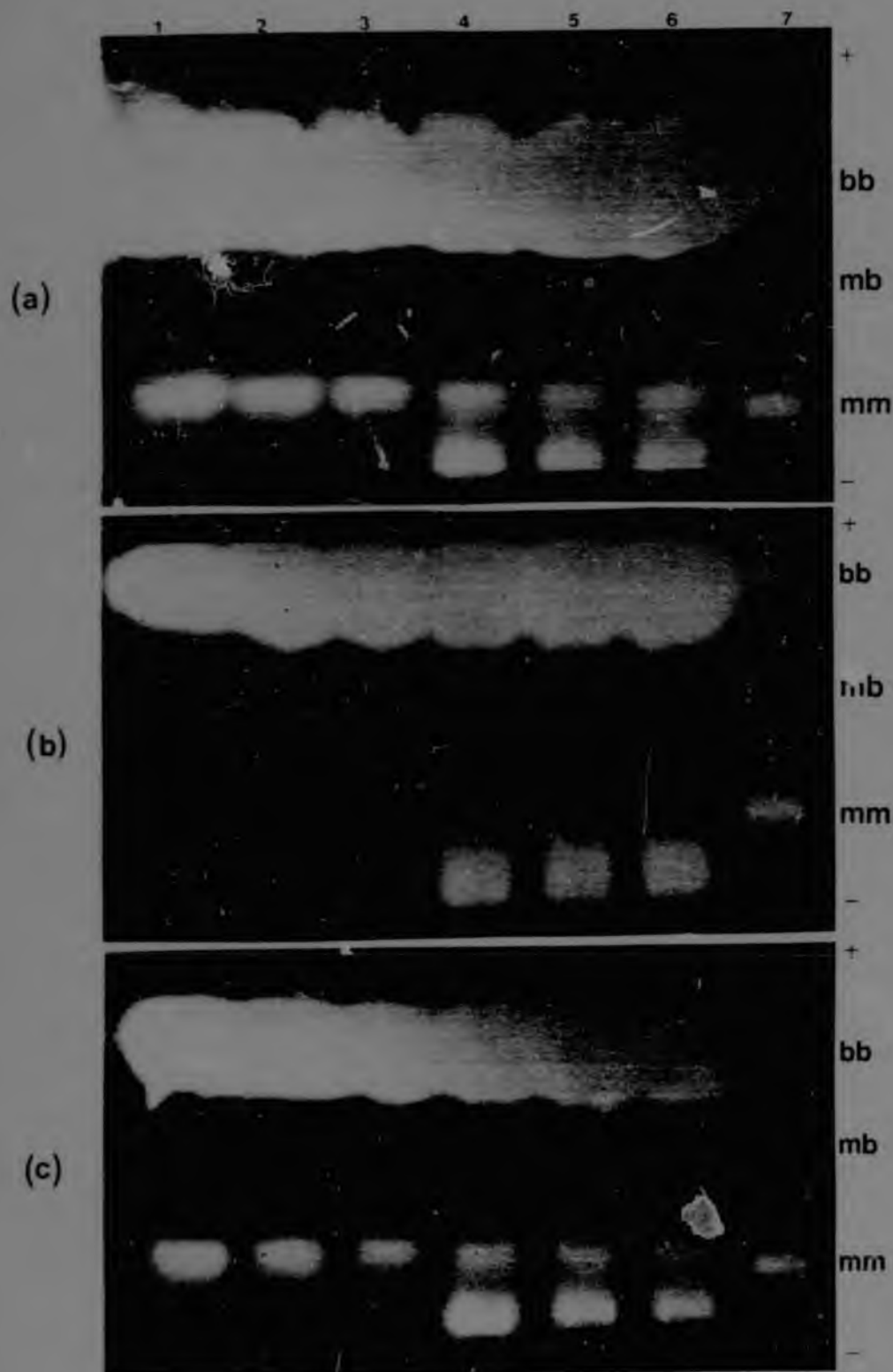


FIG 7g: ISOENZYME ELECTROPHORESIS OF BRAIN HOMOGENISED IN SIX DIFFERENT BUFFER SYSTEMS AND INCUBATED WITH (a) NOTHING, (b) AB_{CKBB} AND (c) AB_{DEN}.

(Lane 1 = 50 mM TRIS pH 7.4 (A); lane 2 = A + 5 mM B-mercaptoethanol; lane 3 = A + 5 mM B-mercaptoethanol + 1 mM EDTA; lane 4 = 100 mM phosphate buffer pH 7.4 (B); lane 5 = B + 5 mM B-mercaptoethanol; lane 6 = B + 5 mM B-mercaptoethanol + 1 mM EDTA; lane 7 = CK isoenzyme markers)



3.8. NON-DENATURING ELECTROPHORESIS EXPERIMENTS

Fig 8a of a Paragon isoenzyme electrophoresis shows how reliant CK is on inorganic ions and/or small molecular mass molecules for the integrity of the molecule. Lane 1 shows the isoenzyme electrophoresis of an untreated cytosolic centrifugal fraction with an anodic CKBB band and a cathodic band. The lanes 2-4 show the effect of buffer exchanging cytosolic fractions of brain to Tris/BME which would remove all small molecular mass molecules associated with the original homogenate. The S1 is a 630xg spin supernatant, the S2 is a 20,000xg spin supernatant and the S3 is a 100,000xg spin supernatant. The effect of buffer exchanging was to produce three extra CK staining bands of approximate Rf +0,73, +0,49 and +0,11. All three bands were produced in all the buffer exchanged lanes. The same fractions were electrophoresed on a 5% PAGE gel and in this case two CK staining bands were seen - one with an Rf of 0,12 and one with an Rf of 0,91.

When the first antibody made to CKBB was found to be impure, the original aim was to make a new antibody by reinoculation using CKBB further purified by excision from a non-denaturing PAGE gel. The peak of activity from the DEAE Sepharose CL6B chromatography of the 70% ethanol precipitate of brain was therefore submitted to non-denaturing electrophoresis on a 10% gel. Three lanes were cut off the edge of the completed gel and stained for protein by Coomassie Blue, for CK activity using the Beckman Paragon stain overlaid on the gel with a soaked filter paper, and for carbohydrate moieties using a periodic acid Schiff stain. The results are shown in Fig 8b. Most unexpectedly, the CKBB preparation yielded five bands of CK activity on the gel. (Since the aim of this experiment was to purify CKBB for

injection, the approach was changed to that cited for the preparation of Ab-DEN). The Rf values of these bands were 0,13 0,19 0,24 0,31 and 0,40. The PAS stain was negative suggesting that the differences in these forms is not due to differences in carbohydrate residues. The fact that these forms separated from a fraction which contains only CKBB proves that this isoenzyme is capable of assuming many forms of differing charge, of which five species have been described here. In addition, it shows that the forms which developed in Fig 11a are probably derived from the CKBB band, and not from the cathodic band. Note that storage of brain supernatant in the freezer led to the formation of a band equivalent to the MB marker (Rf on Paragon gel of about +0,46) as noted in section 7.

Brain which had been stored in the freezer was defrosted, homogenised, centrifuged at 10,000xg for 30 minutes and chromatographed through DEAE Sepharose CL6B. Every fifth fraction eluted by the salt gradient was electrophoresed on a 7,5% non-denaturing PAGE gel which was then electroblotted to nitrocellulose paper and stained for CK activity and immunostained with Ab-DEN. A major band of CK activity was seen 66 mm from the origin in lanes 8-15 corresponding to fractions 50-90 from the column. These same fractions had an immunogenic band 9 mm from the origin, with the strongest band being in fraction 75 where the CKBB peak elutes. In addition, there were three faint bands of CK activity 15, 30 and 44 mm from the origin. Fractions 25, 30 and 35 had a faint immunogenic band 16 mm from the origin, and fractions 30, 35 and 40 had a faint immunogenic band 25 mm from the origin. A Paragon isoenzyme electrophoresis (which has more sensitivity) of the same fractions yielded a faint cathodic band in fractions 15 to 35. The staining of

the PAGE was not sensitive enough to indicate whether this activity was associated with one or other of the immunogenic bands found in these fractions. The Rf values of these bands could not be calculated because the distance the dye front travelled was unknown.

In a 7.5% non-denaturing PAGE gel (results not shown) of the CKBB peak off a DEAE Sepharose CL6B column of the 70% precipitate of brain, two bands were seen which had both CK activity and immunoreactivity to Ab-DEN. These bands were at 39 and 47 mm from the origin respectively. Note that the Ab-DEN recognises CKBB in the presence of SDS. The fact that a CK peak fraction was run and yielded two forms suggests that CKBB exists in two charged states within this narrow range of elution off the DEAE column. Fibroid supernatant, on the other hand, had one band at 49 mm with CK activity and immunoreactivity to Ab-DEN, one band at 17 mm which had only CK activity and three additional immunoreactive bands at 6, 20 and 22 mm respectively. Four lanes of cancer serum and one of normal serum were run on the same gel: there was a band in each of CK activity 39 mm from the origin associated with immunoreactivity, with a broad region of immunoreactivity between 5 and 10 mm from the origin, which may be a doublet, in the cancer, and immunoreactive bands at 18, 24 and 56 mm from the origin, with the 24 mm band apparently being stronger in the cancer sera.

In a separate experiment, breast tumour cytosol and fibroid supernatant gave two immunoreactive bands - one strong band at 25 mm from the origin which was particularly marked in breast tissue, and one at 37 mm. It is likely that the band 25 mm from the origin is the same protein which gives an ELISA peak around fraction 30 in the DEAE Sepharose column. Lanes of serum from cancer and normal

patients run on the same gel all yielded faint immunoreactive bands at 3, 13 and 15 mm from the origin, whilst the cancer sera appeared to have additional immunoreactive bands at 36 and 48 mm from the origin, particularly in prostate and lung cancer.

In yet another similar 7.5% PAGE run, breast tumour cytosol gave a broad band of immunoreactivity between 26 and 30 mm from the origin, with another band at 38 mm and the possibility of a band at 7 mm from the origin. A parallel stain for CK activity yielded activity at the 38 mm band but none at the 26 mm band. This 26 mm band can therefore be said to be an inactive CKBB molecule, or a molecule with similar primary structure to CKBB. On the same gel, serum fractionated into three probable CKMM species at 6, 9 and 13 mm from the origin, a serum with high CKMB gave an CK activity band at 37 mm and a purified CKBB lane gave an activity band at 60 mm from the origin. The serum lanes gave immunoreactive bands at 2 and possibly 6 mm from the origin but not the three activity bands discounting the possibility that the Ab-DEN is cross reacting with CKMM. Likewise there was no cross reaction in the region of the CKMB activity band. The fact that the breast tumour cytosol has an CK activity at the same point of migration as putative CKMB suggests that this band is either due to CKMB or to the form derived from CKBB which migrates to a similar position to CKMB as described in Fig 8a.

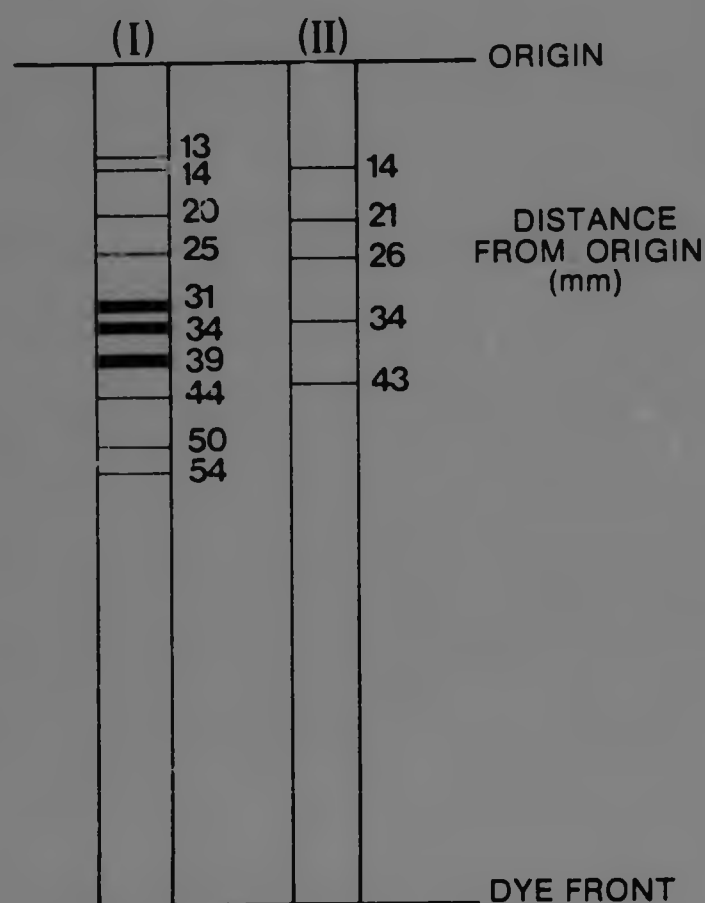
FIG 8a: ISOENZYME ELECTROPHORESIS OF BRAIN CYTOSOLIC FRACTIONS AFTER BUFFER EXCHANGE TO 50 mM TRIS BUFFER pH7,4 CONTAINING 5 mM β -MERCAPTOETHANOL

(i.e. after the removal of low molecular mass proteins and inorganic ions)

(Lane 1 = 630xg brain supernatant with no buffer exchange; lane 2 = 630xg supernatant after buffer exchange; lane 3 = 20,000xg supernatant after buffer exchange; lane 4 = 100,000xg supernatant after buffer exchange)

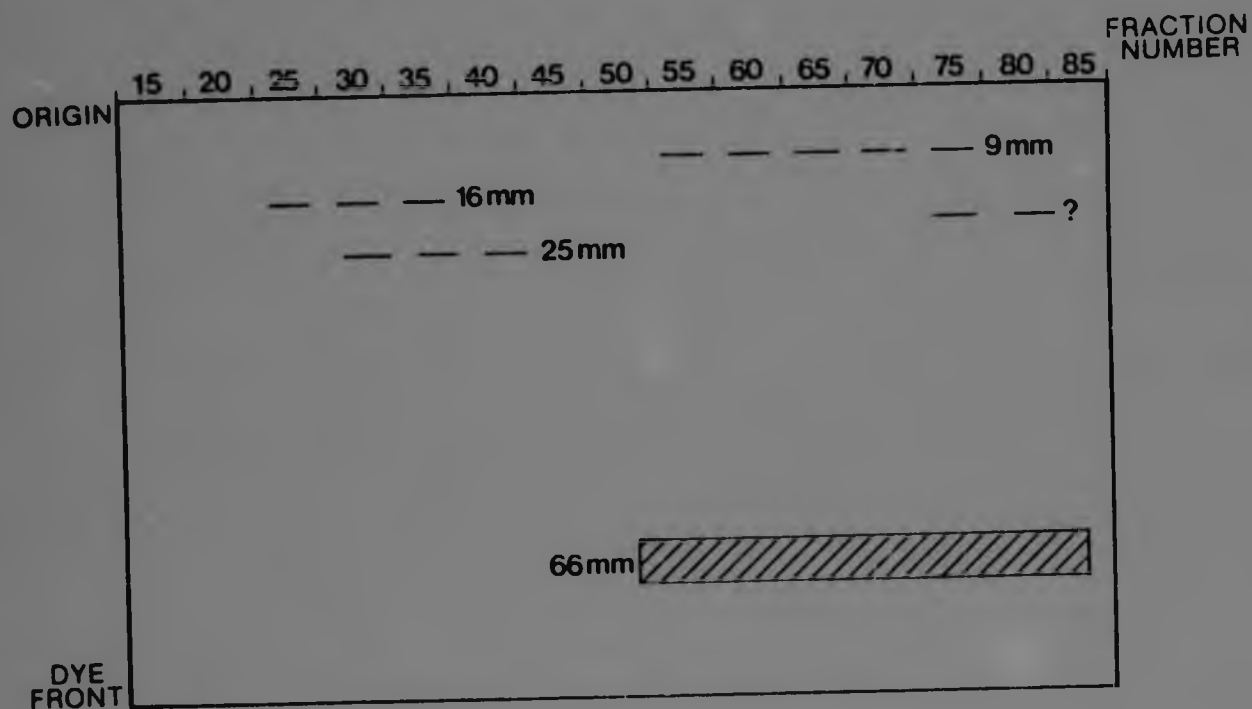


Fig. 8b
NON-DENATURING POLYACRYLAMIDE
ELECTROPHORESIS OF CKBB, purified by 50 - 70%
ethanol fractionation followed by DEAE sepharose CL6B
chromatography. 1 mg protein was added to a
preparative 10% γ gel. After electrophoresis, three vertical
lanes were *



*excised and stained for (I) protein by Coomassie Blue Stain, (II) CK activity using the Paragon activity stain and (III) glycoprotein using the periodic acid Schiff stain (negative, therefore results not shown.)

Fig. 8c
7.5%T NON DENATURING ELECTROPHORESIS OF
EVERY FIFTH FRACTION FROM THE DEAE
SEPHAROSE CL6B CHROMATOGRAPHY OF BRAIN
SUPERNATANT, traced from the electroblot of the gel,
immunostained using Ab-DEN. Thus the series of bands
at 9, 16 and 25 mm from the origin are proteins which react
with Ab-DEN. (The wide hatched band at 66mm is CKBB
activity, as stained with the Paragon activity stain).



3.9. GEL FILTRATION EXPERIMENTS

Fig 9a shows the first experiment in which brain supernatant in Tris/BME was passed through a Sephacryl S200 gel filtration column, with the fractions being analysed for CK activity and reaction to unpurified Ab-CKBB and Ab-DEN by the ELISA technique. There was one CK activity peak at $V_e/V_o = 1,34$. ELISA, using the Ab-CKBB, revealed three main peaks at V_e/V_o of 1,26 1,34 and 1,40 and ELISA using the Ab-DEN revealed a major peak at V_e/V_o of 1,40. Fractions 59 to 67 were electrophoresed on a 10% SDS gel, stained for protein with Coomassie Blue, and immunostained using both antibodies. The gel stained for protein is shown in Fig 9b and the bands which reacted with the antibodies are indicated by the arrows. The immunoblot using the Ab-CKBB reacted with two proteins of molecular mass 45000 and 48000 daltons with the 45000 dalton band being the stronger, and being most dominant in the fraction of the CK peak. The immunoblot with the Ab-DEN also reacted weakly with these bands with the intensity also being greatest in the CK peak. In addition, there was reaction with a 36000 dalton band which was strongest in fractions 60 and 61 (i.e. where peak I from the column was). This experiment shows that the Ab-DEN recognises the 45000 dalton CKBB in the presence of SDS.

It is difficult to say whether the 48000 dalton band represents a higher molecular mass species of CK or the presence of a contaminating antibody which recognises a different protein. It was established that the AB-CKBB was contaminated but the Ab-DEN was raised in a much more particular manner. However, if the 48000 dalton protein is neuron specific enolase which has very similar properties to CKBB, it is not inconceivable that some of this protein

could have been excised from the nitrocellulose in the preparation of the inoculant. However, when the specificity of the antibody (Ab-DEN) was tested originally it reacted with only one band (the CKBB band on an SDS PAGE) in a situation where the 48000 dalton NSE is known to exist. It is therefore a possibility that the 48000 dalton protein is, in fact, a form of CKBB since it appears that the Ab-DEN either does not recognise neuron-specific enolase at all or that the sensitivity of reaction is much lower than the corresponding sensitivity to CKBB.

Fractions 59 to 67 from this same experiment were subjected to non-denaturing electrophoresis on 7.5% PAGE gels and immunoblotted with Ab-DEN and Ab-CKBB (photograph not available). There was no reaction at all with the Ab-DEN which proves that the antigenic determinants recognised by the antibody are concealed in the native conformation. The Ab-CKBB, on the other hand, reacted with five bands (whereas the same fractions on SDS PAGE yielded two). There was a band 70 mm from the origin which had CK activity (shown by overlaying the electroblotted nitrocellulose paper with filter paper soaked in Paragon CK stain) and immunoreactivity. Cathodic to this were three equally spaced immunobands at 51, 58 and 65 mm from the origin. The 51 and 58 mm bands seemed stronger in fractions 64, 65 and 66, whilst the 65 mm band seemed stronger in the fraction 60 and 61. The fact that these bands were equally spaced suggests a sequential modification of the protein. There was also an anodal immunoband at 73 mm - this can be presumed to be the 48000 dalton band since on the ion exchange column this protein elutes just after the active CKBB peak.

Fig 9c shows another gel filtration of brain supernatant in Tris

buffer, with the fractions assessed for ELISA and CK activity. Three peaks of ELISA (Ab-DEN) activity were obtained, with the largest being at $V_e/V_o = 1,06$. There was one CK activity peak at $V_e/V_o = 1,30$. The elution characteristics and molecular masses calculated from the calibration curve in Fig 9d are shown in Table 6.

One thing of note is that in every gel filtration experiment I performed, the molecular mass of CKBB was calculated to be in the region of 180,000 daltons which could represent a tetramer of a 45,000 dalton subunit molecular mass, as calculated by SDS PAGE (Fig 4c). This is in contrast to all the published reports that CK is a dimer of a 42,000 dalton subunit molecular mass with a non-denaturing molecular mass of 80000 daltons (Watts, 1973).

Fig 9e shows the results of two equivalent gel filtrations of brain supernatant in Tris buffer - one in the presence and one in the absence of 5 mM BME. Both columns were assessed for ELISA activity with Ab-DEN and Ab-CKBB, and CK activity. Of particular interest is the observation that the main high molecular mass ELISA peak (using Ab-DEN) is present only in the presence of 5 mM BME. This column only has one CK activity peak at fraction 55 which corresponds to an ELISA peak using Ab-CKBB. The column without BME still indicates three small peaks of ELISA activity with Ab-DEN, but in this case the second peak has a shoulder of CK and ELISA (Ab-CKBB) activity suggesting that in the absence of BME, this particular form is enzymatically active. BME also affects the migration of the proteins through the Sephacryl S200 column. In these two columns, the fraction numbers can be directly compared since they were done on the same packing of Sephacryl S200, and the ELISA and CK activities can be compared since they were done at the same time. It can be seen

that the elution profiles in the absence of BME are shifted in the direction of lower molecular mass. For example, the active CKBB fraction elutes in fraction 55 in the presence and fraction 57 in the absence of BME.

Fig 9f shows the results of the SDS PAGE of peaks 1 to 4 from Fig 9c, stained for protein by Coomassie Blue and immunoblotted using Ab-DEN. Peak 1 - the high molecular mass species formed particularly in the presence of 5 mM BME - in lane 1 reacted immediately and strongly with the Ab-DEN in the immunoblot, staining only one protein band with a molecular mass of 42,000 daltons. This protein, therefore, had the same properties as the purified antigen illustrated in Fig 4f. After about a ten minute delay, there was a reaction on the immunoblot with a protein of estimated molecular mass 36,000 daltons in peaks 2,3 and 4. Lane 2 - corresponding to the antigen peak of approximately 200,000 daltons from Sephacryl S200 - had a faint band of the 42,000 dalton protein (carryover from the peak 1), the 36000 dalton band and a third band of molecular mass 38,000 daltons. This appeared to be the only one of the four protein peaks under investigation which had this third immunoreactive band. Note the strong protein band of 45,000 daltons in lane 3 which can be presumed to be CKBB. Suggested arrangements of subunits are shown in Table 6.

Gel filtration of brain supernatant in phosphate buffer yielded a small CK activity peak at fraction 52 which was not present in an equivalent gel filtration of brain supernatant in Tris buffer (Fig 9g). Both columns yielded a large CK activity peak at fraction 63. It would be reasonable to assume that the CK at fraction 52 is attributable to mitochondrial CK and yet isoenzyme electrophoresis of

TABLE 6: SUGGESTED SUBUNIT ARRANGEMENTS OF CREATINE KINASE SUBUNITS

<u>PEAK</u>	<u>TOTAL MASS</u> (daltons)	<u>SUBUNIT MASS</u> (daltons)	<u>NO. SUBUNITS</u>
1	251,000	42,000	6
2	207,000	42,000	5
		38,000	(5,4)
		36,000	(5,8)
3	182,000	45,000	4
4	162,000	36,000	(4,5)

this fraction showed two CK bands - one equivalent in migration to the CKBB marker, which could be an overlap from the main CKBB peak, and a cathodic band, equivalent in migration to the CKMM marker. The molecular mass of this fraction is estimated to be 234,000 daltons. This is not an accurate measurement however because a void volume marker was not included, since it has been proposed that dextran blue could influence the migration of proteins containing a dinucleotide fold such as CK (Thompson et al, 1975). Note that the fractions cannot be directly compared with those in Fig 9b since these two experiments were performed on different packings of Sephacryl S200.

Fig. 9a SEPHACRYL S200 CHROMATOGRAPHY OF BRAIN SUPERNATANT homogenised in 50 mM TRIS buffer pH 7,4 containing 5 mM β -mercaptoethanol. Fractions assayed for CK activity and ELISA using Ab-DEN and Ab-CKBB

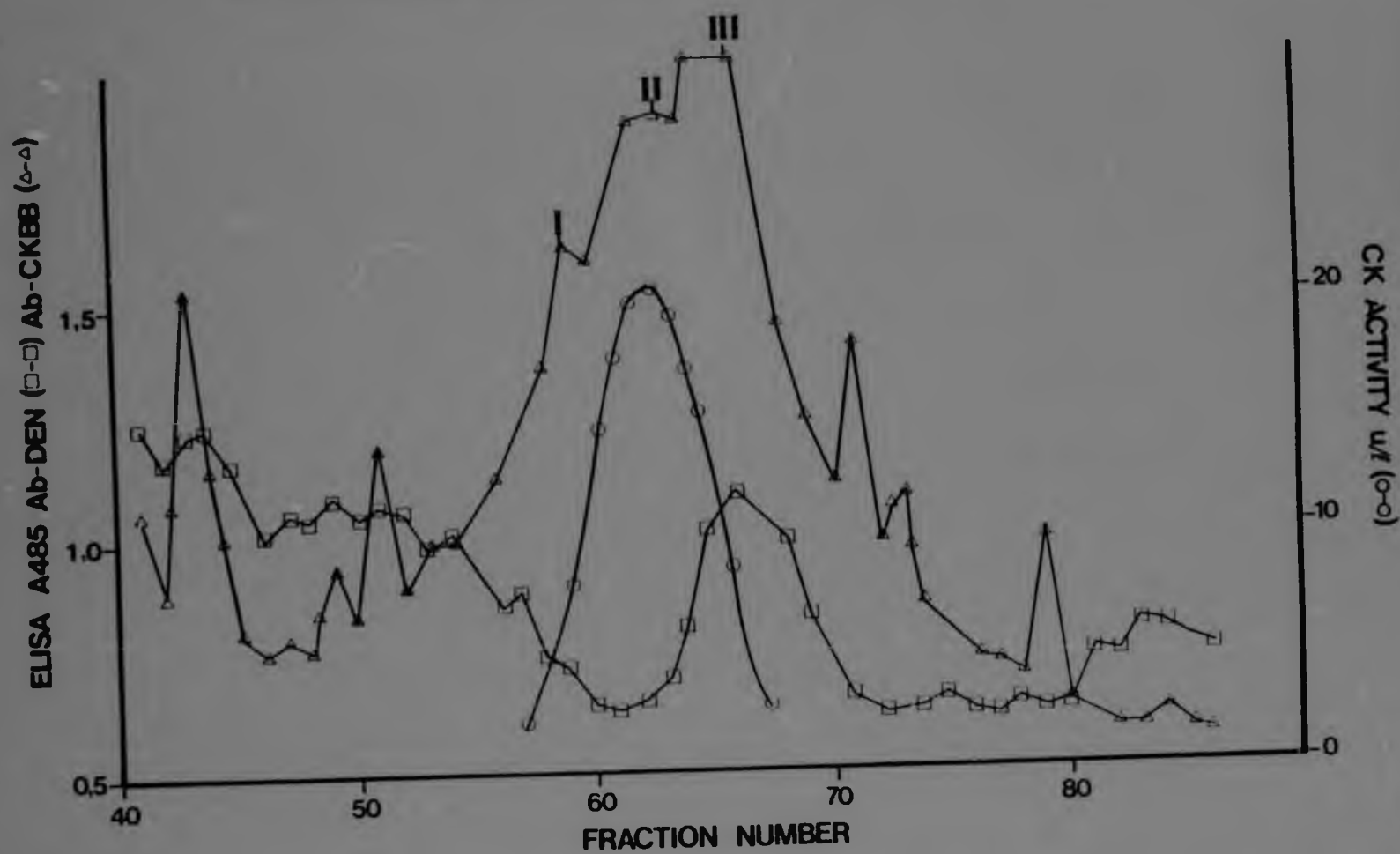
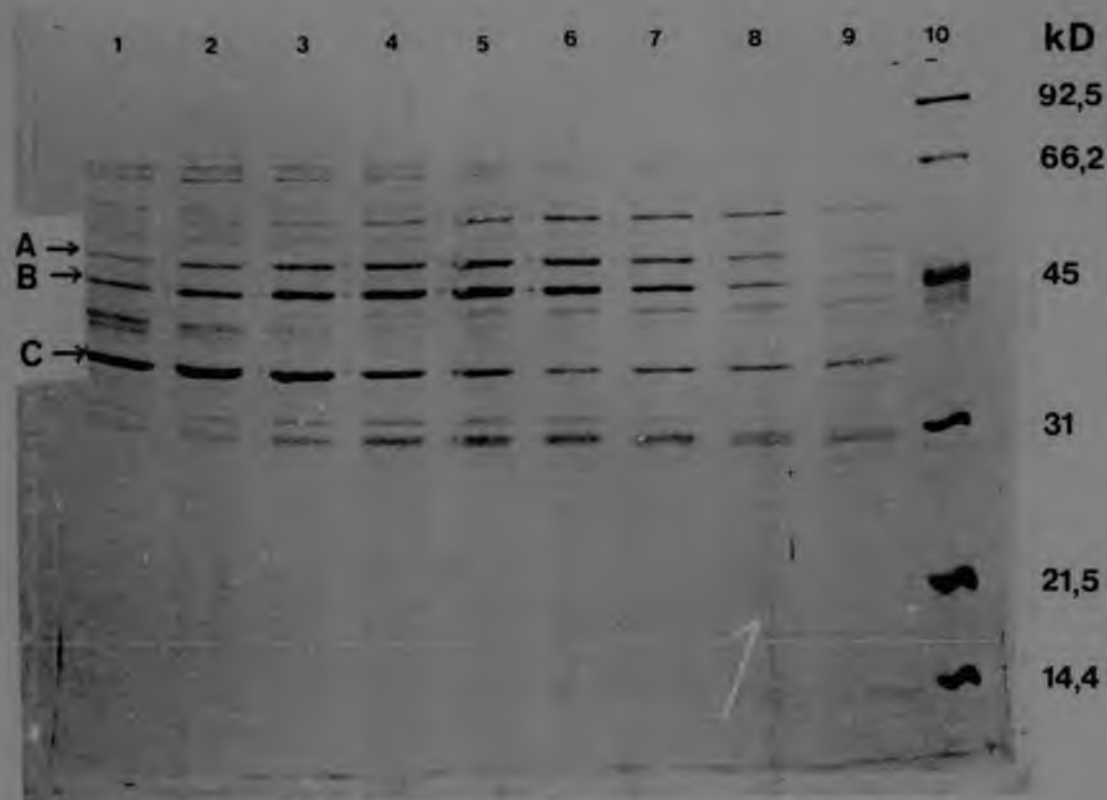


FIG 9b: SDS-PAGE (10%T) OF SELECTED FRACTIONS FROM THE SEPHACRYL S-200 GEL FILTRATION OF BRAIN SUPERNATANT, STAINED FOR PROTEIN BY COOMASSIE BLUE

(Lane 1 = fraction 59, lane 2 = fraction 60, lane 3 = fraction 61, lane 4 = fraction 62, lane 5 = fraction 63, lane 6 = fraction 64, lane 7 = fraction 65, lane 8 = fraction 66, lane 9 = fraction 67, lane 10 = low molecular mass markers)
Bands A and B react with Ab-CKBB immunoblot, bands A, B and C react with Ab-DEN immunoblot.



**Fig.9c SEPHACRYL S200 CHROMATOGRAPHY OF 2 ml
BRAIN SUPERNATANT, homogenised in 50 mM TRIS
buffer pH 7,4 containing 5 mM β -mercaptoethanol;
fractions monitored for CK activity and antigen content
(Ab - DEN)**

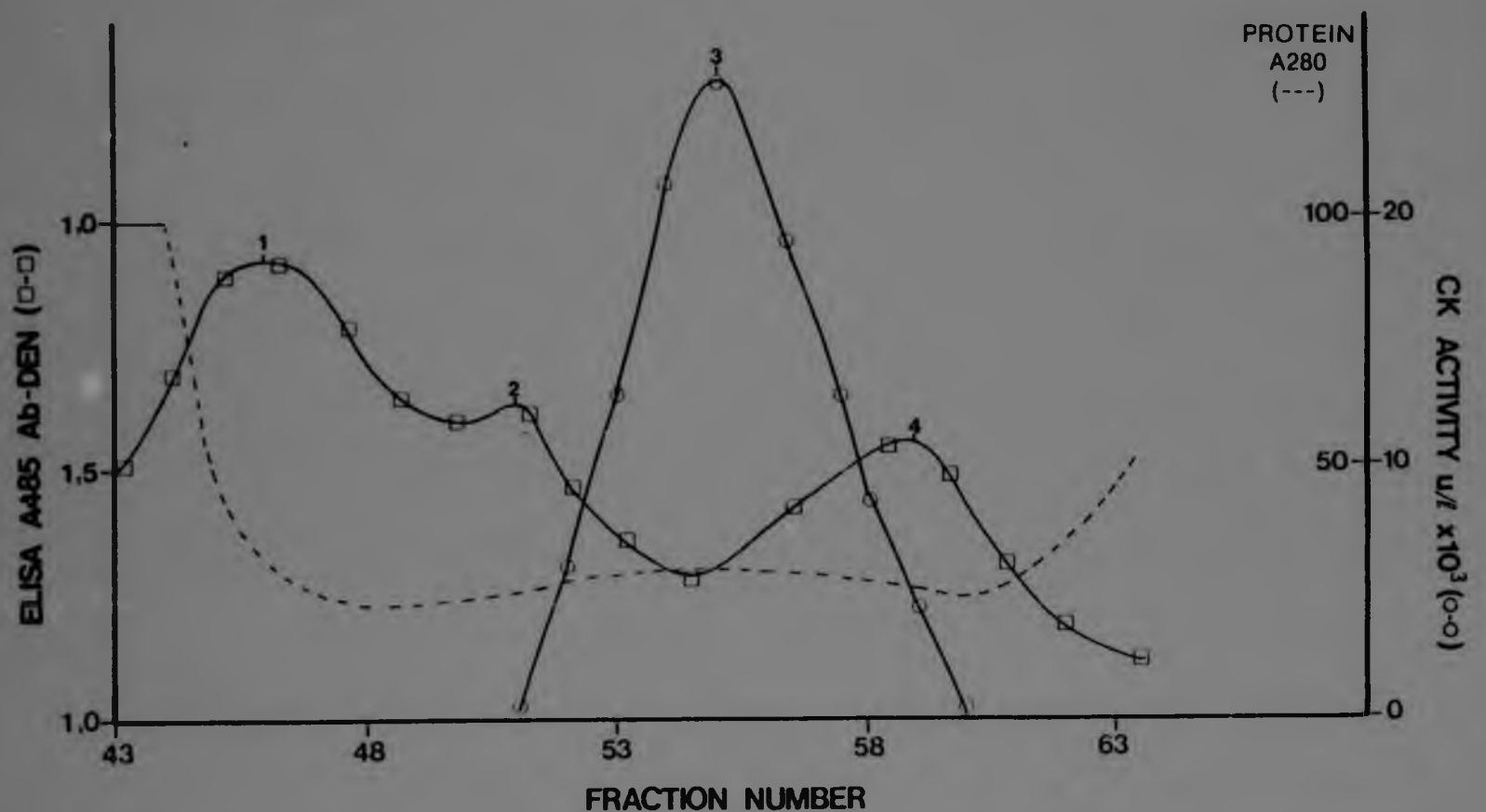


Fig. 9d CALIBRATION OF SEPHACRYL S200 FOR ESTIMATION OF MOLECULAR MASSES. Numbered points refer to peaks in Fig.9c.

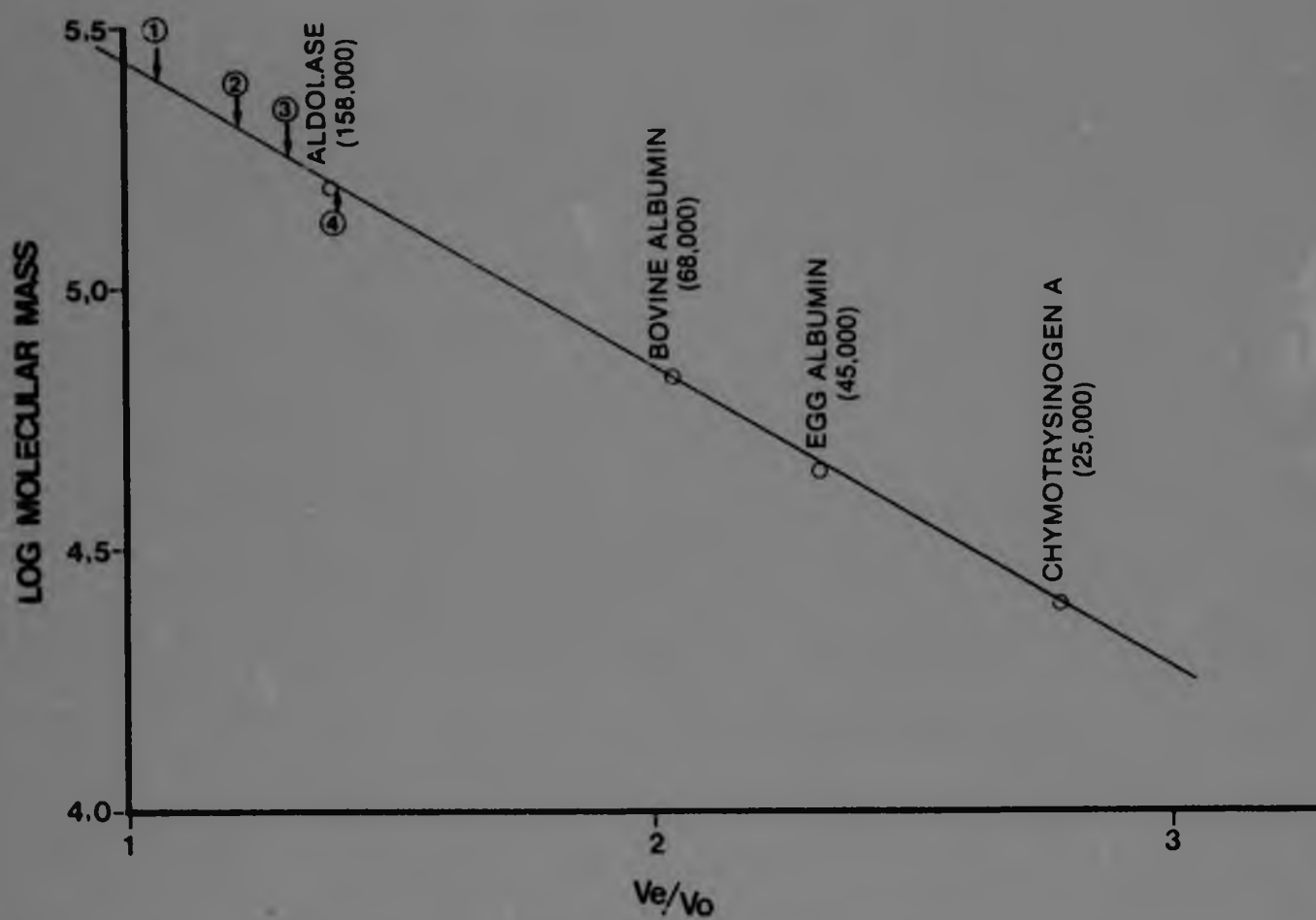


Fig. 3e
SEPHACRYL S200 CHROMATOGRAPHY OF BRAIN
HOMOGENISED IN 50 mM TRIS pH 7,4 WITH (A) AND
WITHOUT (B) THE ADDITION OF 5 mM
 β - MERCAPTOETHANOL

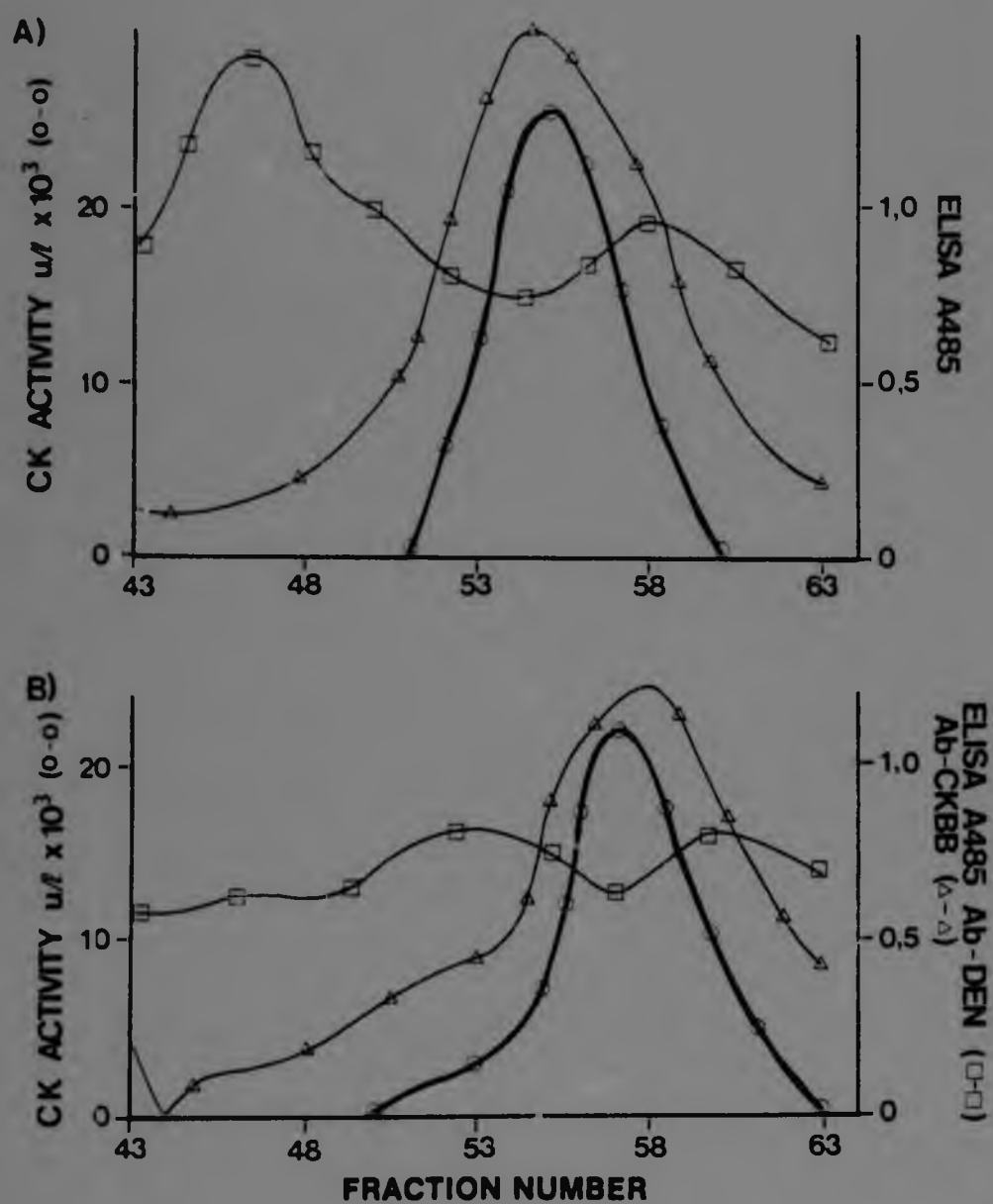


FIG 9f: SDS-PAGE (10%T) OF PEAKS 1-4 FROM THE SEPHA-CRYL S-200 GEL FILTRATION OF BRAIN SUPERNATANT IN 50mM TRIS BUFFER pH 7,4 CONTAINING 5 mM β -MERCAPTOETHANOL

(a) stained for protein by Coomassie Blue

(b) immunoblotted using Ab- α - EN

(Lane 1 = peak 1; lane 2 = peak 2; lane 3 = peak 3; lane 4 = peak 4; lane 5 = low molecular mass markers)

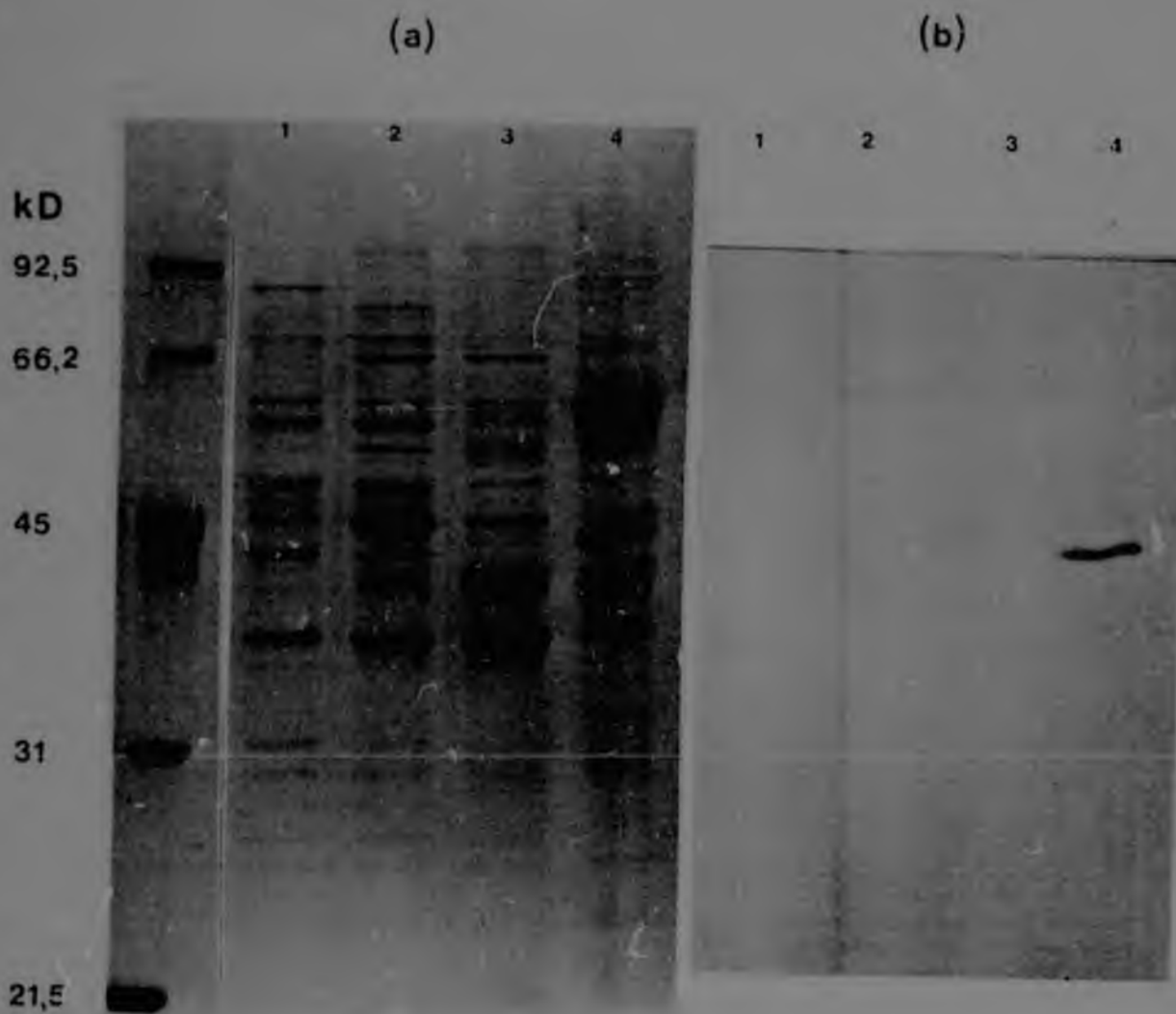
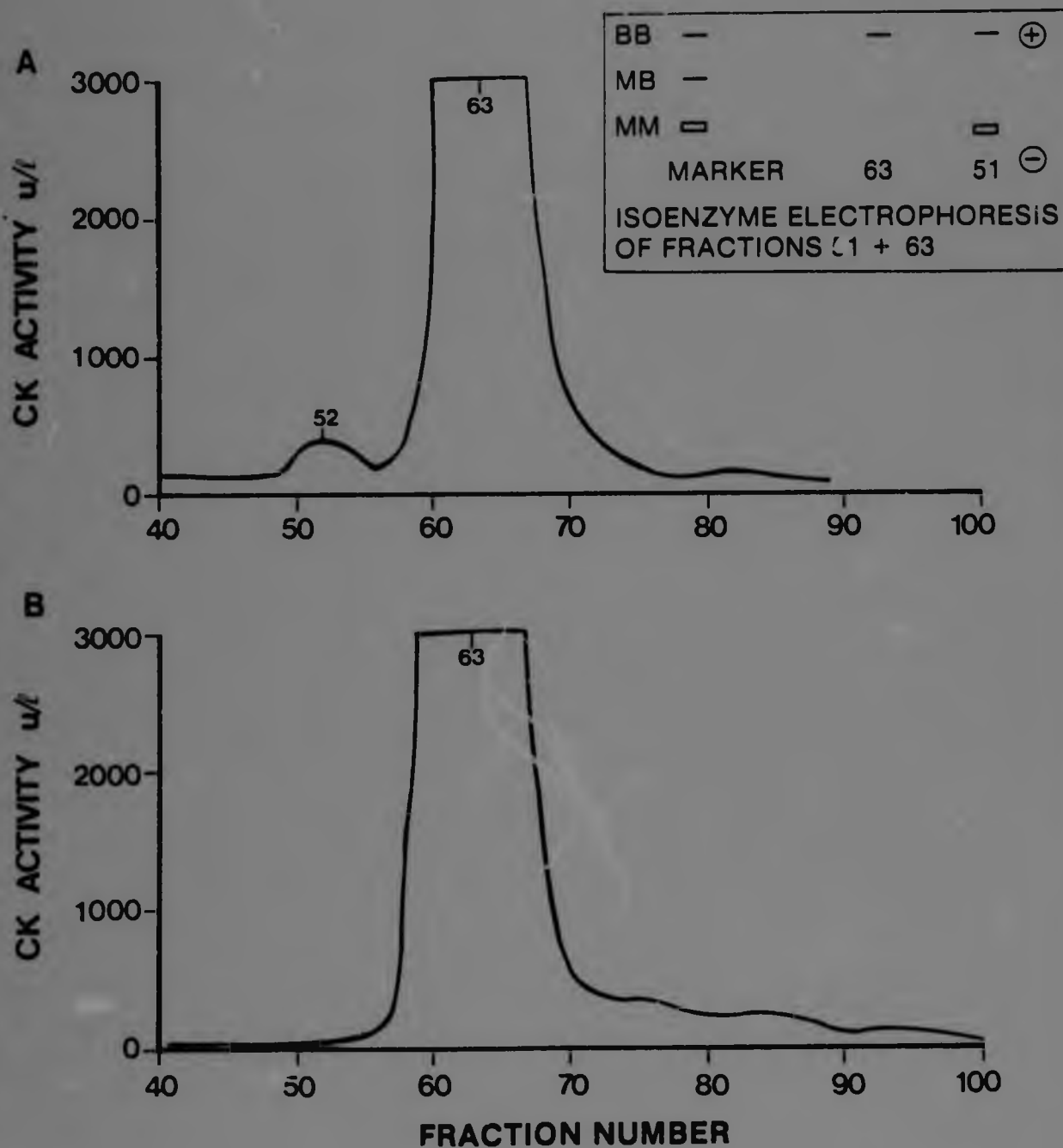


Fig. 9g

SEPHACRYL S200 CHROMATOGRAPHY OF BRAIN SUPERNATANT

(A) Homogenised in 100 mM phosphate buffer containing 5 mM β -mercaptoethanol (2 : 1 v/w)

(B) Homogenised in 50 mM TRIS buffer containing 5 mM β -mercaptoethanol (2 : 1 v/w) 2ml supernatant applied directly without prior buffer exchange.



3.10. EFFECT OF HOMOGENISATION BUFFER ON NUMBER AND NATURE OF CK ISOENZYMES FOUND

Equal quantities of brain (5g) were homogenised in 20 ml 50 mM Tris, 5 mM BME pH 7,4 (Tris/BMF) and 100 mM phosphate, 5 mM BME pH 7,4 buffers respectively, left to stand for an hour and centrifuged at 20,000xg for 30 minutes. 2,5 ml of the respective supernatants were buffer exchanged on a PD10 column equilibrated to Tris/BME and chromatographed on DLAE Sepharose CL6B as described. The fractions were analysed for CK activity, and ELISA using Ab-CKBB (purified) and Ab-DEN.

The first thing to note is that homogenisation of brain in phosphate buffer (Fig 10a) yields an activity peak (fraction 81) which has a lower total CK activity (1020 U/l) than the equivalent homogenisation in Tris (1800 U/l) (Fig 10b). However, the protein elution profile (A_{280}) of the Tris column contains a peak at fraction 89 which the phosphate column does not have. See Table 7.

TABLE 7: COMPARISON OF PROPERTIES OF FRACTION 81 HOMOGENISED IN PHOSPHATE OR TRIS BUFFER

<u>FRACTION 81</u>	<u>TRIS</u>	<u>PHOSPHATE</u>
CK ACTIVITY (U/l)	1800	1020
PROTEIN (A_{280})	0,032	0,021
RATIO CK/ A_{280}	56250	48571

TRIS CK / PHOSPHATE CK = 0,57

TRIS 'SPECIFIC ACTIVITY' / PHOSPHATE 'SPECIFIC ACTIVITY' = 0,86

Therefore, the difference in CK activity obtained for fraction 81 in the tris and phosphate columns can, in part, be explained by the difference in the protein concentration. A fraction with more protein in it would possibly contain more CK molecules than the fraction with less protein and would therefore exhibit a higher CK activity.

The first section of the two columns is shown in Fig 10c in enlarged detail. Homogenisation in phosphate buffer yielded a basic protein peaking in fraction 27 which had CK activity and which was recognised by the purified antibody to CKBB. There was a smaller peak with similar properties at fraction 64. From Fig 7b, these two peaks can be presumed to represent breakdown products of CKBB. Note that fraction 29 from the phosphate column gave an isoenzyme band equivalent in migration to CKBB, whereas the CK at fraction 30 in Fig 7b had a migration equivalent of CKBB. Fraction 27 from the phosphate column was concentrated 2.5 times in a Amicon chamber and 50 μ l incubated with Ab-CKBB and Ab-DEN respectively followed by the measurement of CK activity. The CK activity in the presence of Ab-CKBB was 197 U/l, and that in the presence of Ab-DEN was 404 U/l, a factor of 2. This confirms that the Ab-CKBB reacts with the CK isoform from fraction 27.

Both the Tris column (fraction 35) and the phosphate column (fraction 34) had an ELISA peak reacting to Ab-DEN of roughly similar intensity. This ELISA peak had coincident CK activity in the Tris column with an adjacent peak, presumably at fraction 27 as per the phosphate column. The interesting thing to note is that both the ELISA and CK peaks at fractions 34 and 27 respectively appear to be recognised by the Ab-CKBB (purified) lending credence to the

assumption that the ELISA antigen is indeed a CKBB or CKBB-like molecule.

On the basis that the antigen peak is indeed a form of CKBB as established in section 7, we can say that the presence of phosphate or Tris ions does not affect the antigenicity of the protein in this situation since the ELISA peaks are equivalent intensities. However, the different buffers may affect the CK activity, though it is impossible to establish whether the CK activity in fraction 34 of the phosphate column is the tail of the CK peak at fraction 28 or CK activity from the antigen.

To summarise the findings from these experiments, homogenisation of brain in phosphate buffer yielded four protein peaks of interest within the range studied for the DEAE Sepharose CL6B column - three peaks with CK activity which reacted with anti-CKBB, and which are therefore presumed to be isoforms of CKBB, and one peak which reacted with Ab-DEN. Homogenisation in Tris buffer under identical conditions yielded two protein peaks of interest - one with low CK activity which reacted with Ab-DEN and one with high CK activity which reacted with Ab-CKBB.

An experiment was performed to establish the best buffer in which to homogenise brain to maintain the isoenzyme pattern and the best CK enzyme activity possible. As noted in the introduction, there is controversy as to whether the addition of chelators and thiol agents is desirable. Six portions of exactly 5 g each of brain were weighed out and hand homogenised in the following buffers (all pH 7.4) respectively:

- 1) 50 mM Tris
- 2) 50 mM Tris + 5 mM BME
- 3) 50 mM Tris + 5 mM BME + 1 mM EDTA
- 4) 100 mM phosphate
- 5) 100 mM phosphate + 5 mM BME
- 6) 100 mM phosphate + 5 mM BME + 1 mM EDTA

The isoenzyme patterns obtained with these homogenisations have already been presented in Fig 7g. The CK enzyme activity was estimated immediately, and then at intervals after storage in the refrigerator and the freezer. Because of an unavoidable between run discrepancies in the measurement of CK activity, the activity results were not compared directly. Rather, each batch of results was expressed as a percentage of the value obtained for buffer 2 (Tris/BME) which in this case was the highest. Note that these results have not been adjusted for protein content and this may only represent a higher protein concentration. To indicate the level of activity obtained, the results obtained with the initial estimation and after storage at -20°C for 11 days are represented in Table 8. The comparative stability of the CK in the various buffers expressed as a percentage of the activity in buffer 2 for each estimation are shown in Table 9.

TABLE 8: CREATINE KINASE ACTIVITY OBTAINED BY HOMOGENISATION OF HUMAN BRAIN IN SIX DIFFERENT BUFFER SYSTEMS, AT HOMOGENISATION AND AFTER STORAGE FOR 11 DAYS AT -20°C.

	<u>CK (U/l)</u>	<u>CK (U/l)</u>
	t=0	t=11 days (-20°C)
buffer 1	1068	1087
buffer 2	1242	1175
buffer 3	1029	510
buffer 4	1036	973
buffer 5	975	918
buffer 6	965	863

TABLE 9: COMPARATIVE STABILITY OF CREATINE KINASE FROM HUMAN BRAIN HOMOGENISED IN DIFFERENT BUFFER SYSTEMS AND STORED AT DIFFERENT TEMPERATURES (expressed as percentages of the results obtained for buffer 2 for each set of conditions)

<u>temperature</u>	25°C	25°C	4°C	-20°C	-20°C
<u>time</u>	0	2,5 hrs	5 days	5 days	11 days
buffer 1	86	91	92	98	93
buffer 2	100	100	100	100	100
buffer 3	83	90	77	71	43
buffer 4	83	74	78	78	83
buffer 5	79	68	72	71	78
buffer 6	78	66	72	69	73

It can be seen that the Tris series of buffers gave slightly higher activities than the phosphate series. Tris/BME was undoubtedly the best buffer in terms of maintenance of activity. The worst buffer for storage in the freezer was Tris/BME/EDTA where the level of activity was only 43% of that in Tris/BME. The addition of EDTA to the phosphate series also led to consistently lower activities, though not of a high order of magnitude. It was noted in section 7 that storage of these homogenates in the freezer led to the formation of the band which migrates like CKMB on isoezyme electrophoresis, particularly so in the buffer 3. So it is reasonable to conclude that the chelation of cations leads to a destabilisation of the CKBB molecule.

Another interesting observation is that when the homogenates were thawed after storage in the freezer, the proteins in the phosphate buffer series were not precipitated. However, when the fractions obtained by gel filtration (Sephacryl S200) of brain homogenised in phosphate/BME buffer were frozen and defrosted, the proteins were always precipitated. This did not occur in equivalent experiments using Tris/BME. These two facts taken together suggest that homogenisation in phosphate buffer modifies CKBB - and that protection against denaturation is offered by some other moiety of different molecular mass which is separated from the CKBB during the gel filtration.

Fig. 10a
DEAE SEPHAROSE CL6B CHROMATOGRAPHY
OF BRAIN SUPERNATANT, homogenised in 100 mM
phosphate buffer pH 7,4 containing 5 mM β - mercapto-
ethanol. Fractions assayed for CK activity and
Immunoreactivity to Ab-DEN and Ab-CKBB.

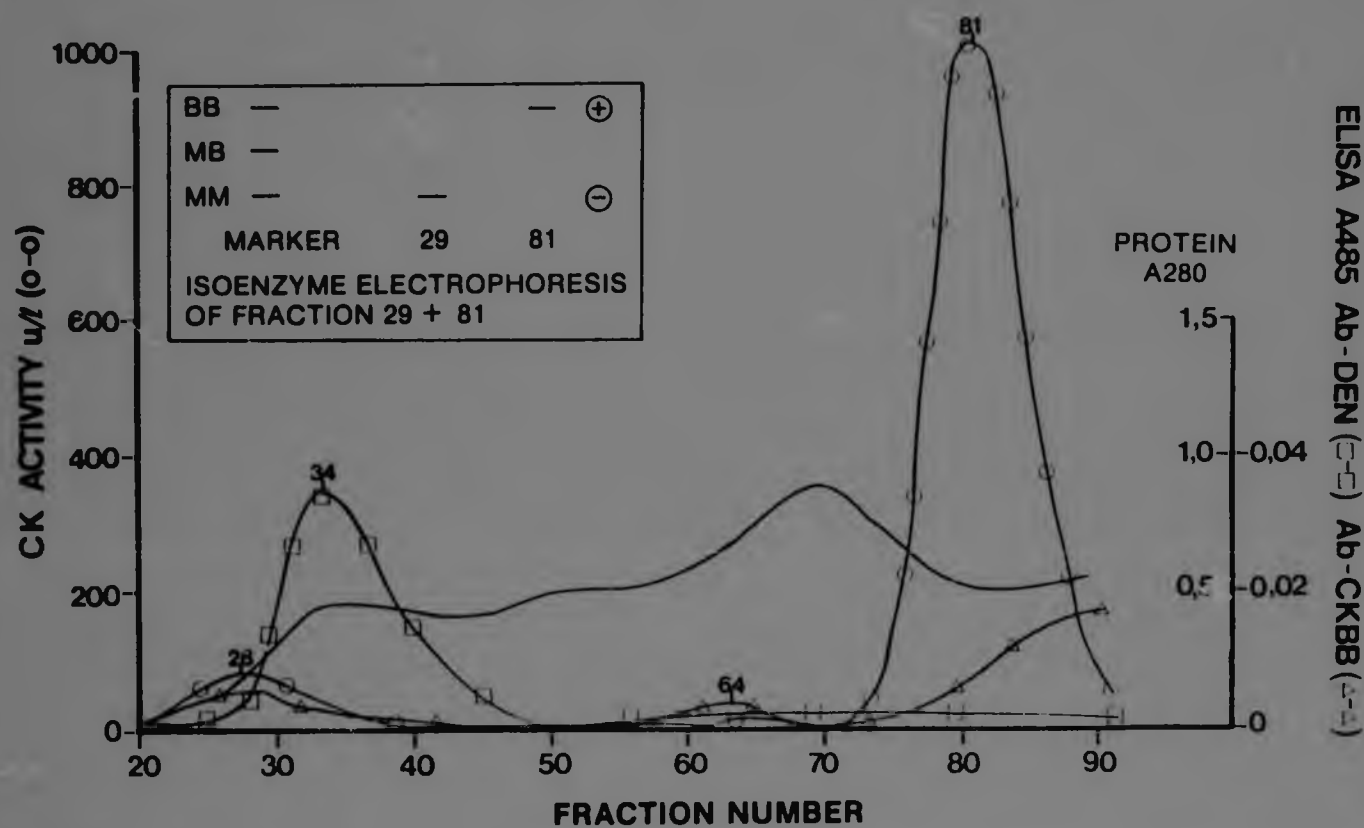


FIG. 10b
DEAE SEPHAROSE CL6B CHROMATOGRAPHY OF
BRAIN SUPERNATANT, homogenised in 50 mM TRIS
 buffer pH 7,4 containing 5 mM β - mercaptoethanol.
 Fractions assayed for CK activity and immunoreactivity
 to Ab-DEN and Ab-CKBB.

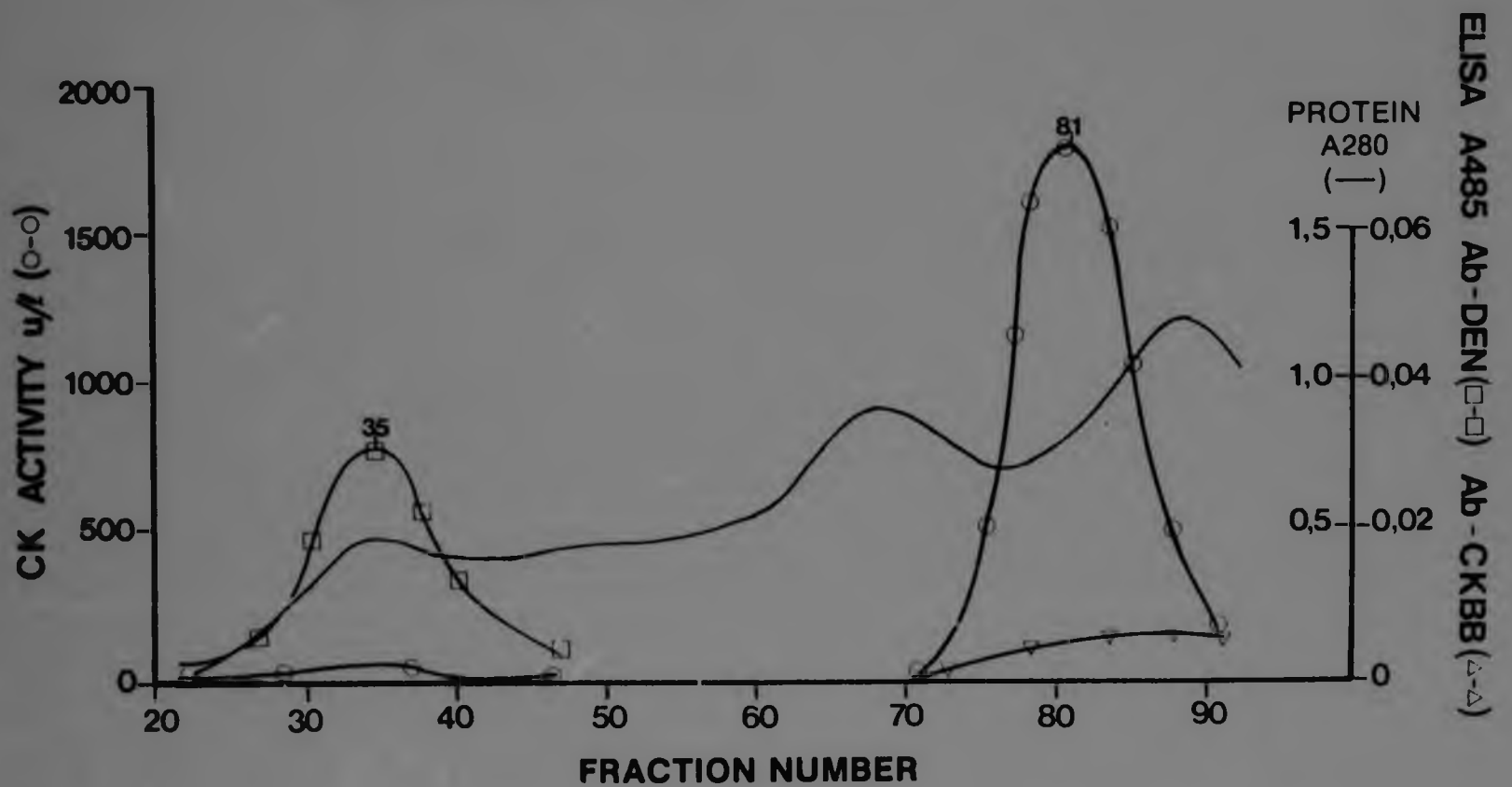
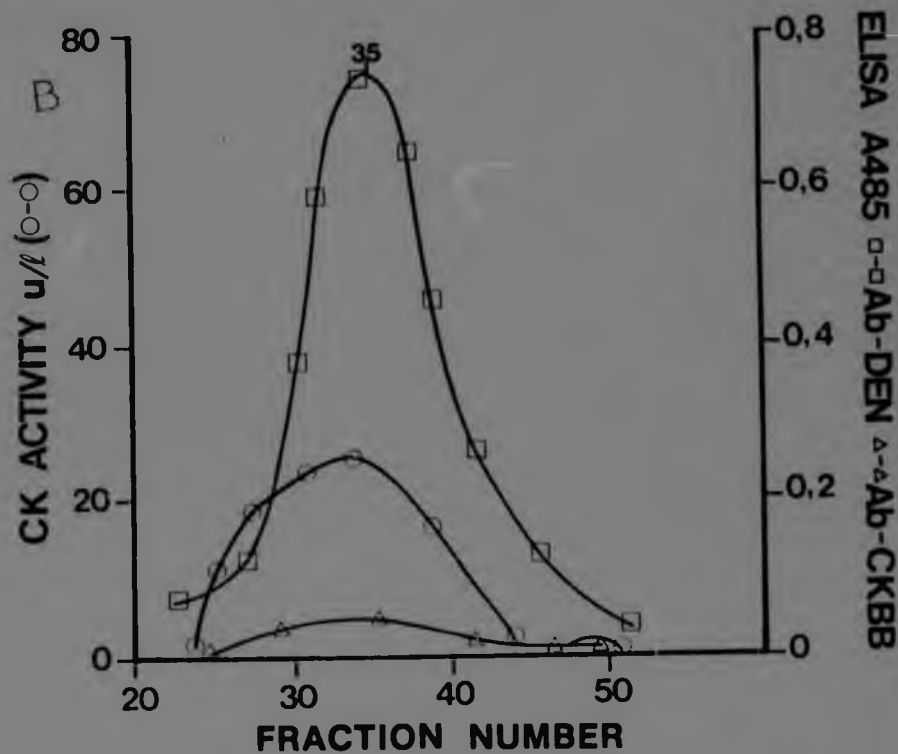
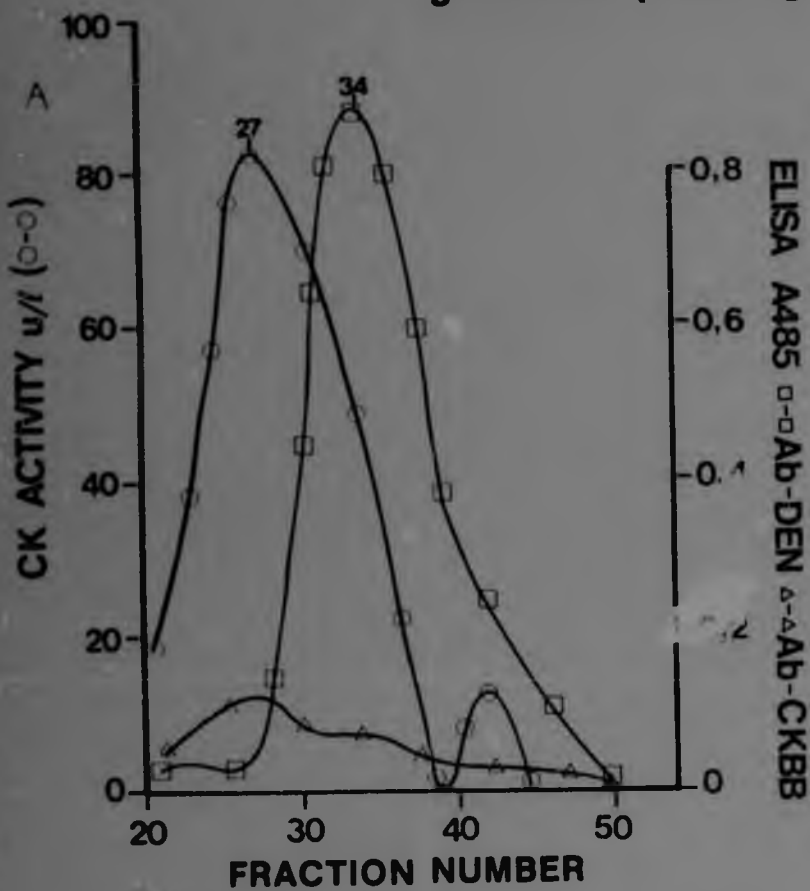


Fig. 10c
DEAE SEPHAROSE CL6B CHROMATOGRAPHY OF
BRAIN SUPERNATANT. Closer details of fractions 20 - 50
from (A) the 100 mM phosphate buffer homogenisation
(from Fig. 10a) and (B) the 50 mM TRIS buffer homo-
genisation (from Fig. 10b).



3.11. IS THE ANTIGEN CYTOSOLIC OR MEMBRANE BOUND?

To see which CK isoforms were possibly membrane bound forms, the 12,000xg precipitate from 21,4 g brain homogenised in Tris/BME was washed three times with Tris/BME, then suspended in 100 mM phosphate, 5 mM BME buffer on ice for 15 minutes and recentrifuged at 12,000xg. This treatment is widely used to release MtCK from mitochondria by inducing mitochondrial swelling (Jacobus and Lehninger, 1973).

The isoenzyme electrophoresis of the centrifugal fractions from this experiment is shown in Fig 11a. The pooled supernatants in Tris buffer (lane 3) contained three CK bands on isoenzyme electrophoresis with a major anodic CKEB band and two cathodic bands with relative Rf values of -0,10 and -0,22 respectively. The phosphate released fraction (lane 4) contained a major cathodic band of Rf value -0,27, with a small band of CKBB and the possibility of masked bands equivalent to those in lane 3. Note that the supernatant from the second wash was not entirely free of CKBB which probably accounts for the presence of the CKBB (a cytosolic protein) in the phosphate released fraction.

The supernatant containing the proteins released from the membranes had a CK activity of 1655 U/l in 34 ml (i.e. 56,27 I.U. in total, or 2,62 I.U. per gram of brain) and was chromatographed on DEAE Sepharose CL6B. The fractionation of the phosphate released proteins on DEAE Sepharose CL6B is shown in Fig 11b, and the isoenzyme electrophoresis of the peak fractions off this column is shown in Fig 11c. The majority of the CK activity passed unretarded through the column and gave a broad band of activity cathodic to CKMM (Rf -0,29) on isoenzyme electrophoresis. This protein is presumed to be mitochondrial CK. The proteins retained on the column yielded two

peaks with CK activity upon salt elution, corresponding to a cathodic band (fraction 27, R_f -0,05) and an anodic CKBB band (fraction 80). Notice that there is a small amount of immunoreactivity associated with fraction 27 as shown by the ELISA results.

Another similar experiment was performed to assess whether the antigen was cytosolic or particulate, but in this instance the homogenisation buffer contained 0,32 M sucrose and the centrifugal precipitates were solubilised in 1,2% sodium dextrocholate. The isoenzyme electrophoresis of the centrifugal fractions is shown in Fig 11d. The 100,000xg supernatant (lane 2) contained a major anodic CKBB band, and two cathodic bands of R_f -0,09 and -0,19 which passed unretarded through a DEAE Sepharose CL6B column (lane 6). The 100,000xg precipitate, which includes the mitochondrial fraction since there was no 20,000xg spin, when solubilised by deoxycholate yielded a major cathodic band (R_f -0,14) and a small band of CKBB which may represent cytosolic contamination. The discarded fraction shown in lane 6 was dialysed overnight against Tris/BME, taken to 50% ethanol and the resulting precipitated proteins were chromatographed through DEAE Sepharose CL6B. The immunoreactivity of each fraction was roughly estimated to be 4+ in the discarded fraction of the first column (i.e. lane 6), 2+ in the discarded fraction of the second column (i.e. the 50% ethanol precipitate of lane 6) and surprisingly 3+ in the 50% ethanol supernatant from this step. This can be interpreted to mean that in the presence of sucrose, all the immunoreactive proteins appear to pass straight through an ion exchange column and are therefore very basic in charge. One would expect all these proteins to be precipitated by 50% ethanol, but after overnight dialysis against Tris/BME, there was significant

immunoreactivity in the 50% ethanol supernatant. Therefore, it was concluded that sucrose affects the mobility of proteins, and was not included in the buffers again.

Fig 11e shows the isoenzyme patterns achieved in a similar experiment to above, this time with Tris/BME buffer containing no sucrose. (It should be noted that this specimen of brain did not have very good enzyme activity.) The 100,000xg supernatant (lane 1) gave the familiar pattern of a major CKBB band and two cathodic bands (Rf -0,12 and -0,24 respectively). The 50% ethanol precipitate (lane 3) contained a strong band of the Rf -0,12 band, which was virtually unnoticeable in the 50% ethanol supernatant (lane 4). The discarded fraction from the DEAE Sepharose chromatography of the 100,000xg supernatant contained only one band of Rf -0,12. This contrasts with lane 6 in Fig 11d where there are two cathodic CK bands in a similar fraction. The proteins released from the 100,000xg precipitate (note that this includes the mitochondrial fraction) by phosphate yielded a band also of Rf -0,12. The similar fraction in lane 4 of Fig 11d had an Rf of -0,14, but in Fig 11c the Rf was -0,29. Since these fractions each represent mitochondrial CK, this is evidence of two types of migration behaviour as noted by Wevers et al (1982).

Another fractionation experiment was performed on a fresh (i.e. unfrozen post mortem) specimen of brain with a classical centrifugal procedure as outlined in Fig 11f. The supernatant and precipitant fractions known as S1-3 and P1-3 respectively were submitted to isoenzyme electrophoresis as shown in Fig 11g. Note that the 630xg supernatant contains three cathodic bands (Rf +0,08, -0,08 and -0,32) i.e. one more than the first spin supernatant given in Fig 11e (lane 2) and Fig 11a (lane 3). It is probable that this band (i.e. the Rf

-0,32 band) represents CK bound to mitochondria which at this centrifugal speed would still be in suspension, since the Rf value corresponds to that obtained for the putative MtCK in the other experiments. Note also that the other cathodic bands were distributed on either side of the CKMM marker, whereas in the previous two experiments they were both cathodic to the CKMM. This is probably due to the brain specimen being fresh since it was noted in section 7 that these two bands convert to a more cathodic migration with time. The S2 and S3 fractions (lanes 3 and 4) gave the same number of bands as noted previously. The nuclear pellet apparently released the Rf -0,32 band with phosphate treatment but this is almost certainly due to contamination of this fraction with cytosolic proteins since this precipitate is large and difficult to wash thoroughly. Further evidence for this statement is that the K⁺ concentration of this fraction was 9,7 mM, whereas that of the P2 and P3 fractions was 0,9 mM. Potassium can be used as an indicator of the efficiency of washing of the intracellular organelles since potassium is an intracellular ion. The P2 fraction, the mitochondrial pellet, revealed one main cathodic band of Rf -0,12 with a trace of the +0,12 band. Surprisingly, the CKBB was stronger than one would expect and this is probably due to cytosolic contamination. Note the putative MtCK had an Rf of -0,12 which is similar to that in Fig 11e (lane 6). It is reasonable to assume, therefore, that fresh mitochondrial CK has an Rf of about -0,12, whereas aged MtCK has an Rf of about -0,30. The P3, synaptosomal, pellet appeared to release a small but sharp band of the +0,12 band. If I were to repeat this experiment, I would dialyse the pelleted fractions extensively before releasing the proteins with phosphate to

exclude any possibility of cytosolic contamination.

Fig 11h shows the DEAE Sepharose CL6B chromatography of the proteins released from the mitochondrial (P2) pellet. There is a very small peak of CK at fraction 30 of 11 U/l, which is not coincident with the ELISA activity which appears to be a tail of activity from the discarded fraction. The discarded fraction gave an ELISA reading of $A_{485} = 0,500$ so we can conclude that any antigenic material that was in this fraction, whether released from the mitochondria or due to cytosolic contamination, passed unretarded through the DEAE column. This indicates that the fraction 30 ELISA fraction noted previously (e.g. Fig 3a) is (i) not released directly from the mitochondria or (ii) is in the more basic conformation of two possible mitochondrial forms.

Table 10 shows a summary of the findings of the centrifugal fractions. If the results are manipulated to give a "specific activity" of the 'antigen' based on the ELISA and CK readings for each fraction, it would appear that the 'antigen' is enriched in the mitochondrial (P2) fraction with a value that is twice that of the P3 fraction and more than ten times that of the cytosolic fractions. This would suggest a membrane associated locality.

The centrifugal fractions were run on a 10% SDS PAGE gel in duplicate and stained for protein by Coomassie Blue, and immunoblotted using the Ab-DEN in an attempt to clarify further the question of cytosolic or particulate distribution. The results are shown in Fig 11i. Rather than clarifying the situation, these gels served to rather confuse it, since the immunoblot stained at least 15 proteins which react with the Ab-DEN. In the supernatant fractions, the Ab is seen to react with about 7 main bands of assessed molecular

TABLE 10: PROPERTIES OF THE SUBCELLULAR FRACTIONS OBTAINED BY DIFFERENTIAL CENTRIFUGATION OF BRAIN HOMOGENATE.

<u>Fraction</u>	<u>Volume</u>	<u>Activity</u>	<u>Total CK</u>	<u>ELISA</u>	<u>'Total ELISA'</u>	<u>'Specact'</u>	<u>K[±]</u>
	(mL)	(U/L)	(UNITS)	(P ₄₈₅)	(vol.x ELISA)	(*)	(mM)
S1	82	343	28,1	0,80	65	2,3	
S2	39	308	27,4	0,82	73	2,7	
S3	85	311	26,4	0,77	65	2,5	17,6
P1	48	225	10,8	1,26	60	5,6	9,7
P2	12	37	0,44	0,97	12	27,3	0,9
P3	6	48	0,29	0,77	4,5	15,5	0,9

(* = Total ELISA / Total CK activity)

masses 68000, 59500, 58000, 51500, 48000, 44000 and 41000 daltons. Notice that it is difficult to do accurate molecular mass determination on the immunoblot since (i) the points of reference, the markers, are another gel, and (ii) the handling of the gel in the electroblotting process can slightly alter the dimensions of the gel. However, one can conclude that the strongest reacting bands are all of a larger apparent molecular mass than the purified 'antigen' which was found to be 42000 daltons in Fig 4f. If we reflect that the Ab-DEN was formed to a narrow band excised off an SDS gel, it is in the first place highly unlikely that these higher molecular mass bands reflect contamination of the antibody. The gel filtration of brain homogenate shown in Fig 9e, which admittedly only spans a certain range of non-denaturing molecular masses, shows cross reaction with only two proteins. There are therefore two possible explanations for

these findings. Firstly, as summarised in section 1.7.1. of the introduction, CK has been shown to give different molecular masses on SDS under circumstances which can only be due to small charge differences not size differences, and therefore molecular mass determinations by SDS PAGE can be misleading. Secondly, if the molecular masses are indeed as calculated, this could reflect the stepwise degradation or alteration of an original higher molecular mass species. Since the molecular masses calculated are very close to each other, reflecting a range of about 25000 daltons, it is unlikely that these results are reflecting the breakdown of a parent molecule into smaller intrinsically coded ones. There is the possibility that the antigenic determinant recognised by Ab-DEN is a common sequence in closely related proteins but one would then have expected to see more evidence of cross contamination.

**FIG 11a: ISOENZYME ELECTROPHORESIS OF SOLUBLE
AND PARTICULATE PROTEINS FROM HUMAN BRAIN**

(Lane 1 = first wash of 20,000xg precipitate with 50 mM Tris buffer pH 7.4; lane 2 = second wash of same; lane 3 = pooled supernatants from first spin plus washes, lane 4 = proteins released from 20,000xg precipitate by 100 mM phosphate buffer pH 7.4; lane 5 = CK markers)

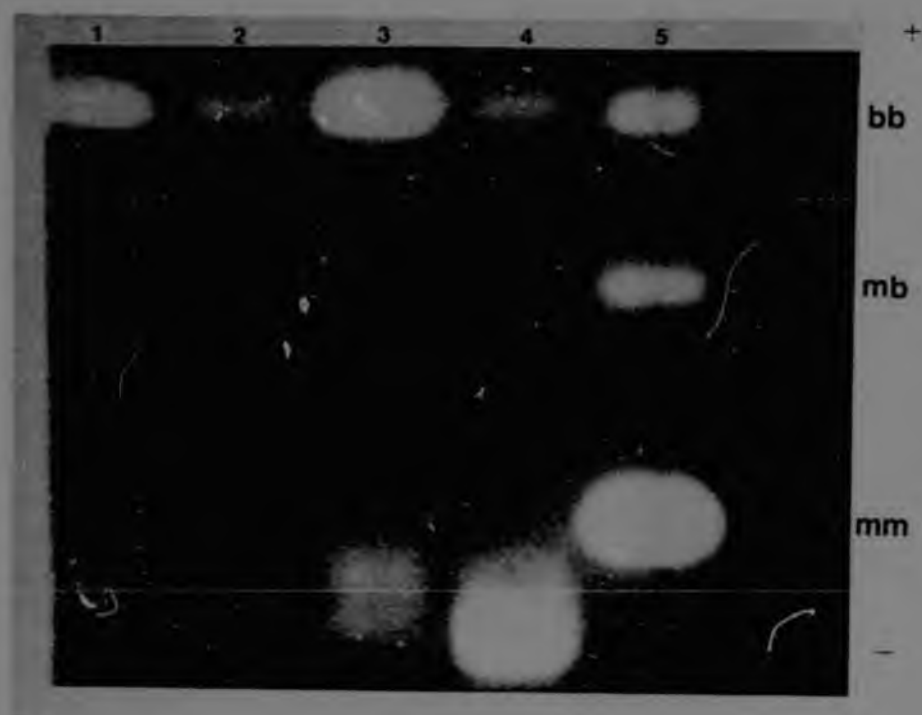


Fig. 11b

DEAE SEPHAROSE CL6B CHROMATOGRAPHY OF PROTEINS FROM 20 000 x g PRECIPITATE OF BRAIN homogenised in 50 mM TRIS buffer pH 7,4 containing 5 mM β - mercaptoethanol. The precipitate was washed three times and the organelle bound proteins were released by 100 mM phosphate buffer pH 7,4 containing 50 mM β - mercaptoethanol. Fractions assayed for CK activity and ELISA using Ab-DEN.

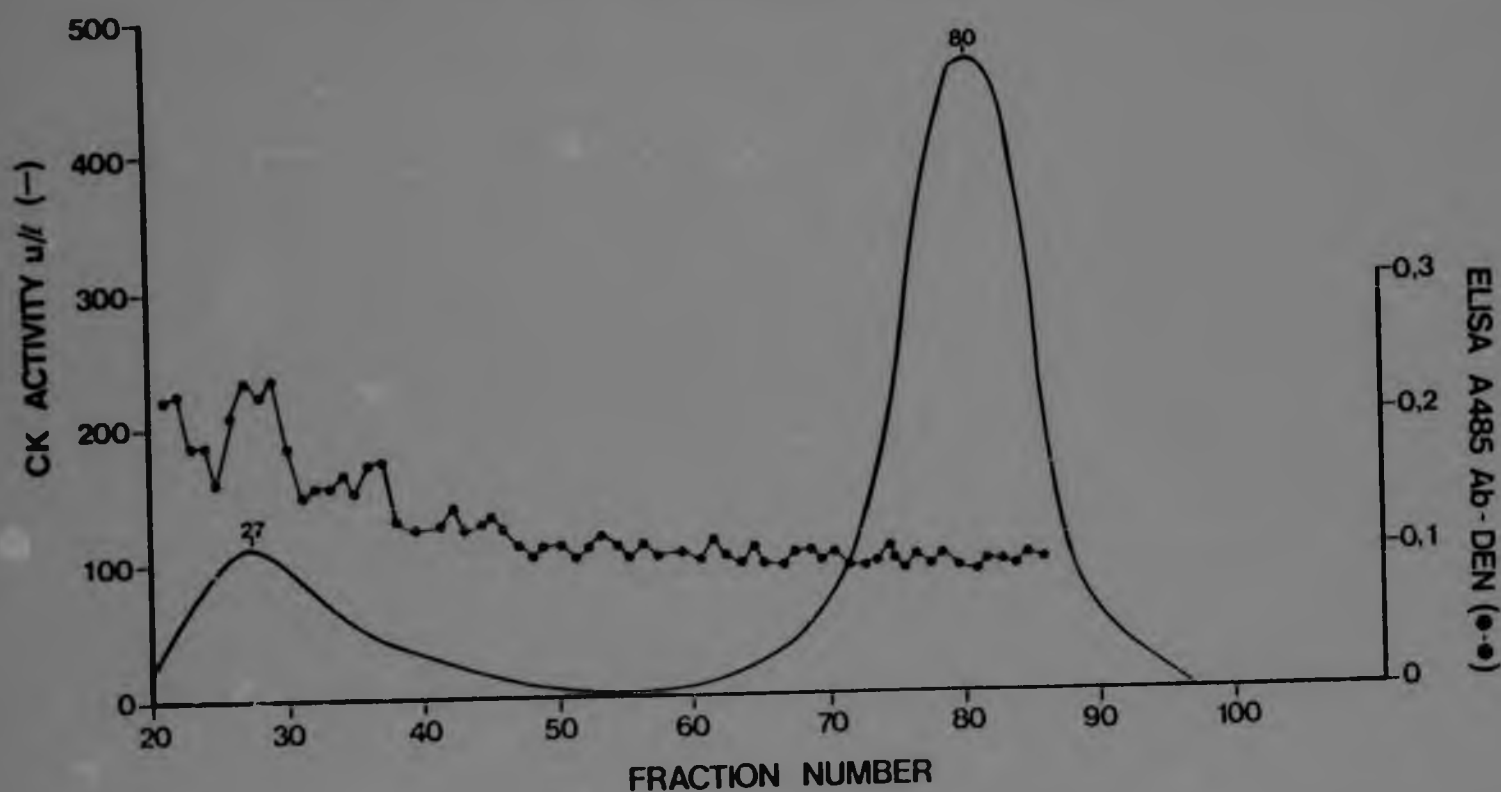


FIG 11c: ISOENZYME ELECTROPHORESIS OF FRACTIONS FROM THE DEAE SEPHAROSE CL6B CHROMATOGRAPHY OF PROTEINS RELEASED BY 100 mM PHOSPHATE BUFFER pH 7,4 FROM THE 20,000xg PRECIPITATE OF HUMAN BRAIN HOMOGENISED IN 50 mM TRIS BUFFER pH 7,4 CONTAINING 5 mM β -MERCAPTOETHANOL

(Lanes 1 and 5 = CK markers; lane 2 = fraction 27; lane 3 = fraction 80; lane 4 = proteins not retained on the column)

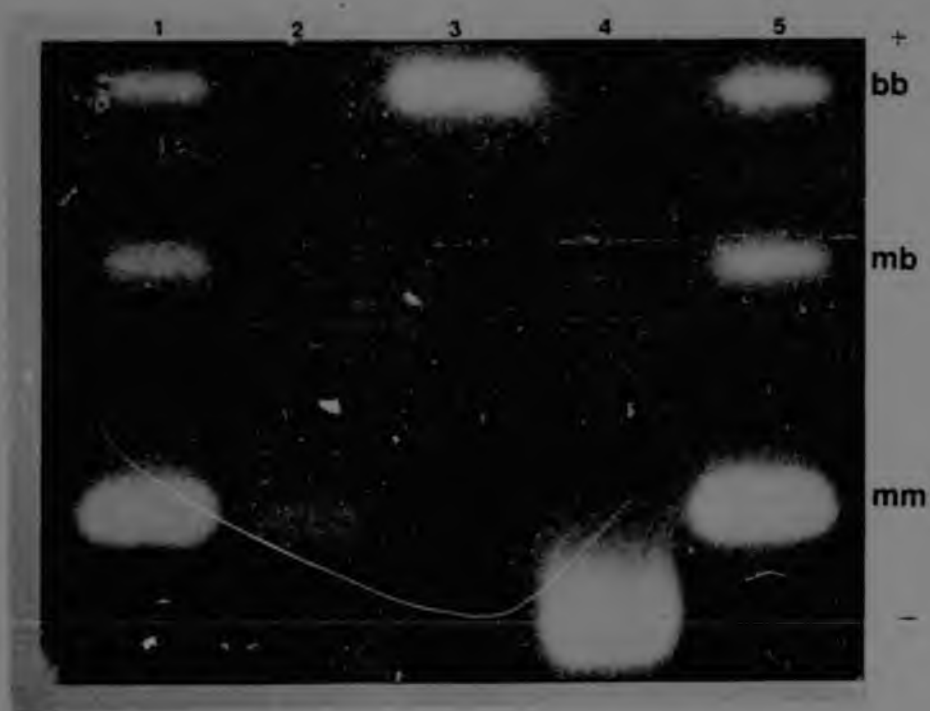


FIG 11d: ISOENZYME ELECTROPHORESIS OF FRACTIONS FROM THE DEAE SEPHAROSE CL6B CHROMATOGRAPHY OF BRAIN HOMOGENISED IN 50 mM TRIS BUFFER pH 7,4 CONTAINING 5 mM β -MERCAPTOETHANOL AND 0,32 M SUCROSE

(Lanes 1 and 5 = CK markers; lane 2 = 100.000xg supernatant; lane 3 = 2.500xg precipitate, released by 1,2% deoxycholate; lane 4 = 100.000xg precipitate, released by 1,2% deoxycholate; lane 6 = proteins from 100.000xg supernatant which were not retained on the column)

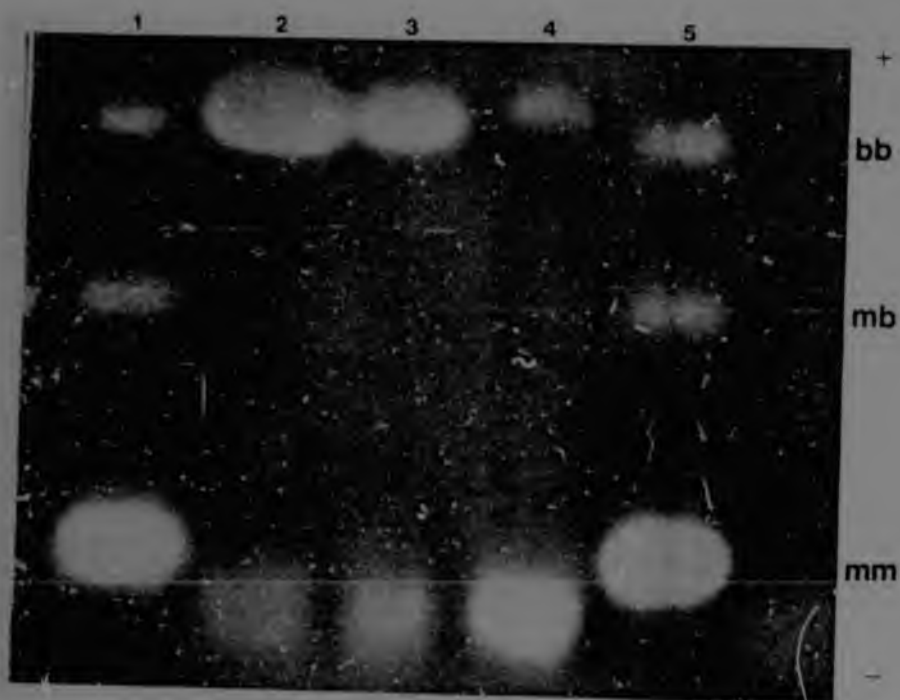
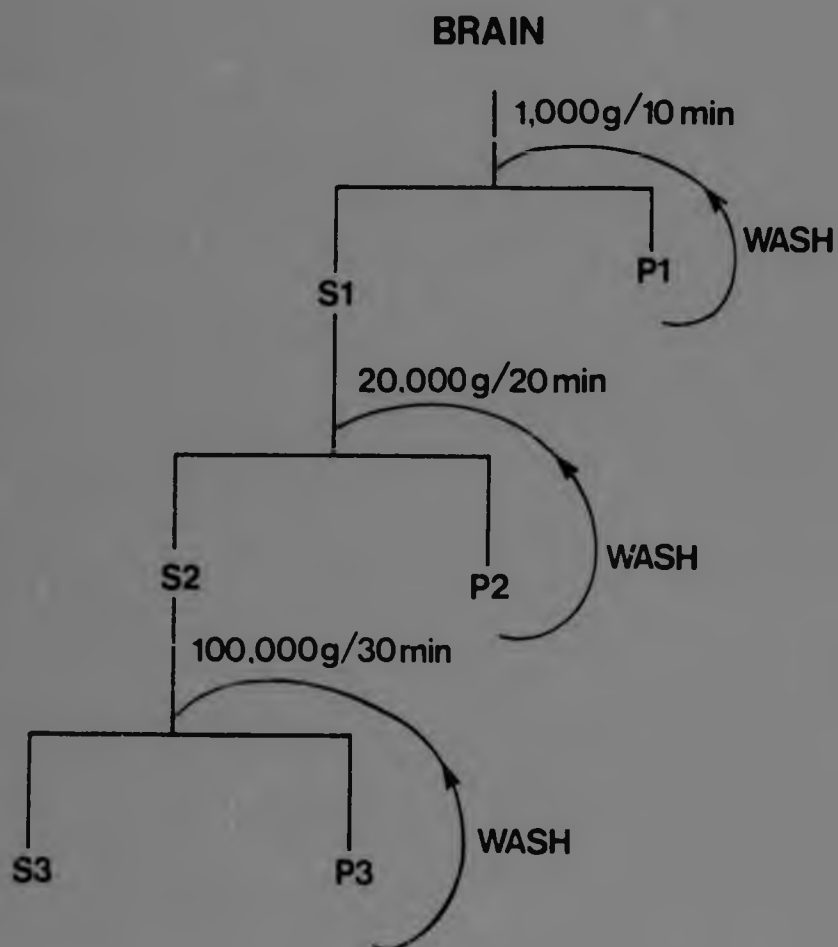


FIG 11e: ISOENZYME ELECTROPHORESIS OF PROTEINS OBTAINED BY FRACTIONATION OF BRAIN HOMOGENISED IN 50 mM TRIS BUFFER pH 7,4 CONTAINING 5 mM β -MERCAPTOETHANOL

This specimen of brain had low specific activity of CK, and was therefore probably not very fresh.
(Lane 1 = CK marker; lane 2 = 100,000xg supernatant of brain homogenate; lane 3 = 50% ethanol precipitate of brain homogenate; lane 4 = 50% ethanol supernatant of brain homogenate; lane 5 = proteins not retained by DEAE Sepharose CL6B chromatography of 100,000xg supernatant; lane 6 = proteins released from 100,000xg precipitate by 100 mM phosphate buffer pH 7,4 containing 5 mM β -mercaptoethanol)



FIG II f: FRACTIONATION OF BRAIN BY DIFFERENTIAL CENTRIFUGATION.



P1 - nuclei, rough membranes

P2 - mitochondria, synaptosomes and plasma membranes

P3 - smooth and rough membranes, and free polysomes

FIG 11g: ISOENZYME ELECTROPHORESIS OF PROTEINS FROM BRAIN OBTAINED BY DIFFERENTIAL CENTRIFUGATION

(Lanes 1 and 8 = CK marker, lane 2 = 630xg supernatant (S1), lane 3 = 20,000xg supernatant (S2); lane 4 = 100,000xg supernatant (S3) lane 5 = 630xg precipitate (P1), released by Triton X-100; lane 6 = 20,000xg precipitate (P2), released by Triton X-100, lane 7 = 100,000xg precipitate (P3); released by Triton X-100)

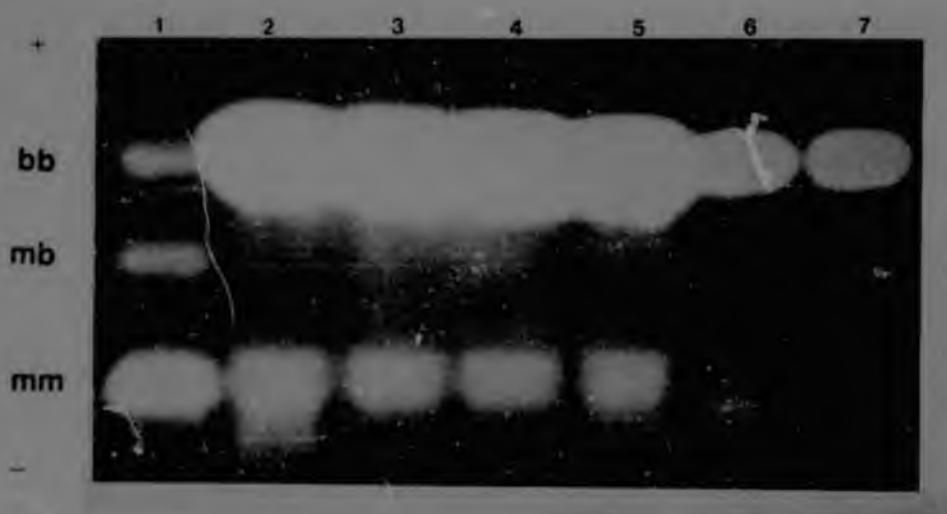


Fig. 11h
DEAE SEPHAROSE CL6B CHROMATOGRAPHY OF
P2 PROTEINS (20 000 x g precipitate of brain
homogenised in 50 mM TRIS buffer pH 7,4 containing
5 mM β - mercaptoethanol). Fractions assayed for CK
activity and ELISA by Ab-DEN.

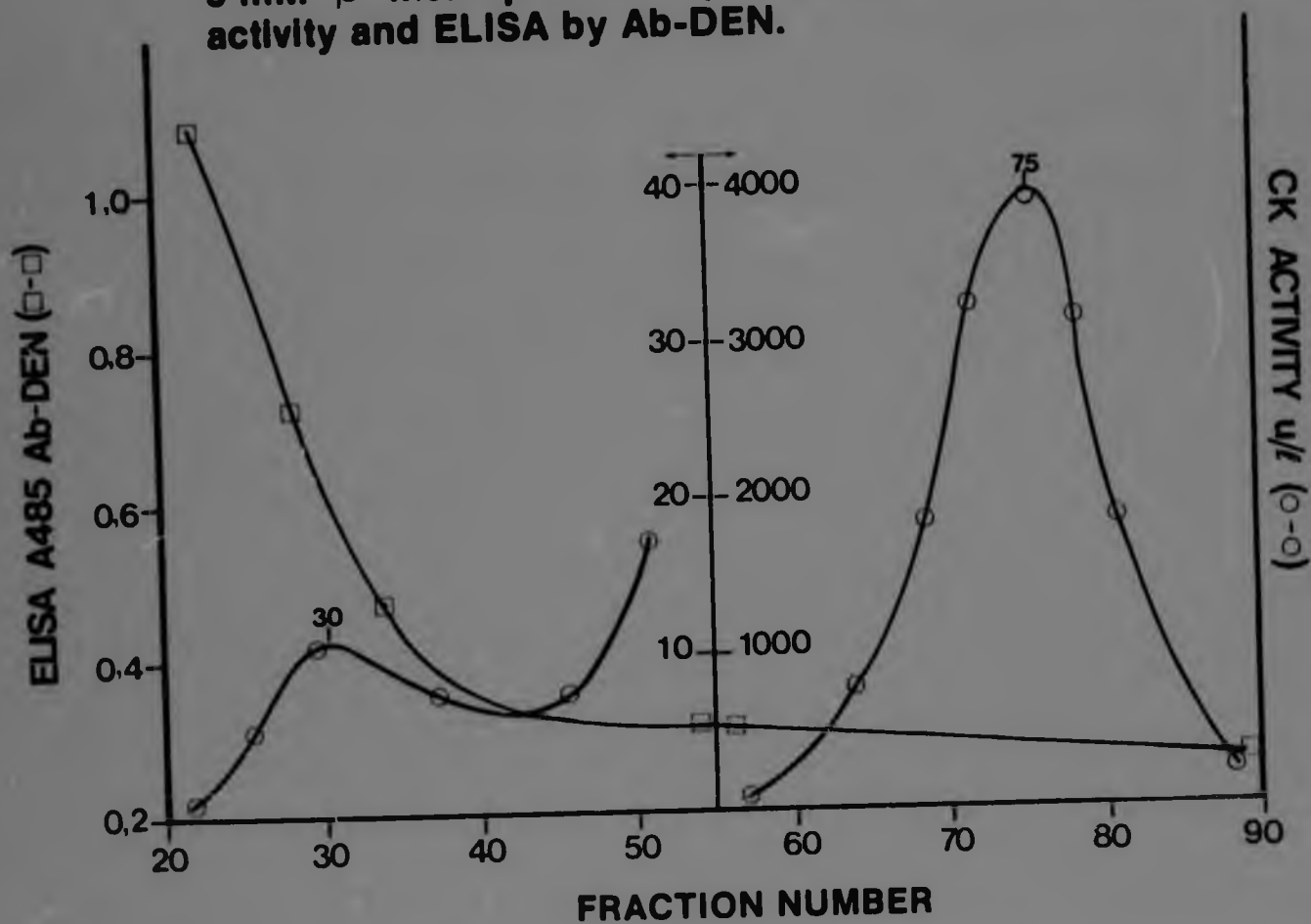
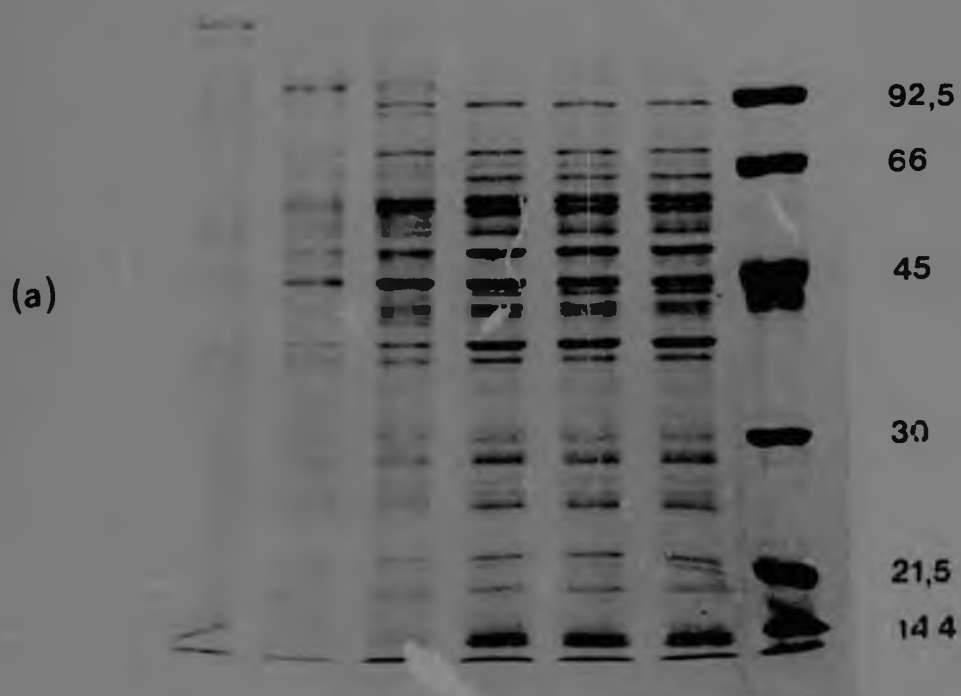


FIG 11i: SDS-PAGE (10%T) OF PROTEINS FROM BRAIN OBTAINED BY DIFFERENTIAL CENTRIFUGATION

(a) stained for proteins by Coomassie Blue

(b) immunoblotted using Ab- α -syn

(Lane 1 = low molecular mass markers; lane 2 = S1 (see Fig 11g); lane 3 = S2; lane 4 = S3; lane 5 = P1; lane 6 = P2; lane 7 = P3)



3.12. EFFECT OF ENZYME MODIFICATION

Fig 12a shows the results of an experiment in which brain supernatant was incubated with various structure altering enzymes and immunoblotted, to see whether a relationship could be established between the 45000 dalton active CKBB and the 42000 dalton 'antigen' protein. The enzymes used were a) neuraminidase, which removes terminal sialic acid residues b) alkaline phosphatase, which removes phosphate groups in a non-specific manner c) endoproteinase Glu-C from *Staphylococcus aureus* V8, which specifically hydrolyses peptide and ester bonds at the carboxyl end of glutamic and aspartic acid residues and d) pronase E, an unusually non-specific protease from *Streptomyces griseus*. The Ab-DEN recognized quite a wide range of proteins in the brain supernatant, with the dominant band being 37000 daltons with two marked high molecular mass bands. The treatment with neuraminidase and alkaline phosphatase appeared to have absolutely no effect of the pattern of bands obtained. Limited proteolysis with *Staph. aureus* eliminated the two high molecular mass bands and reduced the intensity of the 37000 dalton band, with generalised proteolysis virtually eliminating all reaction. None of these treatments led to an enhanced intensity of the 42000 dalton band which one would expect if this band were produced by modification of the 45000 dalton CKBB protein. It is disconcerting that the Ab-DEN recognises so many bands on this immunoblot of brain supernatant. Possible explanations are either an incomplete solubilisation of proteins by SDS, or a common primary structure in several proteins which is recognised by the Ab-DEN.

Another experiment was done to test the effect of alkaline phosphatase. The 50% ethanol fraction of brain (known to contain the

'antigen') was electrophoresed on a 7,5% non-denaturing preparative gel. The gel was sliced into 0,5 cm sections which were leached into Tris/BME buffer and tested for 'antigen' content by ELISA using Ab-DEN. A peak of 'antigen' content was found in the section 0,5-1,0 cm from the origin. This protein fraction was then split into two aliquots: one was incubated at 37°C for 15 minutes with 25 ug alkaline phosphatase (Boehringer 108138) and one was incubated in the absence of alkaline phosphatase as a control. At the end of the incubation, the fractions were boiled with SDS sample buffer and run on a 10% SDS gel. The lane containing 'antigen' only gave one protein band of MW 42000 daltons. The alkaline phosphatase lane only gave a broad protein band in the region of 68000 daltons and the fraction of antigen incubated with alkaline phosphatase gave both these bands in unchanged positions. Therefore, alkaline phosphatase treatment does not change the molecular mass of the antigen.

Fig 12b shows the results of limited proteolysis of four molecular mass species excised from a 10% SDS gel - these are the 45000 dalton band (active CKBB), 42000 dalton band ('antigen'), 47000 dalton band (contaminant of the DEAE Sepharose CL6B CK peak, thought to be neuron-specific enolase) and 53000 dalton band (which reacts with the Ab-DEN). Each band was incubated with 2,5 and 50 ug protease. Unfortunately, there is very little protein in the 42000 dalton lanes which makes it virtually impossible to discern the pattern of fragments. However, there seems to be little relationship between the four bands based on the fragment patterns.

FIG 12a: SDS-PAGE (10%T) SHOWING THE EFFECT OF INCUBATION OF BRAIN SUPERNATANT WITH VARIOUS MOLECULE ALTERING ENZYMES

(a) stained for protein by Coomassie Blue (lane 1 = low molecular mass markers; lane 2 = brain supernatant)
 (b) immunoblotted using Ab_{DBN}. (lane 3 = brain supernatant; lane 4 = brain supernatant + neuraminidase; lane 5 = brain supernatant + alkaline phosphatase, lane 6 = brain supernatant + *Staphylococcus aureus* protease; lane 7 = brain supernatant + pronase)

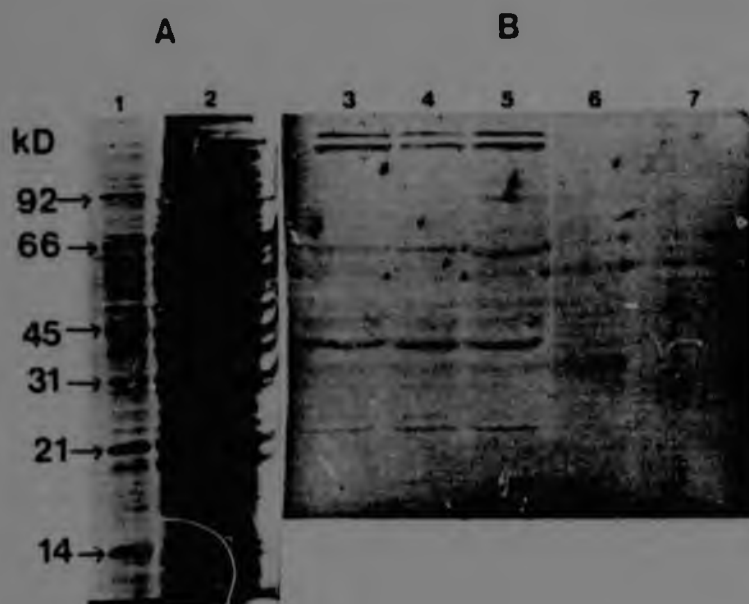
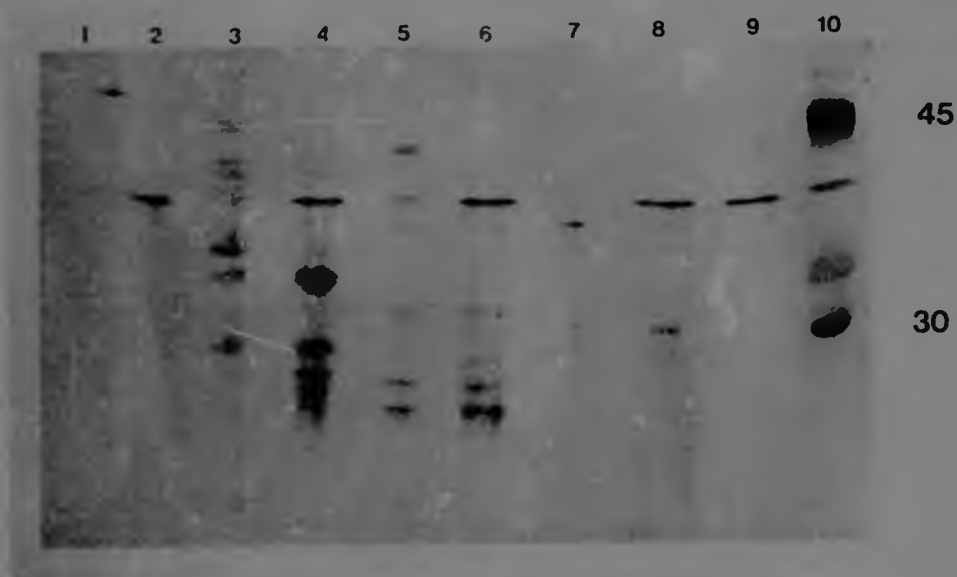


FIG 12b: SDS-PAGE (10%) SHOWING LIMITED PROTEOLYSIS OF SELECTED MOLECULAR MASS PROTEINS USING ENDOPROTEINASE C' U-C

(lane 1 = 42,000 dalton band incubated with 2.5 ug/ml protease;
lane 2 = 42,000 dalton band incubated with 50 ug/ml protease;
lane 3 = 45,000 dalton band incubated with 2.5 ug/ml protease;
lane 4 = 45,000 dalton band incubated with 50 ug/ml protease;
lane 5 = 47,000 dalton band incubated with 2.5 ug/ml protease;
lane 6 = 47,000 dalton band incubated with 50 ug/ml protease;
lane 7 = 53,000 dalton band incubated with 2.5 ug/ml protease;
lane 8 = 53,000 dalton band incubated with 50 ug/ml protease;
lane 9 = protease only; lane 10 = low molecular mass markers)



3.13. DISTRIBUTION OF CKBB IN BRAIN

To establish a rough distribution of CKBB in the brain, 2 g each of cerebellum, cerebrum and white nerve tissue were hand homogenised in 4 ml Tris/BME and centrifuged in a bench top centrifuge for 15 minutes. The specific activities of these fractions is shown in Table 10:

TABLE 10: SPECIFIC ACTIVITY OF CKBB FROM DIFFERENT AREAS OF THE BRAIN

	<u>PROTEIN</u>	<u>CK</u>	<u>SPECIFIC ACTIVITY</u>
	mg/ml	U/ml	U/mg
CEREBRUM	0,464	97,8	210,8
CEREBELLUM	0,816	187,5	229,8
WHITE NERVE TISSUE	0,512	77,6	151,6

It is interesting to note that the specific activities of the cerebrum and cerebellum were not significantly different though there was twice as much protein and therefore CK activity in the cerebellum. The specific activity of the CKBB in the white nerve tissue was about 70% of that in the cerebrum and cerebellum though the protein was about the same order of magnitude.

When these fractions were electrophoresed on the Paragon isoenzyme system (results not shown), they all had a major anodic CKBB band and two cathodic bands (R_f 0,00 and -0,12) which appeared roughly equivalent in the cerebrum and cerebellum, but in the white nerve tissue it appeared that the least cathodic of the pair was the dominant band.

4. DISCUSSION

4.1. PROPERTIES OF Ab-DEN

This study has described the production of an antibody in rabbit against a CKBB denatured by SDS. This antibody has been shown to have the following properties:

4.1.1 NO REACTION WITH CKBB UNDER NON-DENATURING CONDITIONS

Ab-DEN does not recognise active, native CKBB. Evidence is presented for this from the results of many columns (e.g. Fig 9b) where there is a peak of CKBB activity which is not associated with reaction to Ab-DEN as measured by the ELISA method. The CKBB activity peak is, however, associated with an ELISA peak using the Ab-CKBB.

4.1.2 REACTION WITH CKBB UNDER DENATURING CONDITIONS

Ab-DEN does seem to recognise an epitope in the CKBB molecule which is concealed under normal non-denaturing conditions. The evidence for this is that in the presence of increasing amounts of denaturant - SDS (Fig 7d) or urea (Fig 7e) - the immunoreactivity of the CKBB to Ab-DEN, as measured by the ELISA reaction, increases. In addition, if sufficient protein is present on a SDS PAGE gel, then an immunostain with Ab-DEN reacts with CKBB but sensitivity is extremely low. Evidence for this is presented in Figs 9a and 9b, in which fractions from the gel filtration of brain supernatant which did not respond to Ab-DEN in the ELISA (Fig 9a) reacted with Ab-DEN when

electrophoresed on an SDS PAGE gel and immunoblotted (Fig 9b).

4.1.3. REACTION WITH A NATURALLY OCCURRING 'ANTIGEN'

There appears to be a protein occurring naturally which reacts with Ab-DEN. The first evidence of this was obtained when initially brain supernatant (Fig 3a) and then breast tumour cytosol (Fig 3b) were chromatographed through DEAE Sepharose CL6B and all the eluted fractions were monitored for CK activity and immunoreaction to both Ab-DEN and Ab-CKBB. A peak of protein was obtained at the beginning of the gradient which had small, but measureable, CK activity associated with a strong reaction to Ab-DEN.

4.2. PROPERTIES OF THE 'ANTIGEN'

Attempts were then made to isolate and characterise this protein, and it has been shown to have the following properties:

4.2.1 CHARGE

It has been shown in two independent systems that this protein is more negatively charged than CKBB. This was established by column chromatography and electrophoresis.

Column chromatography

The basic nature of the 'antigen' was demonstrated firstly by the fact that it was more readily released than CKBB off an ion exchange column (fig 4a). This confirms the findings of other group who have found a small CK activity peak appearing before the CKBB peak on an anion exchange column (Nealon and Henderson, 1975a; Yasmineh et al, 1976; Lindsey and Diamond, 1978; Kraft et al, 1978).

Electrophoresis

Secondly, my results have shown that the 'antigen' has a cathodic migration after isoenzyme electrophoresis (fig 4d - lane 2, and Fig 7g). This migration could be to one of two positions - either opposite or slightly above ((Fig 4d, Fig 11c, Fig 7gi) the CKMM marker, or below (Fig 7giii, Fig 11e) the CKMM marker. It appears that very fresh brain yields the least cathodic migration, and ageing (or experimental reaction of the 'antigen' with Ab-DEN) results in a form with more cathodic migration. This ties in with the observation that ageing of a CKBB preparation resulted in the formation of more basic forms (Fig 7b).

Isoenzyme electrophoresis of brain homogenate in Tris buffer yielded an anodic CKBB band plus one cathodic band, and in phosphate buffer yielded an anodic band plus two cathodic bands (Fig 7g).

A survey of the literature shows that in the study of the isoforms in human brain found by electrophoretic separation:

- (i) some authors found only one CK band, being an anodic CKBB band (Jockers-Wretou and Phleiderer, 1975; Urdal et al, 1983). In my opinion, the reason for this was that insufficient protein was electrophoresed to reveal the cathodic bands which have significantly less activity than the CKBB.
- (ii) some authors (Murone and Kikuo, 1973; Friedhoff and Lerner, 1977; Chandler et al, 1984) found one cathodic CK activity band in addition to the anodic CKBB.
- (iii) some authors found more than one cathodic band in addition to the anodic CKBB. Wevers et al (1982) found two cathodic bands, and Heinbokel et al (1982) found six bands, in addition to CKBB, using isoelectric focusing.

4.2.2 MOLECULAR MASS BY SDS PAGE

The virtually pure preparation of 'antigen' gave a band equivalent in molecular mass to 42000 daltons, and this same band reacted strongly with the Ab-DEN on an immunoblot (Fig 4f). This contrasts with the molecular mass of CKBB which was estimated to be 45000 daltons (Fig 4c).

There is another protein of molecular mass 38000 daltons which reacts with Ab-DEN. This is either present in much smaller quantities or reacts with less sensitivity to the Ab-DEN (Fig 9e). Whether this is another protein altogether or another form of CKBB-like protein is hard to say but it seems likely to be the latter. Supporting evidence for this statement is that purification of CKBB by hydroxylapatite chromatography yielded an CKBB of molecular mass 38000 daltons (Fig 5b), and Leykam et al (1983) purified a CKBB from human brain of molecular mass 37000 daltons using HPLC as the final purification step (they did not say whether this purified CKBB had activity).

I would suggest that the 38000 dalton form is the one which gives the faster cathodic migration of the two possible migratory positions for the 'antigen', and the 42000 dalton form is that which gives the slower cathodic migration. Fig 7c shows that the discarded fraction of the 50% ethanol fraction of brain chromatographed through DEAE Sepharose CL6B contains a band of estimated molecular mass 38000 daltons which reacts with Ab-DEN as indicated by the single band reacting in this lane in the immunoblot. The CK from this discarded fraction has been shown to be the most cathodically migrating form (Fig 11e, lane 5). Note that when the 'antigen' (the 42000 dalton form) is retained on the ion exchange column and then eluted, its

migration on isoenzyme electrophoresis (Fig 11c) is slower than the form which is discarded from the ion exchange column.

The lower subunit molecular mass antigen was also detected (Fig 9b) on a SDS PAGE immunoblot of 'antigen' peaks (determined by ELISA using Ab-DEN of the eluted fractions) from a gel filtration column of brain supernatant.

Although these results indicate the presence of proteins of differing molecular masses, the published results summarised in section 1.7.1. make caution in this matter advisable. Firstly, all CKBB sequences from different species so far analysed have contained 380 or 381 amino acids, though the corresponding CKBB proteins have yielded molecular masses as determined by SDS PAGE in the range 37000-50000 daltons. Secondly, experiments with alkylating and reducing agents (Barrantes et al, 1983) yielded bands with an apparent difference in molecular mass of 3000 daltons, which was could not be accounted for by the small molecular masses involved in the transfer of alkyl or hydrogen groups.

4.2.3 MOLECULAR MASS BY GEL FILTRATION

Gel filtration of brain homogenised in Tris/BME yielded three peaks of protein which reacted with the Ab-DEN as determined by the ELISA reaction (Fig 9c). The estimated molecular masses of these forms were 251000, 207000 and 162000 daltons. There was an active CKBB peak of estimated molecular mass 182000 daltons which reacted with the Ab-CKBB but not with the Ab-DEN. The largest of the peaks reacting with Ab-DEN was dependent on the presence of BME since it was not present in an equivalent experiment where BME was omitted from the Tris buffer (Fig 9e). In addition, the presence of BME

affected the elution position of proteins from the gel filtration column to a position of apparently higher molecular mass than in its absence. Wevers et al (1982) also found that BME affected the elution of proteins from a gel filtration column when they were trying to estimate the molecular mass of the faster cathodically migrating mitochondrial band. After the gel filtration column, this protein migrated to a less cathodic position on isoenzyme electrophoresis than before the column.

4.3 EFFECT OF BUFFER COMPONENTS ON NUMBER AND TYPE OF CK ISOFORMS FOUND

There is evidence in the literature that the presence and concentration of the basically charged CK isoforms is a function of the buffer system used for homogenisation. There are two explanations for this - one is post-translational modification caused by the different buffer components, and the other is that different conditions release different amounts of membrane-bound fractions.

Chandler et al (1984) found that extraction of cerebral cortex in 50 mM Tris buffer, pH 7,4, led to 95% CKBB and 5% of the cathodically migrating band, whereas extraction in 100 mM ammonium acetate buffer, pH 9,5, led to 80% CKBB and 20% of the cathodically migrating band. Both these buffer systems released more or less the same total creatine kinase activity (60,100 and 55,120 U/g wet tissue respectively), whereas extraction of putamen in ammonium acetate released only 15 U/g wet tissue, of which 20% was CKBB and 80% was the cathodically migrating band.

Wevers et al (1982) found that the intensity of the two cathodic

bands increased with extraction in phosphate buffer, which suggests a membrane-bound location.

My results have also shown that the buffer employed in an experiment affects both the number and the characteristics of the CK isoforms found. For example, in Fig 10a where brain homogenised in phosphate buffer pH 7.4 is chromatographed through DEAE Sepharose CL6B, three peaks of protein which have both CK activity and react to purified Ab-CKBB were found in addition to one band which reacted with Ab-DEN but had no or negligible CK activity. A parallel experiment (Fig 10b) with brain homogenised in Tris buffer pH 7.4 yielded one band which reacted to Ab-CKBB with high CK activity, and one band which reacted to Ab-DEN with low CK activity.

The fact that homogenisation in phosphate buffer yields an extra cathodically migrating band was shown in Fig 7g, and the high molecular mass of this extra form was established in Fig 9a.

My gel filtration experiments showed that the dominant high molecular mass form of protein which reacts with Ab-DEN (estimated to be 251000 daltons) was found in the presence of 5 mM BME and to a negligible extent in its absence (Fig 9d) and that the presence of BME shifted the elution of the proteins (including active CKBB) to an earlier elution position off the gel filtration column.

4.4 NATURE OF THE 'ANTIGEN'

The existence of an additional CK isoform in human brain which is not active CKBB has been established beyond reasonable doubt by the results presented in this study and by consensus of the published literature. The nature and subcellular distribution of this form is,

however, open to considerable debate.

My isoenzyme electrophoresis pattern of brain homogenised in phosphate buffer (Fig 7g) confirms the pattern published by Wevers et al (1982) with an anodic CKBB band plus two cathodic bands. My results show that in the presence of Tris buffer, however, only one of these cathodic bands is found. I suggest that the slower migrating cathodic form is the 42000 dalton 'antigen' which has been characterised above, and the faster migrating cathodic form is mitochondrial CK. This suggestion is supported a) by the fact that the faster cathodic form is only found in the presence of phosphate, and phosphate is known to release Mt-CK by mitochondrial swelling (Font, 1981), and b) by the fact that the Ab-DEN reacts with the slower band but not with the faster band (Fig 7g). Wevers et al found that both cathodic forms were found in the presence of Tris buffer but became more pronounced in the presence of phosphate.

These two cathodic forms under discussion should not be confused with the two possible migration positions for the 'antigen' noted in section 4.2.1. All these forms can be differentiated on the basis of the extent of their migration under isoenzyme electrophoresis, and the mitochondrial form had an Rf which was reproducibly further towards the cathode than either position of the 'antigen'.

There is general agreement in the literature that this cathodic K isoform found in the brain is not CKMM based on immunological and electrophoretic studies (Jockers-Wretou and Phleiderer, 1975; Tsung, 1976; Lerner and Friedhoff, 1980; Wevers et al, 1982; Urdal et al, 1983; Chandler et al, 1984). To my knowledge, only two groups have supported the idea of CKMM in the brain - Lindsey and Diamond (1978) who found that the cathodic CK peak was inhibited by anti-CKMM

antibodies and concluded that 30% of the CK in the brain is CKMM, and Heinbokel et al (1982) who found that three of the seven bands which they found in the brain by isoelectric focusing reacted with anti-CKMM. Both these groups used commercial antiserum to CKMM supplied by Merck, which suggests a cross reaction of this particular antiserum with the CK form which I have called 'antigen'.

I think it would be reasonable to assume that the 'antigen' is not adenylate kinase which elutes from the ion exchange column slightly after the basic CK form (Lindsey and Diamond, 1978), since the molecular mass of adenylate kinase is 21000 daltons (a mass which was never found in this study), and since the method for measuring CK activity includes a blank to exclude the possibility of 'activity' being attributable to adenylate kinase. It is unlikely to be an aggregate of adenylate kinase since the rigorous denaturing conditions (boiling for five minutes in the presence of SDS) would break down any aggregation.

Subcellular location of the 'antigen'

The similarity in properties between membrane bound creatine kinases (for example, in the mitochondria and Torpedo neuroelectocyte synapses) and the 'antigen' could suggest that the 'antigen' is also a membrane bound protein. Mitochondrial CK is basically charged and has a cathodic migration on isoenzyme electrophoresis (Wevers et al, 1982; Kanemitsu et al, 1982). Membrane-associated CK from Torpedo neuroelectocyte synapses migrates to a cathodic position similar to human muscle CKMM on isoenzyme electrophoresis (Gysin et al, 1983).

There is controversy as to whether the Torpedo acetylcholine receptor associated CK is a brain or muscle type. Gysin (Gysin, 1986) claims that on the basis of immunological cross-reactivity, and

comparison between the two isoforms by chromatofocusing, two-dimensional electrophoresis, and apparent molecular mass by SDS PAGE, the membrane bound CK can be concluded to be CKMM. Barrantes et al (1985), on the other hand, found that the receptor associated CK reacted with anti-CKBB, and whilst there was CKMM in the total electrocyte, there was no CKMM associated with the acetylcholine receptor membranes. The membrane location of the CKBB was confirmed by in situ immunocytochemistry using the anti-CKBB. The membrane bound form had a two-fold affinity of ADP binding compared to the soluble CKMM form, suggesting that the association of the CK with the receptor in the membrane is to provide a guaranteed and convenient energy source.

The two research groups just quoted have come to opposite conclusions based on the immunoreactivity of a particular species of creatine kinase to anti-CKMM and anti-CKBB antisera which, in my opinion, serves to illustrate that the epitope which an antibody to CK recognises is vital, since the primary structure of the MM and BB isoforms may be identical in certain regions, but the conformation may allow recognition of only one form. My work has shown that although the antigen injected to raise antibodies in this study was CKBB denatured by electrophoresis on SDS PAGE, the resulting antibody did not recognise native CKBB. It was proved that the antibody did however recognise the CKBB molecule when it was sufficiently denatured to reveal the epitope.

The work discussed above, together with that of Mahadevan (Mahadevan et al, 1984) who identified three forms of phosphorylated CKBB associated with the microtubules in rat brain, establish the precedent of membrane associated CKBB. The phosphorylated forms

identified by Mahadevan et al, however, all had an identical molecular mass as estimated by SDS PAGE. This point is relevant since the subunit molecular masses of the antigenic bands discussed in this work were estimated to be 42000 and 38000 daltons - less than that of the active CKBB at 45000 daltons.

My results have not shown conclusively whether the 'antigen' is membrane bound or cytosolic. The fact that the band can be detected by isoenzyme electrophoresis directly after homogenisation of brain in Tris buffer (Fig 7g) would tend to suggest that the form is cytosolic. The brain used in the experiments was always obtained post mortem. The work of Wevers et al (1982) would suggest, however, that this is not an important factor since they confirmed the results they obtained of two cathodic bands in post mortem human brain using a rat model.

My results of differential centrifugation of brain tissue extracts seemed to indicate that the 'antigen' was associated with the P2 fraction (mitochondria, synaptosomes, plasma membranes). The evidence for this was that a high 'specific activity' for the 'antigen' (about eleven times more than in the cytosolic fractions) was obtained in this fraction than in the other five fractions. The next highest 'specific activity' was in the P3 fraction (smooth membranes, rough membranes and free polysomes) which was half of that in the P2, but still 6 times greater than the cytosolic fractions. The particulate fractions were washed three times and the potassium concentration (an intracellular marker) in P2 and P3 was a twentieth of that of the cytosolic fractions. However, the possibility of cytosolic contamination cannot be ruled out.

In my opinion, Wevers et al (1982) possibly overestimated the

degree of cytosolic contamination in their preparations. Using the Whittaker fractionation procedure they found that "most of the CK liberated [from the mitochondrial pellet] by the desoxycholate treatment turned out to have a cathodic mobility. CKBB in this fraction reflects the cytosolic impurities that are present in spite of the thorough washing steps included in the procedure." The supernatant from washing the pellet had CKBB (+) and cathodic CK (-). whilst the proteins released from the washed pellet had CKBB (+++) and cathodic CK (+++). Exactly the same situation was encountered with the mitochondrial fraction obtained using the Lai fractionation procedure. Their explanation for the increased presence of CKBB in the mitochondrial proteins is "apparently desoxycholate treatment liberated cytosolic proteins. Such cytosolic contaminants have probably been enclosed in vesicles within the M-fraction. These vesicles cannot be removed by repeated washing." They don't seem to entertain what to me is a more reasonable explanation which is that the CKBB activity was also released from the mitochondrial fraction. The synaptosomal mitochondrial fraction, on the other hand, released a trace of CKBB and +++ cathodic CK. This work does prove that cathodically migrating CK is released from mitochondria, but it does not exclude the possibility that one of these CK bands is CKBB or CKBB-like as I am suggesting here.

Friedhoff et al (1977), using the same fractionation procedure used by Wevers et al (1982), found the cathodic band of CK to be associated with the synaptosomal membranes and synaptic vesicles. Wevers et al (1982) found that only minor quantities of the cathodically migrating CK could be recovered from this fraction. The cathodic CK isoform was characterised (Lerner and Friedhoff, 1980) by

isoelectric focusing, antigenicity and column chromatography, and compared to CKMM, CKBB and Mt-CK. The pI of the protein was 7.3 and the estimated molecular mass by gel filtration was 80000 daltons. Of particular interest, as far as this study is concerned, is the fact that the brain particulate protein was shown to be antigenically distinct from CKMM and Mt-CK but was 75% precipitated by antiserum to CKBB. This finding of a CKBB-like cathodically migrating creatine kinase supports my suggestion that the basic CK protein which I have identified in the brain is CKBB or CKBB-like.

The work on a form of CK known as CK-Z has a lot in common with my results. This form was first identified in brain and heart in 1977 by Leroux et al (1977). They found three peaks of CK in brain extract by chromatography on DEAE-cellulose. One of these was excluded from the column (and I would assume this to be mitochondrial CK), one was eluted very early from the column by a salt gradient (this form they called CK-Z and appears to be equivalent to my 'antigen') and one form was eluted later by the salt gradient and was CKBB. Of particular interest is their observations that a) the CK-Z appears to convert to a more basically charged form with time (which is exactly what I have established for the 'antigen' in section 3.7) and b) that CK-Z is not found in the absence of PME (which is what I found in the gel filtration studies in Fig 9e).

Initial investigations of the CK-Z form from heart (Desjardins, 1982) found that it migrated cathodically to CKMM on isoenzyme electrophoresis and was not inhibited by anti-CKMM or anti-CKBB antisera. He concluded that "creatine kinase Z had biochemical properties which were different from those of the other soluble creatine kinase isoenzymes (MM, MB and BB), but similar to those

reported for mitochondrial creatine kinases isolated from other animal tissues."

Another similarity between the results of Desjardins (1982) and mine was the observation that the properties of the CK under investigation depended on the buffer being used. The effects noted differed, but both his results and mine show a marked sensitivity to the components in the buffer. He found that in Tris/BME/EDTA buffer the molecular mass of the CK-Z was 33000 to 38000 daltons, and in phosphate/EDTA buffer the molecular mass was 62000-68000 daltons as assessed by gel filtration on Sephadex G200. In my experiments, a high molecular weight species of 'antigen' was found in the presence of BME, and not in its absence. Also the number and properties of active CK isoforms varied depending on the buffer used.

Desjardins noted that after gel filtration in the presence of phosphate buffer, 87% recovery of CK activity was achieved, whereas in Tris buffer, only 13% recovery of CK activity was achieved, suggesting a greater stability of this form in phosphate buffer. It is interesting that he observed a greater stability in phosphate since I had so much difficulty trying to purify the 'antigen' on hydroxylapatite which uses phosphate for elution of bound proteins. Possibly the passage of the protein solution through the hydroxylapatite depletes the solution of any phosphate ions by competitive binding, thus accounting for my inability to obtain any ELISA (Ab-DEN) peak in the eluted fractions. In addition, purification of active CKBB on a hydroxylapatite column using phosphate buffer yields a CK of molecular mass 37000 daltons whereas purification by cationic exchange using Tris buffer yields a CK of molecular mass 45000 daltons.

In keeping with my observation that the 'antigen' has very low CK activity, he (Desjardins, 1982) note that "A great deal of difficulty was experienced in obtaining sufficient quantities of this enzyme to characterize it further... when it was detected, the activity of the enzyme was extremely low. It was finally realized that this inconsistency was related to the buffer used in the homogenization process, and to the association of this enzyme with the 600xg particulate fraction."

This report of the subcellular location of CK-Z was changed however in a later publication (Desjardins and Pesclovitch, 1983) when it was found that CK-Z accounted for approximately 90% of the CK activity associated with the mitochondrial fraction. This discrepancy is explained by the suggestion that previously the CK-Z was inadequately released from the mitochondrial fraction since in the later study they included 2 mg/ml trypsin in their homogenisation buffer (0,25 mol/L sucrose, 10 mmol/L tricine-potassium hydroxide, and 1 mmol/L EDTA pH 7,4), and in addition the later study used fresh human heart tissue obtained at surgery whereas the previous study used human heart tissue obtained at autopsy. If, indeed, the CK-Z is the same protein as the one which I have been investigating, this constitutes powerful evidence that the 'antigen' is associated with a membrane fraction, probably the mitochondrial.

It seems likely that this 'antigen' is not confined only to the brain. I found a strong peak of 'antigen' in breast tumour cytosol with equivalent properties to the brain form when chromatographed on DEAE Sepharose CL6B. Jockers-Wretou and Phleiderer (1975) observed that 5-15% of the CK activity in heart extracts could not be precipitated by either anti-CKMM or anti-CKBB and concluded that

"this was due to a particulate CK-enzyme known to migrate to the cathode during electrophoresis. This additional enzyme with CK activity neither reacts with anti-CKMM nor with anti-CKBB serum under the experimental conditions used. Small quantities of this particulate enzyme were also found in brain, lung, intestine and thyroid." These tissues are known to have high concentrations of CKBB (Lang, 1981).

It would seem from my results that there are two different types of basically charged CK. One of these is the antigen to Ab-DEN which has been discussed at length above. The other is a breakdown product of active CKBB as shown in Fig 7b. Urdal et al (1983) noted that when autopsy tissue rather than fresh tissue was used for isoenzyme electrophoresis, then cathodic bands were seen, which confirms the observation that ageing produces more basically charged forms.

I have suggested that there are two basic CK forms based on the observation that in autopsy brain which is obtained as quickly as possible and processed without delay, the ELISA peak fractions and CK activity peak fractions in the early part of the salt gradient are separated (Fig 7a). It has been established that ageing of a CKBB preparation produces additional CK activity peaks, the one of interest here being in fraction 30 of the DEAE Sepharose CL6B column (Fig 7b). However, this form had an anodic migration identical to CKBB on isoenzyme electrophoresis. The ELISA (Ab-DEN) peak originally investigated was coincident with the CK activity peak in fraction 30 (Fig 4a). Isoenzyme electrophoresis of this fraction (Fig 4d, lane 2) revealed two CK activity bands - an anodic band plus a cathodic band. The chromatographic separation of the ELISA and CK peaks with fresher material suggests that these are, in fact,

separate entities.

A survey of the relevant literature (Freidhoff and Lerner, 1977; Wevers et al, 1982; Desjardins and Pesclovitch, 1983) together with my results would seem to indicate that the CK isoform under discussion is probably associated with the mitochondria, and is a CKBB like enzyme. The fact that it is found in the supernatant may indicate a loose attachment to the membrane surface, or may be due to the use of autopsy tissue.

4.5 FURTHER WORK

Fresh brain obtained at surgery should be analysed immediately after collection to confirm whether fresh tissue yields the cathodic bands. This would clear up the issue of whether these bands, particularly the slower migrating cathodic band, are present in vivo, or whether they are artifacts caused by ageing or modification.

Further work should be done to establish a definite subcellular location of the 'antigen'. Initial attempts to do this by immunocytochemistry were not particularly successful and this should be repeated.

The characteristics of a purified 'antigen' fractions should be further investigated, in particular the kinetic properties to establish whether it has an enhanced affinity for its substrates as has been shown for other membrane-associated creatine kinases.

Trypsin should be included in the homogenisation buffer after Desjardins and Pesclovitch (1983) to see whether this enhances the amount of 'antigen' detected.

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